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# Myocardial caspase-3 and NF-κB activation promotes calpain-induced septic apoptosis: The role of Akt/eNOS/NO pathway

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ABSTRACT

*Aims:* To explore the potential mechanism that the role of the Akt/eNOS/NO pathway in calpain-induced caspase-3 and NF-κB activation during septic apoptosis.
*Main methods:* Septic rats were stimulated by LPS (8 mg/kg, i.p.). Myocardial calpain, caspase-3, NO, TNF-α and IL-1β levels were detected by ELISA. The levels of Akt/p-Akt, eNOS/p-eNOS, iNOS proteins and number of apoptotic cells were evaluated by immunohistochemistry, western blot and TUNEL method.
*Key findings:* Compared with sham, LPS treatment resulted in 4.1-fold and 1.8-fold increases in myocardial calpain activity and caspase-3 activation, respectively, and a significant increase (6.8-fold) in apoptotic cardiomyocytes was observed. The administration of calpain inhibitors (calpain inhibitor-IV, PD150606 and PD151746) showed that p-Akt and p-eNOS protein levels were correlated with the levels of LPS-induced myocardial calpain and caspase-3 activity. In addition, the quantity of p-Akt protein and NO content were markedly attenuated by wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor. Pretreatment with L-NAME, an NOS inhibitor, induced a decrease in p-eNOS proteins and apoptosis in myocardial tissues, while iNOS proteins were strongly increased in septic rats.
*Significance:* This study suggests that the Akt/eNOS/NO pathway might lead to a novel pharmacological therapy for cardiomyocytes apoptosis in sepsis.

## 1. Introduction

Myocardial dysfunction consists of biventricular systolic and diastolic dysfunction, is one of the chief properties of patients with severe sepsis, and continues to be the leading cause of mortality and morbidity in the intensive care unit [1–5]. However, the mechanism involved in myocardial dysfunction is not fully clear, and there is no effective way to prevent and treat this condition.

Calpains, a large family of calcium-dependent cysteine proteases, play a critical role in the development of myocardial dysfunction, and specific inhibition of calpain in rats with endotoxic shock attenuates circulatory failure and inflammatory factors, improving myocardial systolic and diastolic dysfunction [6–8]. Activated caspase-3 was also confirmed to be involved in septic heart dysfunction since it directly sheared the myocardial contractile proteins Troponin-T, Troponin-I and Troponin-C [7–11]. We demonstrated that gp91phox-NADPH oxidase-mediated calpain-1 activation induces caspase-3 activation and TNF-α expression in cardiomyocytes during LPS stimulation [12], and in addition, myocardial calpain induces caspase-3 activation and apoptosis through activation of the Hsp90/Akt pathway in C57BL/6 septic mice [13]. In a recent study, we reported that myocardial calpain activation promoted TNF-α expression in LPS-induced myocardial dysfunction via IκBα/NF-κB signaling [14]. This finding provides evidence that the eNOS/NO pathway may help suppress the degradation of the apoptotic protein molecule Bcl-2 and the release of cytochrome-c by mitochondria to inhibit caspase-3 activation and cell apoptosis [15–17]. These results strongly indicate that the Akt/eNOS/NO pathway might play a potential role in this pathological process.

In the present study, we observed that the inhibition of calpain reduced caspase-3 activation, NO production, IL-1β and TNF-α levels, thereby preventing apoptosis in septic rats. There was a decrease in p-Akt protein following pretreatment with wortmannin in addition to a

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decrease in p-eNOS and iNOS protein following pretreatment with L-NAME during sepsis. Taken together, the findings of the present study elucidate that inhibition of calpain activity remarkably attenuates caspase-3, NF-κB activation and apoptosis via the Akt/eNOS/NO pathway in LPS-induced septic rats.

## 2. Materials and methods

### 2.1. Animal and reagent preparation

Pathogen-free adult Sprague-Dawley rats (male, 8 weeks, 320 ± 20 g) were housed individually under a 12 h light-dark cycle with food and water available ad libitum. A total of 36 rats were randomized to receive one of the following treatments ($n = 3$). The control rats (sham) were injected intraperitoneally (i.p.) with 100 μl PBS solution, and LPS-induced rats were injected with LPS (8 mg/kg, i.p.), which was obtained from *Escherichia coli* serotype 055:B5 (Sigma, St. Louis, MO, USA) and dissolved in 100 μl PBS solution. Calpain inhibitor-IV (10 mg/kg), PD150606 (10 mg/kg), PD151746 (10 mg/kg), wortmannin (3 mg/kg) and L-NAME (10 mg/kg) plus LPS-treated rats were injected i.p., and all the inhibitors for the in vivo experiments were dissolved in DMSO according to reagent instructions. The rats were injected i.p. with either calpain inhibitors, wortmannin or L-NAME alone 30 min before injecting LPS and were subjected to experiments at 4 h posttreatment in accordance with previous experiments [12–14]. LPS and PD150606 were purchased from Sigma (St. Louis, MO, USA), calpain inhibitor-IV, PD151746, wortmannin and L-NAME were purchased from Selleck (Shanghai blue wood Chemical Co. Ltd., China). Experimental procedures were approved by the Institutional Animal Ethics Committee of Sichuan Provincial People's Hospital.

### 2.2. Calpain activity assay

Calpain activity was assessed by the fluorescence substrate Ac-LLY-AFC (Abnova, AmyJet Scientific Inc., China) when it was cleaved from a peptide substrate. AFC was quantified with a multilabel reader (excitation, 400 nm; emission, 505 nm, Flexstation3, USA), and comparison of the fluorescence intensity between calcium-dependent and calcium-independent reactions allowed the determination of changes in calpain activity. All experiments were conducted in duplicate.

### 2.3. Caspase-3 activity assay

Myocardial caspase-3 activity was measured by a caspase-3 fluorescent assay kit according to the manufacturer's instructions (NanJing Jiancheng Bioengineering Institute, China). Briefly, whole hearts were isolated from rats and homogenized. Duplicate series of protein samples were incubated with the caspase-3 substrate Ac-DEVD-pNA at 37 °C for 4 h before measurements were obtained by using a fluorescent spectrophotometer (excitation at 380 nm, emission at 405 nm, Multiskan MK3, Thermo Scientific).

### 2.4. NO concentration in blood serum and cardiac tissue

Cardiac tissue NO production was determined indirectly by measuring the concentration of the stable end products nitrate and nitrite by using a commercial kit (NanJing Jiancheng Bioengineering Institute, China). Briefly, NO from blood serum or cardiac tissue was converted to nitrite and nitrate, and nitrate was reduced to nitrite. Total nitrite was quantified by a chemiluminescence detector (Multiskan MK3, Thermo Scientific). Absorbance was detected at 550 nm.

### 2.5. Determination of TNF-α and IL-1β levels

The severity and mortality of septic shock are closely related to the levels of inflammatory some cytokines, such as TNF-α and IL-1β, which to some extent represent the severity of inflammation [17]. Therefore, TNF-α levels in blood serum and cardiac tissue were detected by ELISA following the kit instructions (Elabscience Biotechnology Co., Ltd., Wuhan, China).

### 2.6. Western blot analysis

Proteins (40 μg per sample) were subjected to SDS-PAGE using a 5% gel and subsequently electroblotted onto polyvinylidene fluoride (PVDF) membranes. The immunoblots were probed with anti-GADPH (1:1000), anti-Akt (1:2000), anti-p-Akt (1:1000), anti-eNOS (1:1000), anti-p-eNOS (1:1000) and anti-iNOS (1:1000) overnight at 4 °C and then incubated with the corresponding secondary antibodies (1:50000, Boster Biological Technology Co. Ltd., Wuhan, China) at room temperature for 1 h. Blots were developed using enhanced chemiluminescence, and quantification was performed. All of the primary antibodies were purchased from Abcam (Shanghai, China).

### 2.7. Histological preparation and immunohistochemistry

Heart tissues were fixed in a 4% paraformaldehyde-PBS solution over 24 h and subjected to standard histological procedures for paraffin-embedded sections (5 μm thickness). Sections were sliced for immunohistochemical experiments and incubated with anti-Akt, anti-p-Akt, anti-eNOS, anti-p-eNOS and anti-iNOS overnight at 4 °C. The levels of Akt, p-Akt, eNOS, p-eNOS and iNOS proteins in heart tissues were visualized by employing routine immunoperoxidase techniques. Pictures were taken and analyzed by Image Pro Plus 6.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

### 2.8. In situ detection of apoptotic cells

To evaluate the number of cells that underwent apoptosis in the myocardium, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Boster Biological Technology Co. Ltd., Wuhan, China) was performed on the myocardial sections using an in situ apoptosis detection kit (Roche Molecular Biochemicals) following the manufacturer's protocols, as previously described [12]. All of the sections were analyzed by a Leica microscope and Image Pro Plus 6.0 software.

### 2.9. Statistical analysis

All data are presented as the mean ± standard. Differences between two groups were compared by unpaired Student's *t*-test. One-way ANOVA followed by the Student-Newman-Keuls test was used for multigroup comparisons. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Myocardial calpain promotes caspase-3 activation and apoptosis in septic rats

In this project, rats were first injected with calpain inhibitors, and 30 min later, an LPS dose (8 mg/kg, i.p.) was administered to establish a model of sepsis. Administration of calpain inhibitors significantly decreased myocardial calpain (Fig. 1A) and caspase-3 activity (Fig. 1B) and reduced TUNEL-positive staining (4.3 ± 1%, 29.3 ± 7.8%, 6.0 ± 2.3%, 6.0 ± 2.3%, 8.0 ± 2.8%, 7.4 ± 3.3%, respectively, Fig. 1C) in septic rats, whereas administration of calpain inhibitor-IV, PD150606 or PD151746 alone did not affect myocardial calpain in rats.

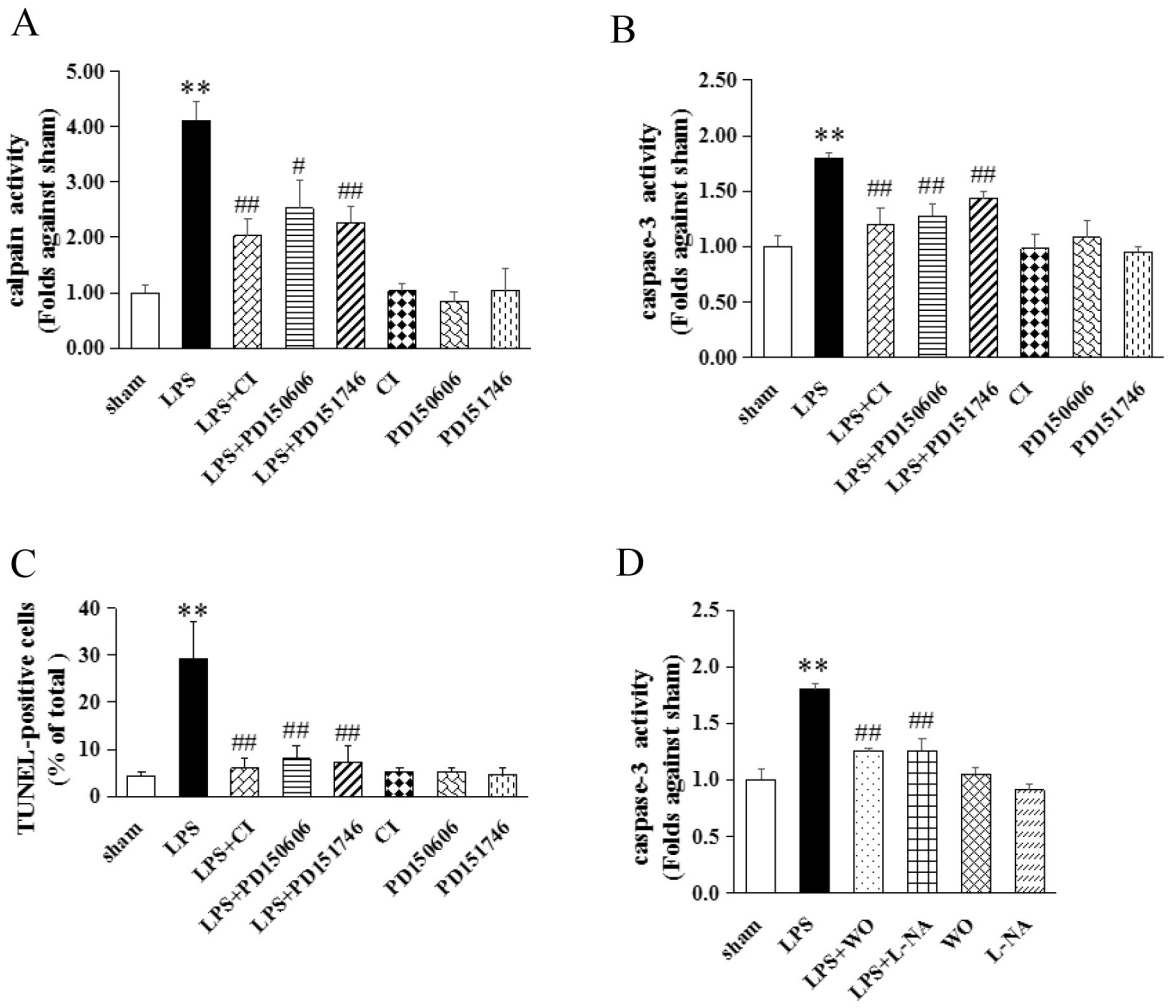


**Fig. 1.** Myocardial calpain expression, caspase-3 activity and apoptosis in septic rats. Rats were treated with LPS (8 mg/kg, i.p.) for 4 h; the myocardial tissues were then extracted, and calpain expression (A), caspase-3 activity (B and D), apoptosis (C and E) were determined among the sham and rats treated with LPS, LPS plus calpain inhibitors, and LPS plus wortmannin or L-NAME. Representative TUNEL-positive cells per field (F). The values were from manual counts of ten randomly selected high-power fields in each section. The data are shown as the mean ± SD ($n = 3$) $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. sham; $^{\#}P < 0.05$ vs. LPS, and $^{\#\#}P < 0.01$ vs. LPS.

### *3.2. Myocardial calpain promotes caspase-3 activation and apoptosis via the Akt/eNOS/NO pathway in septic rats*

The Akt/eNOS/NO pathway has attracted much attention due to its antiapoptotic effects; this pathway boosts cell survival in various conditions, including sepsis. Smith et al. demonstrated that calpain decreased p-Akt levels and inhibited the Akt pathway [18]. To ascertain whether the Akt/eNOS/NO pathway affected apoptosis, an additional four groups of rats were subjected to wortmannin or L-NAME treatment. Both caspase-3 activation (Fig. 1.D) and the number of TUNEL-positive cardiomyocytes (Fig. 1.E) in the LPS plus wortmannin (9.8 ± 2.9%) or LPS plus L-NAME (11.5 ± 3.5%) groups were significantly reduced compared to those in the LPS group (29.3 ± 7.8%). Pretreatment with wortmannin or L-NAME alone had no effect on caspase-3 activation and apoptosis (data not shown). These findings indicate that calpain activity induces caspase-3 activation and apoptosis in septic rats possibly via the Akt/eNOS/NO pathway.

### *3.3. Calpain activity attenuates p-Akt and p-eNOS proteins and enhances iNOS proteins*

To explore the specific mechanism by which calpain activity promotes caspase-3 activation and apoptosis during sepsis, immunohistological staining and western blotting were performed to identify the levels of Akt, p-Akt, eNOS, p-eNOS and iNOS proteins. As shown in Fig. 2, the mean density of the total Akt and eNOS proteins (IOD/area) did not change, but there was a significant decrease in the mean density of p-Akt protein (2B, 2F) and p-eNOS protein (2D, 2G) and an increase in iNOS protein (2E, 2H) in the LPS group compared with the sham. Moreover, there was a decrease in p-Akt, p-eNOS and iNOS protein by cotreatment with wortmannin or L-NAME, respectively, compared with treatment with LPS only. Western blot confirmed that the total Akt and eNOS protein levels did not change, while blockade of PI3K by wortmannin promoted the downregulation of p-Akt proteins (Fig. 3.A and B), and pretreatment with L-NAME decreased the level of p-eNOS and iNOS proteins (Fig. 3.C and D) compared with LPS treatment only. Meanwhile, wortmannin and L-NAME inhibited caspase-3 activity (1.25 ± 0.03, 1.26 ± 0.11, Fig. 1.D) in septic rats compared with LPS treatment only.

### *3.4. Calpain activity increases NO and TNF-α content in LPS-induced rats*

Previous experiments have shown that TNF-α *and* IL-1β induces apoptosis via iNOS expression and NO production in neonatal mouse cardiomyocytes [17]. Therefore, we proposed that calpain activity may increase NO and TNF-α production and aggravate apoptosis. Then, we

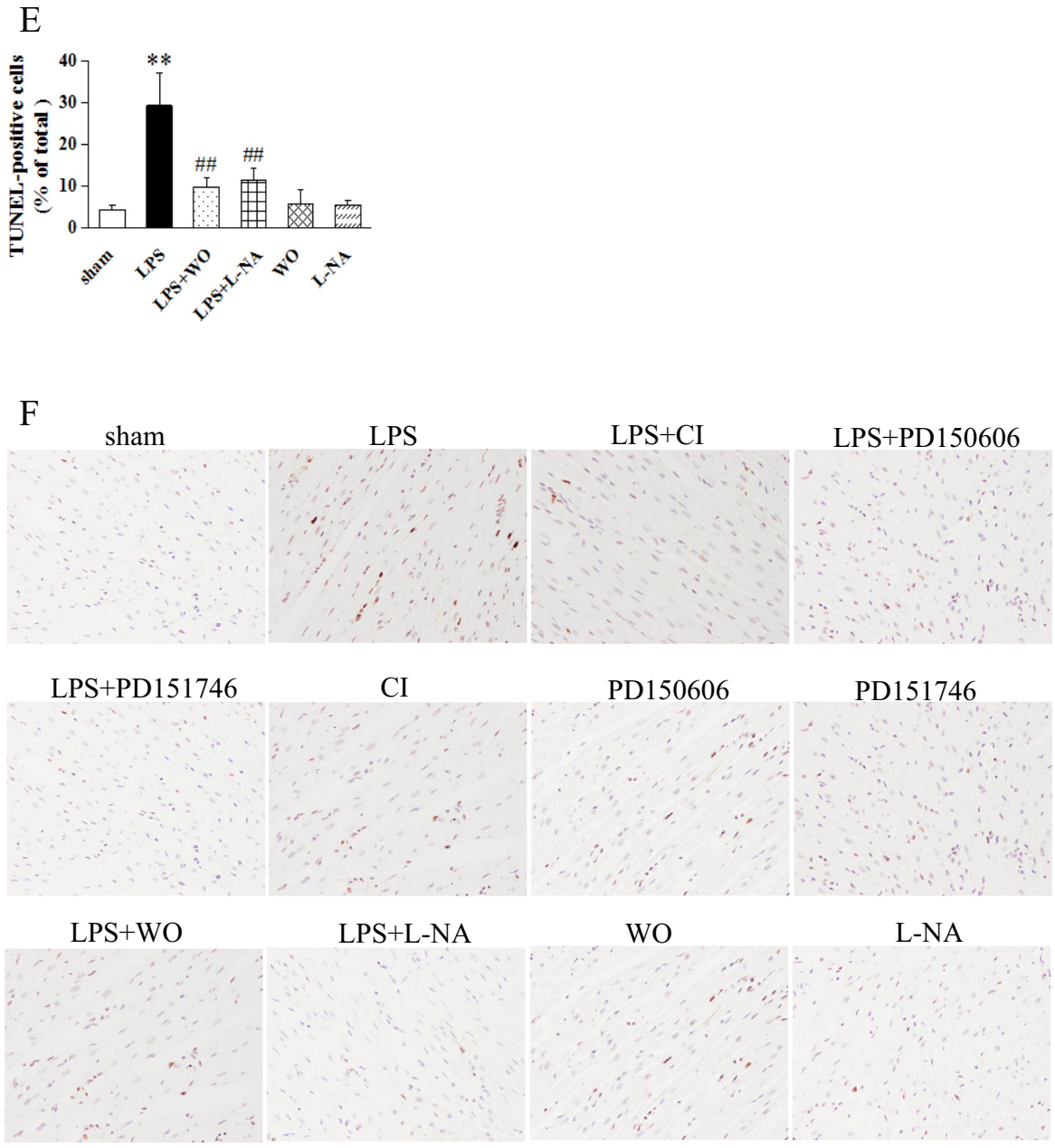


**Fig. 1.** (*continued*)

examined the levels of NO, TNF-α *and* IL-1β. As shown in Fig. 4A-B, the levels of NO, TNF-α *and* IL-1β were all significantly increased in the LPS group compared with the sham and pretreatment with calpain inhibitors strongly inhibited the generation of NO, TNF-α *and* IL-1β. Similarly, pretreatment with wortmannin or L-NAME also sharply decreased the production of NO, TNF-α *and* IL-1β. (Fig. 4A, B) compared with LPS treatment only.

## 4. Discussion

To our knowledge, this is the first study to suggest a direct link between calpain and the subsequent activation of caspase-3 and NF-κB via the Akt/eNOS/NO pathway. These findings make further efforts to explain our previous studies that LPS-induced myocardial calpain activity promoted apoptosis and cardiac dysfunction [12–14].

TNF-α was first discovered as a myocardial inhibitor in patients with sepsis-induced shock [18]. We demonstrated that increased TNF-α in cardiomyocytes directly inhibited myocardial contractile function and that inhibition of TNF-α expression in myocardium could improve cardiac dysfunction in vivo and in vitro in a model of sepsis [12]. Moreover, TNF-α production is aroused by the activation of NF-κB, and inhibition of NF-κB activation could block the expression of TNF-α in myocardium [14]. Subsequently, we demonstrated that calpain activity activates caspase-3 and NF-κB in LPS-treated cardiomyocytes/pulmonary endothelial cells and after inhibition of calpain by drug inhibitors, overexpression of the calpastatin gene in cells or mice blocked the activation of caspase-3 and NF-κB in myocardium and ameliorated the levels of TNF-α, apoptosis, and cardiac dysfunction [12–14,19]. It is suggested that the calpain-induced activation of caspase-3 and NF-κB is involved in the pathological process of sepsis-induced cardiac dysfunction. In addition, a previous study reported that TNF-α *and* IL-1β play a key role in promoting the acute inflammatory response, circulatory failure and septic shock under endotoxin stimulation [18,20].

A growing body of data indicates that endothelial NOS (eNOS) is a

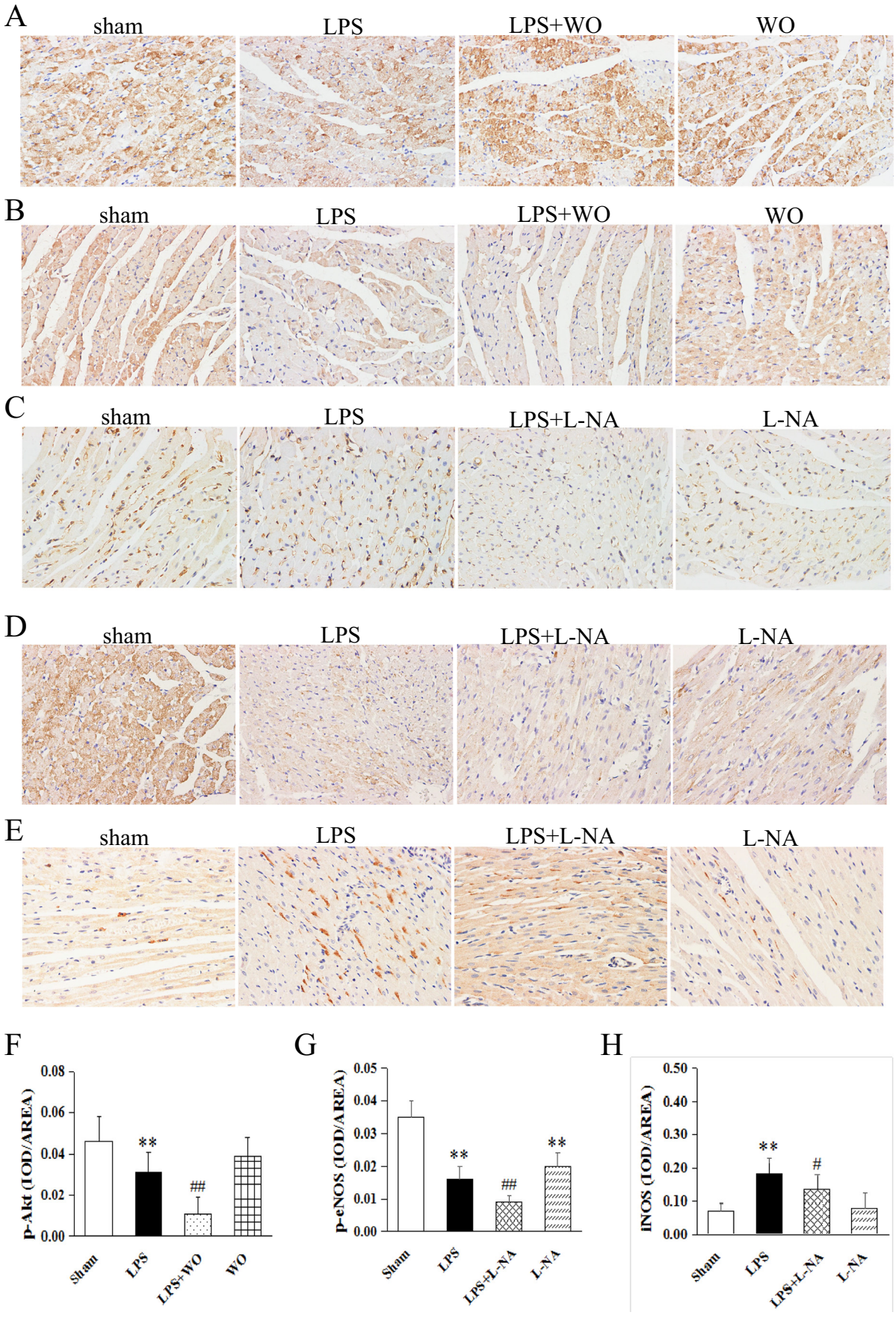


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**Fig. 2.** Immunohistological staining analysis was performed on rats that were treated with LPS (8 mg/kg), LPS plus wortmannin, LPS plus L-NAME and wortmannin or L-NAME alone. Representative immunohistochemical photomicrographs of Akt (A), p-Akt (B), eNOS (C), p-eNOS (D) and iNOS (E) staining. Mean densities (p-Akt, F; p-eNOS, G; iNOS, H) are depicted as the area divided by the IOD (integral optical density). Mean density charts reveal a significant decrease in p-Akt (F) and p-eNOS (G) and a significant increase in iNOS (H) in the cytoplasm of the myocytes treated with LPS. The values were from manual counts of ten randomly selected high-power fields in each section. The data are shown as the mean ± SD ($n$ = 3). $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$ vs. LPS, $^{\#\#}P < 0.01$ vs. LPS.

rate-limiting enzyme for the synthesis of nitric oxide (NO), its downstream effector molecule. High pathological concentrations of NO produced from inducible NO synthase (iNOS) induce apoptosis, whereas a reduction in the concentrations of NO by eNOS attenuates apoptosis [21,22]. Further research has shown that TNF-α induces apoptosis via concentration-dependent iNOS expression and NO production in neonatal mouse cardiomyocytes [23].

Several recent studies have demonstrated that hormones such as estrogen cause eNOS phosphorylation and result in eNOS release through the PI3K/Akt-dependent pathway [24]. Dimmeler showed that eNOS is continuously expressed in cardiomyocytes and is activated by calcium, calmodulin or Akt [25–27]. Akt is downstream of PI3K and is phosphorylated and activated by a number of growth factors, cytokines and hormones. Furthermore, we found that calpain decreases Akt activity by shearing the heat shock protein Hsp90 in septic rats/mice [13,28]. However, the role of Akt in sepsis is debatable. Many researches indicated that PI3K/Akt pathway was activated in the sepsis and cardiac dysfunction [29,30,31]. The results of the Akt inhibitor wortmanner in septic rat were inconsistent with the inhibitors alone in mice as we once reported [13] and were not as we expected, suggesting the existence of compensatory mechanisms or other signaling pathways. The involved mechanism need to be further explored. A recent study also demonstrated that eNOS/NO depressed inflammation and inflammatory factors (TNF-α, COX-2, IL-1, etc.) [32,33]. The decrease in eNOS in the vascular endothelium is often accompanied by an increase in TNF-α in sepsis mice [34], but there have been inconsistent reports [35].

Considering the importance of the Akt/eNOS/NO pathway, in particular, the activation of Akt and eNOS, we specifically investigated the relationship among p-Akt/Akt, p-eNOS/eNOS and iNOS in myocardial tissue under treatment with LPS, LPS plus wortmannin or LPS plus L-NAME. We observed that a decrease in p-Akt protein by pretreatment with wortmannin in addition to a decrease in p-eNOS and iNOS protein by pretreatment with L-NAME in septic rats. In addition, NO production

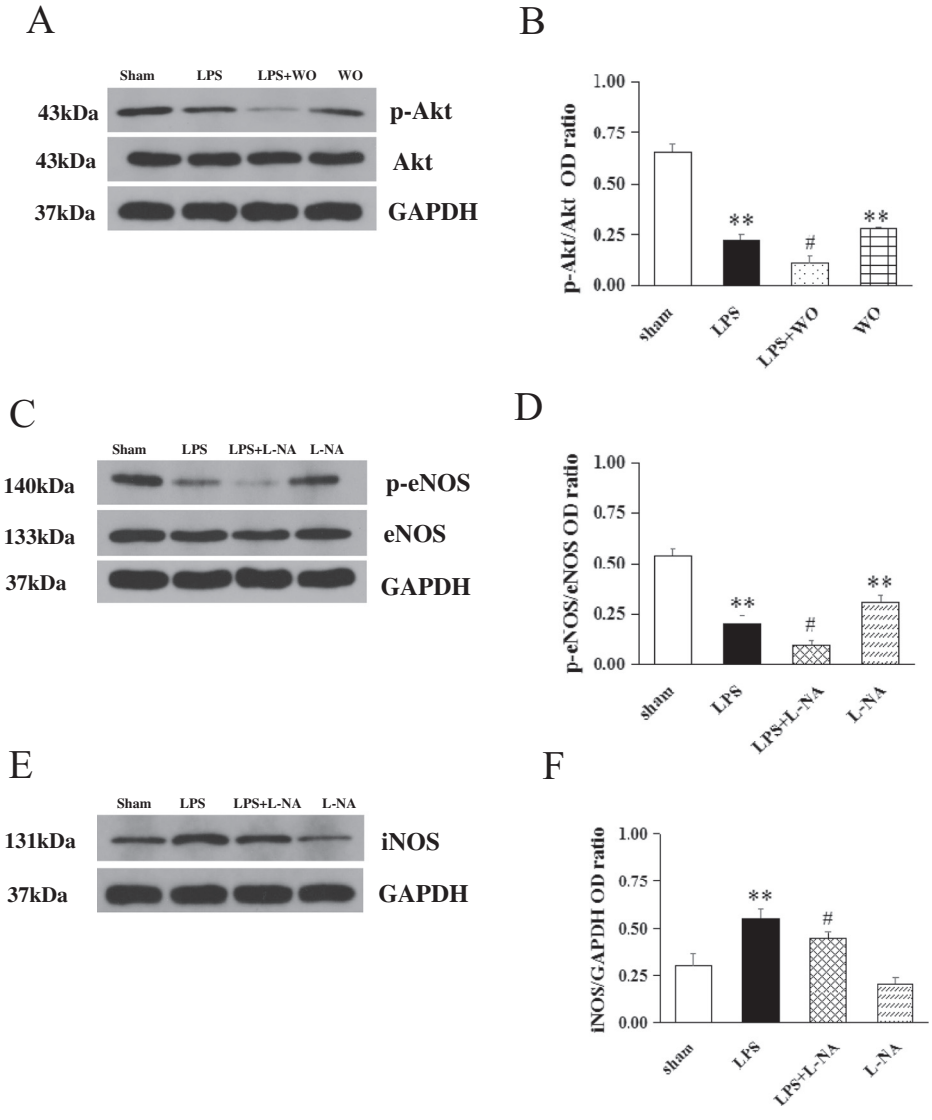


**Fig. 3.** Western blot was performed on rats treated with LPS (8 mg/kg), LPS plus wortmannin, LPS plus L-NAME or wortmannin and L-NAME alone. A, Ratio of p-Akt/Akt. C, Ratio of p-eNOS/eNOS. E, Ratio of iNOS/GAPDH. Densities of the bands were calculated (B and D and F). In short, LPS decreased the expression of p-Akt and p-eNOS but had no effect on total Akt and eNOS. Moreover, in both sham and LPS-induced rats, pretreatment with wortmannin suppressed p-Akt, and pretreatment with L-NAME downregulated p-eNOS and iNOS proteins. The data are shown as the mean ± SD (n = 3). $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. Sham; $^{\#}P < 0.05$ vs. LPS, $^{\#\#}P < 0.01$ vs. LPS.

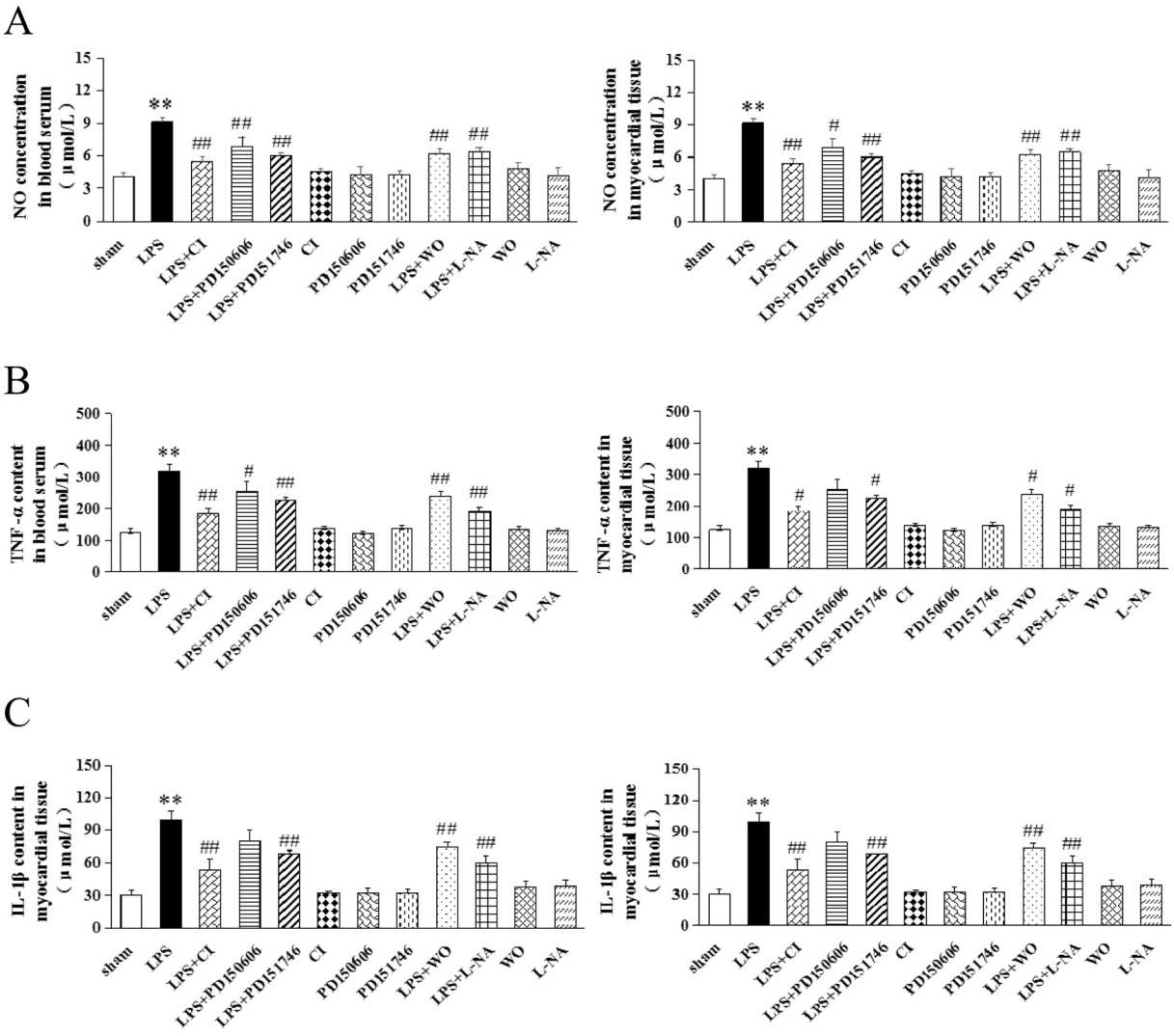


**Fig. 4.** NO,TNF-α and IL-1β production in blood serum and heart tissue. Calpain inhibitors reduced NO, TNF-α and IL-1β levels compared with LPS treatment only (A, B, C). Pretreatment with wortmannin or L-NAME also decreased NO, TNF-α and IL-1β levels (A,B,C). The data is shown as the mean ± SD (n = 3). $^{*}P < 0.05$ vs. sham, $^{**}P < 0.01$ vs. sham; $^{\#}P < 0.05$ vs. LPS, and $^{\#\#}P < 0.01$ vs. LPS.

is dependent on iNOS levels in septic rats, the result is consistent with a previous report [21,22]. Therefore, these findings support the view that calpain activates NF-κB and further excites TNF-α and leads to cardiac dysfunction in sepsis.

In summary, we first found that calpain regulates myocardial caspase-3 and NF-κB activation by adjusting the Akt/eNOS/NO pathway. Activated calpain induces caspase-3 activation and TNF-α expression and further induces apoptosis via Akt/eNOs/NO pathway, as indicated by a decrease in myocardial p-eNOS protein, which induces caspase-3 activity and apoptosis during sepsis. Therefore, the Akt/eNOS/NO pathway may represent a promising approach for the treatment of LPS-induced myocardial dysfunction.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contributions

Rong Luo and Xuepin Chen carried out Western Blot and IHC, participated in the data analysis and drafted the manuscript. Huihui Ma and Chao Yao carried out molecular experiment. Mingjiang Liu and Jianhong Tao conceived of the study, and participated in its design and data analysis. Xiaoping Li participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

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