Over-expression of calpastatin inhibits calpain activation and attenuates myocardial dysfunction during endotoxaemia

Xiaoping Li1,2,3†, Ying Li2,4†, Limei Shan2,4, E Shen2,4, Ruizhen Chen1, and Tianqing Peng2,3,4*

1 Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai, China; 2 Critical Illness Research, Lawson Health Research Institute, London Health Sciences Center, VRL 6th Floor, A6-140, 800 Commissioners Road, Ontario, Canada N6A 4G5; 3 Departments of Pathology, University of Western Ontario, London, Ontario, Canada; and 4 Departments of Medicine, University of Western Ontario, London, Ontario, Canada

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Aims Lipopolysaccharide (LPS) induces cardiomyocyte caspase-3 activation and proinflammatory factors, in particular tumour necrosis factor-alpha (TNF-α) production, both of which contribute to myocardial dysfunction during sepsis. The present study was to investigate the roles of calpain/calpastatin system in cardiomyocyte caspase-3 activation, TNF-α expression, and myocardial dysfunction during LPS stimulation.

Methods and results In cultured adult rat cardiomyocytes, LPS (1 μg/mL) induced calpain and caspase-3 activity, and up-regulated TNF-α expression. These effects of LPS were abrogated by over-expression of calpastatin, an endogenous calpain inhibitor, transfection of calpain-1 siRNA, or various pharmacological calpain inhibitors. Furthermore, blocking gp91phox-NADPH oxidase prevented calpain and caspase-3 activation and decreased TNF-α expression in LPS-stimulated cardiomyocytes. To investigate the role of calpastatin in endotoxaemia, transgenic mice with calpastatin over-expression (CAST-Tg) and wild-type mice were treated with LPS (4 mg/kg, i.p.) or saline in the presence of calpain inhibitor-III (10 mg/kg, i.p.) for 4 h, and their heart function was measured with a Langendorff system. Over-expression of calpastatin significantly attenuated myocardial dysfunction (P < 0.05). Consistently, calpain activity, caspase-3 activity, and TNF-α expression were also reduced in CAST-Tg and calpain inhibitor-III compared with wild-type and vehicle-treated hearts, respectively.

Conclusion gp91phox-NADPH oxidase-mediated calpain-1 activation induces caspase-3 activation and TNF-α expression in cardiomyocytes during LPS stimulation. Over-expression of calpastatin inhibits calpain activation and improves myocardial function in endotoxaemia. The present study suggests that targeting calpain/calpastatin system may be a potential therapeutic intervention for septic hearts.

KEYWORDS Calpastatin; Calpain; Sepsis; TNF-alpha; Caspase-3; Heart; Myocardial dysfunction

1. Introduction

Sepsis and subsequent multiple organ failure remain the major cause of morbidity and mortality in intensive care units. It occurs in 2–11% of all hospital or intensive care unit admissions.⁴ Sepsis-induced myocardial dysfunction is a key manifestation contributing to morbidity and mortality among patients in intensive care units, and 40–50% of patients with prolonged septic shock develop myocardial depression.³ Estimates of mortality due to sepsis range from 20 to 30%, however, mortality increases to 70–90% when there is accompanying myocardial dysfunction.⁴ Thus, myocardial dysfunction is often decisive in determining survival or death in sepsis. Lipopolysaccharide (LPS) of Gram-negative bacteria is an important pathogen responsible for myocardial dysfunction during sepsis through multi-factorial mechanisms.⁵-⁶ LPS induces multiple proinflammatory factors, in particular tumour necrosis factor-alpha (TNF-α), have been shown to play a critical role in the development of myocardial dysfunction in animal models of sepsis.⁵-⁷ LPS also induces cardiac caspase-3 activation, which significantly contributes to myocardial dysfunction in sepsis.⁸-¹⁰ However, the precise mechanisms by which sepsis induces cardiac TNF-α expression and caspases-3 activation, and myocardial dysfunction remain not fully understood and there is no cure available to correct this life-threatening condition.

Calpains belong to a family of calcium-dependent thiol-proteases.¹²,¹³ Fifteen gene products of the calpain family...
are reported in mammals. Among them, calpain-1 (μ-form) and calpain-2 (m-form) are ubiquitously expressed, and other calpain family members have more limited tissue distribution. Both calpain-1 and calpain-2 are specifically countered by the endogenous calpain inhibitor, calpastatin. Calpains are implicated in a wide variety of biological functions including caspases activation/apoptosis and acute proinflammatory process. Studies have shown that calpains are implicated in ischaemia/reperfusion-induced apoptosis in the heart.14–16 It was also shown that over-expression of calpastatin prevented troponin I degradation and improved contractile function in rat hearts subjected to ischaemia/reperfusion.17 These studies suggest that calpain activation may contribute to the progression of heart failure. This was indeed supported by a recent study which demonstrated that cardiac over-expression of calpain-1 is sufficient to cause heart failure in transgenic mice.18 In septic conditions, previous studies showed that treatment with pharmacological calpain inhibitors attenuated myocardial dysfunction in a rat model of endotoxaemia19 and reduced organs injury (kidney, liver, pancreas, lung, and intestine) and dysfunction (kidney) in haemorrhagic shock.20 However, such inhibitors usually lack specificity among cysteine proteases and other proteolytic enzymes.21 Besides, the contribution of calpains to cardiac caspase-3 activation and TNF-α expression has not been shown and whether up-regulation of calpastatin provides a beneficial effect on myocardial function remains to be determined in sepsis.

Therefore, the present study used cultured adult cardiomyocytes and an in vivo model of endotoxaemia to investigate the role of calpain/calpastatin system in cardiomyocyte TNF-α expression, caspase-3 activation, and myocardial dysfunction. We demonstrated that over-expression of calpastatin prevented cardiac caspase-3 activation and TNF-α expression in cardiomyocytes during LPS stimulation, and improved myocardial function in transgenic mice during endotoxaemia. These actions of calpastatin were mediated through the inhibition of calpain activation.

2. Methods

2.1 Animals and adult rat cardiomyocytes culture

This investigation confirms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23). All experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. Breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory and transgenic mice with over-expression of calpastatin (CAST-Tg) were kindly provided by Dr Laurent Baud (the Institut National de la Sante et de la Recherche Medicale, Paris, France)22 through the European Mouse Mutant Archive. A breeding program was implemented at our animal care facilities. Adult male rats (Sprague Dawley, 200 g body weight) were purchased from Charles River Laboratories. Adult rat ventricle cardiomyocytes were isolated and cultured as described in our recent study.23

2.2 Drugs

LPS, calpain inhibitor-III, and PD150606 were purchased from Sigma or Calbiochem. Peptides gp91ds-tat and scramble-tat were synthesized by Prolimmune Ltd. (UK).

2.3 Adenoviral infection of cultured adult rat cardiomyocytes

Cardiomyocytes were infected with adenoviral vectors containing rat calpastatin gene (Ad-CAST, University of Buffalo, USA) or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection of 10 PFU/cell. Adenovirus-mediated gene transfer was implemented as previously described.23 All experiments were performed after 24 h of adenoviral infection.

2.4 Calpain-1 knockdown using small interfering RNA

In order to knock down calpain-1 or calpain-2 expression, a small interfering RNA (siRNA) against rat calpain-1 or calpain-2 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a scramble siRNA was employed as control. Transfection was performed using TransMessenger Transfection Reagent (Qiagen) according to manufacturer’s protocol as described in our previous studies.24

2.5 Calpain activity

Calpain activity was determined by using a fluorescence substrate N-succinyl-LLVY-AMC (Cedarlane Laboratories) as described in our recent study.23 The calpain inhibitor PD150606 was used to determine the specificity of the assay.

2.6 Active caspase-3

As described in detail previously,23 caspase-3 activity in myocardial tissues and cardiomyocytes was measured by using a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories).

2.7 Analysis of tumour necrosis factor-alpha mRNA by real-time reverse transcriptase–polymerase chain reaction

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Gibco-BRL) following the manufacturer’s instructions. Real-time RT–PCR for mouse TNF-α mRNA was performed using the same primers as previously described.23 The primers for rat TNF-α mRNA were as follows: 5’-GTCCCTCTACATGTTCCA-3’ (forward) and 5’-TGCTGGGTGAGGAGCACATAG-3’ (reverse).

2.8 Western blot analysis

Expressions of active caspase-3, calpain-1, calpain-2, and glyceralddehyde 3 phosphate dehydrogenase (GAPDH) protein were determined by western blot analysis using specific antibodies against the relevant corresponding proteins, respectively.23

2.9 Endotoxaemia and isolated mouse heart preparations

Adult mice (male, aged 2-3 months) were intraperitoneally (i.p.) injected with LPS (4 mg/kg) to induce endotoxaemia. After 4 h of LPS treatment, mouse hearts were isolated and perfused in a Langendorff system to measure cardiac function as we previously described.25

2.10 Statistical analysis

All data were given as mean ± SD. Differences between two groups were compared by unpaired Student’s t-test. For multi-group comparisons, ANOVA followed by Newman-Keuls test was performed. A value of P < 0.05 was considered statistically significant.
3. Results

3.1 Calpain activation in cardiomyocytes during lipopolysaccharide stimulation

To determine the effect of LPS on calpain activation, we incubated cultured adult rat cardiomyocytes with LPS (1 μg/mL) or saline. Four hours later, calpain activity and the protein levels of calpain-1 and calpain-2 were measured. LPS increased calpain activity by 78% (Figure 1A). The up-regulation of calpain activity was not due to an increased availability of ubiquitous calpains since LPS treatment did not alter the protein levels of calpain-1 and calpain-2 in cardiomyocytes (Figure 1B).

To clarify the isoform-specific activation of calpain in LPS-stimulated cardiomyocytes, we transfected cardiomyocytes with calpain-1 siRNA, calpain-2 siRNA, or scramble siRNA as control for 24 h, followed by LPS or saline for 4 h. (A) Calpain activity is increased in LPS-stimulated cardiomyocytes. (B) A representative western blot from three different experiments for specific calpain-1 (C1) and calpain-2 knockdown (C2) by siRNA in cardiomyocytes. (D) Calpain activity in siRNA-transfected cardiomyocytes during LPS stimulation. Data are mean ± SD from at least three different cell cultures. *P < 0.05 vs. saline; †P < 0.05 vs. scramble siRNA in LPS.

Figure 1 Calpain activation and expression in LPS-stimulated cardiomyocytes. Cultured adult rat cardiomyocytes were transfected with calpain-1 siRNA, calpain-2 siRNA, or scramble siRNA for 24 h and followed by LPS or saline for 4 h. (A) Calpain activity is increased in LPS-stimulated cardiomyocytes. (B) A representative western blot from three different experiments for calpain-1, calpain-2, and GAPDH protein expression in LPS-stimulated cardiomyocytes. (C) A representative western blot from three different experiments for specific calpain-1 (C1) and calpain-2 knockdown (C2) by siRNA in cardiomyocytes. (D) Calpain activity in siRNA-transfected cardiomyocytes during LPS stimulation. Data are mean ± SD from at least three different cell cultures. *P < 0.05 vs. saline; †P < 0.05 vs. scramble siRNA in LPS.

3.2 Effects of calpain inhibitors in lipopolysaccharide-induced caspase-3 activation and tumour necrosis factor-alpha expression

LPS has been shown to induce caspase-3 activation and TNF-α expression in cultured adult cardiomyocytes.26,27 To investigate the role of calpain in caspase-3 activation and TNF-α expression, we incubated cultured adult rat cardiomyocytes with LPS (1 μg/mL) or saline in the presence of calpain inhibitor-III (CI-III), PD150606, or vehicle for 4 h. (A) Caspase-3 activity. Incubation with CI-III or PD150606 decreases caspase-3 activity in LPS-induced cardiomyocytes. (B) A representative western blot from three different experiments shows that LPS increases the levels of large fragment subunits (17/19 kDa), which are reduced by CI-III or PD150606 in cardiomyocytes. (C) CI-III or PD150606 down-regulates TNF-α mRNA expression induced by LPS in cardiomyocytes. (D and E) Cardiomyocytes were transfected with calpain-1 siRNA or scramble siRNA and followed by incubation with LPS or saline for 4 h. Transfection with calpain-1 siRNA decreases caspase-3 activity (D) and down-regulates TNF-α mRNA (E) in LPS-stimulated cardiomyocytes. Data are mean ± SD from at least three different cell cultures. *P < 0.05 vs. saline; †P < 0.05 vs. vehicle in LPS or scramble siRNA in LPS.

Figure 2 Effects of calpain inhibition on caspase-3 activation and TNF-α expression. Cultured adult rat cardiomyocytes were incubated with LPS or saline in the presence of calpain inhibitor-III (CI-III), PD150606, or vehicle for 4 h. (A) Caspase-3 activity. Incubation with CI-III or PD150606 decreases caspase-3 activity in LPS-induced cardiomyocytes. (B) A representative western blot from three different experiments shows that LPS increases the levels of large fragment subunits (17/19 kDa), which are reduced by CI-III or PD150606 in cardiomyocytes. (C) CI-III or PD150606 down-regulates TNF-α mRNA expression induced by LPS in cardiomyocytes. (D and E) Cardiomyocytes were transfected with calpain-1 siRNA or scramble siRNA and followed by incubation with LPS or saline for 4 h. Transfection with calpain-1 siRNA decreases caspase-3 activity (D) and down-regulates TNF-α mRNA (E) in LPS-stimulated cardiomyocytes. Data are mean ± SD from at least three different cell cultures. *P < 0.05 vs. saline; †P < 0.05 vs. vehicle in LPS or scramble siRNA in LPS.
treatment with calpain inhibitor-III or PD150606 significantly reduced TNF-α mRNA levels in LPS-stimulated cardiomyocytes (Figure 2C). Thus, calpain activation contributes to both caspase-3 activation and TNF-α mRNA expression in LPS-stimulated cardiomyocytes.

3.3 Effects of calpain-1 small interfering RNA on calpain activation, caspase-3 activation, and tumour necrosis factor-alpha expression

To substantiate the results from calpain inhibitors and clarify the role of calpain-1, cardiomyocytes were transfected with calpain-1 siRNA or scramble siRNA for 24 h and followed by LPS (1 μg/mL) for another 4 h. Caspase-3 and TNF-α mRNA were measured. Transfection with calpain-1 siRNA significantly decreased LPS-induced caspase-3 activity (Figure 2D) and reduced TNF-α mRNA levels compared with scramble siRNA in cardiomyocytes (Figure 2E). These results further support the fact that calpain-1 activation induces caspase-3 activation and TNF-α expression in cardiomyocytes during LPS stimulation.

3.4 Role of calpastatin in calpain activity, caspase-3 activity, and tumour necrosis factor-alpha expression

Calpastatin is a highly specific inhibitor of calpain-1 and calpain-2 that does not inhibit the activities of any other protease tested.12,13 Thus, calpastatin expression is an effective means of globally inhibiting cellular calpain-1 and calpain-2 activities. To investigate the role of calpastatin in calpain activity, caspase-3 activity, and TNF-α expression, we over-expressed calpastatin in cardiomyocytes by using Ad-CAST. Cultured adult rat cardiomyocytes were infected with Ad-CAST or Ad-gal as control for 24 h, and then exposed to LPS (1 μg/mL) or saline for 4 h. The effect of Ad-CAST infection on calpastatin up-regulation in cardiomyocytes was verified in our recent study.12,13 Calpain activity, caspase-3 activity, and TNF-α mRNA were not altered by calpastatin over-expression in saline-treated cardiomyocytes. In LPS-stimulated cardiomyocytes, over-expression of calpastatin significantly inhibited calpain activity, decreased caspase-3 activation and reduced TNF-α mRNA levels (Figure 3A–C). Thus, calpastatin over-expression induces calpain inhibition, which in turn prevents caspase-3 activation and TNF-α expression in LPS-stimulated cardiomyocytes.

3.5 Role of gp91phox-NADPH oxidase in calpain activation, caspase-3 activity, and tumour necrosis factor-alpha expression

Our recent study showed that blocking NADPH oxidase inhibited norepinephrine-stimulated calpain activation in cardiomyocytes.23 We also found that deficiency of gp91phox or incubation with NADPH oxidase inhibitors abrogated TNF-α expression in LPS-stimulated cardiomyocytes.23 We therefore hypothesized that gp91phox-NADPH oxidase mediates LPS-induced calpain activation, leading to caspase-3 activation and TNF-α expression in cardiomyocytes. Cardiomyocytes were exposed to LPS (1 μg/mL) or saline in combination with gp91ds-tat (2.5 μM), a peptide which specifically blocks NADPH oxidase assembly and thus inhibits its activation,28 or scramble-tat as control for 4 h.

Co-incubation with gp91ds-tat significantly decreased calpain activity, caspase-3 activity, and TNF-α mRNA in LPS-induced cardiomyocytes compared with scramble-tat (Figure 4). These results suggest that gp91phox-NADPH oxidase also induces calpain activation, which contributes to caspase-3 and TNF-α expression in cardiomyocytes in response to LPS.

3.6 In vivo effects of calpain inhibitor or calpastatin over-expression on caspase-3 and tumour necrosis factor-alpha expression in endotoxaemic hearts

To investigate the in vivo significance of calpain/calpastatin system, CAST-Tg and wild-type mice (male, aged 2–3 months) were given saline or LPS (4 mg/kg, i.p.) in the presence of calpain inhibitor-III (10 mg/kg, i.p.) or vehicle. Four hours later, heart tissues were collected and assayed for calpain activity, caspase-3 activity, TNF-α, and calpastatin mRNA expression. In agreement with a previous report which showed that calpastatin was up-regulated in CAST-Tg hearts,29 transgenic calpastatin mRNA (rabbit calpastatin mRNA) was present in the heart of CAST-Tg but not wild-type mice (Figure 5A), further confirming expression of exogenous calpastatin in CAST-Tg hearts. LPS significantly increased calpain activity, caspase-3 activity, the levels of TNF-α mRNA in both CAST-Tg and wild-type
hearts. However, the levels of calpain activity, caspase-3 activity, and TNF-α mRNA were significantly reduced in CAST-Tg compared with wild-type mice during LPS stimulation (Figure 5B–D). These results suggest that over-expression of calpastatin also inhibits calpain activation, caspase-3 activation, and TNF-α expression in in vivo heart during LPS stimulation. Consistently, administration of calpain inhibitor-III inhibited calpain activation, decreased caspase-3 activity, and down-regulated TNF-α mRNA expression in LPS-stimulated hearts (Figure 5E–G).

3.7 Myocardial dysfunction in wild-type and transgenic mice with calpastatin over-expression during endotoxaemia

To explore the role of calpastatin in myocardial dysfunction during sepsis, CAST-Tg and wild-type mice (male, aged 2–3 months) were injected with LPS (4 mg/kg, i.p.) or saline. Four hours later, cardiac function was assessed in isolated hearts to avoid systemic reflex influences. The heart work was calculated by multiplying the force (g) by the heart rate (b.p.m.). Maximal and minimal first derivatives of force ($+dF/dt_{\text{max}}$ and $-dF/dt_{\text{min}}$) as the rate of contraction and relaxation were analysed. Under physiological condition, there were no differences in heart rate, heart work, and rate of contraction and relaxation between CAST-Tg and wild-type mice. Although there was no change in heart rate, heart work and rate of contraction were significantly reduced in endotoxaemic mice compared with sham animals (Figure 6), indicating myocardial depression. Over-expression of calpastatin significantly increased heart work and rate of contraction and relaxation without affecting heart rate in endotoxaemic CAST-Tg mice compared with wild-type mice (Figure 6). These data demonstrate that over-expression of calpastatin attenuates myocardial dysfunction in endotoxaemia.

4. Discussion

The present study used cultured adult cardiomyocytes and an in vivo model of endotoxaemia to investigate the role of calpastatin in LPS-stimulated calpain activation and myocardial dysfunction. The major findings were as follows. First, gp91phox/NADPH oxidase mediated calpain-1 activation in cardiomyocytes during LPS stimulation, which was inhibited by over-expression of calpastatin. Secondly, over-expression of calpastatin or treatment with calpain inhibitors prevented caspase-3 activation and down-regulated TNF-α expression in cardiomyocytes during LPS stimulation. Finally, cardiac function was restored in endotoxaemia in CAST-Tg compared with wild-type mice. These results suggest that over-expression of calpastatin inhibits caspase-3 activation and TNF-α expression in LPS-stimulated cardiomyocytes and improves cardiac function in
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endotoxaemia. These effects of calpastatin are mediated by the inhibition of calpain.

4.1 Calpain/calpastatin and septic myocardial dysfunction

Calpastatin over-expression has been recently shown to prevent cardiac hypertrophy and vascular remodelling including media hypertrophy, perivascular inflammation, and fibrosis in angiotensin II-induced hypertension. In the present study, we found that after 4 h of LPS injection, wild-type mice developed more severe myocardial dysfunction than CAST-Tg mice. This result suggests a protective effect of calpastatin over-expression on myocardial function in endotoxaemia. We further showed that over-expression of calpastatin blocked calpain activation in LPS-stimulated cardiomyocytes and in vivo endotoxaemic hearts. These results suggest that calpain may be involved in mechanisms underlying septic myocardial dysfunction. Indeed, a previous study has demonstrated that early treatment with calpain inhibitors attenuated myocardial dysfunction induced by endotoxin in rats. Although over-expression of calpastatin inhibits LPS-induced calpain activity, it does not alter basal calpain activity in the heart. This has also been shown in other models. Furthermore, structural and biochemical data indicate that calpastatin might bind preferentially to calcium-activated calpains, suggesting that calpastatin controls calpain activity only under stimulated conditions. Thus, therapeutic strategies focusing on up-regulation of calpastatin may have significant potential for septic myocardial dysfunction, because these appear to limit calpain activation without interfering with basal calpain activity, which is required for normal cardiac protein homeostasis.

Mechanisms by which calpastatin-mediated calpain inhibition restores myocardial function are not fully understood. Studies have suggested that elevated concentrations of proinflammatory cytokines, in particular TNF-α, are major factors that induce myocardial dysfunction in sepsis. It has also been suggested that caspase-3 activation directly contributes to myocardial dysfunction during endotoxaemia. Data from the present study suggest that the inhibition of caspase-3 activation and down-regulation of TNF-α expression may be important mechanisms by which over-expression of calpastatin improves myocardial function in endotoxaemia.

Calpain activation has also been suggested to induce degradation of SERCA2a and impairment of Na⁺/K⁺-ATPase activity, leading to Ca²⁺ deregulation, which occurs in cardiomyocytes during LPS stimulation. In addition, calpain also cleaves myofibrillar proteins such as troponin T, tropinin I, titin, and desmin in cardiomyocytes. Both Ca²⁺ deregulation and degradation of myofibrillar proteins contribute to myocardial dysfunction. Thus, the effect of calpastatin over-expression on septic myocardial dysfunction also may result from the prevention of the cleavages of myofibrillar proteins and Ca²⁺ regulation related proteins by calpain activation. However, further work is needed to clarify whether these mechanisms operate in the improvement of myocardial function induced by over-expression of calpastatin in endotoxaemia.

4.2 Calpain/calpastatin in caspase-3 activation during lipopolysaccharide stimulation

In cultured adult cardiomyocytes, LPS activates caspase-3. This was also shown by the present study. In vivo studies have confirmed that caspase-3 activation is significantly increased in the heart in animal models of sepsis, which is associated with cytochrome c release from mitochondria in sepsis. More importantly, blocking caspase-3 activation by targeting mitochondria-dependent apoptotic pathway, for example over-expression of Bcl-2, or administration of a selective caspase-3 inhibitor significantly attenuates myocardial dysfunction and improves the survival rate in sepsis. These results suggest that caspase-3 activation is an important mechanism in mediating myocardial dysfunction in sepsis. However, the mechanisms by which LPS induces caspase-3 activation remain not fully defined in cardiomyocytes. Studies have suggested that calpain is an important player in cell death signalling. Partial cleavage of pro- or anti-apoptotic proteins by calpain might activate or inactivate, respectively, putative substrates including caspase-3, -7, -8, -9, and -12, Bcl-2, Bcl-xl, Bax, etc. In the present study, calpain was activated in both cultured cardiomyocytes and in vivo hearts in response to LPS stimulation. Over-expression of calpastatin, knockdown of calpain-1, or pharmacological calpain inhibitors prevented cardiac caspase-3 activation during endotoxaemia. These results suggest that calpain is involved in mechanisms underlying caspase-3 activation and thus targeting calpain/calpastatin system may represent a novel therapeutic intervention in sepsis.

4.3 Calpain/calpastatin in tumour necrosis factor-alpha expression induced by lipopolysaccharide

Studies have demonstrated that TNF-α impairs cardiac contractile function in intact animals, isolated hearts, and cardiomyocytes. Thus, treatment with TNF-α antibody
and TNF-α binding proteins abrogates LPS-induced myocardial depression in endotoxemia.\textsuperscript{9,42} These studies support an important role of TNF-α bioactivity in septic myocardial dysfunction. Cardiomycocytes synthesize TNF-α in response to LPS, which may be a major local source of TNF-α in the heart. However, regulation of cardiac TNF-α expression remains not fully understood during LPS stimulation. NF-κB activation is an important mechanism for TNF-α mRNA transcription.\textsuperscript{43} NF-κB exists in the cytosol as a pre-formed trimeric complex. The p50/p65 protein dimer is associated with an inhibitory protein known as IκB. IκB is regulated through phosphorylation by IκB kinases (IKKα and β). Phosphorylated IκB is dislodged from the p50/p65 heterodimer. NF-κB can then migrate to the nucleus and bind to the promoter of TNF-α, thereby initiating its transcription. Calpains have been shown to degrade the inhibitor IκB\textsuperscript{44} and thus induce the nuclear translocation of NF-κB. In this regard, the present study showed that calpastatin inhibition of calpain down-regulated cardiac TNF-α mRNA expression during LPS stimulation. Inhibition of calpain has also been suggested to down-regulate other proinflammatory factors in endotoxemic hearts.\textsuperscript{19} Thus, our data further support an important role of calpain in acute proinflammatory process in the heart.

**4.4 NADPH oxidase mediates lipopolysaccharide-induced calpain activation in cardiomyocytes**

The regulation of calpain activation has not been fully understood. Calpain activity is mainly regulated by altering the Ca\textsuperscript{2+} concentration required for its proteolytic activity. Calpain activity can also be regulated by certain phospholipids, ERK1/2 MAPK, PKC, and PKA.\textsuperscript{12,13} In cardiomyocytes, it remains unknown whether LPS can induce calpain activation. Studies have suggested that LPS induces the [Ca\textsuperscript{2+}]i elevations.\textsuperscript{45} Increase in intracellular Ca\textsuperscript{2+} concentration is an important mechanism for calpain activation. In this regard, the present study demonstrated that LPS increased calpain activity in cardiomyocytes. We further showed that this increase in calpain activity was most likely attributable to calpain-1 activation since knockdown of calpain-1 but not calpain-2 abolished calpain activity induced by LPS. Thus, LPS treatment induces calpain-1 activation in cardiomyocytes.

We have recently demonstrated that LPS activates NADPH oxidase in cardiomyocytes.\textsuperscript{25} The present study further showed that blocking NADPH oxidase abrogated LPS-induced calpain activation. This suggests that LPS induces calpain activation through NADPH oxidase-dependent pathway in cardiomyocytes. NADPH oxidase also has been suggested to induce calpain activation in norepinephrine-stimulated cardiomyocytes.\textsuperscript{23} Thus, NADPH oxidase may be a universal mechanism for calpain activation in cardiomyocytes. However, the mechanisms by which NADPH oxidase regulates calpain activity remain to be determined. It is possible that the activation of NADPH oxidase results in reactive oxygen species production, which activates calpain through regulation of intracellular calcium levels and ERK1/2 pathway,\textsuperscript{25} which merits further investigation. Since nitric oxide (NO) production is induced in septic hearts which contributes to myocardial dysfunction in sepsis,\textsuperscript{36,47} it is also possible that NADPH oxidase-produced superoxide interacts with NO to form peroxynitrite, which induces calpain activation.\textsuperscript{48} On the other hand, calpain activation has been shown to induce hepatic inducible NO synthase (iNOS) during LPS stimulation.\textsuperscript{49} Increased iNOS and NO production will further enhance calpain activation via peroxynitrite formation. Thus, a feed-forward fashion mechanism may operate to induce calpain activation and NO production as well as peroxynitrite formation, all of which contribute to myocardial dysfunction during sepsis.\textsuperscript{46,47}

In summary, gp91\textsuperscript{phox}-NADPH oxidase induces calpain-1 activation, which contributes to caspase-3 activity and TNF-α expression during LPS stimulation. Over-expression of calpastatin prevents calpain activation, leading to the inhibition of caspase-3 activation and down-regulation of TNF-α expression in *in vitro* cardiomyocytes and *in vivo* hearts during LPS stimulation. More importantly, up-regulation of calpastatin improves myocardial function during endotoxemia. Thus, the present study suggests that targeting calpain/calpastatin system may be a potential therapeutic strategy for septic hearts.

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**References**


