



Regular Article

Calpain-1 induces apoptosis in pulmonary microvascular endothelial cells under septic conditions

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ABSTRACT

This study was to investigate the role of calpain in the apoptosis of pulmonary microvascular endothelial cells (PMEC) during septic plasma stimulation. Septic plasma was collected from endotoxemic mice. In cultured PMEC, incubation with septic plasma stimulated calpain activation, increased caspase-3 activity and induced apoptotic cell death. These effects of septic plasma were abrogated by knockdown of calpain-1 but not calpain-2 using specific siRNA. Consistently, treatment with calpain inhibitor-III, or over-expression of calpastatin, an endogenous calpain inhibitor significantly decreased apoptosis induced by septic plasma. Septic plasma also induced NADPH oxidase activation and reactive oxygen species (ROS) production. Inhibiting NADPH oxidase or scavenging ROS attenuated calpain activity and decreased apoptosis in PMEC during septic plasma stimulation. In summary, our study demonstrates that ROS produced from NADPH oxidase stimulates calpain-1 activation, which induces apoptosis under septic conditions. Thus, targeting calpain-1/calpastatin may represent a potential strategy to protect against endothelial injury in sepsis.

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Acute lung injury and its more severe form, acute respiratory distress syndrome, remain an important clinical problem (Wheeler and Bernard, 2007). The most common cause of acute lung injury is bacterial infection resulting in the sepsis syndrome. Lipopolysaccharides or endotoxins (LPS) of Gram-negative bacteria have been suggested to be important pathogens responsible for acute lung injury. The administration of LPS generates ‘circulating factors’ (including cytokines, eicosanoids, etc.) (Simons et al., 1996; Rittirsch et al., 2008), which result in lung injury that is characterized by the presence of apoptosis in the endothelium and epithelium (Li et al., 2004). Apoptosis of endothelial and epithelial cells contributes to the impairment of the barrier function of pulmonary endothelium and epithelium, leading to the development of pulmonary edema. Thus, inhibition of apoptotic cell death in the alveolar endothelium and epithelium prevents the acute lung injury (Kawasaki et al., 2000). However, the intracellular signaling pathways that result in apoptotic cell death are not fully understood in sepsis.

LPS and LPS-induced circulating factors induce NADPH oxidase activation and reactive oxygen species (ROS) production, which has been implicated in sepsis-induced lung injury (Hoesel et al., 2008). An early study showed that administration of NADPH oxidase inhibitor, apocynin attenuated septic lung injury in guinea pigs (Wang et al., 1994). Later on, it was confirmed that NADPH oxidase activation was required for the induction of sepsis-induced lung microvascular injury using mice lacking gp91^{phox} and p47^{phox} (Gao et al., 2002). Although NADPH oxidase-produced ROS has been shown to contribute to apoptosis in human dermal microvascular and umbilical vein endothelial cells (Shin et al., 2004; Li et al., 2007), it remains to be elucidated whether NADPH oxidase mediates apoptosis in pulmonary microvascular endothelial cells (PMEC) under septic conditions as the signaling pathways may differ among endothelial cells in different organs, and between macrovascular and microvascular endothelial cells.

Increased ROS has been shown to induce calpain activation in retinal photoreceptor cells (Sanvicens et al., 2004) and cardiomyocytes (Li et al., 2009). Calpains are a family of calcium-dependent thiol-proteases (Perrin and Huttenlocher, 2002; Goll et al., 2003). Fifteen gene products of the calpain family are reported in mammals. Among them, calpain-1 and calpain-2 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

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calpain inhibitor, calpastatin. Ca^{2+} is required for calpain activation. Calpains respond to Ca^{2+} signals by cleaving specific proteins, frequently components of signaling cascades, thereby irreversibly modifying their function. Calpain has emerged as an important player in cell death signaling. However, it remains unknown whether calpain plays a role in PMEC apoptosis during sepsis.

In the present study, we hypothesized that calpain activation is induced by NADPH oxidase signaling, leading to apoptosis in PMEC during sepsis. To test these hypotheses, we employed an *in vitro* model of PMEC death. Apoptosis was stimulated by septic plasma. The role of NADPH oxidase-mediated calpain activation in PMEC apoptosis under septic conditions was studied.

Materials and methods

Animals and pulmonary microvascular endothelial cells (PMEC)

Breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory. A breeding program was implemented at our animal care facilities. All animals were provided water and food *ad libitum* and housed in a temperature and humidity controlled facility with 12-hour light and dark cycles. All animals were used in accordance with the Canadian Council on Animal Care guidelines and all experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario. PMEC were isolated from adult C67BL/6 mice and cultured as previously described (Farley et al., 2008). All PMEC were used for the present study within 5 generations.

Septic plasma

Adult C57BL/6 mice were injected with LPS (4 mg/kg, i.p.) or saline. Four hours later, animals were euthanized and exsanguinated by cardiac puncture, and the blood processed to obtain plasma (Madorin et al., 2004).

Calpain-1 and calpain-2 knockdown using siRNA

In order to knockdown calpain-1 and calpain-2 expression, a small interfering RNA (siRNA) against calpain-1 or calpain-2 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA) 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 (Shen et al., 2007).

Adenoviral infection of PMEC

Cultured PMEC were infected with recombinant adenoviruses containing rat calpastatin (Ad-CAST, Applied Biological Materials Inc.) or beta-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection (MOI) of 10 PFU/cell. Adenovirus-mediated gene transfer was implemented as previously described (Shen et al., 2007).

Calpain activity

Calpain activity was determined by using a fluorescence substrate N-succinyl-LLVY-AMC (Cedarlane Laboratories) as described in our recent study (Li et al., 2009). The calpain inhibitor PD150606 (Calbiochem) was used to determine the specificity of the assay.

Calpastatin activity

Calpastatin activity was measured by determining the inhibitory effect of cell lysates on calpain-1 activity as described previously (Wei et al., 2005). The calpastatin activity (expressed as % inhibition of calpain activity) was calculated from the difference in calpain activity measured in the absence or presence of cell lysates.

NADPH oxidase activity

NADPH oxidase activity was assessed in cell lysates by lucigenin-enhanced chemiluminescence (20 μg of protein, 100 μM NADPH, 5 μM lucigenin) as described previously (Li et al., 2009).

ROS measurement

The formation of ROS was measured using a ROS sensitive dye, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) as an indicator (Molecular probes) as described in our recent study (Li et al., 2009).

Active caspase-3

As described in detail previously (Feng et al., 2002), caspase-3 activity in PMEC was measured by using a caspase-3 fluorescent assay kit (BIOMOL Research Laboratories).

Annexin V-FITC/propidium iodide (PI) assay

The annexin V kit (Invitrogen) was used according to the manufacturer's protocol to detect phosphatidylserine translocation from the inner to the outer plasma membrane. Briefly, for each assay, cells were trypanosinized and washed with PBS, and incubated in annexin V binding buffer containing annexin V and propidium iodide (PI) for 15 min at room temperature in the dark. The cells were analyzed by fluorescence activated cell sorting (FACS; BD Biosciences), as described previously (Vermees et al., 1995), with acquisition of a total 10,000 events/sample to ensure adequate data. Annexin V-positive but PI-negative (annexin V-positive/PI-negative) cells were identified as apoptotic cells.

Western blot analysis

The protein levels of calpain-1, calpain-2, calpastatin and GAPDH protein were determined by western blot analysis using respective specific antibodies as described in our recent study (Li et al., 2009).

Statistical analysis

All data were given as mean \pm SD. Differences between 2 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.

Results

Calpain activation in PMEC under septic conditions

It has not been shown if calpain is activated in PMEC under septic conditions. To investigate the activation of calpain under septic conditions, PMEC were incubated with normal or septic plasma (20%) for 24 h and calpain activity was then analyzed. This dose of septic plasma was chosen according to a recent report (Joulin et al., 2007). As shown in Fig. 1A, septic plasma significantly increased calpain activity by 107% in PMEC. The detected-calpain activity was blocked when a calpain-specific inhibitor, PD150606 was added in the cell lysates during the assay, confirming that the assay is specifically measuring calpain activity since PD150606 prevents Ca^{2+} binding to calpain but does not significantly inhibit either cathepsins or caspases (Wang et al., 1996). Thus, calpain is activated in PMEC under septic conditions.

To clarify the contribution of calpain-1 and calpain-2 to the increase of calpain activity, we used siRNA to specifically knockdown calpain-1 and calpain-2 expression. PMEC were transfected with calpain-1 siRNA, calpain-2 siRNA or a scramble siRNA as control. Twenty-four hours after transfection, PMEC were incubated with

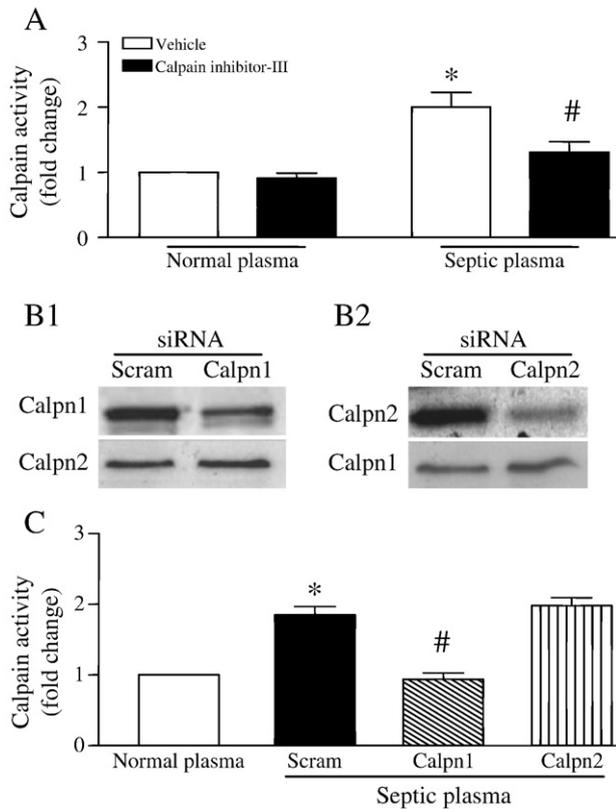


Fig. 1. (A) Effect of septic plasma on PMEC calpain activity. PMEC were incubated with normal or septic plasma in the presence of calpain inhibitor-III or vehicle for 24 h and calpain activity was measured. (B) and (C) Effective knockdown of calpain-1 and calpain-2 expression by siRNA strategy. PMEC were transfected with siRNA for calpain-1 (calpn1), calpain-2 (calpn2) or scramble (Scram) siRNA for 24 h, followed by incubation with normal or septic plasma for another 24 h. The protein levels of calpain-1 (80 kDa) and calpain-2 (80 kDa) were determined by western blot analysis. Calpain-1 siRNA and calpain-2 siRNA specifically down-regulated calpain-1 (B1) and calpain-2 protein (B2), respectively. Calpain activity was measured (C). Data are mean \pm SD from 3 different experiments. * P <0.05 versus normal plasma; # P <0.05 versus septic plasma + vehicle or septic plasma + scram siRNA.

normal or septic plasma (20%) for another 24 h. The efficacy of siRNA inhibition of calpain-1 and calpain-2 protein expression, and calpain activity were analyzed. Transfection of calpain-1 or calpain-2 siRNA specifically down-regulated calpain-1 (Fig. 1B1) or calpain-2 protein (Fig. 1B2) in PMEC, respectively, compared with a scramble siRNA, suggesting a successful knockdown of calpain-1 and calpain-2 expression. In response to septic plasma, calpain activation was completely blocked in calpain-1 siRNA-transfected compared with scramble siRNA-transfected PMEC (Fig. 1C). In contrast, calpain-2 siRNA did not decrease calpain activity in septic plasma-stimulated PMEC (Fig. 1C). These data suggest that septic plasma specifically activates calpain-1 in PMEC.

To examine if the protein levels of calpain were altered in PMEC during septic plasma stimulation, we measured calpain-1 and calpain-2 expression by western blot analysis. Septic plasma did not change the protein levels of calpain-1 or calpain-2 in PMEC (data not shown). The result suggests that calpain activation in PMEC under septic conditions is not due to up-regulated calpain-1 or calpain-2 protein expression.

Role of calpain-1 in PMEC apoptosis under septic conditions

To examine the role of calpain-1 in apoptosis, we transfected cultured PMEC with calpain-1 siRNA or scramble siRNA. Twenty-four hours after transfection, PMEC were incubated with normal or septic plasma (20%) for another 24 h. Septic plasma treatment significantly

increased caspase-3 activity by 81% in PMEC. Transfection of calpain-1 siRNA blocked caspase-3 activation in septic plasma-stimulated PMEC, as compared to the scramble siRNA (Fig. 2A). In contrast, transfection with calpain-2 siRNA did not decrease caspase-3 activation in septic plasma-stimulated PMEC. Apoptosis in PMEC was further determined by binding of annexin V, an early indicator of apoptosis. Since annexin V binds to externalized phosphatidylserine on membranes of early apoptotic cells, we used flow-cytometric analysis of cells stained with FITC-conjugated annexin V and the nuclear stain PI, which only binds to nucleic acids when the cell membrane is damaged, and thus serves as a marker of necrotic cell death. Similarly, septic plasma increased the percentages of annexin V-positive/PI-negative cells by 136%, again indicating that septic plasma induced apoptosis. Knockdown of calpain-1 abrogated the increase in the percentages of annexin V-positive/PI-negative cells (Fig. 2B). These data suggest that calpain-1 signaling contributes to apoptosis in septic plasma-stimulated PMEC.

The role of calpain in apoptosis was further investigated by using a pharmacological calpain inhibitor, calpain inhibitor-III. Cultured PMEC were incubated with normal or septic plasma in the presence of calpain inhibitor-III (10 μ M) or vehicle for 24 h, and calpain activity and apoptosis were then analyzed. Concurrent incubation with

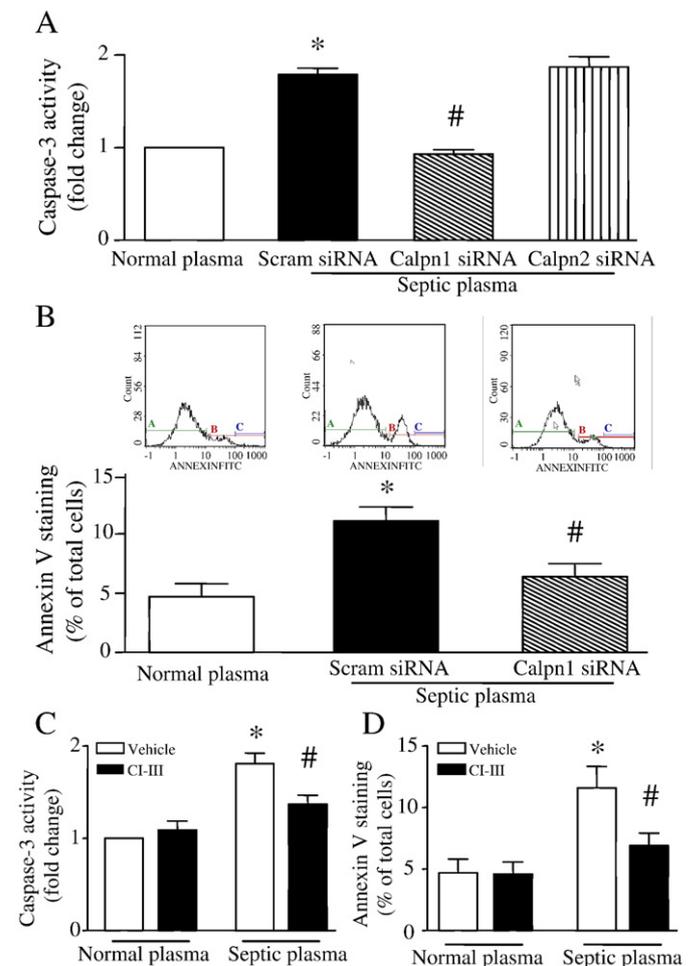


Fig. 2. Role of calpain in PMEC apoptosis under septic conditions. (A) and (B) PMEC were transfected with siRNA for calpain-1 (calpn1), calpain-2 (calpn2) or scramble (Scram) siRNA and then incubated with normal or septic plasma. (A) Caspase-3 activity was measured. (B) Apoptotic cell death was analyzed. Upper panel is a representative flow-cytometric analysis and lower panel is quantification of the percentages of annexin V-positive/PI-negative cells. (C) and (D) PMEC were incubated with normal or septic plasma in the presence of calpain inhibitor-III (CI-III) or vehicle for 24 h. (C) Caspase-3 was measured. (D) Apoptotic cell death was analyzed. Data are mean \pm SD from at least 3 different experiments. * P <0.05 versus normal plasma or normal plasma + vehicle; # P <0.05 versus septic plasma + Scram siRNA or septic plasma + vehicle.

calpain inhibitor-III decreased calpain activity induced by septic plasma (Fig. 1A). In line with the effects of calpain-1 siRNA, calpain inhibitor-III significantly decreased caspase-3 activity and attenuated the percentages of annexin V-positive/PI-negative cells in septic plasma-stimulated PMEC (Figs. 2C and D). These results further confirm an important role of calpain in PMEC apoptosis under septic conditions.

Effects of calpastatin over-expression on calpain activation and apoptosis

Calpastatin is an endogenous calpain inhibitor in cells (Goll et al., 2003). To demonstrate the contribution of calpastatin in apoptosis, we first investigated the effect of septic plasma on calpastatin expression. Septic plasma treatment for 24 h did not change either the protein levels of calpastatin (data not shown) or the calpastatin activity (% calpain inhibition in normal plasma versus septic plasma-treated group: 30 ± 10.32 versus 33 ± 6.52) in PMEC. We then investigated whether over-expression of calpastatin could inhibit calpain activity and prevent apoptosis in PMEC exposed to septic plasma. PMEC were infected with Ad-CAST or Ad-gal for 24 h. Over-expression of calpastatin was verified by western blot analysis (Fig. 3A). Under

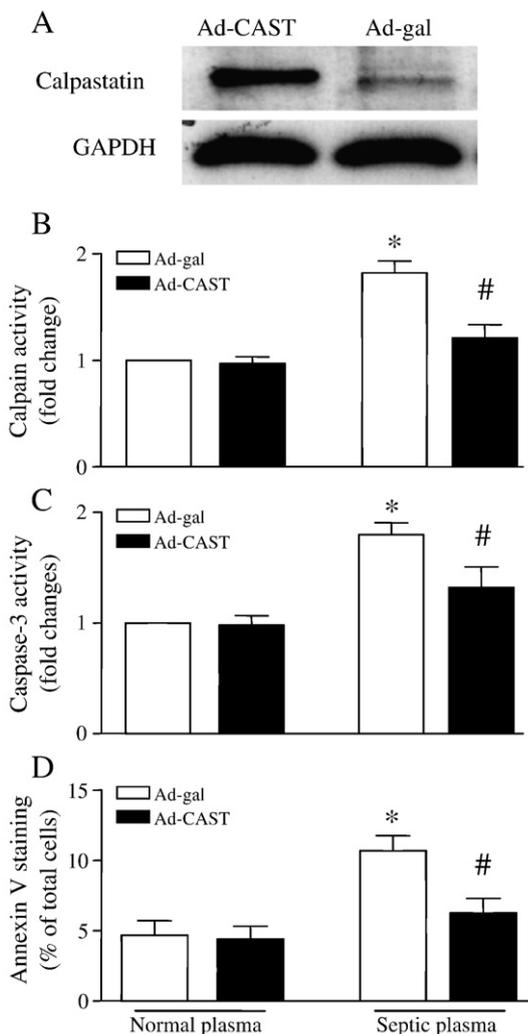


Fig. 3. Effects of calpastatin over-expression on PMEC apoptosis under septic conditions. PMEC were infected with Ad-CAST or Ad-gal for 24 h, followed by incubation with normal or septic plasma for 24 h. (A) Over-expression of calpastatin protein (110 kDa) was determined by western blot analysis. Calpain activity (B), caspase-3 activity (C) and apoptotic cell death (D) were analyzed. Data are mean \pm SD from at least 3 different experiments. * $P < 0.05$ versus normal plasma + Ad-gal; # $P < 0.05$ versus septic plasma + Ad-gal.

control (non-septic) condition, infection of Ad-CAST did not affect calpain activity and apoptosis in PMEC. In response to septic plasma, increases in calpain activity (Fig. 3B), caspase-3 activity (Fig. 3C) and annexin V-positive/PI-negative cells (Fig. 3D) were all significantly attenuated in Ad-CAST-infected compared with Ad-gal-infected PMEC. These results further support the conclusion that inhibition of calpain by calpastatin prevents apoptosis in PMEC during septic plasma stimulation.

Role of NADPH oxidase in calpain activation and apoptosis

We first investigated the effects of septic plasma on NADPH oxidase activation and ROS production. Cultured PMEC were incubated with normal or septic plasma (20%) in the presence of diphenyleneiodonium (DPI, 5 μ M), apocynin (200 μ M) or vehicle for 24 h. NADPH oxidase activity and ROS production were analyzed. Septic plasma significantly increased NADPH oxidase activity by 82% and increased ROS production by 110% in PMEC compared with normal plasma (Figs. 4A and B). Co-incubation with DPI or apocynin inhibited NADPH oxidase activation and blocked ROS production in septic plasma-stimulated PMEC (Figs. 4A and B). These data demonstrate that septic plasma activates NADPH oxidase, leading to ROS production in PMEC.

We then examined if NADPH oxidase-mediated ROS induces calpain activation. Cultured PMEC were incubated with normal or septic plasma in the presence of DPI (5 μ M), apocynin (200 μ M), N-acetylcysteine (NAC, 2.5 mM) or vehicle. Twenty-four hours later, calpain activity was measured. Inhibition of NADPH oxidase with DPI or apocynin significantly decreased septic plasma-induced calpain activity in PMEC (Fig. 4C). Similarly, scavenging ROS by NAC inhibited calpain activation in septic plasma-stimulated PMEC (Fig. 4C). To further confirm the effect of ROS on calpain activation, PMEC were incubated with H_2O_2 (10 μ M) or vehicle for 24 h and calpain activity was measured. Treatment with H_2O_2 increased calpain activity by 153% in PMEC (Fig. 4D). Thus, these results strongly suggest that septic plasma activates calpain, at least in part, through NADPH oxidase/ROS-dependent mechanisms in PMEC.

Finally, we examined the effects of NADPH oxidase activation on apoptosis. Cultured PMEC were incubated with normal or septic plasma (20%) in the presence of NADPH oxidase inhibitors (apocynin or DPI) or vehicle for 24 h. Inhibition of NADPH oxidase with DPI or apocynin attenuated caspase-3 activity and reduced apoptotic cell death in PMEC during septic plasma stimulation (Figs. 4E and F). To determine the contribution of oxidant stress to apoptosis, an antioxidant, NAC was used. Similarly, NAC treatment decreased caspase-3 activity in septic plasma-induced PMEC (Fig. 4E). These data suggest that NADPH oxidase activation and ROS production induce apoptosis in PMEC during septic plasma stimulation.

Discussion

The major finding of the present study is that under septic conditions, calpain-1 is activated, which leads to caspase-3 activation and apoptotic cell death in PMEC. Over-expression of calpastatin blocks calpain activation, inhibits caspase-3 activation and prevents sepsis-induced apoptosis. Thus, the calpain-1/calpastatin system is important in regulating pulmonary microvascular endothelial cell death and survival under septic conditions. Furthermore, blocking NADPH oxidase activation and scavenging ROS attenuates calpain activity and apoptosis in PMEC during septic plasma stimulation. This suggests that NADPH oxidase is upstream of calpain activation in PMEC during sepsis.

Apoptosis of microvascular endothelial cells has been associated with the impairment of endothelial function and lung injury during sepsis (Kawasaki et al., 2000; Stefanec, 2000; Li et al., 2004; Matsuda

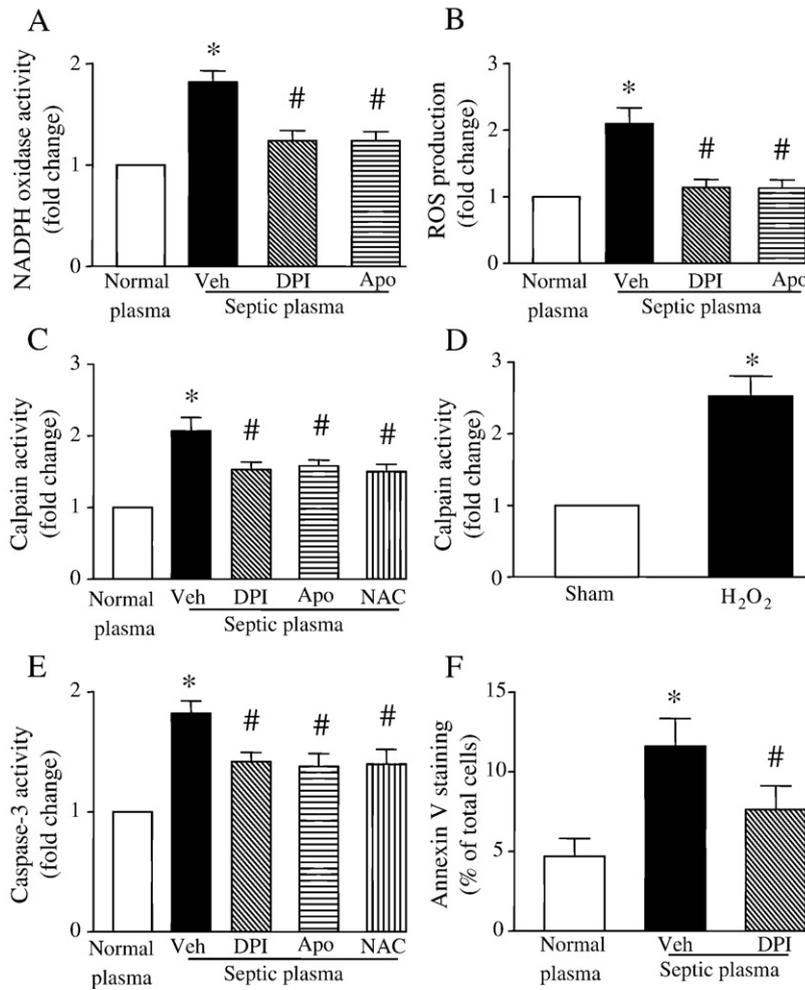


Fig. 4. Roles of NADPH oxidase in calpain activation and apoptosis. PMEC were incubated with normal or septic plasma in the presence of DPI, Apocynin (Apo), NAC or vehicle (Veh) for 24 h. NADPH oxidase activity (A), ROS production (B), calpain activity (C), caspase-3 activity (E) and apoptotic cell death (F) were analyzed. (D) PMEC were incubated with or without H₂O₂ for 24 h and calpain activity was measured. Data are mean \pm SD from at least 3 different experiments. * P <0.05 versus normal plasma or sham; # P <0.05 versus septic plasma + Veh.

et al., 2007). However, the underlying mechanisms by which endothelial cell apoptosis occurs in sepsis are not fully understood. In the present study, we provide direct evidence demonstrating that calpain-1 activation induces apoptosis in PMEC under septic conditions. First, septic plasma induces calpain-1 activation. Second, knockdown of calpain-1 but not calpain-2 prevents apoptotic cell death in septic plasma-stimulated PMEC. Third, pharmacological inhibition of calpain decreases septic plasma-induced apoptosis. Finally, over-expression of calpastatin inhibits calpain activation and prevents apoptosis induced by septic plasma. Interestingly, Ad-CAST did not affect calpain activity in normal conditions. Others have also observed that basal calpain activity remains unchanged in the tissues of calpastatin transgenic mice (Letavernier et al., 2008). These studies suggest that calpastatin controls calpain activity only under stimulated conditions. Consistent with this notion, structural and biochemical data indicate that calpastatin might bind preferentially to calcium-activated calpains (Barnoy et al., 1999; Tullio et al., 1999). Thus, any strategies focusing on up-regulation of calpastatin may have therapeutic potential to protect PMEC under septic conditions. In addition, we noted that calpain-1 siRNA inhibited calpain-1 protein by about 70% whereas it completely blocked septic plasma-induced calpain activity, caspases-3 activity and annexin-V staining in PMEC. It is possible that 70% inhibition of calpain-1 was responsible for the increased calpain activity and apoptosis by septic plasma while the remaining calpain-1 accounted for their basal activity.

The role of calpain in cell death signaling is associated with partial cleavage of pro- or anti-apoptotic proteins, which might activate or inactivate putative substrates including caspase-3, caspase-7, -8, and -9, caspase-12, Bcl-2, Bcl-xl, Bid, Bax, and NF- κ B (Neumar et al., 2003; Tan et al., 2006). The present study suggests that the pro-apoptotic role of calpain-1 is related to caspase-3 activation since blocking calpain abolishes caspase-3 activation. However, the present study cannot exclude the involvement of caspase-3 independent mechanisms, which merits future investigations.

Induction of endothelial cell apoptosis by calpain activation may impair vascular integrity, which increases the permeability of endothelium, contributing to pulmonary edema during sepsis (Kawasaki et al., 2000; Li et al., 2004). In addition, calpain also plays a role in the production of proinflammatory mediators such as cytokines, iNOS and cyclooxygenase-2, all of which contribute to EC dysfunction and microvascular injury in sepsis (Cuzzocrea et al., 2000). Thus, calpain-1 may be a potential therapeutic target for the treatment of endothelial dysfunction and lung injury in sepsis. However, further *in vivo* studies will be needed to clarify the role of calpain-1 in acute lung injury during sepsis, in particular using gene knockout animal models.

Oxidative stress occurs and contributes to endothelial cell dysfunction and lung injury in sepsis. The present study confirms that septic plasma increases NADPH oxidase activity and ROS production in PMEC (Filep, 2007). Moreover, we demonstrate that

NADPH oxidase-produced ROS induces apoptosis. Previous studies have demonstrated that ROS induces calpain activation (Sanvicens et al., 2004; Li et al., 2009). In agreement with these reports, we show that H₂O₂ increases calpain activity in PMEC. We further demonstrate that calpain is activated in parallel with NADPH oxidase activation in response to septic plasma and, more importantly, inhibition of NADPH oxidase or ROS production significantly decreases calpain activation in PMEC. Thus, calpain activation is induced, at least in part, through NADPH oxidase/ROS-dependent pathway in PMEC. Activation of calpain by NADPH oxidase-mediated ROS production has been also demonstrated in cardiomyocytes in our recent study (Li et al., 2009). Thus, ROS produced from NADPH oxidase may be a general mechanism for calpain activation.

However, the signaling mechanisms by which ROS induce calpain activation remain not fully understood. Calpain activity is mainly regulated by altering the Ca²⁺ concentration required for its proteolytic activity. The Ca²⁺ requirement for calpain activity is modulated by several mechanisms. For example, certain phospholipids, with phosphatidylinositol being the most effective, would lower the Ca²⁺ concentration required for autolysis of calpain-1 and calpain-2 (Zalewska et al., 2004). Calpain activity can also be up-regulated through phosphorylation by ERK1/2 MAPK and protein kinase C (PKC) (Kennett et al., 2004), and down-regulated by protein kinase A (PKA) activation (Shiraha et al., 2002). ROS have been shown to regulate intracellular Ca²⁺ through various Ca²⁺ channels (Kowara et al., 2006) and are involved in ERK1/2, PKC and PKA signaling. In this regard, previous studies have shown an alteration of intracellular Ca²⁺, activation of ERK1/2, PKC and PKA in endothelial cells in septic conditions (Cuschieri et al., 2003; Kuklin et al., 2005; Bolon et al., 2008). Thus, it is possible that ROS produced from NADPH oxidase modulate intracellular Ca²⁺, ERK1/2, PKC and PKA, leading to calpain activation in PMEC during septic plasma stimulation, which needs to be clarified in future studies.

In summary, we have shown that septic plasma induces calpain-1 activation, at least in part through NADPH oxidase-dependent pathway in PMEC. Activation of calpain-1 leads to caspase-3 activation and apoptotic cell death, and over-expression of calpastatin inhibits calpain activation and protects PMEC from apoptotic death during septic plasma stimulation. Thus, modulating calpain-1/calpastatin system may be a potential strategy to protect endothelial cells from injury in sepsis.

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