Original Article

Inhibition of calpain alleviates coxsackievirus B3-induced myocarditis through suppressing the canonical NLRP3 inflammasome/caspase-1-mediated and noncanonical caspase-11-mediated pyroptosis pathways

Ying Yu1*, Hui Shi2*, Yong Yu2, Ming Liu1, Minghui Li2, Xiaoxiao Liu2, Yucheng Wang2, Ruizhen Chen2

1Department of General Practice, Zhongshan Hospital, Shanghai Medical College of Fudan University, Shanghai 200032, China; 2Department of Cardiology, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Shanghai Medical College of Fudan University, Shanghai 200032, China. *Equal contributors.

Received August 18, 2019; Accepted April 4, 2020; Epub May 15, 2020; Published May 30, 2020

Abstract: This study aimed to verify the effects of calpain on coxsackievirus B3 (CVB3)-induced myocarditis and to further explore the underlying mechanisms. Transgenic mice overexpressing calpastatin, the endogenous calpain inhibitor, were introduced in the present study. The murine model of viral myocarditis (VMC) was established by intraperitoneal injection of CVB3 into transgenic and wild-type mice. Myocardial injury was measured by H&E staining and ELISA for cTnI. CVB3 replication was assessed via capsid protein VP1 detection and virus titration. The fibrotic factors collagen and TGF-β1 were evaluated by Masson staining and real-time PCR analysis, respectively. Moreover, the levels of NLRP3, AIM2, ASC, cleaved caspase-1, cleaved caspase-11 and the pyroptosis indicators GSDMD p30, IL-1β and HMGB1 were determined by real-time PCR, western blot or immunohistochemical analysis. In addition, peripheral IL-1β and HMGB1 were evaluated by ELISA. We observed that CVB3-infected transgenic mice had lower pathological scores, peripheral cTnI levels, viral loads and expression levels of collagen and TGF-β1 in the heart than CVB3-infected wild-type mice. Furthermore, we found decreased levels of NLRP3, ASC, cleaved caspase-1 and cleaved caspase-11 in the hearts of CVB3-infected transgenic mice. However, after CVB3 infection, the levels of AIM2 in transgenic mice and wild-type mice did not differ significantly. Additionally, calpastatin overexpression significantly reduced the levels of GSDMD p30, IL-1β and HMGB1 in the myocardium as well as peripheral IL-1β and HMGB1. Taken together, these findings indicate that calpain inhibition attenuates CVB3-induced myocarditis by suppressing the canonical NLRP3 inflammasome/caspase-1-mediated and noncanonical caspase-11-mediated pyroptosis pathways.

Keywords: Calpain, NLRP3 inflammasome, caspase-1, caspase-11, pyroptosis, viral myocarditis

Introduction

Viral myocarditis (VMC) is a principal cause of sudden death in young adults and can progress to dilated cardiomyopathy. Coxsackievirus B3 (CVB3) is believed to be the most common cause of VMC. Despite extensive efforts, we still lack effective and specific clinical treatments. Therefore, further exploration of new therapies for VMC is urgently needed.

Different types of cell death, including apoptosis, necrosis, autophagy and pyroptosis, have been reported. In contrast to the other types, pyroptosis is believed to be an inflammatory form of programmed cell death and features gasdermin family-mediated membrane pore formation and subsequent cell lysis, as well as the release of proinflammatory cytokines, mainly IL-1β, IL-18 and HMGB1. Increasing evidence shows that pyroptosis is initiated by the canonical caspase-1-dependent and noncanonical caspase-4/5/11-mediated (human caspase-4/5 and murine caspase-11) pyroptosis pathways [1]. In the canonical caspase-1 signaling pathway, several inflammasomes, such as
The effects of calpain on pyroptosis pathway in viral myocarditis

The nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family (e.g., NLRP3 and NLRP1), PYHIN protein families (e.g., absent in melanoma 2 (AIM2)) and Pyrin proteins, are recruited and activated [2-4]. Upon exposure to stimuli [5, 6] such as bacterial or viral pathogens and double-stranded DNA (dsDNA), NLRP3 and AIM2 can be activated and then bind to the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the protease caspase-1, which results in the cleavage of caspase-1. Subsequently, active caspase-1 cleaves gasdermin D (GSDMD), an executor of pyroptosis signaling, to generate an N-terminal ‘p30’ subunit in order to trigger pyroptosis and release mature IL-1β/18 [7, 8]. In the noncanonical pyroptosis pathway, caspase-11 directly triggers pyroptosis by cleavage of GSDMD and release of HMGB1 [9]. Inflammatory cytokines, such as IL-1β, IL-18, and HMGB1, subsequently promote inflammation [2, 10, 11] during pyroptosis. Recent studies have demonstrated that pyroptosis is implicated in several cardiovascular diseases and conditions, including atherosclerosis, myocardial infarction, diabetic cardiomyopathy and reperfusion injury [12-14]. Additionally, pyroptosis is associated with myocarditis [15]. Specifically, the NLRP3 inflammasome is activated in myocarditis in both patients and mice [16, 17]. Furthermore, blockade of the pyroptosis pathway by the caspase-1 inhibitor Ac-YVAD-CHO significantly attenuates the severity of VMC [17]. These findings confirm that pyroptosis is involved in the pathogenesis of myocarditis.

Calpains, a family of calcium-dependent cysteine proteases, consist of a large 80-kDa catalytic subunit and a small 30-kDa regulatory subunit. The activity of calpains is regulated by the binding of Ca^{2+} and phospholipids and by phosphorylation. In addition, their activity is blunted by calpastatin, their natural and specific endogenous inhibitor [18]. Calpain is involved in a wide variety of cellular processes, including remodeling of cytoskeletal/membrane attachments, caspase-mediated apoptosis and acute inflammation [18]. Our previous studies [19, 20] demonstrated that calpain facilitates CVB3 replication and promotes myocardial inflammation, implying that calpain plays pivotal roles in VMC. However, the potential mechanisms underlying calpain-mediated VMC have not yet been elucidated.

In the present study, we introduced transgenic mice overexpressing calpastatin to investigate the effects of calpain on the canonical caspase-1-mediated and noncanonical caspase-11-mediated pyroptosis pathways in a murine model of CVB3-induced myocarditis.

Materials and methods

Virus

CVB3 (Nancy strain), which was maintained by passage in HeLa cells, was maintained in the Key Laboratory of Viral Heart Diseases, Zhongshan Hospital. The viral titer was measured as the median tissue culture infective dose (TCID_{50}) [21].

Mice

The calpastatin transgenic mouse strain (Tg-CAST), which was introduced from the laboratory of Tianqing Peng (Lawson Health Research Institute, Canada), was bred in a standard specific pathogen-free (SPF) environment in the Department of Laboratory Animal Science, Fudan University. All littersmates were genotyped following the protocol of the Peng laboratory [22, 23]. Male inbred experimental mice aged 4-5 weeks were used and divided into a wild-type control group (Con.), transgenic mouse control group (Tg-CAST), CVB3-infected wild-type group (Virus) and CVB3-infected transgenic mouse group (Virus+Tg-CAST). All animal experiments were approved by the Ethical Committee of Fudan University.

Animal model establishment

As described in our previous study [24], mice were intraperitoneally injected with CVB3 (10^{5} TCID_{50}, 300 μl) to establish the murine model of VMC, while control groups received an equal volume of PBS. Mice were sacrificed on day 7 post infection, and hearts and serum were harvested.

Histological analysis

Hearts from anesthetized mice were rapidly excised, fixed in 4% paraformaldehyde, and embedded in paraffin for histopathologic examination. Five-micron-thick sections were prepared. Sections were stained with hematoxylin and eosin (H&E) and Masson trichrome according to standard protocols. Furthermore, immu-
The effects of calpain on pytoprosis pathway in viral myocarditis

Enzyme-linked immunosorbent assay (ELISA)

The levels of peripheral cTnI, IL-1β and HMGB1 were measured with ELISAs (Uscn Life Science Inc., China) following the manufacturer’s instructions.

Western blot analysis

Heart tissue was homogenized in lysis buffer containing protease inhibitor cocktail (Thermo Scientific, USA) on ice. Tissue homogenates were centrifuged at 12,000×g for 10 min at 4°C, and the protein concentration was determined by a Bradford assay (Thermo Scientific, USA). A detailed protocol was described in our previous investigations [20, 25]. In brief, equal amounts of protein were loaded for SDS gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, USA). After blocking, membranes were incubated with primary antibodies against α-fodrin (1:1000, Enzo Life Sciences, USA), VP-1 (1:500, Leica Biosystems Newcastle Ltd., UK), NLRP3 (1:1000, Abcam, UK), AIM2 (1:1000, Cell Signaling Technology, USA), ASC (1:1000, Abcam, UK), caspase-1 (1:1000, Abcam, UK), caspase-11 (1:1000, Abcam, UK), GSDMD (1:1000, Abcam, UK), and IL-1β (1:800, Cell Signaling Technology, USA) overnight at 4°C. Bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:8000, Jackson ImmunoResearch, USA) for 1.5 h at room temperature. Bands were visualized with the SuperSignal West Pico chemiluminescence detection system (Thermo Scientific, USA). β-actin (1:4000, KangChen Biotech, China) was used as the internal control. The levels of target molecules were analyzed by densitometry using Image Lab software (BioRad, USA).

Viral titration

Heart tissues were weighed and homogenized to harvest virus-containing supernatant. Ten-fold serial dilutions of supernatant were added to HeLa cells. Viral titers were determined using a TCID_{50} assay in HeLa cells as previously described [17, 20].

Real-time PCR analysis

According to the manufacturer’s protocol, total RNA was extracted from left ventricular (LV) tissues using Trizol reagent (Invitrogen, USA). A total of 500 ng of RNA per sample was used for reverse transcription and PCR assays (Takara, Japan). The specific primers for TGF-β1, NLRP3, ASC, AIM2 and β-actin were as follows [17, 26, 27]: TGF-β1, 5’-GCAGTGCTGAACCAAGGA-3’ and 5’-AGCAGTGAGCCTGAATCG-3’; NLRP3, 5’-ATTACGGCAGGAGAAAGG-3’ and 5’-TCGCA-GCAAAGATCCACACAG-3’; ASC, 5’-CAGAGTAC-GGCCAGAAGGAC3’ and 5’-TGTTGCTCTG-CAGGAATGCC-3’; AIM2: 5’-GTCCACAGTCTT-CAGTGT-3’ and 5’-CACCCTCATTGTCCCGT-3’; and IL-1β, 5’-CGATGCCACAGGATTCCA-3’. The expression level of each gene was normalized to that of β-actin, which served as the endogenous control. Relative quantification of gene expression was performed via the 2^{-ΔΔCT} method.

Statistical analysis

All data were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and expressed as the means ± SEM. Student’s t test was used for comparisons between two groups. Multigroup comparisons were analyzed by one-way ANOVA with the post hoc Bonferroni test. P<0.05 was considered statistically significant.

Results

Calpain activity and pyroptosis is increased in the pathogenesis of CVB3-induced myocarditis

We established the VMC model by intraperitoneal injection of CVB3 (10^5 TCID_{50}) into male C57BL/6 mice as described in our previous studies [20, 24]. H&E staining showed apparent inflammatory infiltrates and increased pathological scores, indicating successful es-
The effects of calpain on pyroposis pathway in viral myocarditis

1957


tablishment of VMC (Figure 1A and 1B). To determine the role of calpain and pyroptosis in the murine model of VMC, we then examined α-fodrin, the degradation product of calpain-specific substrates, and the pyroptosis executor GSDMD p30 in heart tissues and IL-1β in serum. CVB3 significantly increased the levels of α-fodrin and GSDMD p30 (P<0.001) (Figure 1C-E). The ELISA for IL-1β demonstrated similar results (P<0.001) (Figure 1F). Collectively, these findings suggested that calpain activity and pyroptosis were upregulated in the pathogenesis of VMC.

Calpain inhibition reduces the severity of CVB3-induced myocarditis

Transgenic mice overexpressing calpastatin, the endogenous inhibitor of calpain, were used to investigate the roles of calpain in VMC. As we expected, CVB3-infected transgenic mice had much lower calpain activity than CVB3-infected wild-type mice (P<0.05) (Figure 2E and 2F).

The Kaplan-Meier curve showed that CVB3-infected transgenic mice had a higher survival rate than CVB3-infected wild-type mice (Figure

Figure 1. Calpain activity and pyroptosis was up-regulated in hearts of CVB3-induced VMC mice. A. The histopathological changes of heart tissues were examined using H&E staining. Arrows show representative positive area (×200). B. Quantitive analysis of pathological score. C. The levels of α-fodrin (calpain cleavage product) and GSDMD in myocardium were measured by western blot. D, E. Quantitative analysis of α-fodrin and GSDMD were shown in bar graphs, respectively. F. Peripheral IL-1β levels were assessed by ELISA assays. Data were shown as the mean ± SEM from 3 to 6 independent experiments. *P<0.001 vs. Con.
Figure 2. Calpain inhibition ameliorated the severity of CVB3-induced VMC. A. Survival curve of the mouse in different groups. B. The histopathological changes of heart tissues were examined using H&E staining. Arrows show representative positive area (×200). C. Quantitative analysis of pathological score. D. ELISA assays of peripheral myocardial damage markers of cTnl. E. The protein levels of α-fodrin and VP-1 (CVB3 capsid protein) in heart tissue were determined by western blot. F. Quantification of α-fodrin levels relative to β-actin. G. Quantification of VP-1 levels relative to β-actin. H. Virus load titration in heart tissue. I. The mRNA levels of TGF-β1 in heart tissues were detected by Realtime-PCR. J. Fibrosis deposition in myocardium was evaluated by Masson staining. Arrows indicated the positive area (×100). K. Quantitative analysis of fibrotic volume. Data were shown as the mean ± SEM from 3 to 6 independent experiments. *P<0.01 vs. Con.; #P<0.05 vs. Virus.
The effects of calpain on pyroptosis pathway in viral myocarditis

Furthermore, H&E staining demonstrated that pathological scores were significantly reduced in the heart tissues of CVB3-infected transgenic mice (P<0.01) (Figure 2B and 2C). Similarly, serum cardiac troponin I (cTnI), a sensitive indicator of myocardial necrosis, was decreased in CVB3-infected transgenic mice (Figure 2D).

Considering that CVB3 replication is important in the pathogenesis of VMC, we further assessed the effect of calpain activity on CVB3 replication. We observed a dramatic reduction in CVB3 capsid protein VP1 and viral titer in CVB3-infected transgenic mice (P<0.05) (Figure 2E, 2G, 2H).

Additionally, myocardial fibrosis is the key pathological mechanism affecting the prognosis of myocarditis. Thus, we further evaluated the role of calpain in CVB3-induced myocardial fibrosis. CVB3-infected transgenic mice had markedly fewer areas of fibrotic tissue and significantly lower levels of TGF-β1 than CVB3-infected wild-type mice (P<0.05) (Figure 2I-K).

Taken together, these results confirmed the protective effect of calpain inhibition on CVB3-induced myocarditis.

Inhibition of calpain suppresses the NLRP3 inflammasome-mediated pyroptosis pathway in CVB3-induced myocarditis

To explore the effects of calpain on pyroptosis pathways, we measured the mRNA and protein levels of NLRP3, ASC and AIM2 in the myocardium. CVB3-infected transgenic mice had lower levels of myocardial NLRP3 and ASC (P<0.05) (Figure 3). However, after CVB3 infection, the
expression of AIM2 in transgenic and wild-type mice did not differ significantly. Moreover, the activation of caspase-1 and caspase-11 was evaluated by western blot and immunohistochemical analyses. The levels of cleaved caspase-1 and cleaved caspase-11 in the myocardium of CVB3-infected transgenic mice were significantly reduced compared with those in CVB3-infected wild-type mice \( (P < 0.05) \) (Figure 4).

In addition, we measured the expression of GSDMD by immunohistochemical analysis. Figure 5A shows that CVB3-infected transgenic mice had lower expression of GSDMD in the myocardium. Furthermore, the levels of GSDMD p30, a subunit of GSDMD that induces pyroptosis, HMGB1 and IL-1\( \beta \) were evaluated by western blot analysis. We found that CVB3-infected transgenic mice had lower expression of GSDMD p30, HMGB1 and IL-1\( \beta \) in heart tissues \( (P < 0.05) \) (Figure 5B-E). The ELISAs for peripheral IL-1\( \beta \) and HMGB1 showed similar results \( (P < 0.05) \) (Figure 5F and 5G).

Collectively, these data indicated that in the model of CVB3-induced myocarditis, calpain inhibition had a suppressive effect on the canonical NLRP3 inflammasome/caspase-1 and noncanonical caspase-11-mediated pyroptosis pathways.

**Discussion**

The present study identified the role of calpain in CVB3-induced myocarditis. We found that calpain was activated in the hearts of CVB3-infected mice, accompanied by an increase in pyroptosis. Moreover, inhibition of calpain markedly suppressed the activation of the NLRP3 inflammasome, caspase-1 and caspase-11; reduced pyroptosis; attenuated cardiac inflammation; alleviated cardiomyocyte injury; prevented cardiac fibrosis; and improved survival. Based on these results, we concluded that inhibition of calpain attenuates CVB3-induced myocarditis by suppressing the canonical NLRP3 inflammasome/caspase-1 and noncanonical caspase-11-mediated pyroptosis pathways.
Calpain has been implicated in a variety of diseases and conditions [22, 23, 28], such as endotoxemia, diabetes, and glomerulonephritis. Numerous studies indicate that targeted inhibition of calpain is a promising strategy for treating these diseases and conditions. To explore the effects of calpain on CVB3-induced myocarditis, in our previous study [19], we used the synthetic calpain inhibitor ALLN and found that it had strong inhibitory effects on CVB3 replication in H9c2 cells in vitro. Unlike synthetic inhibitors, calpastatin is an endogenous inhibitor of calpain that specifically suppresses calpain activation [18]. Transgenic mice overexpressing calpastatin, which were introduced in the present study, have normal baseline calpain activity and exhibit a decrease in calpain activity in models of sepsis, diabetes, ischemia and glomerulonephritis [23, 28, 29]. As expected, our results demonstrated that calpastatin overexpression improved survival, reduced pathological scores and myocardial injury and prevented cardiac fibrosis, implying that inhibition of calpain reduced the severity of CVB3-induced myocarditis.

Calpain participates in the regulation of various pathological processes [18], such as apoptosis and autophagy, inflammation and fibrosis. Cell death and inflammation are the main characteristics of VMC. As described in previous studies [20, 30], calpain significantly increases apoptotic myocardial cell death and raises the production of IFN-γ and IL-17 in the local myocardium of VMC mice; these effects account for the mechanisms underlying calpain-mediated VMC. Pyroptosis is a form of inflammatory programmed cell death that requires cleavage and activation of the pore-forming effector protein GSDMD by inflammatory caspases, and the subsequent secretion of IL-1β, IL-18, HMGB1, and other events. A recent investigation reported that cathepsin B, a cysteine proteolytic enzyme, aggravates CVB3-induced myocarditis.

Figure 5. Calpain inhibition reduced GSDMD activation and down-regulated the levels of IL-1β and HMGB1 in CVB3-induced VMC. A. The representative images of immunohistochemical analysis of GSDMD. B. The levels of GSDMD p30, IL-1β and HMGB1 were assessed by western blot. C-E. Quantitative analysis of GSDMD p30, IL-1β and HMGB1 were shown in bar graphs, respectively. F, G. ELISA assays of peripheral IL-1β and HMGB1. Data were shown as the mean ± SEM from 3 to 6 independent experiments. *P<0.01 vs. Con.; #P<0.05 vs. Virus.
through promoting pyroptosis [15]. In addition, calpain drives pyroptotic vimentin cleavage, intermediate filament loss, and cell rupture during pyroptosis [31], indicating that calpain is also involved in the process of pyroptosis. In the present study, we showed that CVB3 infection significantly increased GSDMD p30 expression in heart tissues. Subsequently, we observed increased levels of IL-1β and HMGB1 in heart tissues and serum. However, calpastatin overexpression markedly decreased the levels of GSDMD p30, HMGB1 and IL-1β in mice with VMC. Our data suggested that inhibition of calpain exerted obvious suppressive effects on the pyroptosis pathway in the pathogenesis of VMC. As demonstrated in previous in vivo and in vitro investigations [11, 32, 33], the release of IL-1β and HMGB1 results in widespread inflammation and myocardial fibrosis with reduced cardiac function. The levels of IL-1β and HMGB1 strongly correlate with the severity of CVB3-induced myocarditis, and blockade of IL-1β and HMGB1 is considered an effective cardioprotective approach [34, 35]. The combination of these observations and our findings indicated that inhibition of calpain attenuated VMC via suppressing pyroptosis pathways.

Increasing evidence shows that pyroptosis is tightly regulated. Previous investigations [3, 4] have demonstrated that canonical caspase-1-dependent pyroptosis requires the activation of various inflammasomes, such as the NLRP3 and AIM2 inflammasomes. The NLRP3 inflammasome is reported to be activated by many pathogens, including viruses, and AIM2 specifically recognizes cytosolic dsDNA [5, 6]. In the present study, we found upregulated expression of NLRP3, AIM2, ASC, and caspase-1 p10 in the hearts of mice with CVB3-induced myocarditis, suggesting that CVB3 could activate NLRP3 and AIM2 and, consequently, the downstream caspase-1 pathway. A recent study demonstrated that calpain inhibition protects the kidney against inflaming, which is closely associated with NLRP3 inflammasome activation [36]. Moreover, calpain has been reported to play a pivotal role in the regulation of NLRP3 activation and IL-1β secretion in human macrophages during ATP exposure [37]. These data indicate that calpain participates in the regulation of NLRP3 inflammasome activation. In the present study, we explored the roles of calpain in NLRP3 inflammasome and caspase-1 activation during CVB3 infection. Our data showed that calpastatin overexpression not only reduced the levels of NLRP3 and ASC but also suppressed the expression of caspase-1 p10 in the hearts of mice with CVB3-induced myocarditis, supporting the hypothesis that inhibition of calpain suppresses the assembly of the NLRP3 inflammasome and the activation of caspase-1 in the murine model of VMC. In addition, we assessed the effects of calpain on AIM2. Regrettably, we observed no significant differences in AIM2 protein levels between transgenic mice and wild-type mice post CVB3 infection, indicating that calpain was not involved in the regulation of CVB3-induced AIM2 activation in the model of VMC. However, studies have reported the AIM2-dependent involvement of calpain in the release of IL-1α by plasmacytoid dendritic cells from lung cancer patients [38], suggesting that the effects of calpain on AIM2 may differ in various models. Additionally, several previous investigations [39] revealed that the noncanonical caspase-11 pyroptosis pathway is activated mainly by lipopolysaccharides. In our present study, we found that caspase-11 was activated upon CVB3 infection. Moreover, calpastatin overexpression inhibited caspase-11 activation, indicating that inhibition of calpain could also suppress the noncanonical caspase-11-mediated pyroptosis pathway in CVB3-induced myocarditis.

In summary, our study demonstrates that inhibition of calpain attenuates CVB3-induced myocarditis by downregulating the canonical NLRP3 inflammasome/caspase-1 and noncanonical pyroptosis pathways, which might provide a therapeutic target for VMC.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81600294, 31570904 and 81772109).

Disclosure of conflict of interest

None.

Address correspondence to: Ruizhen Chen, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Shanghai Medical College of Fudan University, No. 180, Fenglin Road, Xuhui District, Shanghai 200032, China. Tel: +86-21-64041990; Fax: +86-21-64041990; E-mail: chen.ruizhen@zs-hospital.sh.cn
The effects of calpain on pyroptosis pathway in viral myocarditis

References


