RIP3/CaMK II-Mediated Cardiomyocyte Necroptosis Plays a Key Role in Coxsackievirus B3-Induced Acute Severe Myocarditis

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Research Article

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Abstract

Recent studies have shown that RIP3/CaMK II plays a key role in myocardial ischemia-reperfusion injury; however, the role of RIP3/CaMK II-mediated cardiomyocyte necroptosis in CVB3-induced severe myocarditis has remained largely unknown. In this study, we found that KN-93(CaMK II–specific inhibitor) significantly reduced myocardial cell necrosis, improved cardiac function, and an improved survival curve. To further understand the mechanisms behind the regulation of necroptosis by RIP3/CaMK II, we added Ti(reactive oxygen species–specific inhibitor) in the model using the double-antibody sandwich immunoassay and BCA methods. After adding Ti, H2O2 content in the CVB3+KN-93+Ti group was slightly lower than that in the CVB3+KN-93 group. However, there was no obvious improvement of cardiac function or survival curve. Collectively, the RIP3/CaMK II pathway plays a significant role in cardiomyocyte death induced by viral infection. KN-93 blocks this pathway and may serve as a new therapeutic option for the treatment of severe viral myocarditis. The mechanism may occur through the direct inhibition of CaMK II and the indirect inhibition of reactive oxygen species.

Introduction

Studies have shown that programmed necrosis (necroptosis) triggered by inflammatory cytokines, such as TNF–α, is a variety of regulatory cell necrosis that is initiated by death receptors and ligands and mediated by the activation of receptor interacting proteins (RIPs). RIPs, as important signaling molecules, initiate and regulate the stress response of cells and determine whether their fate is apoptosis, necrosis, or survival. RIP1 and RIP3, two RIP proteins, play a key role in the programmed necrosis pathway[1]. Recent evidence shows that regulated programmed necrosis plays an important role in the occurrence and development of cardiomyocyte necrosis, and a variety of signal transduction pathways are involved, such as the RIP1-RIP3-MLKL pathway and RIP3 CaMK II-MPTP pathway. However, the underlying mechanism remains unclear [2].

Viral myocarditis is mainly characterized by myocardial degeneration and necrosis, as well as a series of events caused by oxidative stress. Myocardial cells have almost no ability to repair, regenerate, and proliferate. Progressive necrosis of cardiomyocytes and dysfunction of cardiac contraction or relaxation are important factors affecting the prognosis of viral myocarditis. Previous studies have suggested that cardiomyocyte necrosis is an uncontrollable and passive process. Our study shows that controllable programmed necrosis is the main mode of cardiomyocyte necrosis in viral myocarditis, and RIP1/RIP3 necrotic body-mediated programmed necrosis plays an important role in the development of viral myocarditis[3]. Some studies have shown that [4]RIP3 plays a key role in myocardial ischemia-reperfusion injury by mediating the phosphorylation of Ca2/calmodulin-dependent protein kinase II (CaMK II) and inhibiting reactive oxygen species (ROS) to reduce the programmed necrosis of cardiomyocytes, a process that is independent of the RIP1-RIP3–MLKL pathway. The aim of this study was to construct a mouse model of acute severe viral myocarditis induced by Coxsackievirus B3 (CVB3), and to explore the role of RIP3/CaMK II–mediated programmed cardiomyocyte necrosis in severe viral

myocarditis, so as to provide a new theoretical basis and therapeutic target for its prevention and treatment.

Materials And Methods

Animal

All procedures, experiments and animal care were approved by the Institutional Animal Care and Use Committees of Three Gorges University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. A total of 60 male, 4-week-old Balb/c mice (SPF grade) were randomly divided into four groups as follows: normal control group(n = 15); CVB3 group(n = 15); CVB3 + KN-93 (CaMK Ⅱ specific inhibitor) group(n = 15); and CVB3 + KN-93 + Ti (reactive oxygen species specific inhibitor) group(n = 15). The license number was scxk (E) 2008-0004 and the mice average weight was 16–20 g.

Main reagents

The CVB3 Nancy virus and cell lines were introduced from the United States and donated by the Institute of Medicine, School of Medicine, Shanghai Jiaotong University. CaMK II antibody was purchased from CST company of the United States; KN-93, a specific inhibitor of CaMK II, was purchased from Merck; Titanium, a specific inhibitor of ROS, was purchased from Sigma; a hydrogen peroxide detection kit was purchased from Abcam; and a BCA protein concentration determination kit was purchased from Jiangsu Biyuntian Biotechnology.

Main methods

Establishment of CVB3 myocarditis BALB/c mouse model and experimental grouping

Male BALB/c mice were selected at 4 weeks of age. The model group was inoculated with 0.2 ml of CVB3 at a dose of 1000 TCID50 to create an animal model of acute severe viral myocarditis[5]; the normal control group was intraperitoneally injected with 0.2 ml of normal saline. Each mouse in the drug treatment group was injected with different drugs (KN-93 10 μ mol/kg; Ti 20 μ mol/kg, drug dissolved in 0.1% DMSO) by tail vein 4 h after intraperitoneal injection of CVB3 0.2 ml, while mice in the drug control group were injected with 0.1% DMSO according to different drug doses. The above mice except normal control group were treated with intraperitoneal and intravenous injection of drugs for 7 days. Serum cTnI levels were measured on day 7, and cardiac function was assessed by echocardiography at week 1, week 2, and week 4. The 30-day survival rate was determined. The main criteria for the success of the model of acute severe viral myocarditis were: on the seventh day of the model, troponin I increased significantly, cardiac function decreased significantly, and the survival rate was less than 50%[5].

Echocardiography assay

Echocardiography was performed, including left ventricular ejection fraction (EF) and systolic left ventricular internal diameter (LVID, mm) were used to evaluate the induction and degree of severe viral myocarditis. Fractional shortening (FS) was calculated after 7 days of respective treatment using the following equation: FS% = (LVEDD−LVESD)/ LVEDD×100).

Elisa assay

A volume of 3 ml of venous blood was collected from each animal in all groups. Serum concentrations of cardiac troponin I (cTnI) were measured using a Hitachi Automatic Biochemical Analyzer according to the manufacturer's instructions in the laboratory of the First Clinical Medical College of Three Gorges University.

Western blot

The expression of CaMK II protein was detected by Western blot. Myocardial tissues were pulverized and lysed in ice-cold SDS buffer. Lysates were sonicated and protein concentrations were determined using a BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA). Prior to loading, 2.5% 2-mercaptoethanol and 0.0125% bromophenol blue were added and lysates were boiled for 5 min. The lysed proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) by wet transfer at 100V at 4°C for 1h. After that, the film was placed in blocking solution and sealed at 37°C for 1 h. The one-antibody was kept overnight at 4°C. The membrane was incubated with anti-IgG antibody labeled with alkaline phosphatase, and gently shaken at room temperature for 1 h. The immunoblots were visualized using an enhanced chemiluminesence detection kit (Pierce Chemical). The relative intensity of each band was determined by Quantity One Software (USA Bio-Rad Lab, Inc). To avoid inter-assay variations, values were normalized to the control value in each experiment.

Detection of hydrogen peroxide content in myocardial tissue

Immediately after the mice were killed, about 0.1 g of myocardial tissue was taken from each group. After the sample was frozen by liquid nitrogen, it was crushed at 4°C and ground with precooled PBS to prepare tissue homogenate. Then, the total protein content was determined by the BCA method. Before the experiment, the standard curve of hydrogen peroxide was established according to the instructions of the kit. The myocardial tissue homogenate (total protein 3 ug) was taken. After adding hydrogen peroxide detection diluent, the operation was conducted according to the instructions of the kit. The absorbance value was detected at a wavelength of 570 nm, and the concentration of hydrogen peroxide was determined by the standard curve method.

Statistical analysis

Results are expressed as mean ± SD and were analyzed by ANOVA. Comparisons of data between groups were made using a 2-sample t-test, assuming equal variances, otherwise underwent Kolmogorov-Smirnov and homogeneity of variance testing. Comparisons between groups were performed by single factor

analysis of variance (SNK). The difference was statistically significant when $P < 0.05$. All data analyses were performed using the SPSS 13.0 software package.

Results

KN-93 significantly improved cardiac function and survival curves of mice with severe viral myocarditis by inhibiting CaMK II

Compared with the normal control group, the level of serum cTnI in the CVB3 group was significantly increased on the 7th day after viral infection ($P < 0.01$), indicating that myocardial cells were seriously damaged. The survival rate of the CVB3 group was about 40%, suggesting that the model was of severe viral myocarditis. The results of western blot analysis showed that the CaMK II protein level decreased significantly after adding KN-93 ($P < 0.01$), indicating that KN-93 had a significant inhibitory effect on CaMK II. Also, on the 7th day after viral infection, the serum cTnI level of the CVB3 + KN-93 group was significantly lower than that of the CVB3 group ($P < 0.01$), suggesting that KN-93 had a significant myocardial protective effect. Echocardiography results of mice observed for 1 week, 2 weeks, and 4 weeks showed no significant effects on CaMK II in terms of EF, FS, or LVID. The CVB3 + KN-93 group was significantly better than the CVB3 group ($P < 0.01$), and the CVB3 + KN-93 group's 30-day survival curve was also significantly improved ($P < 0.01$), as shown in Fig. 1.

ROS inhibitor Ti effects cardiac function and survival curve of mice with severe viral myocarditis by reducing H_2O_2 content

The results showed that the H_2O_2 content in the heart tissue of the CVB3 + KN-93 group was significantly lower than that of the CVB3 group ($P < 0.01$). After adding ROS inhibitor Ti, H_2O_2 content in the CVB3 + KN-93 + Ti group was slightly lower than that in the CVB3 + KN-93 group ($P < 0.05$), but cTnI was not significantly decreased ($P > 0.05$). Cardiac ultrasound examination at 2 and 4 weeks showed that the cardiac function and survival curves of the CVB3 + KN-93 + Ti group were not significantly improved ($P > 0.05$), as shown in Fig. 2.

Discussion

Programmed necrosis plays an important role in the pathophysiological processes of many diseases, especially in acute inflammation, ischemic injury, and virus infection[6]. It is well known that the pathogenesis of viral myocarditis occurs mainly through sustained damage to myocardial cells and the immune inflammatory response induced by repeated viral infection. Caspase is the key protein that initiates the apoptosis pathway. ATP is not sufficient to maintain the function of caspase in a pathological state, which leads to a disordered apoptosis pathway, and the cells are subsequently prone to programmed necrosis [7]. Our previously study shows that [3] programmed necrosis is the main mode of myocardial cell death in viral myocarditis, Our data indicated that RIP1 was highly expressed in the model and the Nec-1 dramatically reduced the myocardial damage by downregulating the expression of

RIP1. However, it can only reduce cardiomyocyte necrosis and improve the survival rate of mice to a certain extent.. In the latestmyocardial ischemia-reperfusion model, RIP3 plays an important role in opening the mitochondrial permeability transition pore (mPTP) through the phosphorylation of CaMK II, and mPTP functions in the programmed necrosis of cardiomyocytes induced by calcium overload. KN-93, a specific inhibitor of CaMK II, has been found to have clear myocardial protective effects and to be safe and reliable[8]. In this study, we found that the CaMK II protein level decreased significantly after the administration of KN-93, a specific inhibitor of CaMK II. This indicates that the programmed necrosis of cardiomyocytes was significantly inhibited. Moreover, this inhibition significantly reduced cardiomyocyte necrosis. We observed that the reduction of cardiomyocyte necrosis directly led to obvious improvement in cardiac structure and function in mice, as shown by continuous echocardiography for 1–4 weeks. Ultimately,, the 30 day survival rate was significantly improved. In conclusion, these data suggest that RIP3 plays an important role in severe viral myocarditis through CaMK II-mediated programmed necrosis, but the underlying mechanism requires further exploration.

The role of oxidative stress in the occurrence and development of cell necrosis has been well established [9]. Studies have shown that RIP3 not only mediates programmed necrosis through CaMK II-mediated phosphorylation and autophosphorylation, but also leads to cardiomyocyte necrosis by activating ROS [4]. In this study, we found that the H_2O_2 level of the KN-93 group decreased after adding Ti, but there was no significant reduction of myocardial cell necrosis, nor did it improve the cardiac function and 30 day survival curve of mice. These data indicate that the slight decrease of ROS after adding Ti does not bring about an improved prognosis. However, further studies found that even without Ti inhibition, H_2O_2 level in the CVB3 + KN-93 group was significantly lower than that in the CVB3 group, suggesting that KN-93 could significantly reduce the occurrence of ROS by specifically inhibiting CaMK II itself. This also indicates that KN-93 may have largely inhibited ROS in mice in this model. These experimental data confirmed that KN-93 not only specifically inhibited CaMK II and blocked RIP3-mediated programmed necrosis, but also reduced cardiomyocyte necrosis by inhibiting ROS.

These results preliminarily suggest that RIP3/CaMK II-mediated programmed necrosis plays an important role in CVB3-induced severe viral myocarditis in mice. KN-93, an inhibitor of CaMK II, can significantly reduce myocardial cell damage and improve cardiac function and survival curves by blocking RIP3 signal transduction. The mechanism may be achieved by inhibiting CaMK II phosphorylation and ROS. This also suggests that the RIP3/CaMK II pathway plays an important role in this model, but the specific signal transduction mechanism requires further study.

Declarations

Author contributions ZF and WT designed the research study. WT and ZH performed the research. DJW, TL, YJ and LS analyzed the data. ZF wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The study does not involve any human subjects, samples or cell lines.

Ethics approval and consent to participate All procedures, experiments and animal care were approved by the Institutional Animal Care and Use Committees of Three Gorges University and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

Consent for publication All authors agreed on the manuscript.

Conflict of interest The authors declare that they have no conficts of interest with the publication of the manuscript.

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Figures

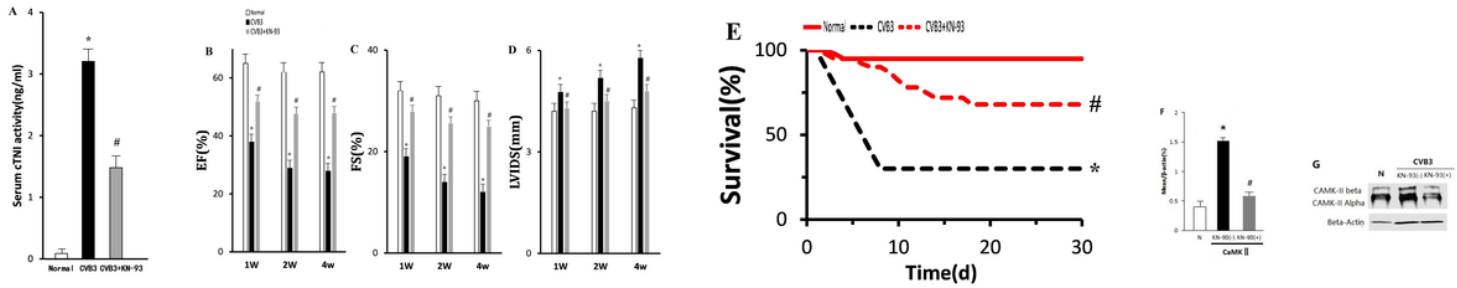

Figure 1

Protective effect of KN-93 on mice with severe viral myocarditis. A: Level of serum cTNI in the normal group, CVB3 infection group, and CVB3+KN-93 group. B–D: Mean values of ejection fraction (EF), fractional shortening (FS), and systolic left ventricle internal diameter (LVID) in the normal group, CVB3 infection group, and CVB3+KN-93 group at 1, 2, and 4 weeks. E: Kaplan-Meier survival curves of the normal group, CVB3 infection group, and CVB3+KN-93 group. F, G: Western blot results in the CVB3+KN-93 group. Mean±SD. n = 5. *P < 0.01 vs. normal group; P < 0.01 vs. CVB3 group.

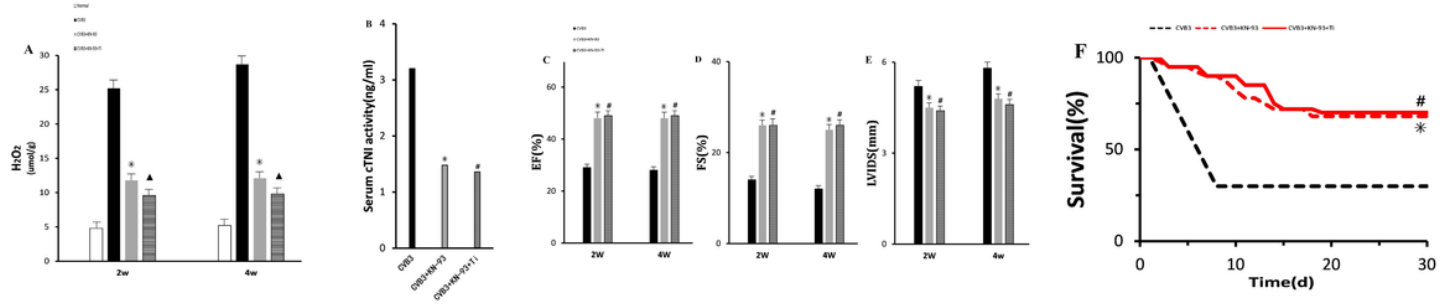

Figure 2

Protective effect of Ti on mice with severe viral myocarditis. A: Content of H2O2 was measured in the normal group, CVB3 group, CVB3+KN-93 group, and CVB3+KN-93+Ti group. B: Serum levels of cTNI in the CVB3 group, CVB3+KN-93 group, and CVB3+KN-93+Ti group. C–E: Mean values of ejection fraction (EF), fractional shortening (FS), and systolic left ventricle internal diameter (LVID) in the CVB3 group, CVB3+KN-93 group, and CVB3+KN-93+Ti group at 2 and 4 weeks. F: Kaplan-Meier survival curves of the CVB3 group, CVB3+KN-93 group, and CVB3+KN-93+Ti group. Mean±SD. n = 5. *P < 0.01 vs. CVB3 group; ▯ P > 0.05 vs. CVB3+KN-93 group; ▲P < 0.05 vs. CVB3+KN-93 group.