Parecoxib attenuates inflammation injury in septic H9c2 cells by regulating the MAPK signaling pathway

XIN QIAN, SHIJUAN XIONG, QI CHEN, JIAXING ZHANG and JUAN XIE

Department of Pharmacy, Guizhou Provincial People's Hospital, Guiyang, Guizhou 550000, P.R. China

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Abstract. Parecoxib, a non-steroidal anti-inflammatory drug, has been reported to possess protective effects against sepsis. However, its detailed role and underlying mechanisms in septic cardiomyopathy remain unclear. Therefore, the goal of the present study was to clarify the function and to investigate the mechanisms of parecoxib in lipopolysaccharide (LPS)-treated H9c2 rat cardiomyocytes. TNF-α, IL-1β and IL-6 expression levels in parecoxib-treated H9c2 cells stimulated with LPS were assessed using ELISA. Parecoxib-treated H9c2 cells stimulated with LPS were tested for viability using the Cell Counting Kit-8 assay. Western blotting analysis and 5-ethynyl-2'-deoxyuridine were used to evaluate cell proliferation. Apoptosis was assessed using TUNEL and western blotting. To assess the protein expression of the MAPK signaling pathway, western blotting was performed. The data showed that parecoxib significantly and dose-dependently reduced the inflammatory responses of LPS-treated H9c2 cells. Parecoxib also significantly and dose-dependently increased the proliferation and inhibited the apoptosis of LPS-treated H9c2 cells. In addition, parecoxib significantly suppressed the activation of the MAPK (p38, JNK and ERK) signaling pathway. The current study indicated that parecoxib could be a viable therapeutic option for septic cardiomyopathy.

Introduction

Sepsis, an infection-caused systemic inflammatory response syndrome, is a common, clinically acute and severe disease (1). Millions of patients worldwide suffer from sepsis every year, and the mortality rate ranges from 30 to 70% (2). The pathogenesis of sepsis is very complex. At present, it is hypothesized that certain infection factors rapidly activate the collective non-specific immune system and release a great number of pro-inflammatory factors, such as TNF-α and IL-6, inducing the occurrence and development of an uncontrolled inflammatory reaction, which can cause multiple organ dysfunction (3-5).

The heart is an especially susceptible target organ of sepsis (6). A study has shown that 40-50% of patients with sepsis have organic myocardial damage in the early stage, manifesting as arrhythmia, hypotension and heart failure, and ~20% of patients also have concealed myocardial damage (7). The mechanisms of myocardial injury caused by sepsis are complex, including the involvement of inflammatory factors, the dysfunction of myocardial energy metabolism, the dysregulation of nitric oxide, the notably increased production of oxygen free radicals, increased apoptosis and excessive activation of the renin-angiotensis system (8-10). Among them, endotoxin, also known as lipopolysaccharide (LPS), activates the immune response of monocytes-macrophages and produces a large number of inflammatory factors, which play a key role in myocardial injury (11). TNF-α causes myocardial injury by activating sphingomyelinase and proteolytic enzyme, as well as inhibiting calcium influx (12). Injection of TNF-α in healthy individuals leads to decreased myocardial contractility and reversible ventricular enlargement (13). IL-6 and IL-1β cause myocardial injury by activating nitric oxide synthase and type 2 cyclooxygenase (COX) (14).

Non-steroidal anti-inflammatory drugs, which have been confirmed to alleviate postoperational pain, possess therapeutic actions, such as decreasing the activity of COX-1 and COX-2 (15). Parecoxib can effectively cross the blood-brain barrier and improve acute mild and moderate pancreatitis by functioning as a selective COX-2-specific inhibitor (16,17). In addition, parecoxib can upregulate IFN-γ levels in the plasma of brain tumors to further improve systemic T helper type 1 (Th1) immune responses (18). In addition, it also possesses analgesic effects, for example, by reducing IL-1β, IL-6 and TNF-α levels in the spinal cords of mice (19). The MAPK signaling pathway has been demonstrated to serve an important role in septic cardiomyopathy (20). Nevertheless, the impacts and mechanisms of parecoxib on septic cardiomyopathy remain unclear. Therefore, the present study focused on examining the effects and processes of parecoxib in LPS-treated H9c2 rat cardiomyocytes through a number of *in vitro* experiments. The findings indicated that parecoxib might represent a unique therapeutic target for the treatment of sepsis brought on by LPS.

Correspondence to: Dr Juan Xie, Department of Pharmacy, Guizhou Provincial People's Hospital, 83 Zhongshan East Road, Nanming, Guiyang, Guizhou 550000, P.R. China
E-mail: xiejuan945@sohu.com

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Materials and methods

Cell culture and treatment. To investigate the mechanism of sepsis-induced myocardial injury, H9c2 rat cardiomyocytes were selected for use in the present study (21,22). Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.) was used to cultivate H9c2 cells, which were supplied by the American Type Culture Collection, at 37˚C with 5% CO_2. Parecoxib (50, 100 and 200 μM, diluted with DMSO; ChemeGen) was administered to H9c2 cells at 37˚C for 30 min following stimulation with LPS (10 μg/ml; Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C (23). The groups were as follows: Control (H9c2 cells without LPS treatment), LPS (H9c2 cells with LPS treatment), LPS + parecoxib low (50 μM), LPS + parecoxib medium (100 μM) and LPS + parecoxib high (200 μM).

Cell counting kit (CCK)-8 assay. H9c2 cells from various groups (Control, LPS and LPS + parecoxib low/medium/high) were injected into 96-well plates ($5x10^3$ cells/well). The culture plate was placed in an incubator for preculture at 37˚C with 5% CO_2 for 48 h. A total of 10 μl of CCK-8 solution (MilliporeSigma) was added to each well. Then, the culture plate was incubated for a further 2 h. The Swiss-made Tecan Infinite M200 microplate reader was used to detect the absorbance (or optical density, OD) value at 490 nm.

5-Ethynyl-2'-deoxyuridine (EdU) assay. Cell proliferation capacity was measured using a Cell-Light EdU Apollo567 *In Vitro* Kit (Guangzhou RiboBio Co., Ltd.). H9c2 cells ($4x10^4$ cells/well) from different groups were inoculated into 24-well plates. The medium was removed by adding 20 μM of EdU working solution at 25˚C for 2 h. After being fixed with 4% paraformaldehyde at 25˚C for 15 min, DAPI (100 ng/ml; Sigma-Aldrich) was added to each well and the cells were incubated at 25˚C for 30 min in the dark. Images were observed using a fluorescence microscope (Nikon Corporation).

TUNEL assay. H9c2 cells from the different groups were cultivated for 48 h at 25˚C and fixed in 100% methanol at 25˚C for 2 h to test for apoptosis. Following the addition of 3% H_2O_2 for 10 min at 25˚C, the cells were then treated with equilibration buffer from the TUNEL cell apoptosis detection kit (cat. no. KGA704; Nanjing KeyGen Biotech Co., Ltd.) for 10 min at 25˚C, Terminal Transferase (cat. no. 3333566001; Roche Diagnostics GmbH) for 15 min at 25˚C and Anti-Digoxigenin-POD (poly) (cat. no. 11633716001; Roche Diagnostics GmbH) for 30 min at 25˚C. Cells were incubated with DAB (10 mg/ml; Sigma-Aldrich; Merck KGaA) at 25˚C for 30 min. Slices were rinsed several times with bi-distilled water and sealed using neutral balsam (cat. no. 10004160; Sinopharm Chemical Reagent Co., Ltd.) and imaged using a fluorescence microscope (Olympus Corporation). The cell numbers were counted in three random fields of view. The number of cells in the visual field was roughly 100-200.

ELISA. To detect cell inflammation, the expression levels of TNF-α, IL-1β and IL-6 in H9c2 cell culture supernatants were determined using Rat TNF-α (cat. no. EK-M27765), IL-1β (cat. no. EK-R36877) and IL-6 (cat. no. EK-R36902) ELISA kits (all purchased from EK-Bioscience Biotechnology Co., Ltd.), according to the manufacturer's protocol. A standard curve was constructed based on the concentration and OD value of the standard, and then the sample concentration was calculated using the standard curve equation.

Western blotting. H9c2 cell protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology), and a BCA kit was used for protein determination (Beyotime Institute of Biotechnology). Protein (45 μg per lane) was separated by SDS-PAGE (12%) and then transferred to PVDF membranes (MilliporeSigma). The membranes were blocked with 5% skim milk for 60 min at 4˚C. Subsequently, membranes were treated overnight with primary antibodies at 4˚C, namely anti-proliferating cell nuclear antigen (PCNA; 1:2,000 dilution; cat. no. 10205-2-AP; ProteinTech Group, Inc.), anti-Ki-67 (1:2,000 dilution; cat. no. 27309-1-AP; ProteinTech Group, Inc.), anti-Bax (1:2,000 dilution; cat. no. 50599-2-Ig; ProteinTech Group, Inc.), anti-Bcl-2 (1:2,000 dilution; cat. no. 12789-1-AP; ProteinTech Group, Inc.), anti-Cleaved Caspase-3 (1:1,000 dilution; cat. no. ab2302; Abcam), anti-Cleaved Caspase-9 (1:1,000 dilution; cat. no. ab2324; Abcam), anti-phosphorylated (p)-JNK (1:2,000 dilution; cat. no. 80024-1-RR; ProteinTech Group, Inc.), anti-JNK (1:2,000 dilution; cat. no. 24164-1-AP; ProteinTech Group, Inc.), anti-p-p38 (1:2,000 dilution; cat. no. 28796-1-AP; ProteinTech Group, Inc.), anti-p38 (1:2,000 dilution; cat. no. 14064-1-AP; ProteinTech Group, Inc.), anti-p-ERK (1:2,000 dilution; cat. no. 28733-1-AP; ProteinTech Group, Inc.), anti-ERK (1:2,000 dilution; cat. no. 16443-1-AP; ProteinTech Group, Inc.) and anti-β-actin (1:5,000 dilution; cat. no. 20536-1-AP; ProteinTech Group, Inc.). β-actin was regarded as the endogenous control. Then, the membranes were then treated for 1 h at room temperature with an HRP-labeled secondary antibody: Goat Anti-Rabbit IgG (H+L) HRP [1:5,000 dilution; cat. no. GAR007; MultiSciences (Lianke) Biotech Co., Ltd.]. Lastly, protein blots were visualized using an enhanced chemiluminescence kit (ECL; MilliporeSigma) and quantification was performed using ImageJ Software (National Institutes of Health; version 4.3).

Statistical analysis. Data was presented as the mean ± the standard deviation. All experiments were repeated three times. One-way ANOVA followed by Tukey's post hoc test was used to assess the differences between groups. Data was analyzed using GraphPad Prism (GraphPad Software, Inc.; version 5.0). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Parecoxib ameliorates inflammatory responses of LPS-induced H9c2 cells. H9c2 cells were pretreated with parecoxib at 37˚C for 24 h at doses of 50, 100 and 200 μM following 24-h LPS (10 μg/ml) stimulation. Then, ELISA was conducted to evaluate the impacts of parecoxib on inflammatory factor levels. As shown in Fig. 1, TNF-α, IL-1β and IL-6 protein levels were significantly upregulated in H9c2 cells induced by LPS, while parecoxib treatment significantly ameliorated the inflammatory responses of LPS-treated H9c2 cells. Parecoxib treatment notably ameliorated the inflammatory responses of LPS-treated H9c2 cells in a dose-dependent manner.

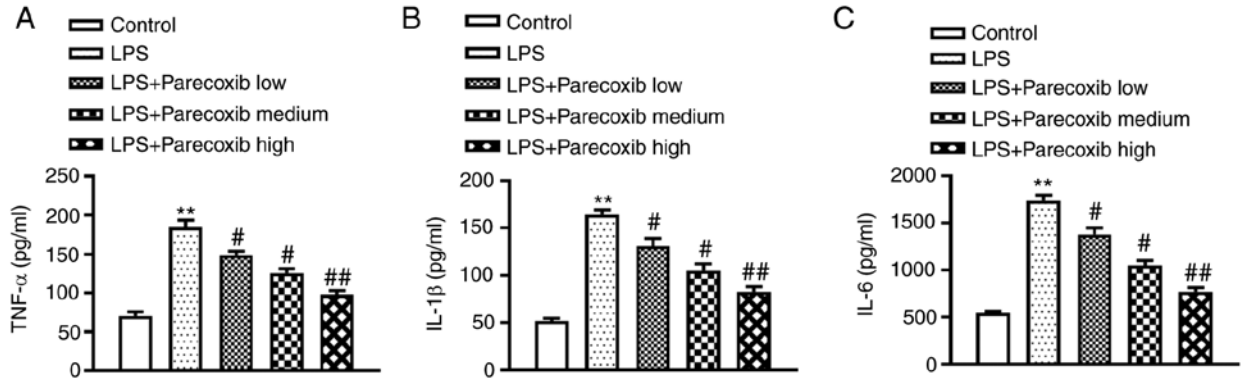

Figure 1. Inflammatory responses of H9c2 cells exposed to LPS are reduced by parecoxib. Expression levels of (A) TNF-α, (B) IL-1β and (C) IL-6 in parecoxib-treated H9c2 cells stimulated with LPS were assessed using ELISA. All results are shown as the mean ± SD. n=3. **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the LPS-only group. LPS, lipopolysaccharide.

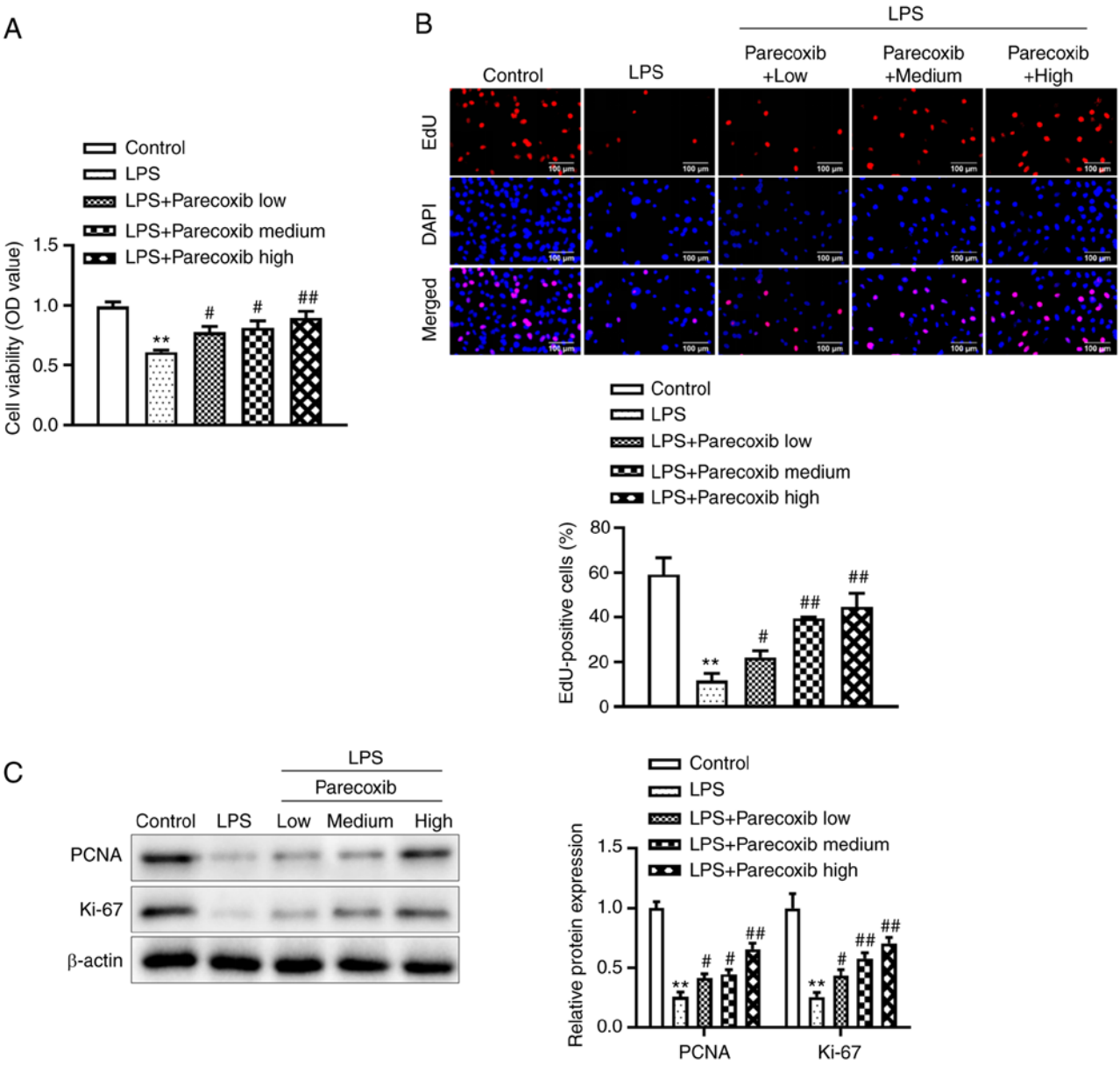

Figure 2. Proliferation of H9c2 cells treated with LPS is increased by parecoxib. (A) Using the Cell Counting Kit-8 assay, the viability of H9c2 cells stimulated by LPS and varying concentrations of parecoxib was evaluated. (B) EdU assay was used to measure the proliferation of H9c2 cells treated with parecoxib and stimulated with LPS (scale bar, 100 μm). (C) Using western blotting, the expression levels of proliferation-related proteins, such as PCNA and Ki-67, in H9c2 cells from various groups were assessed. All results are shown as the mean ± SD. n=3. **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the LPS-only group. LPS, lipopolysaccharide; EdU, 5-Ethynyl-2'-deoxyuridine; OD, optical density; PCNA, proliferating cell nuclear antigen.

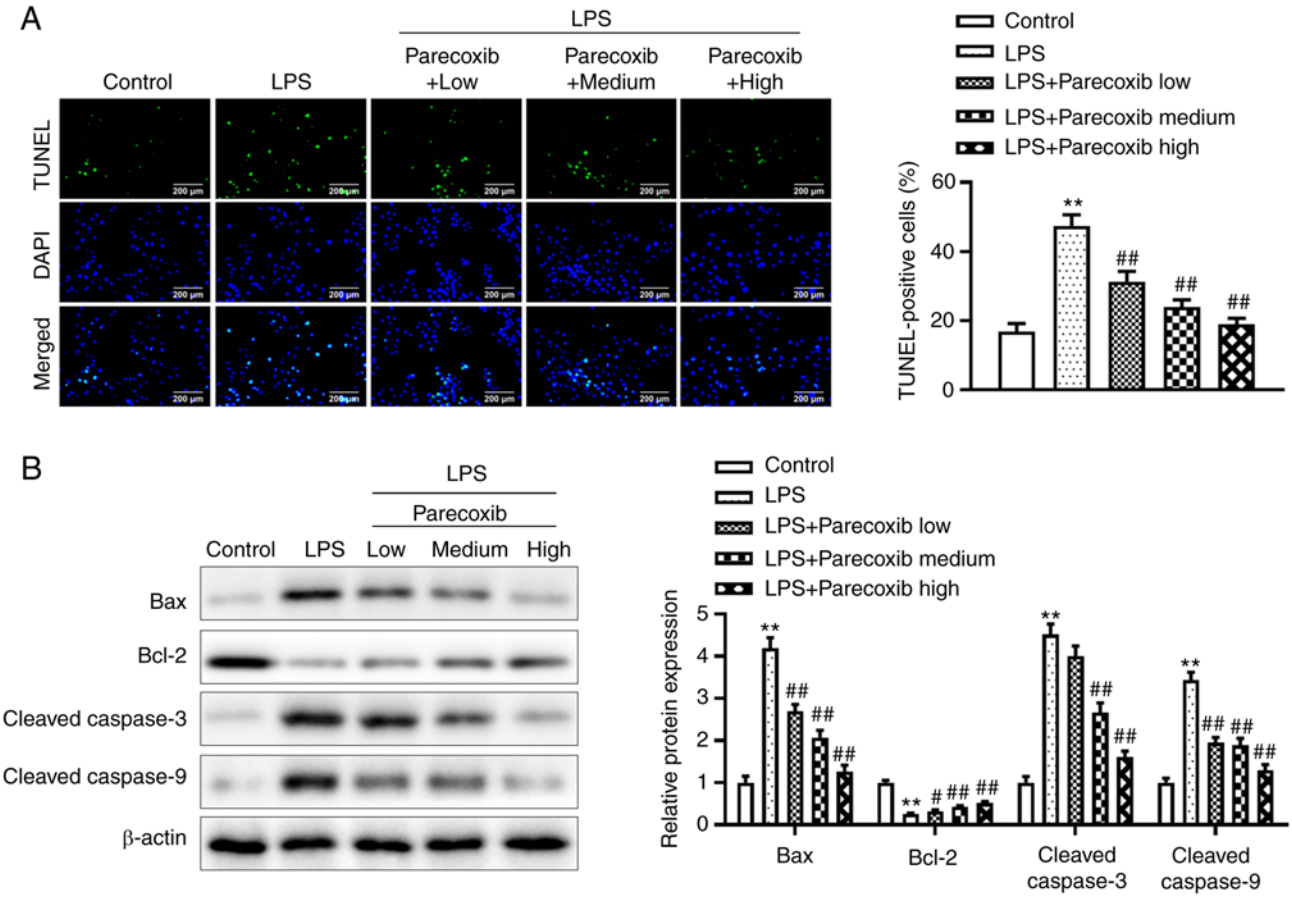

Figure 3. Parecoxib prevents LPS-treated H9c2 cells from undergoing apoptosis. (A) Parecoxib-treated H9c2 cells challenged with LPS had their apoptosis measured using a TUNEL assay (scale bar, 200 μm). (B) Using western blotting, the expression levels of apoptosis-related proteins, such as Bcl-2, Bax, Cleaved caspase-3 and Cleaved caspase-9, in H9c2 cells from various groups were assessed. All results are shown as the mean ± SD. n=3. **P<0.01 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the LPS-only group. LPS, lipopolysaccharide.

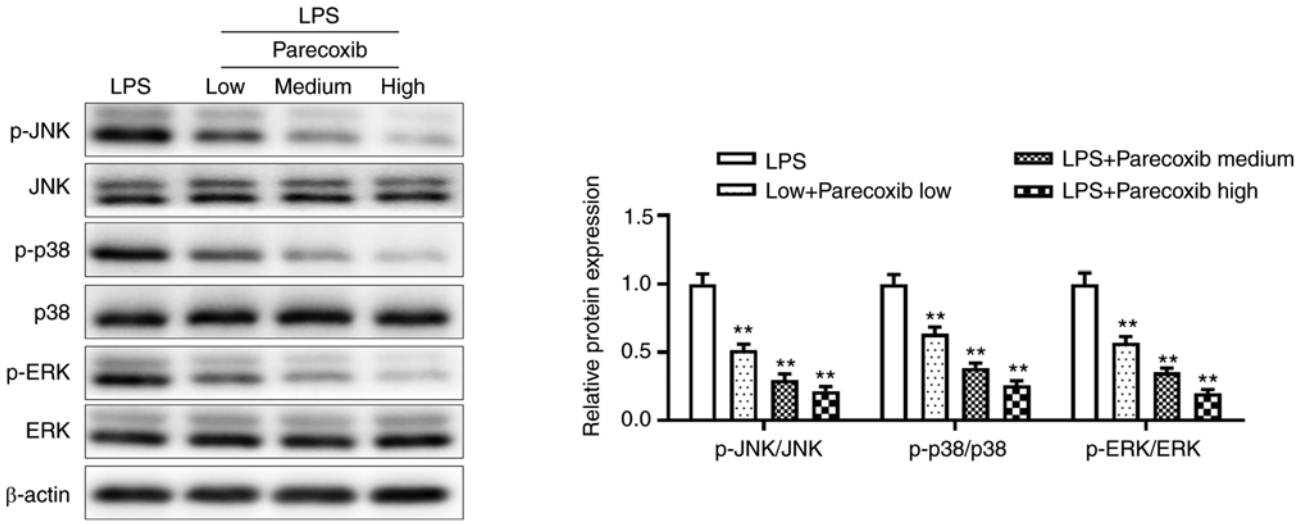

Figure 4. Parecoxib inhibits the production of MAPK signaling pathway proteins by LPS-treated H9c2 cells. To assess the protein expressions of the MAPK signaling pathway in parecoxib-treated H9c2 cells stimulated with LPS, western blotting was performed. All results are shown as the mean ± SD. n=3. **P<0.01 vs. the LPS-only group. LPS, lipopolysaccharide.

Parecoxib increases the proliferation of LPS-induced H9c2 cells. To improve understanding of the effects of parecoxib on the proliferation of H9c2 cells treated with LPS, a CCK-8 assay was performed. Fig. 2A illustrates that relative to the control group, LPS significantly decreased H9c2 cell viability, while parecoxib significantly reversed the LPS-induced decrease in H9c2 cell viability. Similarly, the EdU assay indicated that parecoxib reversed the inhibitory effects of LPS on H9c2 cell

proliferation, as shown in Fig. 2B. Furthermore, western blotting was conducted to assess the effects of parecoxib on proliferation-related protein levels. As expected, PCNA and Ki-67 protein levels were significantly reduced in the H9c2 cells treated with LPS, while parecoxib significantly reversed the inhibitory effects of LPS (Fig. 2C). These data suggested that parecoxib reversed the decrease in proliferation of H9c2 cells treated with LPS.

Parecoxib inhibits the apoptosis of LPS-induced H9c2 cells. The TUNEL assay was adopted for determining the effects of parecoxib on the apoptosis of H9c2 cells induced by LPS. As shown in Fig. 3A, relative to the control group, LPS significantly promoted the apoptosis of H9c2 cells. Notably, parecoxib treatment significantly reduced the promoting effects of LPS on the apoptosis of H9c2 cells in a dose-dependent manner. Additionally, western blotting was conducted to investigate apoptosis-related protein levels. As shown in Fig. 3B, LPS significantly increased Bax, Cleaved caspase-3 and Cleaved caspase-9 protein levels, and also decreased Bcl-2 protein levels, while parecoxib significantly reversed the effects of LPS. These results indicated that parecoxib inhibited the apoptosis of H9c2 cells induced by LPS.

Parecoxib suppresses the expression of the MAPK signaling pathway of LPS-treated H9c2 cells. To ascertain whether parecoxib protects against myocardial injury by regulating the MAPK signaling pathway, western blotting was performed. As shown in Fig. 4, parecoxib significantly inhibited the protein expression of the MAPK signaling pathway, compared with the LPS-only group. These results indicated that parecoxib suppressed the expression of the MAPK signaling pathway of H9c2 cells induced by LPS.

Discussion

As severe sepsis progresses, sepsis-induced cardiac dysfunction, one of its symptoms, is the primary factor contributing to the rise in mortality among patients with sepsis, and cardiovascular dysfunction leads to the decline of quality of life and a poor prognosis (24). In addition, the pathogenesis of sepsis remains unclear, and the inflammatory hypothesis was once considered to be the main explanation for the progression of sepsis (25). A study has found that TNF-α and ILs exert notable impacts on the inflammatory responses of the body, and that these inflammatory factors can cause myocardial injury via various mechanisms, such as activating sphingomyelinase on the cell membrane, inhibiting calcium transport inside and outside the cell membrane, and reducing the release of sarcoplasmic reticulum Ca^{2+} ions (26).

Moreover, TNF-α, IL-1β and IL-6 activate myocardial protein hydrolase, and subsequently degrade cardiac troponin and other key contractile proteins, resulting in myocardial cell contractility damage (27). It has been reported that continuous infusion of TNF-α interferes with the synthesis of cardiac proteins and eventually reduces the synthesis of myocardial fiber and sarcoplasmic proteins, which may be related to the effects of TNF-α on mRNA translation efficiency (28). In addition, an increasing number of studies have shown that pro-inflammatory factors and anti-inflammatory factors are involved in the progression of sepsis (29-31). When the dynamic balance of pro-inflammatory and anti-inflammatory reactions is disrupted, the body becomes injured (32). The present study demonstrated the protective effects of parecoxib on cardiomyopathy inflammatory responses. Parecoxib inhibited TNF-α, IL-1β and IL-6 levels in H9c2 cells induced by LPS. Additionally, Lancel *et al* (33) found that myocardial cell apoptosis directly leads to sepsis-induced myocardial dysfunction. As expected, parecoxib reduced LPS-treated H9c2 apoptosis in the present study.

The MAPK signaling pathway plays an important role in myocardial injury resulting from sepsis, by affecting the expression of numerous inflammatory genes (34). MAPKs are serine and threonine kinases that are activated in signaling cascades and subsequently move from the cytoplasm to the nucleus so as to regulate the activity of transcription factors, transmit signals into the nucleus and participate in cell differentiation, proliferation and death (35). LPS, the pathogenic factor of Gram-negative bacteria, activates the MAPK signaling pathway, increases the expression of MAPK2/3 in lymphocytes, promotes the rapid synthesis of TNF-α in ribosomes and enhances the cellular immunity mediated by Th1 cells, which further improves humoral immunity (36). Moreover, a study has shown that SB 203580 reduces the production of pro-inflammatory factors by inhibiting p38 MAPK activation in septic mice (37). In addition, several drugs can play a protective role in septic organs by inhibiting the MAPK signaling pathway (38). For example, *Carthamus tinctorius L.* improves H9c2 cardiomyocytes by suppressing JNK1/2-NFκB signaling (39). Gas6 has also been shown to attenuate TNF-α expression and apoptosis in LPS-induced H9c2 cells by inhibiting the MAPK pathway (40). Therefore, the development of MAPK-specific inhibitors is currently an area of research for the treatment of septic cardiomyopathy. The current study demonstrated that in LPS-induced H9c2 cells, parecoxib decreased the activation of the MAPK signaling pathway.

In conclusion, parecoxib protected against septic cardiomyopathy by promoting the proliferation and inhibiting the apoptosis and inflammation of LPS-induced H9c2 cells via the inactivation of MAPK. The present study therefore provides a possible treatment strategy for septic cardiomyopathy using parecoxib.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX conceived the study. XQ performed the experiments and drafted the manuscript. SX contributed to data analysis.

QC and JZ designed the methodology and performed data extraction. XQ and JX revised the manuscript and confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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