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USP13 reduces septic mediated cardiomyocyte oxidative stress and inflammation by inducing Nrf2

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KEYWORDS

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Abstract

Background: Sepsis is a common cardiovascular complication that can cause heart damage. The regulatory role of ubiquitin-specific peptidase 13 (USP13) on erythroid 2-related factor 2 (Nrf2) has been reported, but its regulatory role in septic cardiomyopathy remains unclear.

Methods: The Sprague Dawley (SD) rat model of septic myocardial injury was constructed by lipopolysaccharides (LPS). The serum lactate dehydrogenase (LDH) and creatine kinase (CK) levels were detected, the mRNA and protein expression levels of Nrf2 and USP13 in tissues were detected by real-time quantitative reverse transcription PCR (qRT-PCR) and western blot (WB), and the expression of USP13 at the treatment time of 3 h, 6 h, and 12 h was also detected. The cell viability and USP13, Nrf-2 and heme oxygenase-1 (HO-1) expression levels of H9C2-treated cells by LPS and the oxidative stress level and inflammatory response of H9C2 cells were detected by enzyme-linked immunosorbent assay (ELISA) and WB.

Results: The results showed that USP13 was downregulated in septic myocardial injury tissues, and the Nrf2 level was increased *in vitro* after the cells were treated with LPS. Overexpression of USP13 further induced Nrf2 to reduce apoptosis, oxidative stress, and expression of inflammatory factors.

Conclusion: In conclusion, this study demonstrated that USP13 was downregulated in septic myocardial injury tissues, and USP13 overexpression increased Nrf2 levels and reduced apoptosis. Further studies showed that USP13 reduced LPS-induced oxidative stress and inflammation by inducing Nrf2.

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Introduction

Sepsis is a syndrome in which patients develop a systemic inflammatory response due to a dysfunctional immune response. Acute myocardial injury occurs when the disease is severe,^{1,2} and refers to a broad-spectrum myocardial damage caused by sepsis. The causes of myocardial injury include not only serious infections but also possible adverse effects such as ischemia, which directly lead to a high mortality rate.³ Myocardial dysfunction in sepsis is known to increase mortality by 20-50%. In the past few years, myocardial dysfunction caused by sepsis has become the focus of research. Unfortunately, there are currently no effective treatments for septic cardiomyopathy. Therefore, there is an urgent need to study the biological mechanism of acute myocardial injury, and find key proteins or genes to facilitate clinical diagnosis and treatment and development of drugs.⁴

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor with antioxidant property. It has also been reported to modulate immune function.^{5,6} The literature shows that the low expression of Nrf2 in mice reduces the number of innate immune cells through a certain mechanism, which in turn makes the virus unable to bind to reduce the susceptibility.⁷ In addition, Nrf2 can also inhibit inflammatory responses, resist oxidation, and inhibit apoptosis of cells and plays a role in protecting the myocardium. The specific mechanism may be related to the regulation of miR-27a.^{8,9} A hallmark of sepsis is the hyperactivation of apoptotic processes, but the exact mechanism has not been determined. In recent years, many studies have found the relationship between apoptosis and inflammation. Inflammation affects mitochondrial function and mediates the increase in reactive oxygen species that can damage tissues, and Nrf2 plays a role in this mechanism.¹⁰⁻¹²

Ubiquitin-specific protease 13 (USP13) belongs to the deubiquitinase (DUB) superfamily and plays regulatory roles in a variety of diseases.¹³ For example, USP13 acts to counteract the actions of members of the Cbl family of E3 ubiquitin ligases by reducing dependence on peptidases and selectively stabilizing mutant EGFR, thereby improving drug efficacy in non-small cell lung cancer. A recent study showed that USP13 has anti-inflammatory properties that inhibit LPS-induced lung injury.¹⁴ USP13 ameliorates Nrf2 and reduces caspase-3 activation in fibroblast-like synovocytes.¹⁵ Given the close association of USP13 with Nrf2 and oxidative stress responses, we therefore believe that USP13 may also play a key role in the pathogenesis of sepsis-induced myocardial injury. This study aimed to elucidate how USP13 and Nrf2 regulate myocardial injury in sepsis.

Methods

Cells, animals, and treatment

H9C2 cells were acquired from Saibeikang Biotechnology Co., Ltd. (Shanghai, China). DMEM (procell, Shanghai, China) containing 10% FBS (Vica cell) and 1% penicillin-streptomycin (S110JV, Shanghai, China) was used to culture cells. Cells were cultured at 37°C in a cell incubator containing 5% CO₂.

Eight-weeks-old, male Sprague Dawley (SD) rats (Speifu, Chengdu, China) of 250 ± 20 g were housed individually at constant temperature and humidity 12/12 h light-dark cycle with free access to food and water at any time. Rats were acclimated to laboratory conditions for 2 weeks before the experiment. All studies were conducted in accordance with the "Guidelines for Animal Experimentation" drafted by the Ethics Committee of Laboratory Animal Ethics Committee of West China Hospital of Sichuan University (Approval no. 20220414001), and conformed to the ethical standards required by Chinese law and guidelines on experimental animals.

The H9C2 cells were seeded in a six-well plate and were divided into four groups. The method for establishing cell model had been described previously.¹⁶ Briefly, the H9C2 cells were treated with LPS (5 µg/mL) for 0, 3, 6, and 12 h. The USP13 overexpression plasmid was obtained from Beyotime (Nanjing, China). After the plasmid particles were transfected into cardiomyocytes, they were treated with LPS (5 µg/mL) for 12 h, and the cells were grouped as follows: (1) control; (2) LPS; (3) LPS + vector; and (4) LPS + USP13. To investigate the role of Nrf2, cells were treated with 5 µM of the inhibitor ML385, categorized under Group (5) LPS + USP13 + ML385.

Sprague Dawley rats were randomly divided into 2 groups with 10 rats in each group: (1) control group and (2) LPS group. The rats in the LPS group were intraperitoneally injected with 15 mg/kg LPS to establish a sepsis myocardial injury model, and the sham-operated group was intraperitoneally injected with the same volume of normal saline. Six hours after LPS injection, blood samples were collected by cardiac puncture, and cardiac tissue was collected.¹ The animals were sacrificed with sodium pentobarbital (sodium pentobarbital, ip, 80 mg/kg/body weight, BW) 6 h after the LPS treatment. Blood samples were collected by cardiac puncture for further analysis. Heart tissue was then carefully collected, snap-frozen in liquid nitrogen and stored at -80°C.

Antioxidant enzyme activity, serum creatine kinase, lactate dehydrogenase activity in serum and cell supernatant

Commercial kits used in this study were purchased from Beijing Solarbio Biotechnology Co., Ltd. (Beijing, China). Rat serum and cell supernatants were collected for detecting myocardial injury markers, LDH, and CK. All commercial kits were performed according to the manufacturer's instructions. Ultraviolet-visible spectroscopy (Thermo Fisher, Waltham, MA, USA) was used for the experiment.

MTT assay

The cell suspension was seeded into 96-well plates at a concentration of 4 × 10³ cells/mL in 100 µL. MTT assay (Labeled, Beijing, China) was used to detect the cell viability of LPS (5 µg/mL)-treated cells at different treatment times. Cells were then added with 10 µL of MTT and 90 µL of fresh medium for 4 h, after which formazan crystals

would form. Then, the supernatant was carefully aspirated, 110 μ L of formazan solution was added to each well, and the plate was placed in a shaker at low speed for 10 min to fully dissolve the crystals. The absorbance of cells at 490 nm was measured separately using a microplate reader (SpectraMax i3X, Molecular Devices, CA). The experiment was repeated thrice.

Flow cytometry

Cells (10^6 cells/mL) were centrifuged and resuspended with binding buffer (100 μ L). The apoptosis rate was detected using Annexin-V Alexa Fluor-488/PI Apoptosis Detection Kit (Cat no. CA1040; Solarbio, Beijing, China) according to the manufacturer's instructions. After staining, the cells were washed with precooled PBS thrice, and fixed with 1% paraformaldehyde for later analysis. The negative and blank control tubes were set up for comparison. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Quantitative real-time PCR

Total RNA was isolated from cells using an RNA extraction kit (Hunan, China). cDNA was then synthesized using reverse transcription kit (Agbio, Hunan, China). SYBR Green Master Mix (BioRad, USA) was used for qRT-PCR, and β -actin was used as an internal control. Target gene transcription was normalized to β -actin expression (internal control) using the $2^{-\Delta\Delta Ct}$ method to calculate fold induction of target mRNA. All primers used are as follows, USP13: F: TCTCTACGACTCTCCCAATTC, R: CAGACGCCCTCTTACCTTCT, and β -actin: F: ACTTAGTTGCGTTACACCTT, R: GTCACCTTCACCGTTCCA.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were utilized to measure the activity of enzymes including SOD, GPx, GSH, and CAT or the expression of cytokines including TNF- α (Cat no. SEKH-0047; Solarbio, Beijing, China), MCP-1 (Cat no. SEKR-0024; Solarbio, Beijing, China), IL-1 β (Cat no. SEKM-0002; Solarbio, Beijing, China), and IL-6 (Cat no. SEKH-0013; Solarbio, Beijing, China).

Antibody was incubated in medium from H9C2 cells treated by LPS (5 μ g/mL) at 37°C for 12 h. Then the absorbance of each well was detected at 450 nm using a microplate reader (SpectraMax i3X, Molecular Devices, CA).

Western blotting

To obtain total protein from cells, high-efficiency RIPA lysate (Cat no. R0010; Solarbio, Beijing, China) was used, which was centrifuged at $16,000 \times g$ at 4°C for 15 min. The protein concentration was then quantified by the BCA protein assay kit (Cat no. PC0020, Solarbio, Beijing, China). Proteins were denatured at 95°C for 5 min, followed by SDS-PAGE (7.5%). The loading amount of protein per pore was 30 μ g. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane

(Solarbio, Beijing, China) by wet transfer method, blocked with 5% skimmed milk dissolved in tris-buffered saline with 0.1% Tween® 20 detergent (TBST) prepared in advance, and placed in a shaker for 2 h at room temperature. The corresponding primary antibodies were used to incubate the membranes for 2 h at 37°C.

The primary antibodies included USP13 (1:2000; Cat no. 16840-1-AP; Proteintech, Shanghai, China), Nrf2 (1:2000; Cat no. 80593-1-RR; Proteintech, Shanghai, China), HO-1 (1:2000; Cat no. 10701-1-AP; Proteintech, Shanghai, China), COX-2 (1:2000; Cat no. 66351-1-IG; Proteintech, Shanghai, China), and iNOS (1:2000; Cat no. 80517-1-RR; Proteintech, Shanghai, China). The Easysee Western Blot Kit (Beijing TransGen Biotech, Beijing, China) was used to measure chemiluminescence signals. ImageJ version 1.53 was used to analyze the expression of proteins in each group.

Statistical analysis

All data were presented as mean \pm standard deviation (SD) for experiments performed in triplicate. Unpaired or paired Student's t-test or one-way analyses of variance (ANOVA) was utilized to analyze the results, followed by Tukey's test to compare each group. Data were analyzed, and graphs were plotted by using GraphPad Prism version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ indicated a significant difference.

Results

USP13 is downregulated in septic myocardial injury tissue

The SD rat model of septic myocardial injury was successfully constructed. LDH and CK were used to determine lipid peroxidation in serum and cell supernatants,¹⁷ and LPS treatment significantly increased the levels of LDH and CK (Figure 1A). The mRNA and protein expression levels of USP13 and Nrf2 were also investigated. The results consistently showed that LPS treatment significantly reduced the mRNA levels of USP13 and Nrf2 (Figure 1B), as well as their protein expression levels (Figure 1C). These results suggest that the expression of USP13 and Nrf2 is inhibited in LPS-induced septic myocardial injury.

USP13 increases Nrf2 levels in vitro

The changes in the protein expression levels of USP13, Nrf2, and HO-1 in H9C2 cells treated with LPS at different time points were further studied (Figure 2A). The results of WB showed that the expression of these genes gradually decreased with time, and there were significant differences when compared with the control group. At the same time, MTT assay was also used to detect the changes of cell viability after H9C2 cells were treated with LPS at different time points. The results were consistent, indicating that with the extension of time, the cell viability gradually decreased, and there were significant differences compared with the control group (Figure 2B). In order to investigate whether

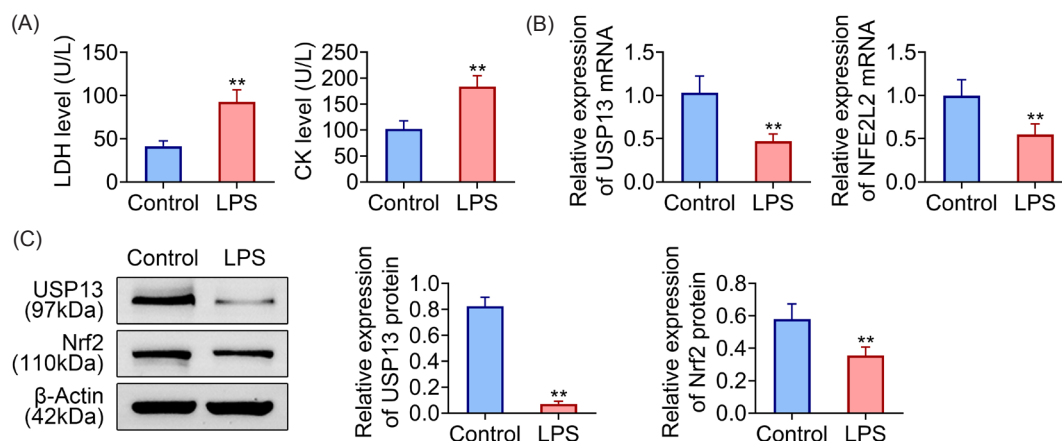


Figure 1 Differences of LDH and CK expression, as well as USP13 and Nrf2, between SD rat model group and control group with LPS-induced sepsis myocardial injury. (A) Differences in the expression levels of LDH and CK among different groups; (B) Differences in mRNA expression of USP13 and Nrf2 among different groups; (C) Differences in protein expression of USP13 and Nrf2 among different groups. N = 3, **P < 0.01 vs control group.

USP13 can regulate the expression of Nrf2, USP13 was over-expressed in H9C2 cells, and the protein expression levels and cell viability were detected as above. WB results showed that LPS decreased the expression levels of these proteins relative to the control group, and USP13 overexpression increased the expression levels of these proteins relative to the LPS + vector group (Figure 2C); MTT assay results showed that when compared to the control group, the overexpression of USP13 reversed the decrease in cell viability caused by LPS (Figure 2D), and the expression levels of these proteins were increased by overexpression of USP13 compared with the LPS + vector group.

USP13 reduces apoptosis by inducing Nrf2

The previous experiments found that USP13 increased the survival rate of cells. In order to further verify whether Nrf2 played a key role, ML385 inhibitor was used to carry out research. The results showed that compared with control, LPS treatment significantly increased the apoptosis rate of cells, whereas compared with LPS + vector group, USP13 overexpression significantly decreased the apoptosis rate, and the inhibition of Nrf2 by ML385 reversed the effect of USP13 (Figure 3). These results indicate that USP13 has a regulatory effect on Nrf2.

USP13 reduces oxidative stress by inducing Nrf2

Previous experiments have identified the regulatory role of USP13 on Nrf2, but whether it affects oxidative stress remains unclear. Cell supernatant SOD, GPx, GSH, and CAT were detected by the ELISA kit, and the activity of cellular antioxidant enzymes was determined. It was found that LPS treatment significantly reduced the activities of these antioxidant enzymes in cells compared with control, while USP13 overexpression improved the antioxidant capacity compared with LPS + vector, whereas the inhibition of Nrf2 by ML385 reversed USP13 effect (Figure 4A). In addition,

WB experiments were used to examine the protein expression levels of COX-2 and iNOS in these groups, which reflect the level of inflammation (Figure 4B). The results showed that compared with control, LPS treatment significantly increased the expression levels of these two proteins in cells; whereas compared with LPS + vector, USP13 overexpression significantly decreased the expression levels of these two proteins in cells. Inhibition of Nrf2 by ML385 reversed the effect of USP13. These data indicated that USP13 could reduce oxidative stress by inducing Nrf2.

USP13 reduces inflammatory factor expression by inducing Nrf2

Finally, the levels of inflammatory factors IL-6, TNF- α , IL-1 β , and MCP-1 in the supernatants of these groups were examined, and the results are shown in Figure 5. Compared with control, LPS treatment significantly increased the expression levels of the above four inflammatory factors in cells, while compared with LPS + vector, USP13 overexpression significantly decreased the expression levels of these cell inflammatory factors. However, inhibition of Nrf2 by ML385 reversed the effects of USP13 overexpression. These findings indicated that USP13 could reduce the expression of inflammatory factors by inducing Nrf2.

Discussion

In this study, by constructing a septic myocardial injury model in SD rats, we found that LPS increased serum LDH and CK levels. These two enzymes are known and widely used as blood markers for screening tissue damage.¹⁸ This result suggested that LPS increased myocardial injury, indicating that the modeling was successful. Subsequently, the mRNA and protein expression levels of USP13 and NFE2L2 were detected, and it was found that LPS reduced their expression. USP13 is a tumor suppressor protein that acts by deubiquitinating and stabilizing mediators such as PTEN.¹⁹

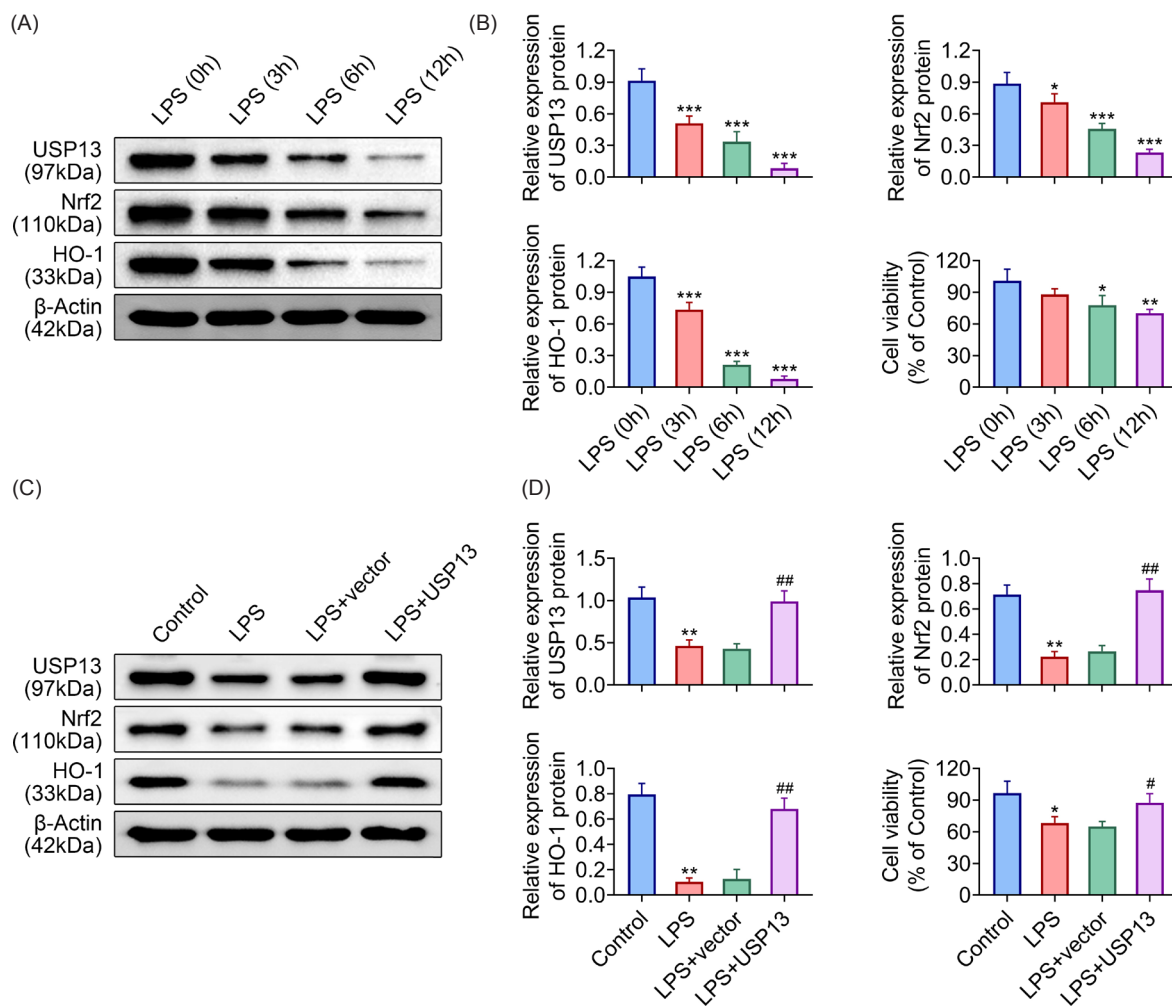


Figure 2 The effects of LPS treatment of H9C2 at 3 h, 6 h, and 12 h or overexpression of USP13 on the protein expression levels and cell viability of USP13, Nrf2, and HO-1. (A) Differences in protein expression among groups under different treatment durations; (B) Differences in cell viability among different groups under different treatment durations; (C) Differences in protein expression among groups under different treatments; (D) Differences in cell viability among groups under different treatments. N = 3, *P < 0.05 vs control or LPS (0 h) group, **P < 0.01 vs control or LPS (0 h) group, ***P < 0.001 vs LPS (0 h) group, #P < 0.05 vs LPS + vector group, ##P < 0.01 vs LPS + vector group.

USP13 has been reported to be able to modulate antiviral activity. Nrf2 is an antioxidant regulator.²⁰ Although the regulation of Nrf2 by USP13 has been widely reported, its role in sepsis-induced myocardial injury has not been elucidated.

The relationship between USP13 and Nrf2 has been widely reported. For example, Li et al. reported that USP13 could enhance P62 stability and aggregate formation, activate autophagy, and inhibit Keap1 as a negative regulator of Nrf2.²¹ Huang et al. found that USP13 has a protective effect, which is related to the inhibition of caspase-3 by Nrf2.²² In this study, the changes of the protein expression levels of USP13, Nrf2, and HO-1 on LPS treatment at different durations were detected. The results showed that their expression and cell viability were decreased gradually over time. The effects of LPS on the expression of these three proteins were consistent. The literature shows that they act together as stress-responsive proteins, and their expression levels reflect resistance to external stimuli.²³ Subsequent overexpression of USP13 increased the expression of these proteins and cell viability, suggesting that USP13 can

regulate Nrf2. To further verify the role of USP13 in regulating Nrf2, Nrf2 inhibitor ML385 was used. The results proved that the overexpression of USP13 reduced apoptosis, and inhibition of Nrf2 by ML385 increased apoptosis, which further supports the regulatory effect of USP13 on Nrf2.

Further, the effects of overexpression of USP13 and inhibition of Nrf2 expression on cellular oxidative stress and inflammatory factor expression were examined. SOD, GPx, GSH, and CAT are enzymes that respond to oxidative stress capacity, and IL-6, TNF- α , IL-18, and MCP-1 reflect the activation of inflammatory response.^{24,25} These enzymes and inflammatory factors have been reported to be activated during sepsis. Our results demonstrate that USP13 can reduce oxidative stress and inflammatory responses by inducing Nrf2. Furthermore, the results showed that USP13 was downregulated in septic myocardial injury tissues, and USP13 was able to induce an increase in Nrf2 levels, which in turn reduced apoptosis, oxidative stress, and inflammatory responses.

However, this study has some limitations. First, the overexpression of USP13 also caused changes in the

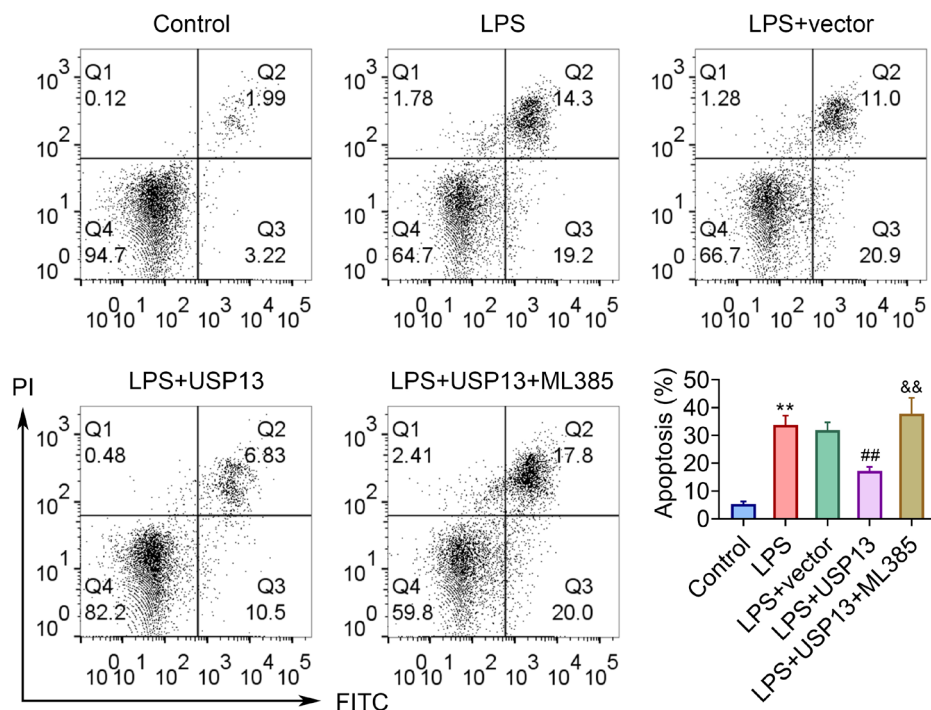


Figure 3 The effect of overexpression of USP13 or inhibition of Nrf2 expression on the apoptosis rate of H9C2 cells. N = 3, **P < 0.01 vs control group, ##P < 0.01 vs LPS + vector group, &&P < 0.01 vs LPS + vector group.

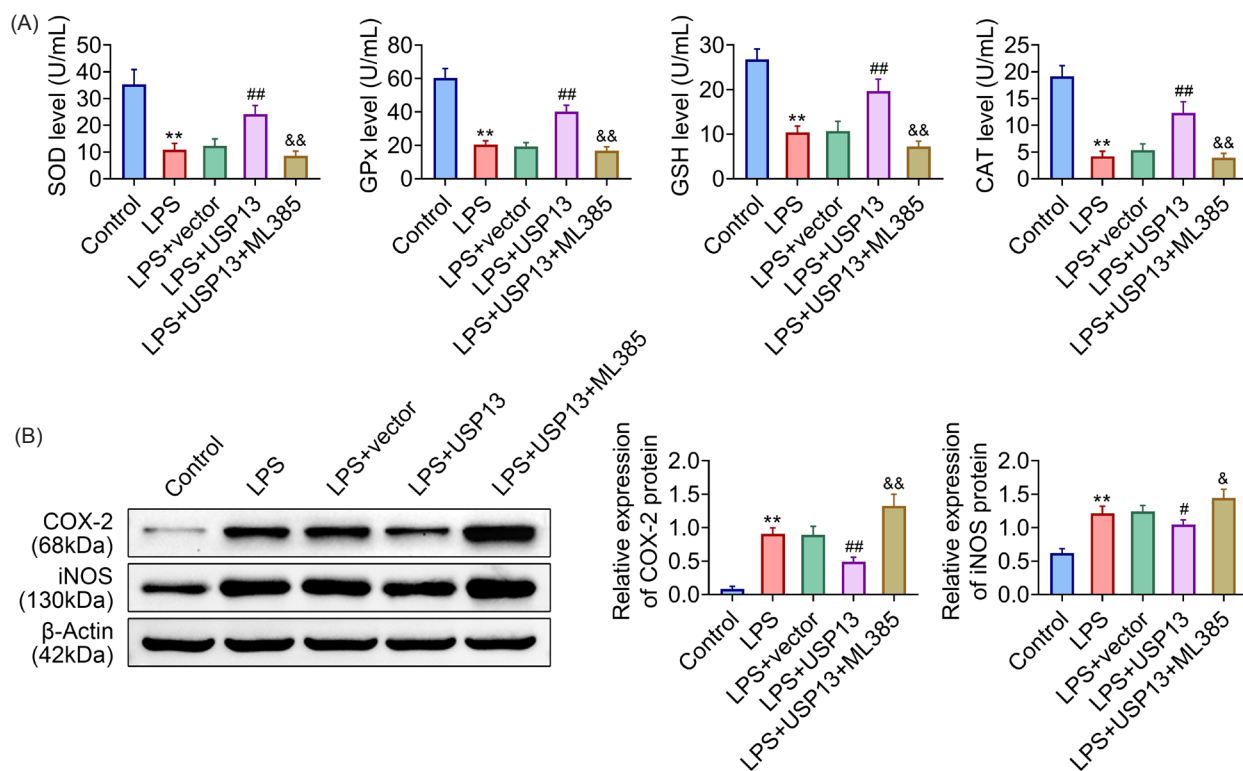


Figure 4 Detection of oxidative stress levels in H9C2 cells overexpressing USP13 or inhibiting Nrf2 expression. (A) Differences in the expression levels of SOD, GPx, GSH, and CAT in the supernatant of cells in each group; (B) Differences in the protein expression levels of COX-2 and iNOS in each group. N = 3, **P < 0.01 vs control group, #P < 0.05 vs the LPS + vector group, ##P < 0.01 vs LPS + vector group, &P < 0.05 vs LPS + vector group, &&P < 0.01 vs the LPS + vector group.

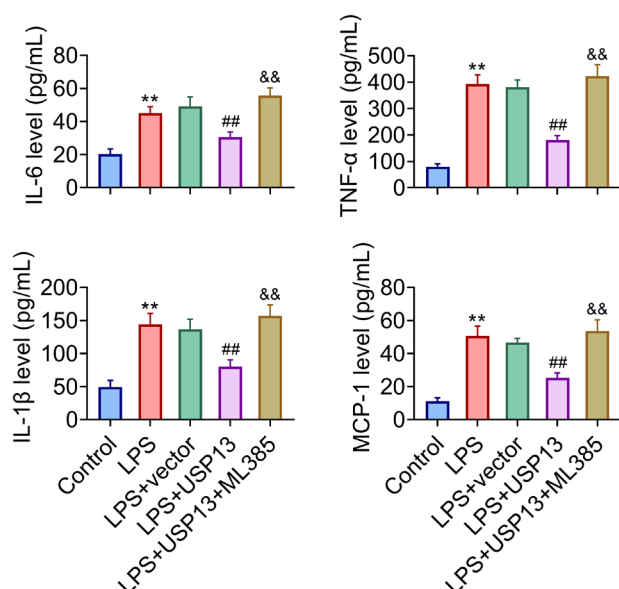


Figure 5 Effects of overexpression of USP13 or inhibition of Nrf2 expression on the expression levels of IL-6, TNF- α , IL-1 β , and MCP-1 in the supernatant of H9C2 cells. N = 3, **P < 0.01 vs control group, ##P < 0.01 vs LPS + vector group, &&P < 0.01 vs LPS + vector group.

expression of HO-1, and whether it also has a similar regulatory relationship with Nrf2 remains to be resolved. Second, the research on Nrf2 and cellular processes after knock-down of USP13 may lead to the lack of convincing of this study. Finally, although the SD rat model was established in this study, the research on rat behavior was not studied, and further research must be carried out in future.

Conclusion

In conclusion, this study found that USP13 was downregulated in septic myocardial injury tissues, and USP13 overexpression could increase Nrf2 levels and reduce apoptosis. Further studies found that USP13 reduced LPS-induced oxidative stress and inflammation by inducing Nrf2.

Funding

No funding was used in this study.

Competing Interests

The authors state that there are no conflicts of interest to disclose.

Ethics Approval

Ethical approval was obtained from the Ethics Committee of Laboratory Animal Ethics Committee, West China Hospital, Sichuan University (Approval no. 20220414001).

Data Availability

The authors declare that all data supporting the findings of this study are available within the paper, and any raw data can be obtained from the corresponding author upon request.

Contribution of Authors

Danyang Wu and Rong Yuan designed and carried out the study. Danyang Wu, Rong Yuan, and Lian Zhang supervised the data collection, and analyzed and interpreted the data. Danyang Wu and Meng Sun prepared the manuscript for publication and reviewed the draft version. All authors have read and approved the manuscript.

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