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The IRE1 signaling pathway is involved in the protective effect of low-dose LPS on myocardial ischemia-reperfusion injury

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ABSTRACT

Aim: The IRE1 signaling pathway is implicated in I/R injury. However, little is known about the involvement of this pathway in low-dose LPS treatment of myocardial I/R injury. Thus, an attempt was made to determine the relationship between the IRE1 pathway and I/R injury using rats or in vitro H9C2 cell myocardial I/R injury models.

Main methods: Sprague-Dawley rats and cultured H9C2 cells were pretreated with low-dose LPS and subjected to myocardial I/R injury models.

Key findings: Low-dose LPS did not affect normal rat or cellular function. Compared with the I/R group, treatment with LPS attenuated myocardial apoptosis, decreased plasma LDH and CK-MB activities, reduced myocardium infarct size, and downregulated caspase-3 expression. Moreover, the protein or mRNA expression levels of the IRE1 signaling pathway-related proteins Grp78, IRE1, p-ASK1, ASK1, p-JNK, and JNK were notably increased during I/R injury but significantly decreased by low-dose LPS treatment both in rats and in H9C2 cells.

Significance: Low-dose LPS exhibited therapeutic effects in myocardial I/R injury. Most importantly, the cardioprotective mechanism of low-dose LPS may be associated with the IRE1 signaling pathway.

1. Introduction

Despite multiple developments of therapeutic approaches, myocardial ischemia reperfusion (I/R) injury is still a vital cause of myocardial dysfunction [1]. Lipopolysaccharide (LPS) is a Toll-like receptor 4 (TLR4) ligand and is a Gram-negative bacterial cell wall antigenic component. Excessive amounts of LPS may be deleterious for myocardial function, causing cardiac dysfunction and exacerbating I/R injury, whereas more moderate doses of LPS have no detrimental effect and also partially reverse the ischemia-induced impairment of myocardial contractility [2]. Previous studies demonstrated that LPS administration reduced myocardial infarct size in a rabbit I/R injury model, which may be associated with a significant downregulation of the spontaneous activation and adhesion of circulating polymorphonuclear neutrophils during reperfusion [3]. Reportedly, the cardioprotective mechanism of LPS may be through the activation of the phosphoinositide 3-kinase/Akt (PI3K/Akt)-dependent signaling pathway, a reduction of cardiomyocyte death and caspase-3 activity, the elevated of protein phosphorylation associated with signal transduction [4], or the triggering of iNOS expression [5]. A previous study revealed that activation of Inositol-requiring enzyme 1 (IRE1) was indispensable for the sustained production of inflammatory mediators, such as interleukin 6 (IL-6). Therefore, we speculated that LPS, which is important in the inflammatory process, may exhibit its cardioprotective effect through the IRE1 signaling pathway [6].

IRE1 is a type I transmembrane glycoprotein of the ER, which functions as a Ser/Thr protein kinase and endoribonuclease (endoRNase) [7]. Under normal conditions, IRE1 is bound to glucose-regulated protein-78 (Grp78). Grp78 senses unfolded protein accumulation and dissociates from three unfolded protein response (UPR) sensors, further triggering the oligomerization and autophosphorylation of the UPR sensors, resulting in their activation [8]. Therefore, activated IRE1 during ER stress may lead to IRE1 autophosphorylation and activation of its endoRNase activity [7]. In I/R injury, the UPR acts as a vital factor in cell death, and UPR suppression is beneficial in myocardial I/R injury. For eukaryotic cells, IRE1 is one of the ER transmembrane effector proteins that mediates one of the three branches of the UPR signaling pathway and is involved in myocardial I/R injury [9]. mRNA expression of a spliced nuclear transcription factor, X-box-binding protein-1 (sXBP1), increases as a result of the activation of the IRE1 pathway, and sXBP1 becomes highly active for ER-resident enzymes and chaperones [10]. Moreover, under severe ER stress conditions, IRE1 recruits apoptosis signal-regulating kinase 1 and TNF receptor associated factor 2, further activating c-Jun N-terminal kinase

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(JNK) and inducing apoptosis [11]. However, how the IRE1 signal pathway is involved in the alleviation of myocardial I/R injury by LPS is not fully understood. In addition, the effect of a low-dose treatment of LPS on myocardial I/R injury has not been studied in the rat model. Therefore, this study will examine the IRE1 signal pathway in myocardial I/R injury in rats and in cardiomyocytes treated by LPS in vitro We evaluated the effects of a more precise LPS treatment and determined whether the IRE1 signaling pathway is involved in the molecular mechanism underlying LPS treatment.

In this study, we evaluated the treatment efficacy of low-dose LPS in myocardial I/R injury in rats and in H9C2 cell models in vitro. Here, we demonstrated that a moderate dose of LPS alleviated myocardial I/R injury, most likely by inhibiting the IRE1 signaling pathway. To the best of our knowledge, this is the first study that evaluated the IRE1 signaling pathway in the LPS treatment of myocardial I/R injury, which may lead to a more comprehensive understanding of new therapeutic approaches.

2. Materials and methods

2.1. In vivo myocardial I/R injury model establishment

For the animal model, 80 adult male Sprague-Dawley rats weighing between 250 and 300 g were purchased from Beijing HFK Bioscience (Beijing, China). Animal treatments and the experimental procedures conducted in this study were approved by a local ethical committee and were followed according to the guidelines of the Institutional Animal Ethics Committee (IAEC). Animals were fed with normal rat chow and were allowed free access to water in a 22 °C environment with 12 h/12 h light and dark cycles. Rats were housed for one week to acclimate to this environment before being subjected to I/R injury. After acclimation, the rats were randomly assigned to four groups: sham (sham operation with vehicle), LPS (sham operation with LPS), I/R (operation with vehicle) and I/R + LPS (operation with LPS). One hour before surgery, the rats were injected with LPS (Sigma-Aldrich) (80 μg/kg) or vehicle. To establish the I/R injured rat model, a left anterior descending coronary artery ligation was utilized [12]. Briefly, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and equipped with tracheal intubation ventilations. The chest was opened, and the left anterior descending coronary artery was ligated by a 6/0 atraumatic suture for 30 min followed by reperfusion or 120 min. Electrocardiography confirmed the presence of ischemia. The sham and LPS groups underwent thoracotomy, except that the suture around the left coronary artery was not fastened.

2.2. In vitro myocardial I/R injury model establishment

For the in vitro myocardial I/R injury model, rat-derived myoblast H9C2 cells (ATCC, USA) were utilized. Cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco, Canada) and penicillin-streptomycin at 37 °C under 5% CO^2 conditions. To establish the myocardial I/R injury model in vitro, myoblasts were deprived of oxygen and glucose by incubation with glucose-free serum in an anaerobic environment with 1% O^2, 5% CO^2 and 94% N^2 at 37 °C for 24 h. The cells were then subjected to normoxic conditions and cultivated in glucose-containing DMEM for reoxygenation. The NC and LPS groups received normal DMEM and normoxia throughout the duration of the entire experiment. Twenty-four hours before model establishment, the LPS and I/R + LPS groups were treated with the LPS solution at a concentration of 50 ng/ml.

2.3. Determination of LDH and CK-MB activities in rat serum by ELISA

At the end of the experiment, plasma from the carotid aorta was collected from the rats for ELISA measurement. An ELISA kit (Kenuodi, Quanzhou, China) was used to measure serum lactate dehydrogenase (LDH) and creatine kinase MB (CK-MB) activities. The procedures in the manufacturer's instructions were strictly followed.

2.4. Measurement of the myocardium infarct size in I/R injured rats

At a specific experimental time point, 2% Evans blue dye (Solarbio, China) was injected in the external jugular vein of the anesthetized rat, and the area at risk (AAR) was confirmed and displayed as nondyed area. Five minutes after injection, the heart tissue was quickly excised and immediately frozen at −80 °C for 10 min. The tissues were transversally sectioned into 1 mm-thick slices and incubated in a 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Solarbio, China) PBS solution for 20 min at 37 °C. The slices were then fixed overnight at 2 °C–8 °C with 10% PBS formation buffer. Finally, the area of infarction (AI) was displayed as white in the AAR which was not stained by TTC. The AI was quantified by ImageJ software [13].

2.5. Apoptosis measurement by TUNEL and FACS assays

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling staining (TUNEL) (Roche, Germany) was used to determine the degree of apoptosis in heart tissues. Heart tissue sections were embedded in paraffin, sectioned in 5 μm-thick slices, and used for apoptotic nuclei staining. Procedures were conducted as described by the manufacturer.

For the in vitro I/R injury model, fluorescence-activated cell sorting (FACS) using Annexin V-FITC/PI (4A Biotech, Beijing, China) staining was utilized to quantitatively examine cellular apoptosis. Briefly, H9C2 cells were incubated with 5 μL Annexin V- FITC/PI at room temperature in the dark. A flow cytometer (ACEA, USA) was then used to analyze the cells from each sample. FlowJo software was utilized to analyze the data obtained.

2.6. Immunohistochemistry and immunofluorescence for Grp78, IRE1, and p-JNK protein expression

Heart tissue paraffin sections of rats were prepared for immunohistochemical analysis. Briefly, the sections were incubated with 3% H_2O_2 in methanol at 37 °C for 10 min to quench endogenous peroxidase activity. Bovine albumin was then used for 20 min to block nonspecific binding. After blocking, the sections were incubated overnight at 4 °C with antibodies against Grp78 (11587-1-AP, Proteintech), IRE1 (27528-1-AP, Proteintech), or p-JNK (24164-1-AP, Proteintech), followed by incubation with a secondary antibody for 20 min at room temperature. Expression was visualized by the DAB kit. Positive results were indicated as brown staining.

For cell immunofluorescence, cells cultured on chamber slides were fixed with 4% paraformaldehyde for 30 min. The fixed cells were then permeated in 0.5% Triton X-100/PBS for 20 min and blocked in 2% bovine serine albumin for 30 min. Cells were continuously incubated with primary antibodies against Grp78, IRE1 and specific antibodies. Finally, the cells were stained with DAPI for 5 min. The images were examined with a confocal microscope.

2.7. Western blot analysis for protein expression

The heart tissues and H9C2 cells were subjected to protein expression analysis by western blotting. The experimental procedures were reported previously [14]. Briefly, the total tissues and cells were lysed using RIPA buffer. Supernatant was obtained by centrifugation at 12,000*g* for 5 min at 4 °C. The BCA protein assay kit (TAKARA, Beijing, China) was used to determine the protein concentrations. Equal amounts of protein were loaded in each well of a 10% polyacrylamide SDS-PAGE gel, gel electrophoresis was performed, and protein was transferred onto PVDF membranes (Millipore, Billerica, MA, USA), followed by blocking with 5% nonfat milk. The membranes were then

Table 1
Primer sequences used in RT-PCR experiments.

Gene	Forward	Reverse
Grp78	5′- TCTGCTGGGCATCTTCTGTTTGGC-3′	5′- TGTAACGCTGTCGGGAGGCTCTTT-3′
IRE1	5′- ACCTTGCTTCATCAGACCACCTCA-3′	5′- GGCTGAAACTATGCCTGCCCACT-3′
Caspase-3	5′- TTTTGGAACGAACGGACCTGTGG-3′	5′- AACCGGGTGCGGTAGAGTAAGCA-3′

incubated with primary antibodies overnight at 4 °C. The primary antibodies were against Grp78 (11587-1-AP, Proteintech), IRE1 (27528-1-AP, Proteintech), p-ASK1 (ab47304, Abcam), ASK1 (28201-1-AP, Proteintech), p-JNK (ab4821, Abcam), JNK (24164-1-AP, Proteintech), caspase-3 (19677-1-AP, Proteintech), and GAPDH (10494-1-AP, Proteintech). The membranes were then washed and conjugated with secondary antibody. Protein signals were detected with the ECL system (Wuhan Sanying, China), and the relative band intensity was quantified by the ImageJ software. An antibody against GAPDH was used as an internal control.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) analysis for mRNA expression

Primers were designed according to the sequences reported in GenBank and synthesized by General Biosystems (Anhui, China). The primer sequences used are listed in Table 1. Total RNA was extracted from rat heart tissues and H9C2 cells by Trizol (Invitrogen). The specific steps were performed as previously reported [15]. PCR amplification was conducted as follows: 40 cycles at 95 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s.

2.9. Statistical analysis

All data were expressed as the mean ± SD (standard deviation, SD), and differences were analyzed by one-way ANOVA. For paired data, differences were analyzed using Student's *t*-test. Differences were considered to be statistically significant when the p value was < 0.05.

3. Results

3.1. Low-dose LPS alleviates infarct size in myocardial I/R injury rats

The CK-MB isoenzyme and LDH secreted from I/R injured myocardial tissues to the blood indicated acute myocardial infarction, and the enzyme levels in the coronary flow increased, suggesting injury rate and cell necrosis [16]. As depicted in Fig. 1A, low-dose LPS administration did not influence the regular CK-MB and LDH activity in rat plasma. In contrast, I/R injury significantly increased both enzymes' activities ($P < 0.001$). However, pretreatment with low-dose LPS decreased enzymatic activity to near normal levels ($P < 0.001$).

We utilized the Evans-TTC method to measure myocardial infarct size under low-dose LPS treatment. The infarction volume was expressed as the ratio of AI to AAR. As shown in Fig. 1B, the infarction volume in I/R injured rats was approximately 42%. Compared with I/R group, the I/R + LPS group showed a notable decrease in myocardium infarct size, with a decrease to 27%.

3.2. Low dose of LPS alleviates apoptosis resulting from myocardial I/R injury

Myocardial I/R injury may lead to apoptosis in cardiomyocytes, resulting in the loss of cardiomyocytes, an acceleration in cardiac dysfunction, or heart failure [17]. First, we used TUNEL staining to evaluate the myocardial apoptosis level in I/R injured rat heart tissues (Fig. 2A). Compared with the sham control rats, LPS did not affect the function of normal cardiomyocytes. Under I/R injury, the apoptosis index elevated by nearly 3-fold compared to the control group. Compared with the I/R injured rats, the numbers of apoptotic cells were significantly lower than those in the I/R group. FACS was then used to quantify apoptotic cell numbers in the I/R injury H9C2 cell model (Fig. 2B). As observed with the TUNEL staining, the number of apoptotic cells notably increased under I/R but decreased with LPS administration.

Reportedly, caspase-3 increases in I/R injury to initiate and activate the downstream apoptotic pathways [9]. In our study, caspase-3 protein expression (Fig. 2C) and mRNA expression (Fig. 2D) were significantly increased in the I/R injury model compared with the sham or the normal control groups. However, protein and mRNA expression were both downregulated in the low-dose LPS treatment groups.

3.3. The protein expression of IRE1 signaling pathway proteins was downregulated by low-dose LPS treatment

Protein expression changes were quantified by immunohistochemistry and western blot assay. In immunohistochemistry experiments, the results from rat tissue (Fig. 3A) show that the relative protein expression of Grp78, IRE1 and p-JNK was increased during I/R injury but was notably decreased with LPS treatment. For the H9C2 cells (Fig. 3B), the Grp78 and IRE1 positive number ratio changes agreed with those in the rat model.

The western blot assay showed more precise protein expression changes of the factors related to the IRE1 signaling pathway. As observed in Fig. 4A, the relative protein expression of Grp78, IRE1, ASK1 and JNK increased after I/R injury. However, the protein expression significantly decreased with the administration of low-dose LPS ($P < 0.001$). The western blot results for the H9C2 cells displayed similar changes in protein expression (Fig. 4B) ($P < 0.001$).

3.4. IRE1 signaling pathway mRNA expression was downregulated under low-dose LPS treatment

An RT-PCR experiment was conducted to verify the results observed by the western blot assay. Both in I/R injured rat tissues (Fig. 5A) and H9C2 cells (Fig. 5B), the levels of Grp78, IRE1significantly increased ($P < 0.001$). With the administration of low-dose LPS, the expression levels were significantly decreased ($P < 0.01$). These results agree with those observed with the western blot assay, verifying that IRE1 signaling was inhibited during LPS treatment.

4. Discussion

The present study verified that low-dose LPS did not induce myocardial injury but ameliorated myocardial I/R injury in rats in both an in vivo and an in vitro model. Furthermore, we also revealed that the IRE1 signaling pathway was involved in the LPS-induced cardiac protective function.

For a myocardium with I/R injury, the elevation of myocardial enzymes in plasma and myocardial infarct size are two important markers of permanent myocardial ischemia [18]. CK-MB isoenzyme and LDH secreted from I/R injured myocardial tissues to the blood indicate acute myocardial infarction. During I/R injury, LDH and CK-MB levels in the coronary flow increases, suggesting injury rate and cardiomyocytes necrosis [16]. In the present study, using the Evans-

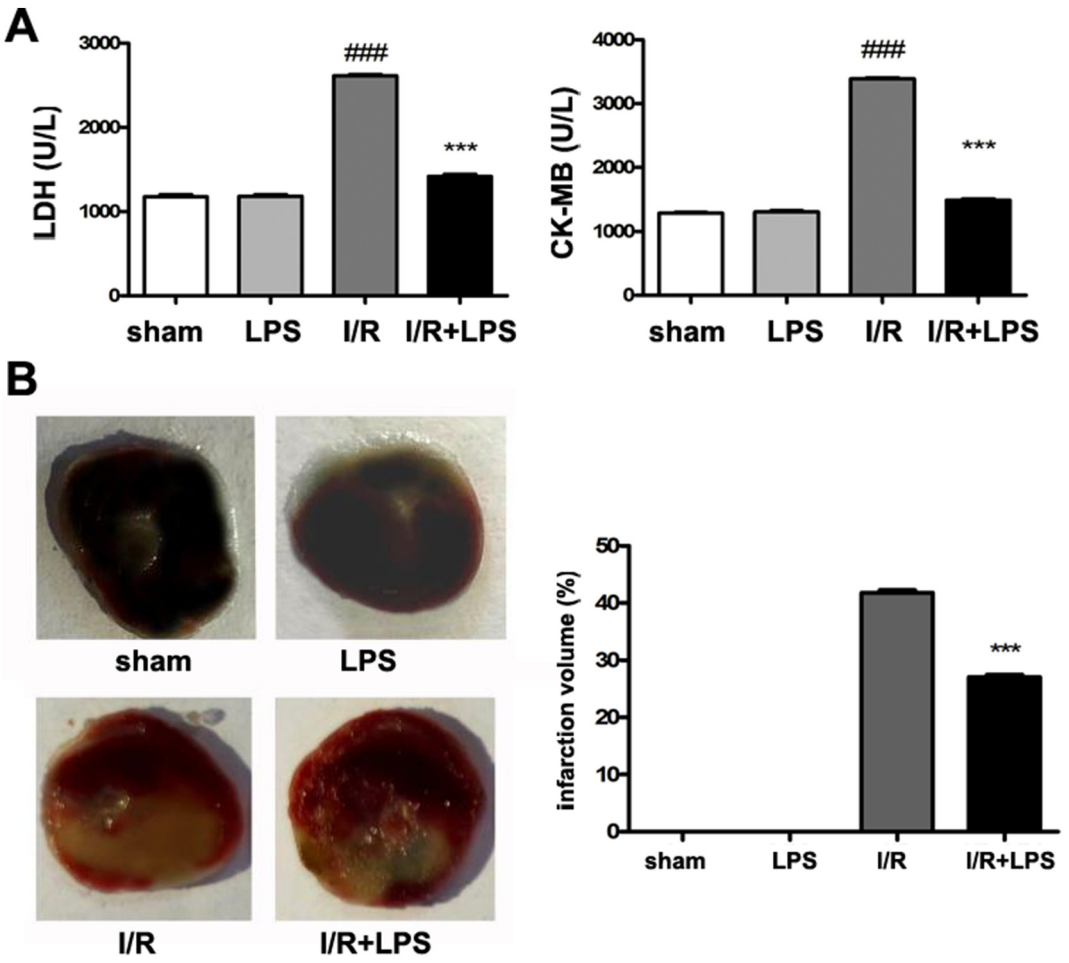

Fig. 1. A low-dose of LPS reduces infarct size in myocardial I/R injured rats. (A) CK-MB and LDH activity in rat plasma. (B) TTC staining for myocardial infarct size of rats with low-dose LPS treatment. ***p < 0.001 compared with the I/R group. ###p < 0.001 compared with the sham group. The experiments were performed three times.

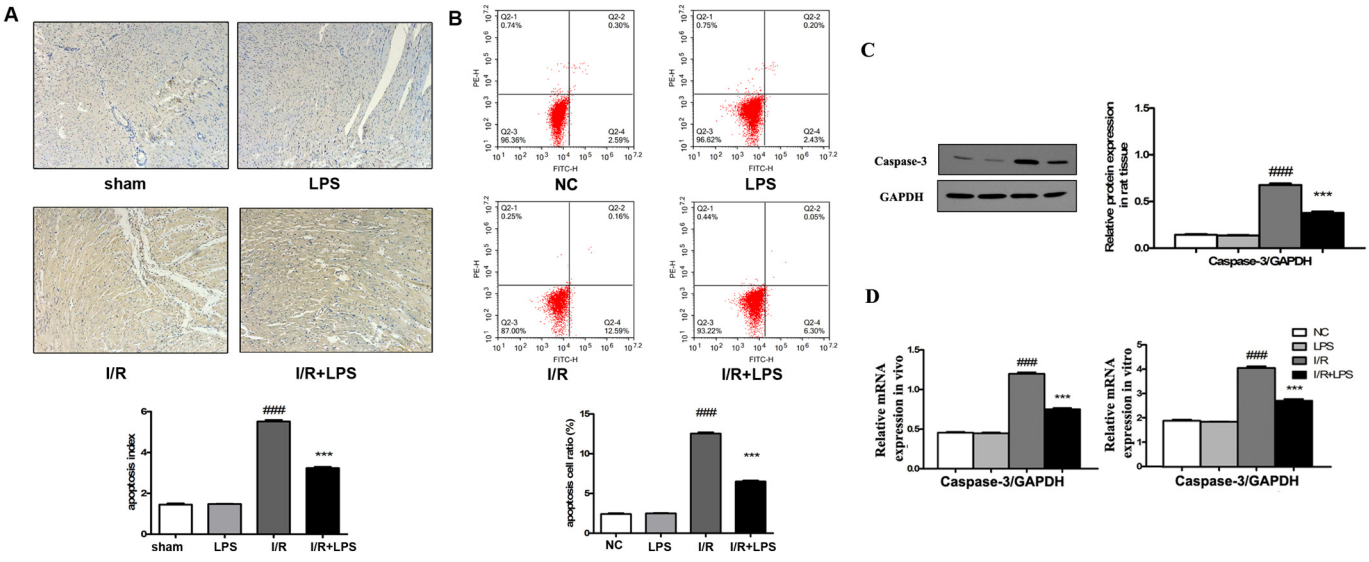

Fig. 2. A low dose of LPS alleviates the apoptosis induced by myocardial I/R injury (A) TUNEL staining for myocardial apoptosis levels in I/R injured rat heart tissues (B) Quantification of the apoptotic cell numbers in H9C2 cells by FACS. (C) Western blot for myocardial caspase-3 relative protein expression in rat tissues. (D) RT-PCR for caspase-3 relative mRNA expression in rat tissue and H9C2 cells. ***p < 0.001 compared with the I/R group. ###p < 0.001 compared with the sham or NC group. The experiments were performed three times.

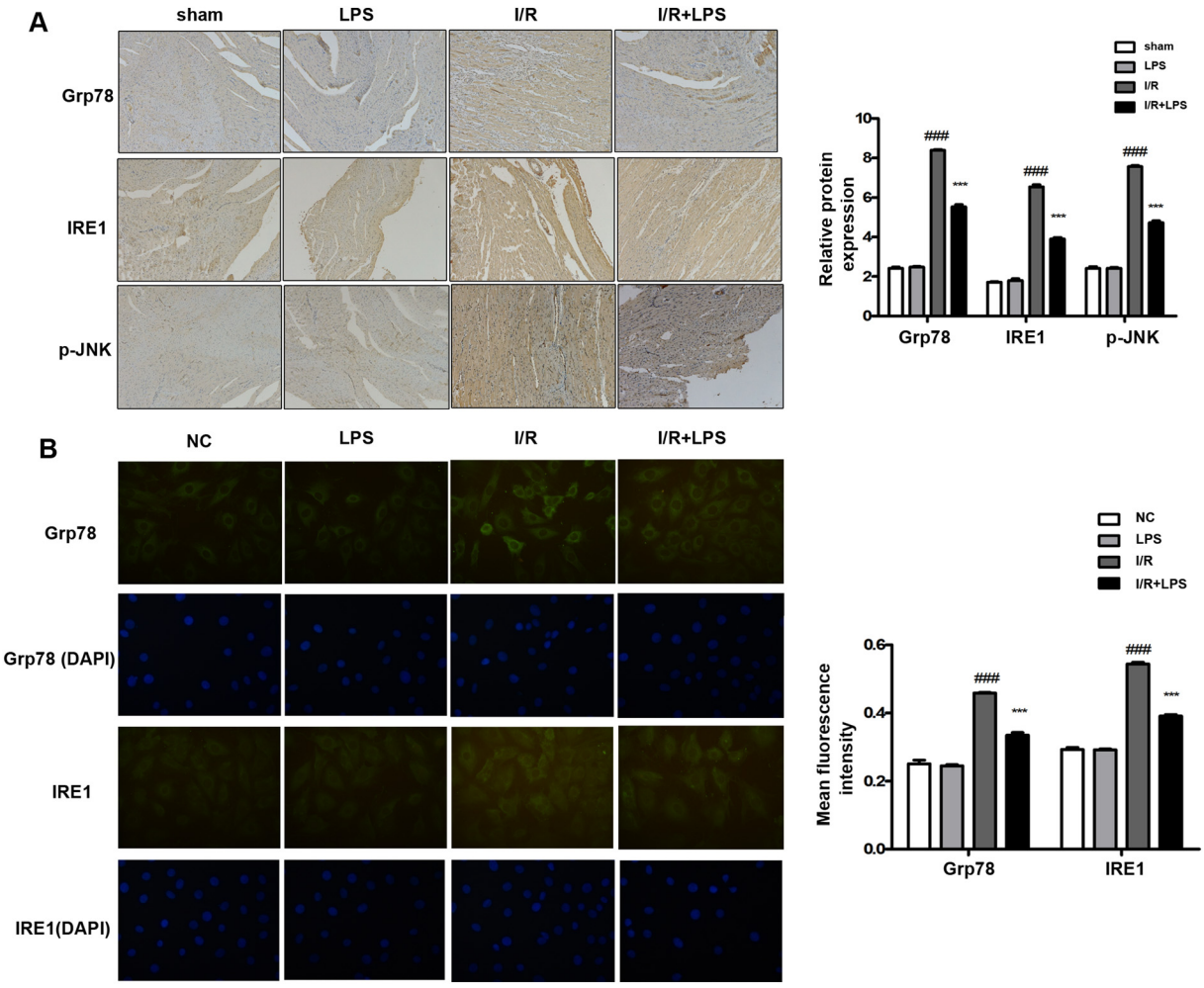

Fig. 3. The IRE1 signaling pathway protein expression was downregulated and detected by immunohistochemistry assay under the low-dose LPS treatment. (A) Immunohistochemistry for myocardial Grp78, IRE1 and p-JNK relative protein expression in rat tissues. (B) Immunohistochemistry for Grp78, IRE1 and p-JNK relative protein expression in H9C2 cells. ***p < 0.001 compared with the I/R group. ###p < 0.001 compared with the sham or the NC group. The experiments were performed three times.

TTC assay (Fig. 1B), we found that infarcted myocardial size was aggravated in I/R injured rat tissue but attenuated by LPS treatment. We also show that pretreatment with LPS downregulated plasma CK-MB and LDH activities (Fig. 1A). Together, these results suggest that low-dose LPS treatment exhibits a cardioprotective effect against myocardial I/R injury.

Cardiomyocyte apoptosis is a common result of ischemia-induced myocardial injury and can lead to heart failure. The morphological

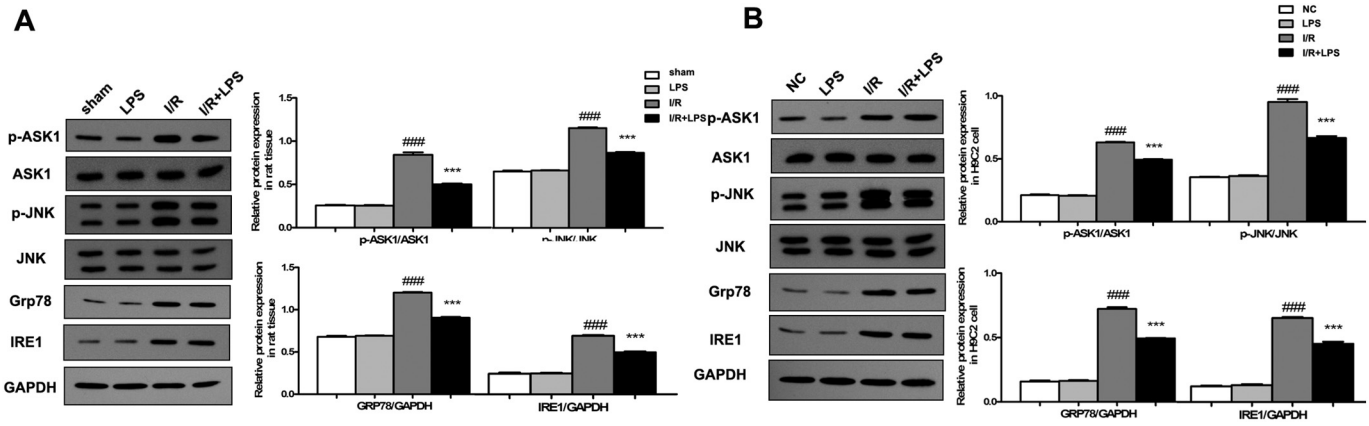

Fig. 4. The IRE1 signaling pathway protein expression was downregulated and detected by western blot assay under low-dose LPS treatment. (A) Western blot for myocardial p-ASK1, ASK1, p-JNK, JNK, Grp78 and IRE1 relative protein expression in rat tissues. (B) Western blot for p-ASK1, ASK1, p-JNK, JNK, Grp78 and IRE1 relative protein expression in H9C2 cells. ***p < 0.001 compared with the I/R group. ###p < 0.001 compared with the sham or the NC group. The experiments were performed three times.

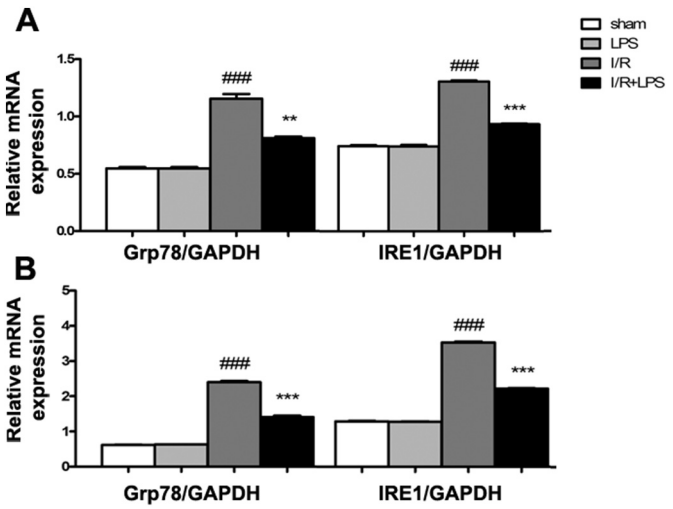

Fig. 5. The IRE1 signaling pathway mRNA expression was downregulated by low-dose LPS treatment. (A) RT-PCR for myocardial Grp78 and IRE1 relative mRNA expression in rat tissues. (B) RT-PCR for Grp78 and IRE1 relative mRNA expression in H9C2 cells. ***p < 0.001 compared with the I/R group. ###p < 0.001 compared with the sham or the NC group. The experiments were performed three times.

features of apoptosis are characterized by caspases activation [19]. Ischemia is related to apoptosis as caspase is activated in an ischemic rat heart, and caspase activation is dependent on the duration of ischemia. Blocking caspase activation is important for apoptosis inhibition [20]. Caspase-3 plays a vital role in ER stress-mediated cell death and was significantly elevated during I/R injury to initiate and activate downstream apoptotic pathways [9]. Data from previous studies indicate that extended myocardial ischemia can lead to apoptosis mediated by the release of cytochrome *c* from mitochondria, further activating caspase-3 [21]. Both TUNEL and FACS were used to determine the apoptotic changes occurring during low-dose LPS treatment in I/R injured rats and in the cardiomyocyte model (Fig. 2). There were more apoptotic cells observed in the I/R injury group. Pretreatment with low-dose LPS notably decreased the apoptotic cell number. Concurrently, the transcript and protein levels of caspase-3 expression in the rat model were also decreased, further verifying the therapeutic effect of LPS in myocardial I/R injury.

IRE1 is one of the branches of the UPR signaling pathway activated during myocardial I/R injury. Enhancement of ER stress is associated with heart failure, and ER stress is also reported to be involved in I/R injury, resulting in cell death [9]. As a Ser/Thr kinase, IRE1 utilizes its endonuclease domain to remove 26 nucleotides of x-box binding protein 1 (XBP-1) mRNA, which leads to stable translation of the XBP-1 transcription factor and acceleration of the endoplasmic reticulum stress (ERS) gene program [22]. IRE1α is the most conserved transducer of UPR and is indispensable for the optimum production of inflammatory cytokines after TLR stimulation. Myeloid-specific IRE1α deletion protects mice from inflammatory arthritis [23]. The accumulation of unfolded proteins in the ER stimulates IRE1 dimerization and trans-autophosphorylation, which facilitates its activation [24]. The mRNA and protein expression of Grp78 were increased with myocardial I/R injury-induced ER stress. Under normal conditions, Grp78 binds to IRE1 to suppress its activity, but during ER stress conditions, Grp78 dissociates from IRE1, allowing IRE1 activation [25]. Activated IRE1α recruits tumor necrosis factor receptor-associated factor 2 (TRAF2) and successively activates JNK and p38 MAPK through apoptosis signal-regulating kinase 1 (ASK1), resulting in the initiation of inflammation and cell death [26,27]. Until our study, no existing study elucidated the role of the IRE1 signaling pathway in the mitigation of myocardial I/R injury. In the current study, IRE1 signal-related gene and protein expression increased during I/R injury, indicating that the signaling was activated in ischemic myocardial disease. With the pretreatment of low-dose LPS, both the transcript and proteins expression levels significantly decreased, suggesting that the IRE1 signaling pathway was involved in the LPS treatment process.

5. Conclusions

In summary, the present study has verified the effect of treatment with low-dose LPS in rat myocardial I/R injury and has provided a novel concept that the IRE1 signaling pathway may be implicated in this therapeutic process. A new therapeutic method, and a more profound understanding of its mechanism in myocardial I/R injury was provided.

Declaration of Competing Interest

The authors have no funding and conflicts of interest to disclose.

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