Crocin inhibits KBTBD7 to prevent excessive inflammation and cardiac dysfunction following myocardial infarction

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Abstract. Myocardial infarction (MI) refers to myocardial ischemic necrosis that is caused by coronary artery disease. Notably, crocin has protective effects on the heart. The present study aimed to i) investigate the protective effect of crocin, an active ingredient in *Gardenia jasminoides* Ellis and *Crocus sativus* L., on myocardial ischemia and ii) to verify the interaction between crocin and kelch repeat and BTB domain-containing protein 7 (KBTBD7), which is a novel member of the BTB-kelch protein family. In the present study, the left anterior descending coronary artery was ligated to establish a myocardial ischemia-reperfusion injury (MIRI) model in rats and the protective effect of crocin on rat myocardial tissue was observed. The levels of the inflammatory cytokines, interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNFα), in the sham, MI model, MI + crocin (100 mg/kg) and MI + crocin (200 mg/kg) groups were compared in the rat myocardial tissue. The TUNEL assay was used to detect apoptosis of myocardial cells. In addition, RAW264.7 cells were stimulated with the inflammatory factors recombinant mouse high mobility group box 1 (rmHMGB1) and recombinant mouse heat shock protein 60 (rmHSP60). The inhibitory effect of crocin on inflammatory cytokine levels was observed using ELISA. Western blotting was used to detect the inhibitory effect of crocin on KBTBD7. The inhibitory effect of KBTBD7 knockdown on MAPK and nuclear factor (NF)-κB signaling pathways was also analyzed. The expression levels of IL-1β, IL-6 and TNFα were significantly decreased in the crocin-treated groups compared with in the model group. Crocin significantly reduced the apoptosis of myocardial cells and significantly inhibited the release of inflammatory cytokines induced by rmHMGB1 and rmHSP60. KBTBD7 was determined to be a target of crocin. Knockdown of KBTBD7 significantly inhibited p38 and NF-κB signaling pathways. Furthermore, the results demonstrated that KBTBD7 knockdown significantly reduced the production of inflammatory cytokines induced by rmHMGB1 and rmHSP60. KBTBD7 knockdown also significantly reduced p38 and NF-κB signaling in the rmHMGB1- and rmHSP60-treated groups. The present study demonstrated the potential protective effect of crocin on MIRI in rats. The underlying mechanism may be through direct inhibition of KBTBD7, thereby inhibiting excessive inflammatory responses and myocardial cell apoptosis following myocardial infarction.

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Introduction

Acute myocardial infarction (MI) leads to myocardial ischemia, and reperfusion of ischemic myocardium is the primary treatment strategy for acute MI (1). Reperfusion therapy is a treatment that reopens completely blocked blood vessels using thrombolytic drugs, interventional therapy, or surgery, improving outcome for patients with myocardial infarction (MI) (2). Although the ischemic myocardium can be restored to normal perfusion, reperfusion can increase the pathological process of patients with MI, resulting in tissue damage and cardiac dysfunction, called myocardial ischemia-reperfusion injury (MIRI) (3). Increased apoptosis of myocardial cells during MI is accentuated by the subsequent reperfusion-induced inflammatory response (4,5). MI caused by coronary artery obstruction leads to myocardial tissue damage and ischemic death, and is a serious threat to human health. A Sprague-Dawley rat model is often used to study the inflammatory response following MI. Macrophages are the main inflammatory cell type that trigger and regulate the inflammatory response following MI (6,7). Macrophages phagocytose apoptotic or necrotic cells and activate the anti-inflammatory process by releasing cytokines (8). The quantity of inflammatory cytokines released is proportional to cardiac function damage and the level of myocardial apoptosis following ischemia (9). Notably, inflammatory responses are activated during MI and reperfusion can significantly increase the myocardial inflammatory response; MIRI is common in clinical practice (10). An effective and feasible therapeutic treatment that can protect ischemic myocardium, limiting MI-associated damage and reducing MIRI, is therefore necessary. Over the last decade, drug preconditioning has received extensive attention in multiple diseases, including MI (11-13).

Traditional Chinese medicines have been extensively investigated because of their potential curative effects (14,15), low toxicity, limited side effects and capacity to act on multiple molecular interactions associated with disease (16). Crocin is an active component found in *Gardenia jasminoides* Ellis and *Crocus sativus* L. (17-19). Crocin reportedly exhibits cardioprotective (20-22) and antitumor properties, and serves an important pharmacological role in the treatment of central nervous diseases (23). Xu *et al* (24) revealed that crocin suppresses the apoptosis of bovine endothelial cells through increasing the ratio of Bcl-2/Bax, leading to an anti-atherosclerotic role. In addition, Bcl-2/Bax mRNA expression has been reported to be increased in response to crocin, thus inhibiting cell apoptosis (25). A previous study investigating platelet apoptosis induced by *Agkistrodon pallas* venom demonstrated that crocin may inhibit the expression of caspase-3 and caspase-9 (26). Furthermore, crocin has been shown to effectively inhibit cardiovascular cell and platelet apoptosis (27,28). However, the pharmacological mechanism of crocin remains unclear.

The BTB-kelch protein family is involved in numerous cellular functions, including transcriptional regulation, cytoskeleton formation, ion channels, protein ubiquitination, angiogenesis and apoptosis (29). Kelch repeat and BTB domain-containing protein 7 (KBTBD7) is located on chromosome 13q14.11, has a cDNA length of 3,008 bases, an open reading frame of 2,052 bases and encodes a protein of 684 amino acid residues (30). The KBTBD7 protein contains a conserved BTB domain and a series of kelch repeats. Homologous proteins have been found in a range of species, from yeast to fruit flies to mice, displaying a high level of inter-species protein conservation. Fragment analysis has demonstrated that the BTB, BTB and C-terminal kelch, and kelch repeat domains exhibit transcriptional activation activity (31). However, few cardiovascular studies have reported on KBTBD7. Moreover, the role of KBTBD7 in MIRI and whether crocin functions through KBTBD7 remain unclear.

In the present study, a rat model of acute MI was established to investigate the protective effect of crocin on acute MI aggravated by reperfusion injuries. The interaction between crocin and KBTBD7 was also examined. The present study has provided the foundations for further MI research and the development of new therapeutics.

Materials and methods

Animal model of MI. Clean-grade Sprague Dawley rats (male; age, 8-10 weeks; weight, 200-220 g; n=40) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Animals were housed at 21-25˚C and 50-60% humidity, under a 12-h light/dark cycle, with free access to food and water. Sprague-Dawley rats were randomly divided into four groups (n=10): Sham operation group, MI model group, MI + crocin (Amyjet Scientific, Inc.) low-dose group (100 mg/kg) and MI + crocin high-dose group (200 mg/kg). Rats in the MI + crocin low-dose group and MI + crocin high-dose group were intraperitoneally injected with crocin once per day (volume 0.2 ml/100 g) for 14 days. Rats in the sham operation and MI groups were intraperitoneally injected with an equal volume of normal saline for 14 days. The rats were anesthetized 30 min after the last administration by intraperitoneal injection of 2% sodium pentobarbital (40 mg/kg). Following endotracheal intubation, an artificial respirator was connected (tidal volume, 1.5-2 ml/100 g; frequency, 48-54 times/min). The right common carotid artery was carefully separated and the left ventricle was intubated. An incision was made between ribs 3 and 4 on the left side of the chest of rats. The pericardium was excised to completely expose the heart. With the exception of the sham operation group, the left anterior descending coronary artery was ligated. After ligation for 30 min, the ligature was loosened for reperfusion for 120 min. Left ventricular end-diastolic pressure, left ventricular systolic pressure and cardiac function were all evaluated following ischemia and reperfusion in each group. At the end of the experiment, rats were euthanized via carbon dioxide; rats were placed within a box and 100% CO_2 was used at a displacement rate of 30% vol/min. After 10 min, the rats were observed to no longer be breathing and had discolored eyes. Minimum CO_2 flow was maintained for a further 2 min following respiratory arrest. Animal death was then determined. All animal experiments were approved by the Ethics Committee of Zhongda Hospital Southeast University (approval no. 190501017; Nanjing, China).

Cell culture. The mouse leukemia RAW264.7 cell line was purchased from the American Type Culture Collection and seeded in RPMI 1640 media (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in an incubator at 37˚C with 5% CO_2. After 24 h, the culture medium was changed to serum-free medium overnight. An inflammatory response model was established using recombinant mouse high mobility group box 1 (rmHMGB1; 1 μg/ml; cat. no. ab255799; Abcam) and recombinant mouse heat shock protein 60 (rmHSP60; 1 mol/ml; cat. no. ab92364; Abcam) for 24 h at 37˚C. Crocin (low dose, 25 μg/ml; high dose, 50 μg/ml) was used to pretreat RAW264.7 cells 1 h before establishment of the inflammatory response model. The cells in the control group were not treated with crocin, rmHMGB1 or rmHSP60.

Cell transfection. RAW264.7 cells ($2x10^5$ cells/well) were transfected with 50 nM small interfering RNA (si)-negative control (NC; 5'-UUCUCCGAACGUGUCACGU-3') and 50 nM si-KBTBD7 (5'-GGAUUAAUAUAGGCACCAU-3'), synthesized by Shanghai GenePharma Co., Ltd. Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) reagent was used for transfection according to the manufacturer's protocol. The transfection was performed at 37˚C for 24 h.

TUNEL assay. Cardiac tissue apoptosis was detected using the TUNEL assay (cat. no.; 40307ES50; Shanghai Yeasen Biotechnology Co., Ltd.) according to the manufacturer's protocol. Briefly, RAW264.7 cells were fixed with 4% paraformaldehyde at 4˚C for 25 min and permeabilized using 0.2% Triton X-100 at room temperature for 5 min. After washing with PBS, the cells were stained with 50 μl TdT buffer at 37˚C for 1 h. The nuclei were stained with 2 μg/ml DAPI at room temperature for 5 min in the dark. Images were captured using a confocal microscope and 10

randomly-selected high-magnification fields were observed. The apoptotic rate was calculated as follows: Apoptotic nuclei/total nuclei x100.

Reverse transcription quantitative-PCR (RT-qPCR). Total RNA was extracted from rat myocardium and RAW264.7 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). An ultraviolet spectrophotometer was used to determine the concentration and purity of the RNA. Total RNA was reverse transcribed into cDNA using the following components: High-Fidelity Script reverse transcriptase, dNTPs, primers, RNA, RT buffer, dithiothreitol and RNase-free water (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed at 37˚C for 15 min followed by 85˚C for 5 sec. qPCR was subsequently performed using the UltraSYBR Mixture (CoWin Biosciences) according to the manufacturer's protocol. The following thermocycling conditions were used for qPCR: Pre-denaturation at 95˚C for 10 min; 35 cycles of denaturation at 95˚C for 15 sec, and annealing and elongation at 60˚C for 1 min; followed by final extension at 95˚C for 15 sec, 60˚C for 1 min, 95˚C for 15 sec and 60˚C for 15 sec. The following primer pairs were used for qPCR: KBTBD7 forward (F), 5'-AGCTCAGTTGTATCG GTAGCA-3' and reverse (R), 5'-CCGGAATAAGGGTCG TAACAGA-3'; interleukin (IL)-1β F, 5'-GGTGTGTGACGT TCCCATTAGAC-3' and R, 5'-CATGGAGAATATCACTTG TTGGTTGA-3'; IL-6 F, 5'-TAGTCCTTCCTACCCCAATTT CC-3' and R, 5'-TTGGTCCTTAGCCACTCCTTC-3'; tumor necrosis factor α (TNFα) F, 5'-AAGCCTGTAGCCCACGTC GTA-3' and R, 5'-GGCACCACTAGTTGGTTGTCTTTG-3'; Bax F, 5'-AGTGTCTCAGGCGAATTGGC-3' and R, 5'-CAC GGAAGAAGACCTCTCGG-3'; Bcl-2 F, 5'-ACTGAGTAC CTGAACCGGCATC-3' and R, 5'-GGAGAAATCAAACAG AGGTCGC-3'; and GAPDH F, 5'-AGGTCGGTGTGAACG GATTTG-3' and R, 5'-TGTAGACCATGTAGTTGAGGT CA-3'. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (32) and normalized to the internal reference gene GAPDH.

Western blotting. After 30 min of ischemia and 120 min of reperfusion, 100 mg myocardial tissue was immediately extracted from the ischemic area in the left ventricle of the rats. Tissue was subsequently washed with 1X PBS before tissue lysis solution was added. The tissue was then incubated with RIPA buffer (Beyotime Institute of Biotechnology) on ice for 30 min. Total protein content was determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Inc.). In addition, RAW264.7 cells were collected, cells were cleared on ice, and total protein was extracted using RIPA buffer and was quantified using a BCA assay. Total protein (20 μg) was separated by SDS-PAGE on 12% gels. After blocking with 5% skim-milk at room temperature for 1 h, the PVDF membranes were incubated with primary antibodies against HMGB1 (1:1,000; cat. no. ab227526; Abcam), HSP60 (1:1,000; cat no. ab190828; Abcam), p38 (1:1,000: cat. no. 8690; Cell Signaling Technology, Inc.), phosphorylated (p)-p38 (1:1,000; cat. no. 4511; Cell Signaling Technology, Inc.), JNK (1:1,000; cat. no. 9252; Cell Signaling Technology, Inc.), p-JNK (1:1,000; cat. no. 9251; Cell Signaling Technology, Inc.), p65 (1:1,000; cat. no. 8242; Cell Signaling Technology, Inc.), p-p65 (1:1,000; cat. no. 3033; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) overnight at 4˚C. Horseradish peroxidase-labeled secondary antibodies (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) were then added to incubate with the membranes at room temperature for 2 h. Protein bands were visualized using ECL (Shanghai Yeasen Biotechnology Co., Ltd.). The ratio of gray values was used for semi-quantitative analysis with GAPDH as the loading control using ImageJ software (version 1.8.0; National Institutes of Health).

ELISA. The RAW 264.7 cell supernatant solution was collected. ELISA was performed according to the manufacturers' instructions. The levels of cytokines in each treatment group were determined using the standard curve and optical density. The following ELISA kits were used: IL-1β ELISA Kit [cat. no. EK201B/3; Hangzhou Multi Sciences (Lianke) Biotech, Co., Ltd.], TNFα ELISA Kit [cat. no. EK282/4; Hangzhou Multi Sciences (Lianke) Biotech, Co., Ltd.], IL-6 ELISA Kit [cat. no. EK206/3; Hangzhou Multi Sciences (Lianke) Biotech, Co., Ltd.], HSP60 ELISA Kit (cat. no. EM1136; FineTest), and HMGB1 ELISA Kit (cat. no. EM0382; FineTest).

Statistical analysis. Data were analyzed using SPSS 19.0 software (IBM Corporation) from three independent experiments. Data are presented as the mean ± standard deviation. Unpaired Student's t-test was used for comparisons between two groups. One-way ANOVA and Tukey's post-hoc test was used for comparisons between multiple groups (33-35). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Crocin inhibits the inflammatory response and myocardial apoptosis caused by MIRI. A rat model of MIRI was constructed to study the pharmacological effects of crocin, the chemical structure of which is shown in Fig. 1A. To assess the inflammatory response and the degree of myocardial injury, mRNA expression levels of IL-1β, IL-6 and TNFα in myocardial tissue were determined. The mRNA expression levels of IL-1β, IL-6 and TNFα were significantly increased in the MI group compared with those in the sham group. However, the mRNA expression levels of IL-1β, IL-6 and TNFα were significantly decreased following crocin treatment compared with in the MI group (Fig. 1B-D). Furthermore, the TUNEL assay was used to detect apoptosis. Apoptosis was observed following myocardial I/R in the MI group, this was significant compared with the sham group; however, the apoptotic rate in the low and high crocin treatment groups was significantly reduced compared with that in the MI group (Fig. 1E). The mRNA expression levels of the apoptosis-related protein Bax and antiapoptotic protein Bcl-2 were also detected. The mRNA expression levels of Bax in the myocardial tissue of the MI group were significantly increased compared with those in the sham group; however, Bax expression was significantly reduced following treatment with high and low doses of crocin compared with in the MI group (Fig. 1F). The mRNA expression levels of Bcl-2 were significantly downregulated in the MI group compared with those in the sham group, and were significantly upregulated following high and low levels

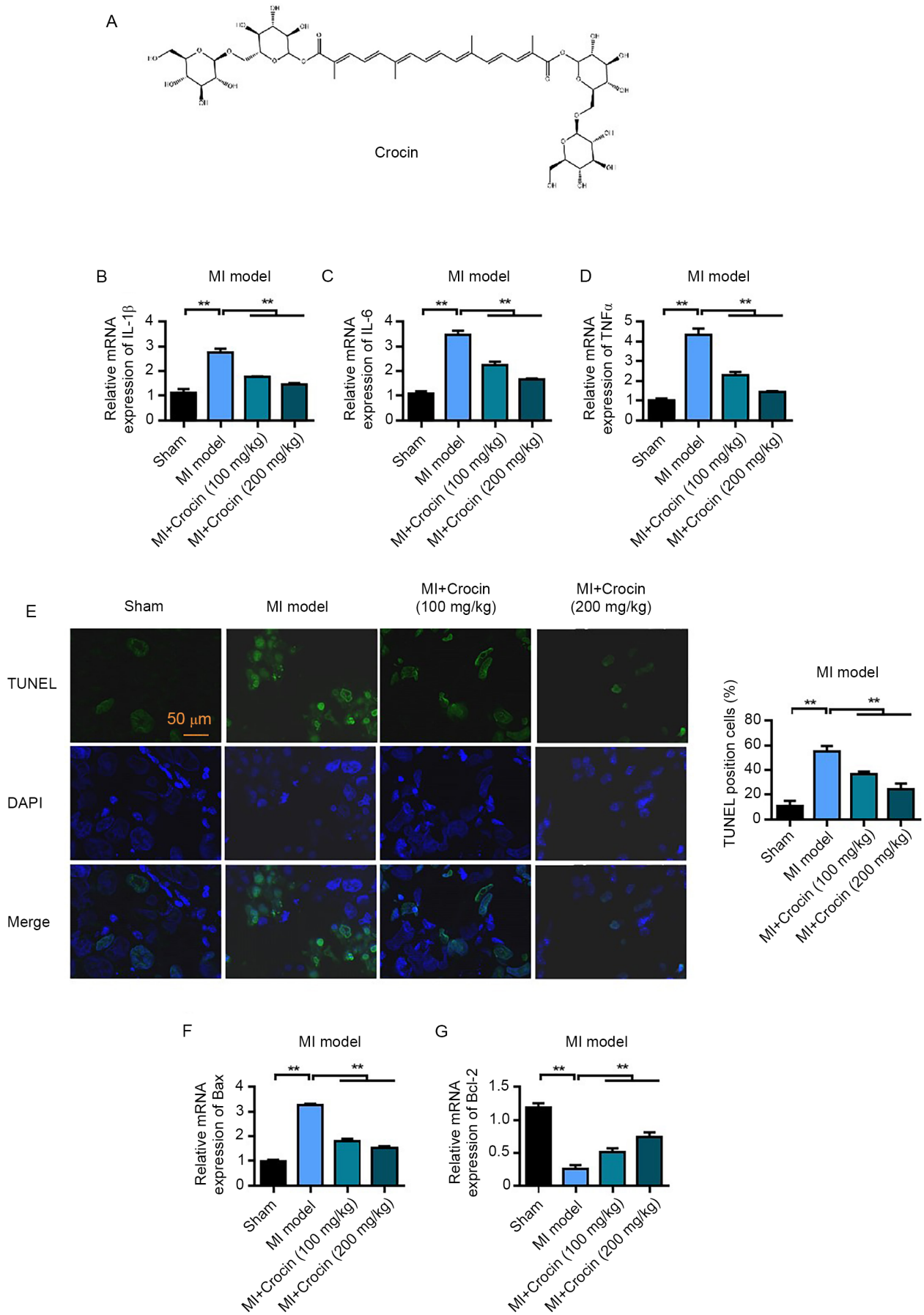

Figure 1. Crocin inhibits inflammation following myocardial infarction. (A) Molecular structure of crocin. RT-qPCR detected (B) IL-1β, (C) IL-6 and (D) TNFα mRNA expression levels in rat myocardial tissue. (E) TUNEL assay detected rat myocardial tissue apoptosis. RT-qPCR detected (F) Bax and (G) Bcl-2 mRNA expression levels in rat myocardial tissue. Results were obtained from three independent experiments. Data are presented as the mean ± SD (n=10). **P<0.01. Magnification, x400. RT-qPCR, reverse transcription-quantitative PCR; MI, myocardial infarction; IL, interleukin; TNFα, tumor necrosis factor α.

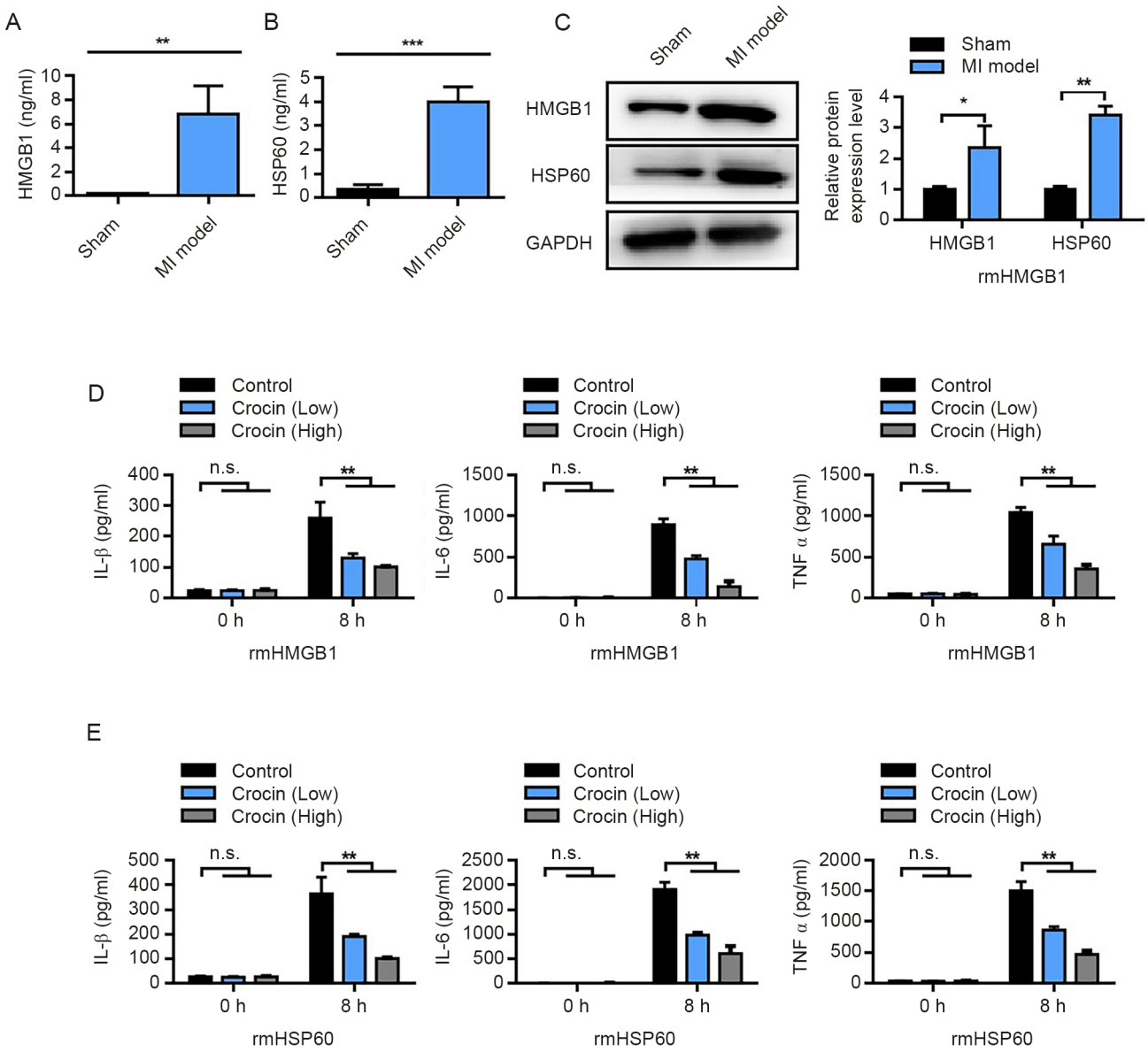

Figure 2. Crocin inhibits the production of damage-associated molecular pattern-triggered inflammatory cytokines. ELISA was used to detect the levels of (A) HMGB1 and (B) HSP60 in rat heart tissue homogenates. (C) Western blotting was performed to detect the protein expression levels of HMGB1 and HSP60 in rat heart tissue. ELISA was used to detect the effect of crocin inhibition on (D) rmHMGB1 (1 μg/ml) and (E) rmHSP60 (1 mol/ml) induced inflammatory factor levels in the RAW 264.7 cells supernatant. Results were obtained from three independent experiments. Data are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001. HMGB1, high mobility group box 1; HSP60, heat shock protein 60; rm, recombinant mouse; MI, myocardial infarction; n.s., not significant; IL, interleukin; TNFα, tumor necrosis factor α.

of crocin treatment compared with in the MI group (Fig. 1G). These results therefore suggested that crocin could inhibit myocardial apoptosis caused by MIRI.

Crocin inhibits the production of damage-associated molecular pattern (DAMP)-triggered inflammatory cytokines. Changes in the levels of HMGB1 and HSP60 in myocardial tissue following MI were determined. HMGB1 and HSP60 levels, measured using ELISA, were significantly increased in the MI group compared with those in the sham group (Fig. 2A and B). Western blotting results also demonstrated that the protein expression levels of HMGB1 and HSP60 were significantly increased in the MI group compared with those in the sham group (Fig. 2C). Subsequently, rmHMGB1 and rmHSP60 were used to stimulate an inflammatory response in RAW264. 7 cells and the effect of crocin on these macrophages was observed. The results demonstrated that the levels of IL-1β, IL-6 and TNFα following rmHMGB1 treatment were significantly downregulated by the addition of crocin, in a dose-dependent manner, compared with the control (no crocin treatment) (Fig. 2D). These results were further reflected upon RAW 264. 7 cells treatment with rmHSP60 (Fig. 2E). These results indicated that crocin may inhibit the inflammatory response triggered by HMGB1 and HSP60.

Activation of MAPK and NF-κB signaling pathways is inhibited by crocin. Western blotting was used to detect the expression levels of proteins involved in the MAPK and NF-κB signaling pathways following treatment with rmHMGB1, rmHSP60 and crocin. The results demonstrated that the protein expression

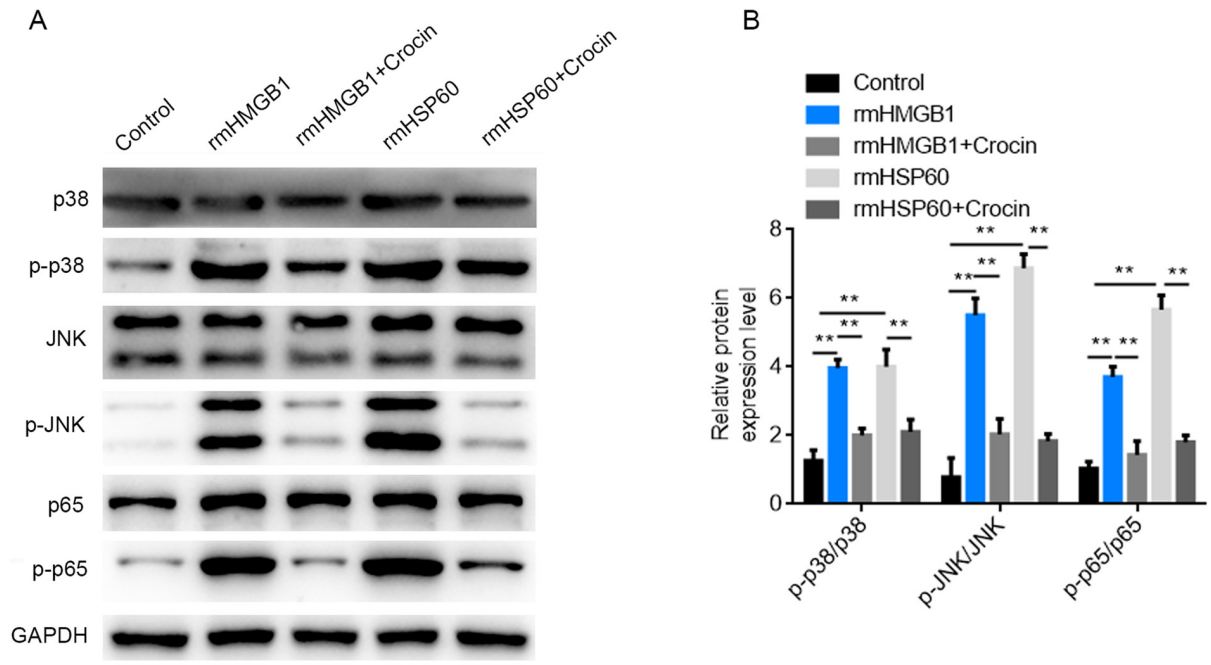

Figure 3. Crocin inhibits p38 and NF-κB signaling pathways under damage-associated molecular pattern-induced conditions. (A) Western blotting was used to detect MAPK and NF-κB-associated protein expression levels. (B) Semi-quantification of western blotting results. Results were obtained from three independent experiments. Results were obtained from three independent experiments. Data are presented as the mean ± SD. **P<0.01. rmHMGB1, recombinant mouse high mobility group box 1; rmHSP60, recombinant mouse heat shock protein 60; n.s., not significant; p, phosphorylated; NF-κB, nuclear factor-κB.

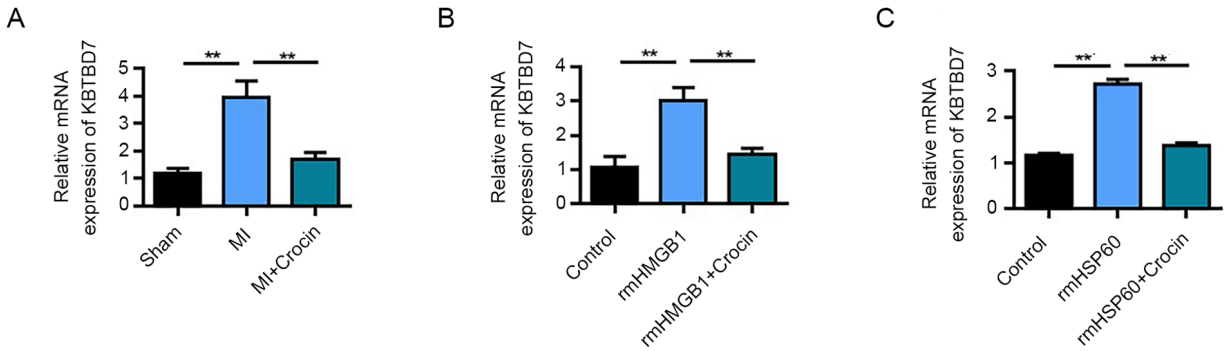

Figure 4. Crocin-targeted inhibition of KBTBD7. (A) mRNA expression levels of KBTBD7 were upregulated during myocardial infarction and inhibited by crocin. (B) mRNA expression levels of KBTBD7 were upregulated when cells were treated with rmHMGB1 and inhibited by crocin. (C) mRNA expression levels of KBTBD7 were upregulated when cells were treated with rmHSP60 and inhibited by crocin. Results were obtained from three independent experiments. Results were obtained from three independent experiments. Data are presented as the mean ± SD. **P<0.01. KBTBD7, kelch repeat and BTB domain-containing protein 7; rmHMGB1, recombinant mouse high mobility group box 1; rmHSP60, recombinant mouse heat shock protein 60; MI, myocardial infarction.

levels of p-p38, p-JNK and p-p65 were significantly increased following treatment with rmHMGB1 and rmHSP60 compared with those in the control group. However, the protein expression levels of p-p38, p-JNK and p-p65 were significantly reduced following high-dose crocin treatment compared with cells that received no crocin (Fig. 3A and B). Therefore, the results indicated that rmHMGB1 and rmHSP60 macrophage stimulation may activate the p38 and NF-κB signaling pathways. The results also suggested that crocin may suppress the production of inflammatory cytokines through inhibition of the MAPK and NF-κB signaling pathways.

Crocin targets and inhibits KBTBD7. KBTBD7 plays a crucial role in MI, especially in the response to inflammation and regulation of myocardial function (6). Therefore, the present study investigated whether crocin could function through regulating KBTBD7. qPCR detection demonstrated that KBTBD7 was significantly upregulated following MI compared with in the sham group. Furthermore, high-dose crocin treatment significantly downregulated KBTBD7 mRNA expression levels compared with those in the MI group (Fig. 4A). In RAW264.7 cells, rmHMGB1 induction significantly upregulated KBTBD7 mRNA expression levels compared with in the control, whereas crocin inhibited KBTBD7 mRNA expression levels in rmHMGB1-treated cells (Fig. 4B). Similar results were also determined in the rmHSP60-induced RAW264.7 cells (Fig. 4C). These results therefore suggested that KBTBD7 is a target of crocin.

KBTBD7 knockdown reduces the production of DAMP-induced inflammatory cytokines. The effect of KBTBD7 on

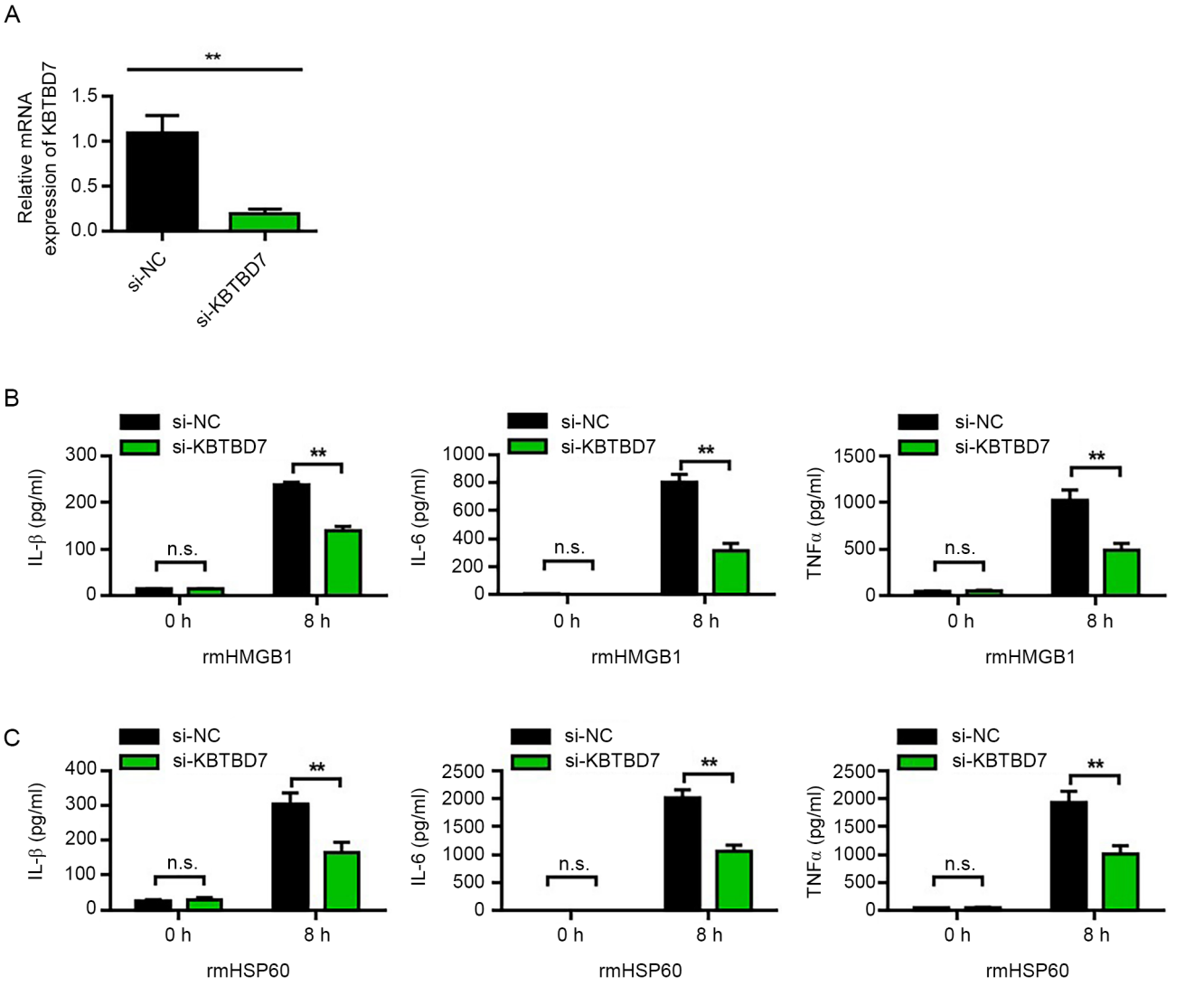

Figure 5. Knockdown of KBTBD7 reduces damage-associated molecular pattern-induced inflammatory cytokine production. (A) Transfection efficiency of si-KBTBD7 was verified. Knockdown of KBTBD7 reduced (B) rmHMGB1 (1 μg/ml) and (C) rmHSP60 (1 mol/ml) induced increases in inflammatory factors. Results were obtained from three independent experiments. Results were obtained from three independent experiments. Data are presented as the mean ± SD. **P<0.01. KBTBD7, kelch repeat and BTB domain-containing protein 7; si, small interfering RNA; rmHMGB1, recombinant mouse high mobility group box 1; rmHSP60, recombinant mouse heat shock protein 60; NC, negative control; n.s., not significant; IL, interleukin; TNFα, tumor necrosis factor α.

DAMP-induced inflammatory cytokines in RAW264.7 cells was further observed. The transfection efficiency of si-KBTBD7 was first analyzed. The results demonstrated that si-KBTBD7 could significantly reduce mRNA expression levels of KBTBD7 compared with si-NC (Fig. 5A). Furthermore, the results demonstrated that KBTBD7 knockdown significantly decreased the levels of the rmHMGB1- and rmHSP60-induced inflammatory cytokines compared with si-NC (Fig. 5B and C).

KBTBD7 knockdown reduces DAMP-induced p38 and NF-κB signaling activation. The molecular mechanism by which KBTBD7 affects the inflammatory response was investigated. Western blotting was used to detect the expression levels of proteins associated with the MAPK and NF-κB pathways following rmHMGB1, rmHSP60 and si-KBTBD7 treatments. The results demonstrated that the protein expression levels of p-p38, p-JNK and p-p65 were significantly increased following rmHMGB1 and rmHSP60 treatment compared with those in the control group. However, KBTBD7 knockdown significantly reduced the protein expression levels of p-p38, p-JNK and p-p65 compared with the rmHMGB1 and rmHSP60 only treatments (Fig. 6A and B). These results indicated that rmHMGB1 and rmHSP60 stimulation activated the p38 and NF-κB signaling pathways, whereas KBTBD7 knockdown potentially inhibited the production of inflammatory cytokines.

Discussion

Ischemic cardiomyopathy is a complex heart disease worldwide (36). The primary and most common cause of ischemic cardiomyopathy is coronary atherosclerosis, which causes lumen stenosis or occlusion (37). Early reperfusion therapy is an important strategy to save dying myocardial tissues; however, reperfusion itself can also cause injury. Reperfusion can induce the production of high levels of reactive oxygen species, resulting in the peroxidation damage of proteins, lipids and chromosomes, calcium overload, leukocyte accumulation,

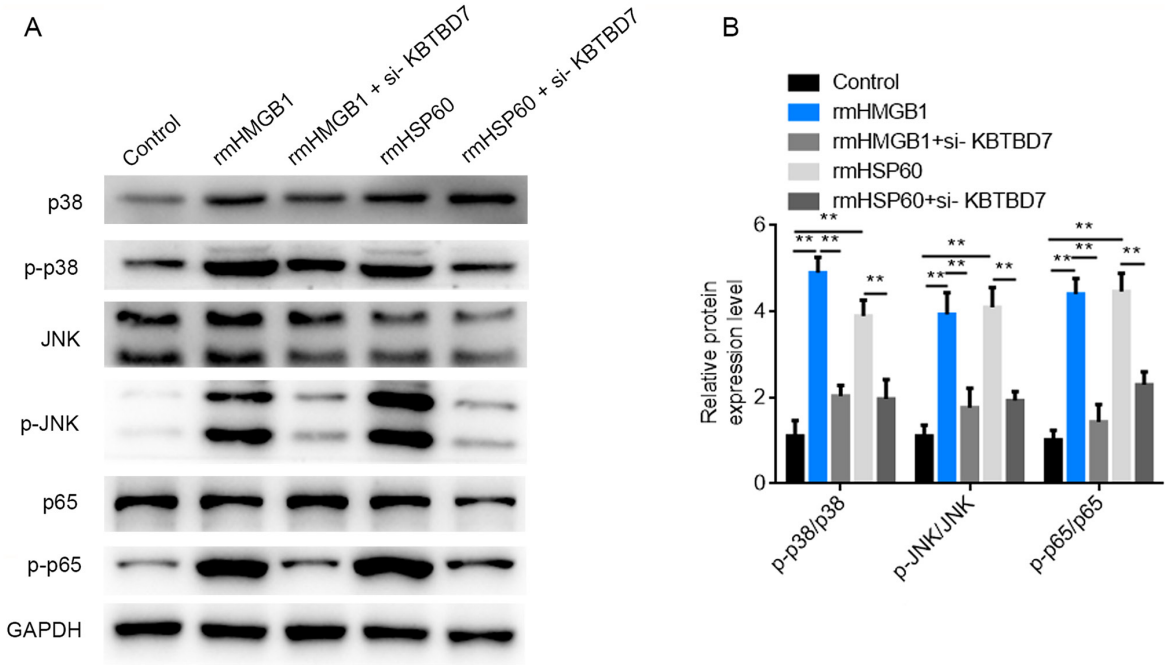

Figure 6. Knockdown of KBTBD7 reduces the activation of p38 and NF-κB signaling pathways under damage-associated molecular pattern-induced conditions. (A) Western blotting was used to detect MAPK and NF-κB-associated protein expression levels. (B) Semi-quantification of western blotting results. Results were obtained from three independent experiments. Results were obtained from three independent experiments. Data are presented as the mean ± SD. **P<0.01. KBTBD7, kelch repeat and BTB domain-containing protein 7; rmHMGB1, recombinant mouse high mobility group box 1; rmHSP60, recombinant mouse heat shock protein 60; n.s., not significant; p, phosphorylated; si, small interfering RNA.

abnormal pH and ATP energy metabolism disorder (38). The inflammatory response plays an important role in this phenomenon by triggering the release of numerous pro-inflammatory factors, such as TNFα, IL-6 and IL-1β, which can lead to tissue damage and in severe cases to apoptosis and necrosis (39). Therefore, inhibiting the inflammatory response can reduce MIRI to a certain extent (40).

Crocin is a traditional Chinese medicine extract with strong bioactivity (17). Crocin has been reported to exhibit a variety of pharmacological activities, such as antioxidative, anticancer, anti-inflammatory and anti-atherosclerosis effects, and cardiovascular protection (41). Crocin has also been shown to act on mitochondria and inhibit mitochondrial dysfunction in cardiovascular-related tissues and cells (42-44). Crocin has previously been reported to reduce the hydrogen peroxide-induced mitochondrial membrane potential of platelets (44) and increase the release rate of cytochrome *c* into rat heart tissues (42). Crocin may also act on cardiovascular diseases via reducing platelet aggregation and apoptosis, oxidative stress and cardiovascular apoptosis (45,46). In the present study, the literature and preliminary experiments were used to determine the concentrations of crocin to use in animals and cells (47-50). The preliminary results demonstrated that the current dose of crocin (200 mg/kg in mice) would reflect the therapeutic effect of crocin on myocardial ischemia.

Following MIRI, DAMPs are released, which consequently activate toll-like receptors expressed in macrophages, triggering a series of inflammatory responses. Notably, HMGB1 and HSP60 are major DAMP triggers (51,52). HMGB1 is a highly conserved and widely present protein in the nuclei of eukaryotic cells (53,54). Under normal physiological conditions, HMBG1 has the function of a nuclear-binding protein, whereas in the pathological state, it can enter the intercellular space from the nucleus through passive release and active secretion, thus triggering an inflammatory response (55). As a pro-inflammatory protein, HMGB1 plays an important role in MIRI and is associated with inflammatory factors, such as TNFα, IL-6 and inducible nitric oxide synthase (56). Inhibition of HMGB1 activity has demonstrated the protective effects of HMGB1 on cardiopulmonary function injury (57). The present study further investigated the inhibitory effects of crocin on HMGB1 and HSP60-induced inflammatory responses. The results demonstrated that treatment with HMGB1 and HSP60 markedly upregulated the levels of IL-1β, IL-6, and TNFα in RAW 264.7 cells. Conversely, the levels of these pro-inflammatory cytokines were significantly inhibited by crocin. The cardiac protective effect of crocin may be related to the inhibition of inflammatory responses. The release of inflammatory factors is closely related to the occurrence and development of MIRI (58). During and after MIRI, a high level of inflammatory cytokines, such as TNFα and IL-6, are released into the blood, which can cause neutrophils to adhere to endothelial cells (59). This phenomenon results in no reflow after reperfusion and a large amount of oxygen free radicals are generated in response (60). In addition, macrophages are encouraged to secrete a large amount of inflammatory factors, which eventually cause or aggravate the degree of MIRI (61). In the present study, crocin treatment reduced the levels of TNFα and IL-6 in the serum of rats with MIRI. This result suggested that crocin may protect against MIRI in rats by reducing inflammatory responses.

The MAPK and NF-κB signaling pathways serve key roles in inflammatory responses in MIRI; therefore, inhibition of

the MAPK and NF-κB signaling pathways serves an important role in reducing the inflammatory response (62,63). The present study revealed that crocin suppressed the production of inflammatory cytokines, which may be mediated through inactivation of the MAPK and NF-κB signaling pathways.

The BTB protein is a transcription factor in eukaryotes, which is closely related to the development of various multicellular organisms, including fruit flies and vertebrates. Yang *et al* (6) demonstrated that microRNA-21 inhibited the inflammatory response in the early stages of MI by targeting KBTBD7, and inhibiting p38 and NF-κB signaling activation, thereby preventing excessive scarring and improving cardiac function. In the present study, crocin reduced the inflammation caused by MIRI and the apoptosis of myocardial cells. The protective effect of crocin may be realized by inhibiting the expression of KBTBD7.

In conclusion, the present study demonstrated that crocin protects against MIRI by inhibiting myocardial inflammatory response and regulating the expression of inflammatory factors. The anti-inflammatory effects of crocin were possibly related to the inhibition of KBTBD7. The present study has provided a new therapeutic approach for MIRI and lays the foundations for further research on the myocardial-protective mechanism of crocin and the development of novel treatments. Moreover, KBTBD7 may be a potential target of MI therapy.

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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

CY designed the study and wrote the manuscript. CY, ZC and QZ performed all experiments. ZC is responsible for data analysis. CY and QZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were operated according to the Guide for the Care and Use of Laboratory Animals (64) and were approved by the Ethics Committee of Zhongda Hospital Southeast University (approval no. 190501017; Nanjing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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