Original Article Cryptotanshinone alleviates myocardial ischemia and reperfusion injury in rats to mitigate ER stress-dependent apoptosis by modulating the JAK1/STAT3 axis

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Abstract: Objective: Myocardial ischemia is the stoppage or insufficiency of blood flow to the myocardium, depriving cells of oxygen supply which leads to their apoptosis or death. Currently, the management of patients has improved, making it possible to reduce myocardial infarction injury with new strategies of reperfusion and pharmacologic treatment. Methods: A rat model of myocardial ischemia and reperfusion injury (MIRI) was created and subjected to cryptotanshinone (CRY) with or without JAK1 inhibitor filgotinib (FILGO) treatment. H&E staining was used for histopathologic evaluation of heart injury, and TTC staining was employed for evaluation of the infarct size. Western blotting and immunofluorescence were used to measure the protein expression and qRT-PCR for determining mRNA expression. Results: CRY significantly reduced the area of the infarct, the number of apoptotic cells, and the concentrations of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) induced by ischemia/reperfusion (I/R). Subsequent analysis showed that CRY repressed the expression of caspase-12, CHOP, and GRP78, but enhanced the phosphorylation of JAK and STAT3. However, FILGO treatment markedly abolished the beneficial effect of CRY pretreatment on cardiomyocyte damage, apoptosis, cardiac function, and inhibition of endoplasmic reticulum stress (ERS)-dependent apoptosis marker proteins. Conclusion: CRY may alleviate MIRI by inhibiting ERS-dependent apoptosis by activating the JAK1/STAT3 signaling pathway.

Keywords: Apoptosis, cryptotanshinone, endoplasmic reticulum, JAK1/STAT3 signaling, myocardial ischemia and reperfusion injury

Introduction

Myocardial infarction (MI) represents a high percentage of cardiovascular diseases (over 40% in men) and is the leading cause of morbidity and mortality globally [1]. Remarkable advances in the treatment, care, and prevention of acute myocardial infarction have been achieved since the early 1980s, with the increasing use of pharmacologic treatments and the advent of several coronary reperfusion strategies. The decrease in mortality can also be explained by secondary prevention and the discovery of a sensitive marker of cardiac muscle ischemia, troponin, which allowsbetter classification of risks and early diagnosis [2]. However, myocardial tissue reperfusion after ischemia can induce myocardial ischemia and reperfusion injury (MIRI), which leads to additional damage to the myocardium [3]. This damage accounts for more than half of myocardial infarctions, worsening cardiac function and worsening the prognosis [4]. Despite the proposal of numerous pathophysiologic mechanisms underlying the pathogenesis of MIRI (including myocardial apoptosis, inflammation, calcium overload and reactive oxygen species (ROS)-related injury), few evidence-based strategies for the prevention of MIRI have been elucidated [5-8]. Thus, there is a need to develop new pharmacologic treatment strategies to alleviate MIRI while improving the prognosis of patients with myocardial infarction.

The endoplasmic reticulum (ER) is an important part of the secretory pathway, which provides the proteins entering it with a favorable environment for the acquisition of a correct conformation. The proteins then undergo numerous post-translational modifications under the supervision of chaperone proteins. A previous study indicated that the ER was involved in cell apoptosis regulation without activating an inflammatory process based on the fragmentation of DNA and cell organelles [9]. Additionally, disruption of ER homeostasis induced by ischemia/hypoxia or nutrient deprivation can cause imprecise protein synthesis and assembly. However, under certain pathophysiologic conditions, abnormally conformed proteins accumulate in the ER, which leads to the induction of an adaptive response called unfolded protein response (UPR) [10]. This process allows the cell to reabsorb the accumulated proteins in the lumen of the ER by the attenuation of protein synthesis. It also reduces the protein overload observed in the ER by a specific transcriptional program, thus, allowing an increase in the expression level of chaperone proteins or proteins involved in the ER-associated protein degradation (ERAD) mechanism. If the homeostasis of the ER is not restored, then the second phase of the program leading to apoptosis is initiated [11]. This integrated adaptive response is controlled mainly by three transmembrane proteins residing in the ER: PERK, ATF6, and inositol enzyme-1 (IRE1). The first of these three proteins to have been identified is IRE1. It is the only one present in eukaryotes, from yeast to humans. IRE1, therefore, represents the most conserved arm of the UPR. Under normal physiologic conditions, GRP78 usually binds tightly to IRE1. ERS acts as a stress response for the body to respond to external stimuli, and is involved in tumorigenesis and development. Among these, glucose-regulated protein 78 (GRP78) plays a role in regulating cells. As a role of proliferation and metabolic capacity, GRP78 can increase the expression of apoptotic protein Caspase-12 through the IRE1/tumor necrosis factor receptor-associated factor 2 (TRAF2) signaling pathway, and induce apoptosis. Using transgenic animal models, all of the above data have shown that the activation of glucose-regulated protein-78 (Grp78) and the IRE1 and ATF6 pathways are beneficial in the ischemia-reperfusion response.

Nevertheless, the deletion of C/EBP homologous protein (CHOP) protects the heart by decreasing infarct size, demonstrating that ER stress can have a harmful role [12]. In addition, the state of the ER in cardiomyocytes is altered particularly by pathologies increasing the susceptibility to ischemia-reperfusion. For example, in a diabetic rat model, the increase in the Grp78 and CHOP markers is associated with an aggravation of cell death following ischemiareperfusion. However, this effect is abolished by the deletion of CHOP [13]. This suggests that the activation of ERS may be involved in the process of myocardial ischemia-reperfusion injury.

Cryptotanshinone (CRY) is a lipophilic active compound isolated from the roots of Salvia miltiorrhiza, that has been associated with apoptosis by a pharmacologic processes network. CRY has been used for centuries in traditional Chinese medicine as an anti-cancer and anti-inflammatory agent, antioxidant, and treatment against cardiovascular disease [14]. CRY has anti-inflammatory, neuroprotective, cardioprotective, visceral protective, anti-metabolic disorders and other properties. STAT3-related signaling pathways perform a crucial role in the CRY-mediated induction of tumor cell apoptosis and proliferation [15]. In addition, CRY exerts anti-inflammatory effects by inhibiting NO and inducible NO synthase expression by the ERBdependent pathway to enable cardioprotective effects [16]. A previous study demonstrated that CRY could suppress oxidative stress and apoptosis though the Akt-GSK-3β-mPTP signaling pathway to exert cardioprotective effects [17]. Studies have shown that JAK/STAT3 signaling pathway plays a role in endoplasmic reticulum stress injury during myocardial ischemia-reperfusion, which could affect signal transducer and the activator of transcription 3 (STAT3) which is a component of cardioprotection [18]. Activation of JAK-STATs upregulates iNOS through the ischemic preconditioning pathway, thereby promoting the heart to adapt to ischemic stress. The JAK-STAT pathway consists of a series of receptor-associated cytoplasmic tyrosine kinases (JAKs) that phosphorylate tyrosine residues in STAT homology [19]. In addition, CRY was considered early on as a drug that alleviates I/R myocardial damage. In vivo and in vitro bioassays have confirmed the inhibitory properties of CRY in I/R-induced cardiomyocyte apoptosis [20]. Over the past 20 years, considerable progress has been made in improving thrombolysis and angioplasty techniques thatreduce mortality due to infarction by promoting reperfusion of the artery responsible for the necrosis. Today, one of the challenges is to develop new treatments to protect against or limit I/R damage following myocardial infarction. However, whether CRY attenuates myocardial I/R injury by inhibiting ERS through JAK-STAT3 signaling remains unclear. Therefore, using a rat model of MIRI, we aimed to assess whether treatment with CRY could alleviate MIRI by regulating ERS-related apoptosis proteins and the underlying mechanism.

Materials and methods

In this study, male Sprague Dawley (SD) wildtype rats (SPF grade, 211-253 g) were used. All institutional and national guidelines for the care and use of laboratory animals were followed and approved by the institutional ethics committee of Shanghai Sixth People's Hospital (approval No. 2022-0282). The animals were placed in an animal room with regulated temperature conditions and humidity with 12-hour alternating daytime/night cycles. Food and drink were accessible ad libitum. All the following procedures were conducted after anesthesia. Rats were injected intraperitoneally with an overdose of pentobarbital (150 mg/kg) to make them die from cardiac arrest and respiratory failure, and then tissue samples were taken to be tested.

MIRI model

A rat model of myocardial I/R injury was established according to a previously reported strategy [21]. Anesthesia of rats was performed by intraperitoneal administration of sodium pentobarbital (60 mg/kg). Electrocardiogram was recorded, and ventilation was maintained with a tidal volume of 15 mL/kg with air ambient and a respiratory rate of 70 times/min. The ligation of the left coronary artery was performed with 6-0 suture over a tube at a width of 3-4 mm and a depth of about 1.5-2 mm. The successful establishment of the MIRI model was confirmed by the pallor of the myocardium at the far end of the tied point and a continuous increase of the ST segment. The ligation of the left coronary artery was maintained for 30 min, resulting in ischemia. After this, the ligature was relaxed, and reperfusion lasted for 24 h. Reperfusion was confirmed by a lowering of ST segment and the transformation of the ischemic myocardium from a pale to ruddy state. The same protocols were applied for the Sham group, except that the left coronary artery was not ligated.

Experimental grouping and treatment

Five experimental groups were studied (n=10 per group): sham control group; ischemia and reperfusion (I/R) group, I/R + CRY (40 mg/kg), I/R + CRY (40 mg/kg) + FILGO (0.3 mg/kg) and I/R + FILGO (0.3 mg/kg). FILGO, a specific inhibitor of JAK1, was dissolved in DMSO to a final concentration of 0.3 mg/mL before use. After establishment of the MIRI model, Sham and I/R groups were administered an equivalent amount of 0.9% saline for 3 days. Each treatment group was administered for 3 days on the basis of the I/R model group. The rats in the I/R + CRY group were administered with CRY (40 mg/kg) by intraperitoneal injection on the basis of the model group, and the rats in the I/R + CRY + FILGO group were intraperitoneally injected with CRY (40 mg/kg) and FILGO (0.3 mg/kg). I/R + FILGO group rats were intraperitoneally injected with FILGO (0.3 mg/kg). Then the rats in each group were allocated for histopathologic evaluation, biochemical and molecular assay.

Histopathologic examination of the myocardial tissue

After the MIRI model and treatment, the hearts were collected, and the blood was rinsed. The myocardial tissue was fixed in buffered formalin for at least 48 h. Next, the heart samples were successively dehydrated in gradient concentrations of ethanol, paraffin-embedded and then sectioned. Sections were selected, affixed to slides, and stained with H&E staining. Images of the whole tissue were taken in white light with a microscope equipped with a camera. The images were acquired using the software ImagePro.

Assessment of infarction size

After ischemia-reperfusion, the heart was stripped of its connective tissue and then frozen at -80°C. The heart was then cut crosswise in 6 slices and incubated for 20 min in phosphate buffer containing 1% sodium chloride triphenyl tetrazolium (TTC in 0.2M Tris buffer, pH 7.4) (Sigma-Aldrich, St. Louis, USA) at 37°C.

Primer name	Primer sequence
GRP78 forward	5'-CCAATGACCAAAACCGCCTG-3'
GRP78 reverse	5'-AGCTTTCCAATTCATTCCTCGTG-3'
CHOP forward	5'-TCTGCCTTTCGCCTTTGAGA-3'
CHOP reverse	5'-AAGCTAGGGATGCAGGGTCA-3'
Caspase-12 forward	5'-TGGTCTTTATGTCCCACGGC-3'
Caspase-12 reverse	5'-CTGCAGGCTTGCATGATGAG-3'
GAPDH forward	5'-GACTCTACCCACGGCAAGTT-3'
GAPDH reverse	5'-TGGGTTTCCCGTTGATGACC-3'

Table 1. List of primers employed in this study

Reducing the TTC by cutting the tetrazolium cycle led to formazan formation, a compound colored red. Thus, the viable cells were stained red and necrotic cells remained white. The slices of heart were then fixed in 10% formalde-hyde for 24 h and photographed. The photos were analyzed using ImageJ software. The size of the infarct area was presented as a percentage of the size of the ventricles.

Measurement of myocardial injury markers

Lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were used in the early diagnosis of myocardial infarction to estimate the time of onset of the infarction, its size and extent, and diagnose recurrence of ischemia or infarction. These cytoplasmic enzymes escape from damaged myocardial cells, providing information on the severity of destruction of cell membrane integrity. The CK-MB and LDH were measured through blood plasma samples obtained using available assay kits (Nanjing Jiancheng Bioengineering Institute, China).

TUNEL apoptosis assay

To locate myocardial apoptotic cells on tissue sections, we used the TUNEL method (terminal transferase-mediated dUTP nick end labeling) [22]. The TUNEL method was carried out on frozen sections (5 μ m sections) according to the supplier's instructions (Roche) to detect the DNA fragments produced during apoptosis. A positive control (tissue treated with DNase I 3 U/mL (Qbiogene)) and a negative control (omission of DNA transferase step) were used simultaneously. The calculation of TUNEL-positive cardiomyocytes was based on five selected sections (×400) of each piece. Finally, we calculated the apoptosis rate as the percentage of positive TUNEL cells to total myocardial cells.

Heart function assessment

The linear dimensions of the left ventricle were obtained by measuring the direct view of the various structures from the minor axis medial cut, which intersects the ventricle left at the level of the two posteromedial and anterolateral pillars of the mitral valve. After cardiac puncture, a catheter tip (Millar Mikro-Tip[®] pressure catheter, connected to power lab data acquisition system; NIBP, AD Instruments, Australia) was inserted into the left ventricle. Thus, LVEDd. LVEDv. LVEDId and LVESv were measured. The ejection fraction (EF) and fractional shortening (FS) of left ventricular were estimated from the linear dimensions of the ventricle left according to the following formula: (EF= (LVEDv - LVESV) *100%/LVEDv; FS= (LVEDd -LVEDId) *100%/LVIDd).

Quantitative real-time PCR (qRT-PCR)

The Trizol kit (Invitrogen, USA) was used for extracting and purifying total RNA from myocardial samples. Then, reverse transcription was achieved using 1 g of extracted RNA into complementary DNA using the TaKaRa (Japan) cDNA synthesis kit. The cDNA was thereafter used for RT-PCR amplification with the SYBR Green Supermix (Bio-Rad, United States) and the CFX96 Touch Real-Time PCR Detection Systems thermal cycler (Bio-Rad, USA). The reaction conditions were as follows: denaturation at 95°C for 15 m; 40 denaturation cycles at 94°C for 10 s, then annealing at 55°C for 30 s; finally, extension to 70°C for 30 s. Each reaction was performed in duplicate. The GAPDH gene was used as a control. The relative values of gene expression were evaluated by the comparative quantification method ($2^{-\Delta\Delta CT}$). Primer sequences for RT-PCR are shown in Table 1.

Immunofluorescence staining

In order to assess the cardioprotective effects of CRY on the I/R, immunofluorescence staining was used to detect the expression of α -actinin in I/R rats. First, paraffin sections were dewaxed and dehydrated by gradient concentrations of alcohol, washed three times using PBS (37°C), then fixed in PBS and incubated at 4°C overnight with anti- α -actinin (1:100). Secondly, the sections were washed and labeled with a fluorescein isothiocyanate

(FITC)-IgG antibody. Thirdly, the sections were washed 3 times in PBS and subjected to 4',6-diamidino-2-phenylindole (DAPI) staining for 2 min. Lastly, they were again washed in PBS and detected with a fluorescence microscope.

Western blotting

Total proteins from myocardial tissue were extracted using the Beyotime kits (China). After centrifugation, the supernatant liquid was collected to determine the protein concentration of each sample. Then, we chose to work with 10% SDS-polyacrylamide gels, mostly depending on the molecular weight of the proteins studied. After the separation in the gel, the proteins were transferred and exposed to a nitrocellulose membrane. The membrane was blocked in a solution rich in milk (casein) for 1 h to limit the non-specific hybridization of proteins on the membrane. The proteins of interest were revealed using primary antibodies directed against the protein of interest. The membrane was incubated overnight with antibodies (1:1000) against GRP78 (11587-1-AP, Proteintech, USA), Caspase-12 (ab62463, Abcam, USA), CHOP (15204-1-AP, Proteintech, USA), JAK1 (344, CST, USA), p-JAK1 (74129, CST, USA), STAT3 (9139, CST, USA), p-STAT3 (9145, CST, USA), CYT-C (10993-1-AP, Proteintech, USA) and C-Caspase-8 (8592, CST, USA). Secondary antibodies were specifically bound to the primary antibody. These primary antibodies were coupled to a peroxidase, catalyzing the following reaction at the place where the protein was located. The application of photographic film made it possible to identify the band corresponding to the protein of interest and correlate its expression level with the intensity of the obtained signal. We used the protein Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference.

Statistical analysis

Data in this study are expressed as the mean \pm standard deviation ($\overline{x} \pm$ sd). One-way ANOVA followed by Dunnet's posttest were used for detecting significance in the discrepancy between groups, and P<0.05 was used as significance threshold. Statistical analyzes were performed using software GraphPad Prism.

Results

CRY attenuates I/R injury in the myocardium

The efficacy of CRY on myocardial injury was evaluated by treatment with different doses (20, 40, and 60 mg/kg) of CRY. Histopathologic changes were analyzed by H&E staining, and observation under the light microscope showed changes in the morphology of the myocardial cells (Figure 1A). In the Sham group, the myocardial fibers remained intact; moreover, the myocardial cell structures were clear, without necrosis. On the contrary, in the I/R group, the heart tissue was incompletely muddled. accompanied by the shedding of the cells, which fell apart and lysed. However, treatment with different doses of CRY alleviated the effect of I/R on the myocardial structure (Figure 1A). Evaluation of the damage score indicated significant damage in the I/R group compared to the control group; however, the treatment with CRY relieved this effect dose-dependently (Figure 1B). Significant changes were observed when CRY concentration of 40 mg/kg was used; consequently, we retained this concentration as the optimal dose for the experiments that followed.

CRY reduces the size of myocardial infarction and alleviates myocardial damage

The infarct size was measured using double staining with Evans blue and TTC. The infarct size was significantly increased in the I/R group compared to the sham group (Figure 2A). We also noticed that the infarct size was categorically reduced by 40 mg/kg CRY in the CRY40 + I/R group compared to that of the I/R group (Figure 2A). In addition, co-treatment with CRY and FILGO significantly increased the effect of CRY on myocardial infarction size compared to the single treatment of CRY (Figure 2B). Also, FILGO alone significantly decreased the infarct size compared to the I/R + CRY40 group. Further measurements indicated that I/R injury induced the damage of heart tissue as reflected by the upregulation of the plasma levels of LDH as well as CK-MB (Figure 2C, 2D). The treatment of CRY was followed by lower levels of CK-MB and LDH compared to the I/R group (Figure 2C, 2D). Most notably, FILGO treatment increased the effect of CRY on the plasma levels of LDH and CK-MB compared to the I/R group (Figure 2C, 2D). In addition, there were



Figure 1. Histopathologic evaluation of the effect of CRY on myocardial tissue during ischemia-reperfusion. A: Representative images of H&E staining after CRY administration (×200); B: Damage score on the myocardial tissue. Compared with the sham group, *P<0.05, ****P<0.0001; compared with the I/R group, ###P<0.001, ####P<0.0001, n=10 per group. CRY, Cryptotanshinone; I/R, ischemia/reperfusion.

significant differences in LDH and CK-MB levels between the group treated with FILGO alone and the I/R group (P<0.05); (Figure 2C, 2D).

CRY alleviates myocardial I/R injury, promotesα-actinin expression, and inhibits myocardial apoptosis

Immunofluorescence was used to appraise the effect of CRY on the expression of α -actinin in I/R rats. As shown in Figure 3A, 3B, I/R significantly inhibited the expression of α -actinin but this effect was alleviated by CRY treatment. In addition, the treatment by FILGO promoted the effect of CRY on the expression of α -actinin in I/R animals (Figure 3A, 3B). The expression of α-actinin was induced by FILGO treatment alone compared to that of the I/R group but inhibition of the expression was still showing after FILGO treatment alone compared to that of the sham group (Figure 3A, 3B). Furthermore, cardiomyocyte apoptosis was evaluated by the TUNEL staining. As we can see in Figure 3C, 3D, minimal TUNEL-positive cells could be seen in the Sham group. Besides, I/R injury significantly increased myocardial cell apoptosis, raising the number of TUNEL-positive cells in the myocardial tissue (Figure 3C, 3D). On the contrary, treatment by CRY alleviated the effect of I/R on cell apoptosis (**Figure 3C**, **3D**). A dramatic increase in the number of apoptotic myocardial cells was recorded after FILGO treatment, which promoted the effect of CRY.

CRY restricts heart dysfunction caused by I/R injury, and the restraint of JAK1 enhances its protective effects against I/R

Systolic heart failure is characterized by an abnormality of systolic function with dilation of the cardiac chambers and eccentric remodeling based on two entities which are ejection fraction (EF) and fractional shortening (FR). According to our study, as shown in Figure **4A-C**, the I/R injury caused a deterioration in systolic cardiac function. In contrast, administration of CRY showed marked improvement in EF (36.6%) and FS (25.6%) (Figure 4B, 4C). Also, the beneficial effects of CRY on cardiac function were promoted by FILGO (Figure 4A-C).

CRY reverses myocardial ERS-dependent apoptosis induced by I/R injury by inhibiting the JAK1/STAT3 signaling pathway

To elucidate the action of CRY on ERS, the mRNA and protein levels of GRP78 (a particular



Figure 2. FILGO increased the effect of CRY on the MIRI. A: Representative images of Evans and TTC staining; B: Ratios of myocardial infarct size; C: CK-MB enzyme levels; D: LDH levels. Compared with the sham group, **P<0.01, ****P<0.001, ****P<0.001; compared with the I/R group, *P<0.05, **P<0.01, ****P<0.001, ****P<0.0001; compared with the I/R group, *P<0.05, **P<0.01, ****P<0.001, ****P<0.0001; compared with the I/R group, FILGO, filgotinib; CRY, Cryptotanshinone; MIRI, myocardial ischemia and reperfusion injury; CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase.

ERS biomarker) was assessed in each group of heart tissues using messenger RNA and protein as samples during polymerase chain reactions. **Figure 5A**, **5B** showed that after pretreatment with 40 mg/kg CRY, GRP78 was markedly downregulated, suggesting that CRY attenuated myocardial ERS prompted by I/R injury. Nevertheless, co-treatment with CRY and FIL-GO increased the effect of CRY on the level of GRP78. There was no important change in the expression of GRP78 between the group treated with FILGO alone and the I/R group. Taken together, these results suggested that CRY improves ERS induced by MIRI through the JAK1/ STAT3 signaling pathway. To confirm this assumption, we further explored the expression of caspase-12 and CHOP through RT-PCR and western blot (**Figure 5B-E**). The I/R injury significantly stimulated the caspase-12 and CHOP expression levels (**Figure 5B-E**). In addition, CRY administration significantly hindered CHOP and capase-12 expression levels compared to



Figure 3. CRY alleviates MIRI-promoted α -actinin expression and inhibits myocardial apoptosis in I/R. A: The α -actinin green fluorescence images from the studied groups (×400); B: α -actinin intensity rate. Compared to the sham group; C: The TUNEL staining images from the studied groups (×400); D: Apoptosis rate. Compared to the control group, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; compared with the I/R group, *P<0.05, #*P<0.05, **P<0.01; compared with the I/R + CRY40 group, *P<0.05, **P<0.01, n=6 per group. CRY, Cryptotanshinone; MIRI: myocardial ischemia and reperfusion injury.

the I/R group (Figure 5B-E), and this effect was increased by FILGO treatment (Figure 5B-E). We noticed that the CRY treatment facilitated STAT3 and JAK1 phosphorylation, and this effect was significantly increased by FILGO treatment (Figure 5F, 5G). These results showed that CRY inhibited the expression level of p-JA-K1 and p-STAT3, which were the key proteins in JAK-STAT signaling pathway, while FILGO treatment markedly increased the beneficial effects of CRY pretreatment on ER stress-dependent apoptosis. Our results also showed that compared to group I/R, treatment with CRY could not change cytochrome C expression and the level of cleaved caspase-8 protein (Figure 5H, 5I).

Discussion

Myocardial ischemia and reperfusion injury (MIRI) is a pathological condition with which patients can have short-term ischemia and hypoxia, and may experience cardiac ischemia and hypoxia during revascularization. MIRI may lead to cardiac ultrastructural changes and cardiac dysfunction. The high level of ROS during reperfusion induces unprovoked necrosis of myocardial cells and reduce myocardial contractile function. The only treatment option for ischemia is reperfusion. It is essential for preventing heart attack, but it occurs abruptly, with a rapid return of oxygen and nutrients, causing other types of myocardial consequenc-





Figure 4. CRY ameliorates cardiac function after MIRI. A: Evaluation of heart function in each group; B: Ejection fraction rate; C: Fractional shortening. Compared to the control group, **P<0.01, ***P<0.001, ****P<0.001; compared to the I/R group, ##P<0.01, ###P<0.001; compared to the I/R + CRY40 group, \$P<0.05, \$P<0.01, n=6 per group. CRY, Cryptotanshinone; MIRI, myocardial ischemia and reperfusion injury.

Cryptotanshinone inhibits MIRI by JAK1/STAT3



Figure 5. CRY reverses myocardial ERS-dependent apoptosis induced by I/R injury via inhibiting the JAK1/STAT3 axis. A: GRP78 measured by qRT-PCR; B: GRP78 and caspase-12 measured by western blot; C: CHOP measured by qRT-PCR; D: Caspase-12 measured by qRT-PCR; E: CHOP measured by western blot; F: STAT3 and p-STAT3 measured by western blot; G: JAK1 and p-JAK1 measured by western blot; H: Cytochrome C measured by western blot; I: Cleaved Caspase-8 analyzed by western blot. Compared to the control group, **P<0.01, ***P<0.001, ****P<0.001; compared with the I/R group, #P<0.05, #P<0.01; compared with the I/R + CRY40 group, \$P<0.05, \$P<0.01, n=6 per group. CRY, Cryptotanshinone; I/R, ischemia/reperfusion.

es [23]. In addition, reperfusion after ischemia has been reported to increase levels of pro-

inflammatory mediators in the plasma membrane and neutrophil infiltration into the infarct area, leading to contractile dysfunction and ischemic damage in rat model of ischemia-reperfusion [24, 25]. As demonstrated by numerous clinical trials, the availability of therapies has increased significantly, but their effectiveness is still limited by not knowing their mechanism or action or the complexity of the pathogenesis of myocardial infarction [26-28]. It has been reported that ischemia and reperfusion induce various responses such as decreased oxygenation and production of radicals from endothelial cells to several injuries due to microcirculation [29]. Therefore, finding effective means or drugs to inhibit myocardial cell apoptosis and reduce myocardial ischemiareperfusion injury is aan urgent issue that needs to be solved in the treatment of myocardial ischemia. CRY has been known for centuries in traditional Chinese medicine for its anticancer, anti-inflammatory and antioxidant properties, as well as its ability to relieve cardiovascular disease [30]. However, the effects of CRY on myocardial cells of MIRI model rats and the mechanisms by which CRY might alleviate MIRI are very ill-defined. Here, we showed, for the first time, that CRY improved heart function and reduced heart infarction in the rat model of MIRI. Furthermore, we found that CRY inhibited ERS-depend apoptosis of myocardial cells by triggering the JAK1/STAT3 axis, indicating that activation of the JAK1/STAT3 axis and enhancement of ERS may be implicated in the inhibitory role of CRY on myocardial apoptosis.

A previous study found that inflammatory response and apoptosis are closely related to MIRI injury, while ERS is an important signaling pathway that induces the inflammatory response and apoptosis [31]. Furthermore, apoptosis is characterized by condensation and fractionation of chromatin, shrinkage of cells, and fragmentation into apoptotic bodies, which can be eliminated by macrophages [32]. Although the signaling pathways for apoptosis and necrosis (linked to mitochondrial swelling caused by the opening of the mitochondrial permeability transition pore) are different, they involve identical steps, such as activation of caspases [33]. Thus the chronology of their activation and the preponderance of one mechanism over another during ischemia or reperfusion are still poorly defined [34]. As for the signaling pathways that control cell apoptosis, apoptotic signaling associated with ERS has

been recognized as one of the essential mechanisms of MIRI [35]. Publications have shown activation of ER stress in ischemia or after ischemia-reperfusion [36, 37]. However, its beneficial or harmful role is not clearly defined, and the role also depends on its duration of activation. The ERS sensor protein, Grp78, plays an essential role in the cardiovascular system since its complete deletion causes many problems in embryonic development, leading to the death of the embryo in utero. In this study, our results demonstrated that CRY significantly suppressed the expression of caspase-12, CHOP and GRP78, which indicated that CRY inhibited the level of ERS in the MIRI model rats. The endoplasmic reticulum is a membranous organelle that is ubiquitous in eukaryotic cells, and endoplasmic reticulum stress (ERS) is an important pathologic pathway regulating inflammatory response and apoptosis [38]. MIRI also induces ERS and aggravates MIRI injury. Excessive ERS can destroy the protein synthesis process, resulting in protein unfolding or misfolding and accumulation in the endoplasmic reticulum, and induce the up-regulation of GRP78 and p-JNK expression, so they can be used as ERS detection indicators [39]. CHOP, as a pro-apoptotic signaling molecule existing in the endoplasmic reticulum organelle, can cut and activate Caspase-12, and activated cleaved Caspase-12 that is the activation stimulus factor of Caspase-3, which in turn activates cells apoptosis [40]. In addition, the overexpression level of Grp78 induces cardiac remodeling, which can lead to heart failure [41, 42]. Like Grp78, the IRE1 pathway is also active in ischemia, particularly with the overexpression of XBP1 shown in vivo and in vitro, e.g., in the hearts of patients in ischemia [43, 44]. The specific cardiac deletion of XBP1 increases the size of infarction following ischemia-reperfusion and alters cardiac function, while its overexpression decreases the size of the infarct and improves cardiac function. Using models of transgenic animals, all of the above data showed that activating Grp78 and the IRE1 and ATF6 pathways was beneficial in the ischemiareperfusion response. Nevertheless, the deletion of CHOP protected the heart by reducing the size of infarcts, thus demonstrating that ER stress could have a harmful role. In addition, overexpression of Grp78 in neonatal rat ventricular myocytes diminished damage due to ischemia-reperfusion through attenuation of the production of ROS and activation of the STAT3 signaling pathway [12, 31]. However, CRY profoundly downgraded the expression of caspase-12, GRP78, and CHOP at gene and protein levels, with increase in the other markers implicated in the severity of MIRI. JAK/ STAT3 signaling is an important transduction pathway involved in various pathologic processes such as inflammation, immunity, apoptosis and oxidative stress in the body. In addition, study has shown that after activating JAK/ STAT3 signals, cells first activate JAK, further phosphorylate STAT3, transfer extracellular signals to the nucleus, regulate the expression of target genes, and exert biological effects [45]. Studies have confirmed that in myocardial ischemia-reperfusion rats, activation of JAK/STAT3 signaling can significantly enhance the expression level of anti-apoptotic protein Bcl-2 and reduce apoptosis [46]. A previous study has shown that in cardiomyocytes injured by hypoxia-reoxygenation, activation of JAK/STAT3 signaling can significantly inhibit cellular oxidative stress and reduce ROS accumulation [47]. Interestingly, in our study, JAK1 inhibitor was used in model animals to inhibit JAK1/STAT3 signaling, while CRY could promote the phosphorylation level of JAK1 and STAT3, and exert a protective effect on the heart. Our results indicated that CRY inhibited the JAK1/STAT3 signaling pathway to alleviate myocardial ischemia-reperfusion injury. Therefore, we anticipated that the myocardial therapeutic or protective properties of CRY were mainly driven by the mitigation of the stress of the endoplasmic reticulum and that of the apoptotic signaling pathways by the JAK1/STAT3 signaling pathway.

Our study also revealed a primary role of CRY in decreasing p-JAK1 and p-STAT3 levels after administration of CRY in I/R. The role of the JAK1/STAT3 axis in the improvement process of MIRI was explored through the use of CRY and FILGO in IR. The JAK1/STAT3 inhibition with FILGO treatment impeded JAK1 and STAT3 phosphorylation induced by CRY. The outcome of the acceleration of CRY effects by FILGO was the improvement of cardiac function and induction or ERS-associated apoptosis as demonstrated by the changes in the expression profiles of CHOP, GRP78, and caspase-12. Our results imply that JAK1/STAT3 restriction and inhibition of ERS-associated apoptosis of the myocardial cells may be involved in regulating mechanisms of CRY on MIRI. The present study is the first to convey these findings. Nevertheless, CRY is routinely considered a typical STAT3 inhibitor. However, in our study, the results showed that CRY dramatically reduced the expression level of STAT3, especially phosphorylated STAT3. Therefore, we will further study these hypotheses in the future, such as the possible molecular mechanism of CRY on STAT3, and how it affects the expression of STAT3 and p-STAT3. However, in this study, our results indicated that CRY could have cardioprotective effects on MIRI in vivo. Further studies are required to demonstrate this protective effect and underlying mechanisms of CRY on MIRI in vitro. For instance, previous report showed that CRY could attenuate ischemia and reperfusion-induced microcirculatory disturbances through the inhibition of NF-KB-activation during ischemia and reperfusion [48]. In addition, further large multicentric human studies are required to verify the prospective effect of CRY in ameliorating cardiac injury in patients with MIRI, whose underlying mechanism needs to be further investigated to treat MIRI clinically. Further mechanisms of CRY in MIRI should be explored in the future.

In summary, our results revealed a protective effect of CRY on heart infarction induced by I/R. It should also be noted that CRY could alleviate myocardial ERS induced by cardiomyocyte apoptosis by the JAK1/STAT3 signaling pathway. These results give a foundation for the clinical application of CRY. However, further clinical studies are needed to confirm its therapeutic potential.

Disclosure of conflict of interest

None.

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