Cucurbitacin B protects against myocardial ischemia-reperfusion injury through activating JAK2/STAT3 signaling pathway

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ABSTRACT

Cucurbitacin B, a tetracyclic triterpenoid compound extracted from various plants, has been proven to exert a vital role in various diseases. However, the effect of cucurbitacin B on myocardial infarction (MI) and ischemia-reperfusion (I/R) injury is still relatively unclear. The main purpose of the present study was to investigate the effect of cucurbitacin B on cell apoptosis and oxidative damage after myocardial I/R injury in vitro and in vivo and elucidate the molecular mechanisms underlying its role. The 56-day-old adult mice and 1-day-old neonatal mice cardiomyocytes were used to construct I/R or oxygen-glucose deprivation/reoxygenation (OGD/R) injury models. The oxidative injury, western blot and TUNEL assay were performed to evaluate cardiomyocyte damage in the present study. In vitro, we confirmed that cucurbitacin B could attenuate LDH release, oxidative stress and cell apoptosis in cardiomyocytes exposed to OGD/R. Besides, we confirmed in an adult I/R mouse model that cucurbitacin B can improve cardiac repair and block cell apoptosis in the acute phase (24 h) post-myocardial I/R injury, as well as promote long-term cardiac function and fiber scar area after 28 days of I/R. Mechanically, we clarify that cucurbitacin B exerts cardiomyocyte protective effects through activating the JAK2/STAT3 signaling pathway. In conclusion, our study elucidates for the first time the protective role of cucurbitacin B in cardiac I/R injury, which provides a novel perspective for better prevention of I/R injury through the JAK2/STAT3 signaling pathway.

Keywords: Myocardial ischemia-reperfusion; Cucurbitacin B; Apoptosis; Oxidative stress; JAK2/STAT3 pathway

Introduction

Acute myocardial infarction (AMI), as the most severe type of ischemic heart disease (IHD), can lead to countless irreversible myocardial deaths in the infarcted area, as well as severe damage to myocardial tissue at the border of the infarction(1, 2). Timely reperfusion therapy, such as thrombolysis and percutaneous coronary intervention (PCI), can reconstruct the coronary arterial blood flow and greatly reduce the area of myocardial necrosis, which is the key to the treatment of AMI(3). However, sudden opening of the coronary artery can cause dysfunction of cardiomyocytes, activation of inflammatory factors, and oxidative damage, which can exacerbate I/R injury of the ischemic myocardium(4). Previous studies have shown that nearly 50% of necrotic myocardium after reperfusion is caused by reperfusion, which significantly weakens the benefits of reperfusion therapy(5, 6). Therefore, reducing I/R injury has become an important link in the prevention and treatment of AMI.

The mechanism of myocardial injury caused by myocardial I/R therapy mainly includes the following aspects: Firstly, reperfusion injury leads to excessive free radical production, which exceeds the body's clearance capacity, thereby exacerbating myocardial injury(7). Secondly, during ischemia and hypoxia, myocardial intracellular acidosis causes a large amount of Ca²⁺ to deposit in mitochondria, causing damage to mitochondrial function(8). Additionally, ischemia leads to energy metabolism disorders in myocardial cells, further exacerbating free radical production and mitochondrial damage, in addition, I/R injury can exacerbate endothelial cell antioxidant system damage and cause a series of gene expression disorders, exacerbating myocardial damage(9). These factors mentioned above can exacerbate oxidative stress damage to myocardial cells, initiate the process of myocardial cell death, and thus hinder the benefits of I/R therapy(10). Therefore, how to attenuate myocardial cell damage caused by reperfusion therapy is currently a difficult research topic.

Cucurbitacin is a tetracyclic triterpenoid compound extracted from various plants in the Cucurbitaceae and other families and genera, with nearly 50 species discovered(11). Among them, cucurbitacin B is the most widely used and has extensive pharmacological activities in inflammation, cell proliferation, oxidative stress, apoptosis damage, immune regulation, and other aspects(12). In the field of cardiovascular research, there have been some reports on cucurbitacin B, for instance, cucurbitacin B can significantly inhibit pressure overload-induced myocardial hypertrophy and fibrosis in mice by increasing autophagy.
gy(13). Interestingly, cucurbitacin B can also significantly reduce the release of LDH, oxidative stress, and inflammatory damage, reducing neuronal apoptosis and cerebral infarction area, thereby improving cerebral I/R-induced brain injury(14). However, the role and mechanism of cucurbitacin B in myocardial I/R injury are currently unclear.

In the present study, we investigated the function of cucurbitacin B on OGD/R or I/R-induced oxidative stress and apoptotic damage *in vitro* and *in vivo*. In addition, we elucidated that the JAK2/STAT3 signaling pathway mediates oxidative stress and apoptotic damage in cardiomyocyte induced by I/R. Our results reveal that cucurbitacin B can serve as therapeutic targets in the treatment of cardiac I/R injury.

### Materials and Methods

#### Animal model construction

The animals used in the present study for in vivo experiments were approved by the Laboratory Animal Research Committee of Soochow University (Approval NO.SUDA20200197A01). Using postnatal 56-day (P56) adult male mice, intraperitoneal injection of 1.2% Avertin (Sigma-Aldrich, St. Louis, USA) to full anesthesia. Fix the limbs and provide tracheal intubation and a small animal ventilator for auxiliary ventilation. Separate the subcutaneous tissue between 3-4 ribs on the left side of the precordium to expose the heart, and use the suture needle of 7-0 suture to pass through the myocardial layer on the left side of the middle part of LAD. Subsequently, place the PE10 catheter on the surface of the heart, parallel to LAD, and use a ligature suture to lift the heart. After 45 minutes of ischemia, the knots were untied and the PE10 tube was removed to achieve reperfusion with or without intraperitoneal administration. Finally, remove the endotracheal tube and place it on a constant temperature table until it is fully awake and normal activity is fully restored before returning to the cage.

#### Cell culture and treatment

Digestion of neonatal 1-day-old mouse myocardial tissue using trypsin (Sigma-Aldrich, St. Louis, USA) and protease II (Worthington, USA), extraction of primary myocardial cells through centrifugation, differential adhesion, and other operations. After culturing cells in a DMEM (Invitrogen, Gibco, USA) medium containing 10% HS and 5% FBS for 48 hours, OGD/R model construction and drug treatment were performed. Treat with corresponding concentrations of cucurbitacin B (Rongehe, Shanghai, China) for 48 hours in control and OGD/R cardiomyocytes. In the rescue experiment, cucurbitacin B treatment was given after OGD/R, with or without AG490 (Selleck, Shanghai, China) treatment for 48 hours. Finally, LDH release, oxidative stress levels, and the proportion of apoptotic cells were measured.

#### MTT assay

Digestion, centrifugation, and counting of pre-processed myocardial cells in the logarithmic phase, followed by 100 μL with the density of 10^3/well was inoculated on a 96-well plate and then incubated at 37°C and 5% CO2 for 24 hours. Observe the growth status and density of cells under a microscope, and take holes with uniform density for testing. Measure the absorbance at 570nm using an enzyme-linked immunosorbsorbent assay and perform statistical analysis.

#### Detection of oxidative stress levels and LDH release

Use the corresponding commercial oxidative stress kits (Beyotime, Shanghai, China) to detect the oxidative stress markers and LDH release after different treatments, including the production of reactive oxygen species (ROS), the level of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD). The specific operations were carried out according to the production instructions.

#### Western blot

Put ventricular muscle tissue into EP tube and put it on ice, then add 300 μL RIPA protein lysate and protease inhibitor into each tube, then sterilize and wipe the tissue with scissors, and cut the tissue as much as possible with scissors, and keep it on ice during the cutting process. For the extraction of protein from adherent parietal cells, add an appropriate amount of protein lysate into the cell culture dish and transfer the protein lysate to 1.5 ml EP tube and place it on ice. After testing the protein concentration using BSA standards, add 5 X Loading Buffer protein loading buffer, mix well, and then boil in a 99°C metal bath for 10 minutes. Cool on ice and use for Western blot experiments. Protein was separated by 10% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membrane. After 2h BSA blocking, the membranes were cultured with primary antibodies of p-JAK2, JAK2, p-STAT3, STAT3, Bax, Bcl-2, caspase-3 and β-actin (1:1000, Abcam, MA, USA) overnight at 4 °C. After incubating the secondary antibody for 2 hours, prepare the developer according to the ECL developer instructions and perform exposure development. Use the chemiluminescence imager (ProteinSimple, San Jose, CA, USA) to expose the bands and collect statistical image results.

#### TUNEL assay

For *in vitro* apoptosis staining, select myocardial cells with satisfactory cell growth status and density and undergo steps such as cleaning, fixation, membrane breaking, and sealing. Use a Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) reagent kit (Solarbio, Beijing, China) and follow the operating instructions for staining. For myocardial tissue, the collected heart is paraffin-embedded, and sliced for hydration, antigen repair, blocking, and antibody incubation. After cleaning the antibodies, use the TUNEL reagent kit and follow the instructions for staining. Finally, use a Zeiss microscope (LSM510META, Carl Zeiss, Jena, Germany) for photography and statistical analysis.

#### TTC staining

Twenty-four hours after I/R surgery, the heart was removed and residual blood was immediately squeezed out of the heart in PBS to preserve the intact heart. Place the heart tissue in a -20 °C refrigerator and embed it in OCT until completely solidified, wrapping the heart. Cross section of the heart from the apex to the bottom of the heart, with intervals greater than 500 μm between different sections. After staining with TTC solution (Solarbio, Beijing, China), fix the myocardial tissue sections with 4% neutral paraformaldehyde for 10 minutes, and then place the tissue sections under a stereomicroscope for photography.
Under the microscope, the tissue in the infarcted area of myocardial ischemia appears white, while the myocardial tissue in non-ischemic normal areas appears red.

**Transthoracic ultrasound analysis**

On the 28th day after I/R surgery, echocardiography was performed on different groups of mice. At the Animal Experimental Center of Suzhou University, professional technicians first anesthetizate mice by inhalation, and then use small animal heart ultrasound instruments to detect the ejection fraction (EF) and left ventricular short axis shortening rate (FS) of each group of mice. Finally, a statistical analysis of the results is conducted.

**Masson immunofluorescence staining**

After 28 days of cardiac ultrasound examination post I/R surgery, the heart was removed for paraffin embedding. Perform paraffin removal, Weigert iron hematoxylin staining, alcohol differentiation, and water washing on tissue sections. Finally, use the Masson staining kit (Solarbio, Beijing, China) to stain the tissue slices, seal and take photos, and calculate the scar area.

**Statistical analysis**

All statistical data were analyzed using SPSS 22.0 (IBM Corp., Armonk, USA) and mean ± standard error of the mean (SEM) using GraphPad Prism 8.0 software (La Jolla, CA, USA). For the mean comparison between the two groups, an unpaired T-test is performed when the data is normal distribution, otherwise, a hypothesis unequal variance t-test is performed. For comparisons among multiple groups, a single-factor analysis of variance (Tukey multiple comparison test) was used to determine statistical differences. When P<0.05, the difference is considered statistically significant.

**Results**

**Cucurbitacin B suppresses LDH release and oxidative stress in OGD/R-induced cardiomyocytes**

Cucurbitacin B is derived from plants in the Cucurbitaceae family and belongs to a class of tetracyclic triterpenoids with 19 methyl groups appearing at the C-9 position, Figure 1A shows its chemical structure. Cell viability analysis revealed that less than or equal to 1 μM does not alter the stability of cardiomyocytes (Fig 1B). To investigate the effect of cucurbitacin B on myocardial injury induced by I/R injury, we first constructed an OGD/R injury model using cardiomyocytes, As shown in Fig 1C, cucurbitacin B displayed a concentration-dependent inhibition on OGD/R induced LDH release in cardiomyocytes. Moreover, the oxidative stress damage indicators ROS and MDA induced by OGD/R were markedly decreased, while the SOD products inhibited by OGD/R were significantly elevated by cucurbitacin B (Figure 1D-1F).

**Cucurbitacin B inhibits apoptosis in cardiomyocytes exposed to OGD/R**

To explore the role of cucurbitacin B on cardiomyocyte apoptosis, cardiomyocytes were cultured with OGD/R for 24 hours with or without cucurbitacin B treatment. We used TUNEL reagent to stain myocardial cells and found that the number of apoptotic positive cells significantly increased in the OGD/R group, but could be significantly reversed by cucurbitacin B (Fig 2A-2B). Similarly, we further tested the effect of cucurbitacin B on apoptosis-related proteins of caspase-3, Bax and Bel-2 using the western blot experiment, and the results showed that the increased ratio of Bax/Bel-2 and the upregulation of cleaved-caspase-3 induced by OGD/R were evidently blocked by cucurbitacin B (Fig 2C-2E).

**Cucurbitacin B hinders acute injury and apoptosis post-cardiac I/R injury**

To further investigate the role of cucurbitacin B on myocardial injury post I/R injury, we constructed myocardial I/R injury models by ligating LAD for 45 minutes and then releasing the ligation cord to achieve reperfusion. Before reperfusion, a single dose of cucurbitacin B was administered through intraperitoneal injection, while the control group was only injected with water solvent. The injection
of cucurbitacin B improved acute phase I/R injury, as evidenced by the improvement in the myocardial infarction area evaluated by TTC staining analysis. As shown in Fig 3A-3B, the cucurbitacin B group mice had significantly less myocardial infarction area than the control solvent group. In addition, TUNEL staining results showed that cucurbitacin B markedly alleviated the cell apoptosis level in the infarct border myocardial area caused by I/R injury than the control group (Fig 3C-3D).

Cucurbitacin B promotes cardiac function and fiber scar repair post-I/R damage

To further explore whether cucurbitacin B can improve long-term cardiac function prognosis and reduce fibrotic scar area, we conducted cardiac function evaluation and Masson staining 28 days after I/R injury with or without cucurbitacin B treatment. On the 28th day after the I/R injury, transthoracic echocardiography results showed that compared to the control solvent group, cucurbitacin B significantly increased LVEF and FS levels, indicating that cucurbitacin B improved I/R-induced cardiac function damage (Fig 4A-4C). The hearts of two groups of mice were then collected and Masson staining was performed.

We found that the myocardial scar area in the cucurbitacin B group was significantly reduced compared to the control solvent group, indicating that cucurbitacin B has the effect of promoting fibrous scar repair (Fig 4D-4E).

Bioinformatics prediction of cucurbitacin B targets

To further investigate the therapeutic targets of cucurbitacin B for myocardial I/R injury, we used drug disease target prediction bioinformatics analysis. Using Swiss target prediction (http://www.swisstargetprediction.ch/), SuperPreD (https://www.genecards.org/), OMIM (https://www.omim.org/) and DisGeNET (https://www.disgenet.org/) retrieve disease targets related to “cucurbitacin B” and "myocardial I/R injury", and ultimately obtain 51 intersection target genes between myocardial I/R injury and cucurbitacin B (Fig 5A). Subsequently, the DAVID database was used for GO gene functional enrichment analysis to obtain drug disease intersection genes. Using P<0.05 as the standard, we screened 119 significantly enriched biological functions of cucurbitacin B in the treatment of myocardial I/R injury, mainly involving biological processes such as inflammatory response, signal transduction, protein phosphorylation, gene expression, cell apoptosis, gene expression regulation, and cell proliferation (Fig 5B). Using the DAVID database for pathway enrichment analysis, a total of 92 pathways related to the treatment of myocardial I/R injury with cucurbitacin B were enriched, including NOD-like receptors, PI3K-Akt, chemokines, and other signaling pathways (Fig 5C-5D). Finally, through drug component target data, core target and network interaction analysis (Fig 5E-5F), potential core targets were screened, including STAT3, TLR4, FYN, ITGB3, ITGB1, CASP8, HS-P90AB1, NFKB1, STAT1, PIK3R1.

Cucurbitacin B exerts cardiomyocyte protective effects through JAK2/STAT3 signaling pathway

Among the core target genes analyzed above, we found that STAT3 is most significantly associated with cucurbitacin B and is involved in cell apoptosis and oxidative stress regulation in various diseases. JAK2/STAT3, as a

Figure 3. Cucurbitacin B hinders acute injury and apoptosis post-cardiac I/R injury. A-B. TTC staining analysis was examined to evaluate acute phase I/R injury, n=6 in each group. C-D. TUNEL staining in vivo was performed to calculate the proportion of apoptotic positive cells in different treatment groups, n=6 in each group. ***p < 0.001.

Figure 4. Cucurbitacin B promotes cardiac function and fiber scar repair post-I/R damage. A-C. The cardiac function index of LVEF and FS were evaluated by transthoracic echocardiography. D-E. Masson staining was performed to calculate the scar area of myocardial fibers in different groups. n=6 in each group, ***p < 0.001.

Figure 5. Bioinformatics prediction of cucurbitacin B targets. A. A total of 51 intersection target genes between myocardial I/R injury and Cucurbitacin B were obtained. B. the DAVID database was used for GO gene functional enrichment analysis to obtain drug disease intersection genes. C-D. The DAVID database for pathway enrichment analysis and a total of 92 pathways related to the treatment of myocardial I/R injury with Cucurbitacin B were enriched. E-F. Core target and network interaction analysis were used to reveal potential downstream targets.
Cucurbitacin B exerts cardiomyocyte protective effects through the JAK2/STAT3 signaling pathway. A-C. Western blot analysis was conducted to examine the protein expression of (p)JAK2 and (p)STAT3. D-F. The level of ROS, MDA and SOD was used to detect the oxidative stress damage level. G-I. The caspase-3 expression and Bax/Bcl-2 ratio were detected by western blot analysis. Each experiment was repeated 3 times, n=3 in each group, *p < 0.05, **p < 0.01, ***p < 0.001 vs OGD or OGD/R group, #p < 0.01 and ##p < 0.001 vs OGD/R+CuB group.

Discussion

Multiple reasons can cause myocardial ischemia, resulting in hypoperfusion and hypoxia of myocardial tissue, and even leading to acute myocardial infarction. After reperfusion, the production of reactive oxygen species (ROS) increases after the restoration of blood ischemic tissue perfusion. ROS leads to oxidative stress response, resulting in endothelial cell dysfunction, DNA damage, and inflammatory response. Inflammatory cascade and oxidative stress lead to structural damage to endothelial cells, leading to cell death(17). Reperfusion injury is a dynamic change that can last for hours to days(18). Therefore, understanding the mechanism of myocardial ischemia-reperfusion injury and exploring therapeutic targets may provide new strategies for future treatment and prevention of myocardial infarction.

Oxidative stress and apoptotic damage are the main pathological changes in I/R injury, and a series of studies have confirmed that inhibiting oxidative stress and apoptotic damage can significantly alleviate myocardial damage caused by myocardial infarction I/R(19). Abnormal mitochondrial function after I/R injury is a major source of ROS production, which can promote programmed cardiomyocyte death by releasing cytochrome C into the cytoplasm and subsequently activating the apoptotic signaling pathway(20). In the case of myocardial injury, the generation of ROS is uncontrolled, leading to an abnormal increase in ROS levels and exacerbating myocardial damage. Bcl-2, Bax and Caspase are the most representative genes that mediate cell apoptosis(9). Among them, Bax and Bcl-2 regulate apoptosis mainly by mediating the release of cytochrome C, and the Bax/Bcl-2 ratio can better reflect its regulatory role in cell apoptosis(21). In addition, Caspase-3, as an enzyme marker for cell apoptosis, is an important protease in the caspase enzyme cascade reaction, mainly mediating the process of cell apoptosis through enzymatic digestion and activation(22). In this study, it was confirmed that OGD/R or I/R-induced oxidative stress was significantly activated in vitro and in vivo, as well as caspase-3 activity and Bax/Bcl-2 ratio were significantly increased. Cucurbitacin B treatment can significantly alleviate these myocardial injuries caused by ischemia-reperfusion, indicating that cucurbitacin B has a clear myocardial protective role.

Cucurbitacin B has various pharmacological effects such as anti-tumor, anti-inflammatory, antioxidant stress, and apoptotic damage(23). Research shows that cucurbitacin at low concentrations can effectively inhibit phenylephrine-induced in vitro cardiomyocyte hypertrophy, which is achieved by inhibiting the production of connective tissue growth factor and transforming growth factor β mediated by the smad signaling pathway(24). In addition, cucurbitacin B can alleviate pressure overload-induced myocardial hypertrophy and myocardial interstitial fibrosis(13). However, there is currently no report on the role of cucurbitacin B in myocardial infarction I/R injury and the mechanisms through which these effects are exerted. In this study, we screened different downstream targets through bioinformatics analysis, among which the JAK2/STAT3 signaling pathway with strong correlation attracted our great attention(25). The JAK2/STAT3 signaling pathway plays an important role in various cardiovascular pathophysiological processes, including heart failure and cardiomyopathy(13). Recently, various studies have shown that the activation of the JAK2/STAT3 signaling pathway plays an important role in protecting against myocardial I/R injury, for example, Smith et al. found that knockout of STAT3 in mice did not play a protective role in ischemic damaged hearts, indicating the importance...
of STAT3 in the protective mechanism of preconditioning(26). Nagata et al. found that JAK/STAT signaling pathway activators have a cardioprotective effect in myocardial I/R and as a negative feedback regulator of this signaling pathway, Suppressor Of cytokine Signaling 3 (SOCS3) can exacerbate myocardial ischemia-reperfusion injury by inhibiting the JAK/STAT pathway(27). To further investigate the protective mechanism of cucurbitacin B on myocardial I/R injury, we first confirmed in mouse cardiomyocytes that cucurbitacin B can reverse the inhibitory effect of I/R injury on JAK2/STAT3. On this basis, further use of JAK2/STAT3 inhibitor AG490 was conducted, and rescue experiments confirmed that AG490 can block the protective effect of cucurbitacin B on OGD/R induced myocardial oxidative stress and apoptosis damage. These results indicate that cucurbitacin B exerts a molecular mechanism of protecting against myocardial I/R injury by activating the JAK2/STAT3 signaling pathway.

Conclusion
We demonstrated that cucurbitacin B promotes cardiac function and myocardial repair post I/R injury and attenuates OGD/R induced cardiomyocyte LDH release, oxidative damage and apoptosis via JAK2/STAT3 signaling pathway. Our study reveals for the first time the protective effect of cucurbitacin B in cardiac I/R injury, which provides a new insight into better prevention of I/R injury via the JAK2/STAT3 signaling pathway.

Declarations
Author contribution statement
Chao Chen, Yuqiong Chen and Mingzhu Xu conducted the experiments and wrote the paper. Lin Chen and Junrong Gong performed some in vitro experimentsZhongqi Sun analyzed and organized the data Yafei Li, and Tingbo Jiang conceived, designed the study and revised manuscripts.

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Data availability statement
Data will be made available on request.

Declaration of interest’s statement
There are no conflicts of interest.

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