European Review for Medical and Pharmacological Sciences 2020; 24: 4430-4439

STRAP reduces endoplasmic reticulum stress and apoptosis in cardiomyocytes and attenuates myocardial ischemia-reperfusion injury by activating PI3K/PDK1/Akt signaling pathway

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Abstract. – OBJECTIVE: Myocardial ischemia-reperfusion injury (MIRI) is a common problem in heart-related diseases. The aim of this study was to explore the protective effects of STRAP on cardiomyocytes in the MIRI process and its mechanisms.

MATERIALS AND METHODS: We used SD rats to construct a MIRI model and increased the expression of STRAP in myocardial tissue by Entranster to detect the effect of STRAP on rat myocardial tissue. In addition, we cultured rat cardiomyocyte cell line H9c2 cells and constructed a hypoxia-reoxygenation model to detect the protective effect of STRAP on H9c2 cells. LY294002, an inhibitor of the PI3K/PDK1/Akt signaling pathway, was used to validate the mechanism by which STRAP protects cardiomyocytes.

RESULTS: Overexpression of STRAP significantly reduced the activity of MDA in myocardial tissue and increased the activity of SOD. STRAP also substantially lowered CK and LDH levels in rat serum and increased Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity. In addition, overexpression of STRAP considerably reduced endoplasmic reticulum stress (ERS) and apoptosis levels in H9c2 cells. However, LY294002 attenuated the protective effect of STRAP on cardiomyocytes.

CONCLUSIONS: STRAP reduces ERS and apoptosis in cardiomyocytes by activating the PI3K/PDK1/Akt signaling pathway, thereby reducing myocardial MIRI.

Key Words:
STRAP, Endoplasmic reticulum stress, Apoptosis, Myocardial ischemia-reperfusion injury, PI3K/PDK1/Akt signaling pathway.

Introduction

Myocardial infarction is one of the serious diseases that threaten human health and is the leading cause of death in humans[1]. In the United States, millions of people suffer from myocardial infarction each year, and only about 700,000 of them receive various surgeries[2]. After the occurrence of acute myocardial infarction, coronary artery occlusion causes myocardial ischemia and necrosis, forming fibrous scar tissue without contraction function, leading to ventricular remodeling, and eventually developing into congestive heart failure. Timely recovery of blood perfusion is a fundamental measure to treat myocardial infarction and save ischemic myocardium, which is also the main cause of myocardial ischemia-reperfusion injury (MIRI)[3]. MIRI mainly refers to the injury of cardiomyocytes with reversible injury during the ischemia period after the recovery of blood perfusion that aggravates the injury of cardiomyocytes, and even causes irreversible injury. The main manifestation is that the infarct area is not decreased due to the restoration of blood flow. Instead, the expansion of the infarct area, reperfusion arrhythmia, myocardial stunning, etc., occur, which affect the prognosis of patients[4].

The cause of ischemia-reperfusion injury (IRI) is currently unclear. It is generally believed that oxidative stress caused by large numbers of free radicals and reactive oxygen species (ROS) during ischemia-reperfusion is one of the main factors leading to IRI[5]. In addition, when MIRI occurs, due to factors such as depletion of blood, oxygen, and nutrients by calcium ions, the function of the endoplasmic reticulum is disordered, resulting in endoplasmic reticulum stress (ERS), which in turn increases the damage of cardiomyocytes[6].

There are multiple signal transduction pathways involved in MIRI. Among them, the phosphatidylinositol 3-hydroxy kinase (PI3K)/phosphoinositide-dependent kinase 1 (PDK1)/protein kinase B (Akt) signaling pathway plays

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an important role in myocardial protection. Activated Akt can increase myocardial cell growth and survival, enhance myocardial contractile function, and promote coronary angiogenesis by activating mTOR and inhibiting GSK3, FOXO, etc[7]. Activation of Akt has protective effects on ischemic myocardium when exercise and cardiac load increase. Transplantation of rat mesenchymal stem cells overexpressing Akt into rat ischemic myocardium can reduce fibrotic area and improve cardiac function. Overexpression of Akt in the myocardium reduces myocardial cell death and decrease cardiac function when myocardial ischemia occurs[8]. Therefore, the PI3K/PDK1/Akt signaling pathway plays an important protective role in MIRI. Our previous study found that STRAP, one of the WD40 superfamily proteins, can activate the PI3K signaling pathway by regulating PDK1[9]. STRAP is a serine threonine kinase receptor-associated protein. In recent years, STRAP has been found to bind to a variety of receptors to play a pivotal role in cell proliferation and metabolism and be involved in the regulation of transduction of multiple signaling pathways[10].

Therefore, in this study, we used the rat model of MIRI and the rat cardiomyocyte cell line H9c2 to explore the effect of STRAP on MIRI and its mechanism.

Materials and Methods

Animals and Grouping

A total of 30 SD rats were used in the study. All rats were specific pathogen free (SPF) grade and were housed in a barrier facility. The rats in this experiment were divided into four groups: control group, MIRI group, MIRI+NC group, and MIRI+STRAP group. Except for the control group, the remaining three groups of rats underwent surgery to make a MIRI model. In addition, rats in the MIRI+NC group were transfected with Lenti-NC one week prior to surgery as a negative control, while rats in the MIRI+STRAP group were transfected with Lenti-STRAP *in vivo* one week prior to surgery to increase the expression of STRAP in cardiomyocytes. This investigation was approved by the Animal Ethics Committee of Peking University Shenzhen Hospital Animal Center.

Operative Procedure of Rat MIRI

The rats were anesthetized with 4% paraformaldehyde (10 μl/g). After the rats were anesthetized, we removed the fur from the chest and abdomen of the rats and disinfected them with 75% alcohol. We used scissors to cut the chest of the rat and expose the heart. After pinpointing the anterior descending coronary artery of the rat heart, we ligatured the anterior descending coronary artery for half an hour using a suture. The darkening of the anterior wall of the rat heart indicates that the myocardial ischemia model is successful. Then, we loosened the suture and restored the blood supply. It was observed that the color of the anterior wall of the rat's heart recovered rosy to indicate blood flow recovery. Four hours after the blood flow was restored, we sacrificed the rats through the aortic hemorrhage.

Entranster

We transfected rat myocardial tissue with Lenti-STRAP using the Entranster™-*in vivo* kit (Engreen, Beijing, China). After the rats were anesthetized, we cut the rat's chest and exposed the heart. A mixture of Lenti-STRAP and Entranster was then injected into the pericardium of the rat. Finally, we sutured the surgical incision of the rat and continued to place it in a cage.

Cell Culture and Treatment

H9c2 cells, a rat cardiomyocyte cell line, were used in this study. Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin plus streptomycin was used to culture H9c2 cells. The cells were then cultured in an incubator with 5% CO_2 at 37°C. LY294002, an inhibitor of the PI3K/PDK1/Akt signaling pathway, was used to inhibit the activity of the PI3K/PDK1/Akt signaling pathway in H9c2 cells. We constructed the MIRI model at the cellular level by hypoxia reoxygenation (HR) as follows: we cultured the cells in a culture dish, and after the cell density reached 90%, we changed the medium with phosphate-buffered saline (PBS), and placed it in an incubator filled with N_2. After 4 hours, we replaced the PBS in the culture dish with the medium and placed it in an incubator with 5% CO_2 at 37°C.

Cell Transfection

After the cell growth density reached 50-60%, the cells were transfected with Lenti-NC and Lenti-STRAP. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection. Lenti-STRAP was used to increase the expression of STRAP in H9c2 cells, while Lenti-NC was used as a negative control.

Western Blot

H9c2 cells were cultured in 6-well plates. After treating the cells, we lysed the cells with cell lysate and then removed the cell debris by centrifugation. The protein concentration was detected by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). We added 20 μg of protein to each well on the gel. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked non-specific antigens with 5% skim milk. We then used a primary antibody to incubate the PVDF membrane overnight at 4°C. After washing the PVDF membrane the next day, we incubated the PVDF membrane for 2 hours at room temperature using a secondary antibody. After washing the PVDF membrane again, we used enhanced chemiluminescence (ECL) to detect protein expression.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

H9c2 cells were cultured in 6-well plates. After treating the cells, we extracted total RNA from the cells using TRIzol (Invitrogen, Carlsbad, CA, USA). A spectrophotometer was used to detect the RNA concentration. The Reverse Transcription kit was used to reversely transcribe RNA into cDNA, and then we used the SYBR® Green kit (TaKaRa, Komatsu, Japan) to amplify cDNA. The reaction system volume was in total 25 μl, pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing at 60°C for 45 sec, extension at 72°C for 3 min, with 35 cycles, and then extension at 72°C for 5 min. PCR products were stored at 4°C. GADPH was used as an endogenous control. $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression level. The primer sequences of mRNA are shown in Table I.

Malondialdehyde (MDA), Superoxide Dismutase (SOD), Creatine Kinase (CK), Lactate Dehydrogenase (LDH) Activity Assay

We took rat myocardial tissue and used lysate to dissolve myocardial tissue. Then, we used MDA and SOD activity assay kits to detect MDA and SOD activity in myocardial tissue by the manufacturer's instructions. In addition, we collected rat blood and obtained serum by centrifugation. Finally, we used CK activity assay kit and LDH activity assay kit to determine CK and LDH levels in rat serum.

Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase Activity Assay

The apical tissue of the rat was taken out and ground to powder in PBS under low temperature conditions. ATP becomes ADP and inorganic phosphorus during the decomposition process. Therefore, we can detect the activity of ATPase by measuring

Table I. RT-PCR primer sequences.

Name	sense/anti-sense	Sequence (5'-3')
CHOP	sense	CAGCGACAGAGCCAGAATAAC
	anti-sense	ACCGTCTCCAAGGTGAAAGG
ATF-6	sense	TTTACGGACTTGCATGCACACGT
	anti-sense	TTGCAGCACTACGAGTAATCG
GRP-78	sense	TACCCCAGATTGAAGTCACCT
	anti-sense	TTCTCGGCGTCATTGACCA
caspase3	sense	CAGAATCATAAGCCCCTGGA
	anti-sense	TCTGCGAGTCAGGCATTTG
caspase8	sense	GGGAAGTGTTTTCACAGGTT
	anti-sense	TTCTTGCTTCCTTTGCGGAAT
caspase9	sense	TTCTTGAGCAACACCCTC
	anti-sense	CGCATACACTGTCTACCT
Bax	sense	CAGTTGAAGTTGCCATCAGC
	anti-sense	CAGTTGAAGTTACCATCAGC
Bcl-2	sense	GACTGAGTACCTGAACCGGCATC
	anti-sense	CTGAGCAGCGTCTTCAGAGACA
Akt	sense	CAGGTTCACCCAGTGACAACTCA
	anti-sense	CACGAGACAGGTGGAAGAAGAGC
GAPDH	sense	ACAACTTTGGTATCGTGGAAGG
	anti-sense	GCCATCACGCCACAGTTTC

the content of inorganic phosphorus. The Na^+-K^+-ATPase activity assay kit and Ca^{2+}-Mg^{2+}-ATPase activity assay kit were used in this experiment.

Immunocytofluorescence (IF) Staining

We cultured H9c2 cells using 24-well plates. When the cell growth density reaches 50~60%, we treated the cells. At the end of the treatment, we removed the cells from the incubator and fixed them with 4% paraformaldehyde. We then soaked the cells for 15 minutes using 0.2% Triton-PBS. 10% goat serum was used to block non-specific antigens in the cells. Later, we incubated the cells with primary antibody dilution at 4°C overnight. The next day, after washing the cells with PBS, we incubated the cells for 1 hour at room temperature using fluorescent secondary antibody, washed the cells, and incubated the cells for 15 minutes using 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). Finally, we observed and recorded the staining results using a fluorescence microscope.

Flow Cytometry

We used the Annexin V-FITC kit to detect the level of apoptosis in H9c2 cells. After the cells were treated, we discarded the medium and washed the cells with PBS. We then collected each group of cells and added 5 μl of Annexin V-FITC and 5 μl of PI. Subsequently, we incubated the cells in the dark for 15 minutes. Finally, we added 400 μl of loading buffer to each group and then measured the apoptosis rate by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Statistical Analysis

SPSS 21.0 (SPSS IBM Corp., Armonk, NY, USA) software was used to analyze the experimental data of this study. In addition, we drew and analyzed the chart through GraphPad. The measurement data were presented as mean ± standard deviation. Comparison between multiple groups was done using one-way ANOVA test followed by the post-hoc test (Least Significant Difference). All experiments were repeated more than 3 times. $p<0.05$ was considered to be statistically significant.

Results

STRAP Reduced Oxidative Stress and MIRI

MDA and SOD are key indicators of the level of oxidative stress in cells. We examined the activity of MDA (Figure 1A) and SOD (Figure 1B) in myocardial tissue and found that the MDA level in the myocardial tissue of MIRI group and MIRI+NC group was higher than that of control group and the SOD level was lower than that of control group, indicating that after the MIRI model was constructed, the level of oxidative stress in myocardial tissue was significantly increased, and the ability to resist oxidative stress decreased. However, after transfection of rat myocardium with Lenti-STRAP, MDA level declined, and SOD level was increased. In addition, CK (Figure 1C) and LDH (Figure 1D) levels in rat serum were also significantly reduced after transfection of Lenti-STRAP, indicating that STRAP reduces myocardial injury in rats. Na^+-K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity are key indicators of myocardial function. Our study found that STRAP significantly attenuated the reduction of Na^+-K^+-ATPase (Figure 1E) and Ca^{2+}-Mg^{2+}-ATPase (Figure 1F) activity by MIRI.

Overexpression of STRAP Significantly Reduced ERS Levels in H9c2 Cells

CHOP, ATF-6, and GRP-78 are key molecules in the ERS-related pathway. By detecting these indicators, we can determine the ERS level in the cells. Western blot (Figure 2A) and RT-PCR (Figure 2B-2D) showed that the expressions of CHOP, ATF-6, and GRP-78 in H9c2 cells in HR group and HR+Lenti-NC group were increased, indicating that HR significantly increases ERS level in H9c2 cells. The expressions of CHOP, ATF-6, and GRP-78 in H9c2 cells in HR+Lenti-STRAP group were substantially lower than that in HR group and HR+Lenti-NC group, indicating that overexpression of STRAP significantly decreases ERS level in H9c2 cells. The results of IF staining (Figure 2E) also indicate that STRAP reduces the expression of CHOP.

Overexpression of STRAP Remarkably Reduced the Apoptosis Level in H9c2 Cells

ERS increases the apoptosis level in cells, so we examined the effect of STRAP on the apoptosis level in H9c2 cells. The results of Western blot (Figure 3A) and RT-PCR (Figure 3B-3F) showed that the expressions of caspase-3, caspase-8, caspase-9, and Bax in H9c2 cells of HR group and HR+Lenti-NC group were significantly higher than those of control group and the expression of Bcl-2 was decreased. However, the expressions of caspase-3, caspase-8, caspase-9, and Bax were

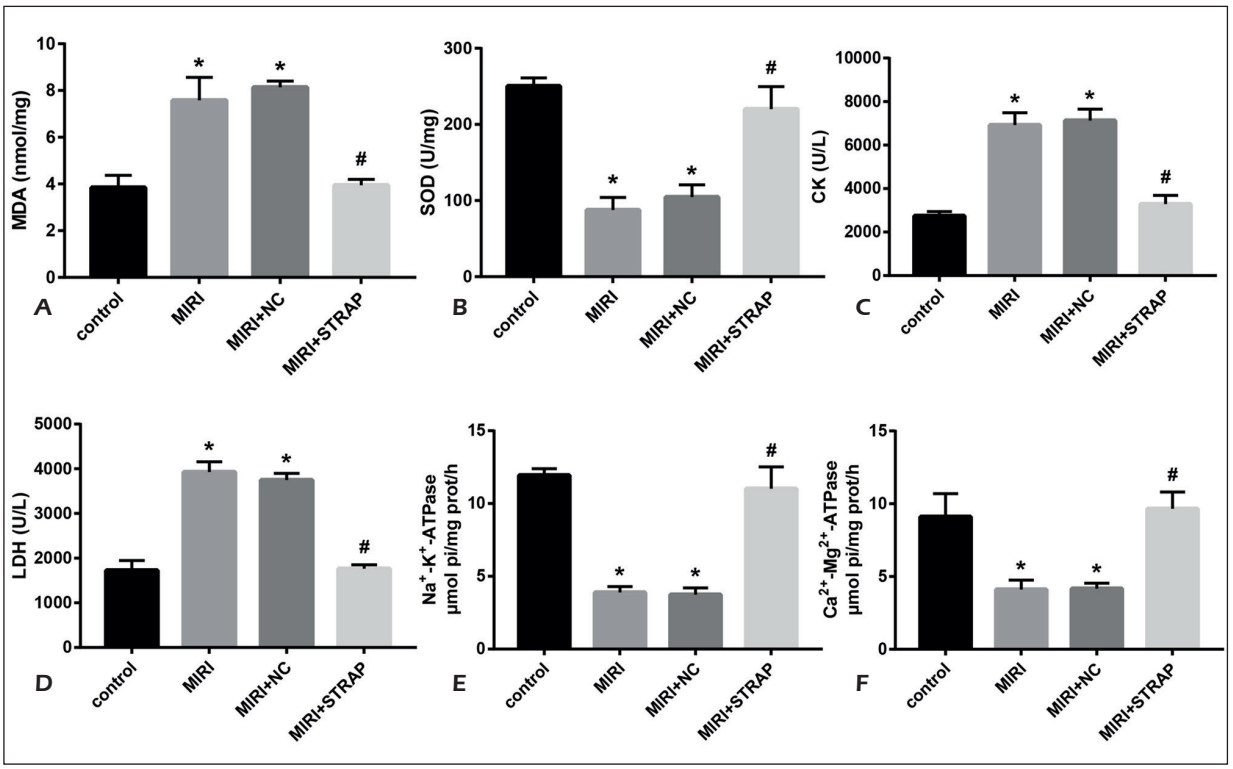

Figure 1. STRAP reduced oxidative stress and MIRI. **A**, MDA activity assay. **B**, SOD activity assay. **C**, CK activity assay. **D**, LDH activity assay. **E**, Na^+-K^+-ATPase activity assay. **F**, Ca^{2+}-Mg^{2+}-ATPase activity. ("*" means there is a statistical difference with control group and "#" means there is a statistical difference with MIRI+NC group).

decreased in H9c2 cells of HR+Lenti-STRAP group, and the expression of Bcl-2 was increased. Flow cytometry (Figure 3G) revealed that the overexpression of STRAP reduced the apoptosis rate of H9c2. The results of IF staining (Figure 3H) also indicate that STRAP can reduce the expression of caspase-3.

STRAP Increased the Activity of the PI3K/PDK1/Akt Signaling Pathway

Previous studies have shown that STRAP can promote PDK1 activity and activate the PI3K/PDK1/Akt signaling pathway, so we examined the effect of STRAP on the PI3K/PDK1/Akt signaling pathway. The results of Western blot and RT-PCR showed that overexpression of STRAP increased Akt expression and phosphorylation in both rat myocardial tissue (Figure 4A, 4B) and H9c2 cells (Figure 4C and 4D). The results of IF staining (Figure 4E) suggest that the expression of Akt in H9c2 cells can be elevated by STRAP as well.

Inhibition of the PI3K/PDK1/Akt Signaling Pathway Attenuated the Protective Effect of STRAP on H9c2 Cells

LY294002 is an inhibitor of the PI3K/PDK1/Akt signaling pathway. We used LY294002 to verify the protective effect and mechanism of STRAP on H9c2 cells. The results of Western blot (Figure 5A) and RT-PCR (Figure 5B-5F) showed that the expression of CHOP, ATF-6, GRP-78, and Bax increased, and the expression of Bcl-2 decreased after stimulation with LY294002, implying that the protective effect of STRAP on H9c2 cells is attenuated by LY294002.

Discussion

The endoplasmic reticulum is not only a subcellular organelle for eukaryotic protein synthesis and folding, lipid synthesis, and intracellular calcium storage, but also a subcellular organator that regulates cell stress and apoptosis, thus main-

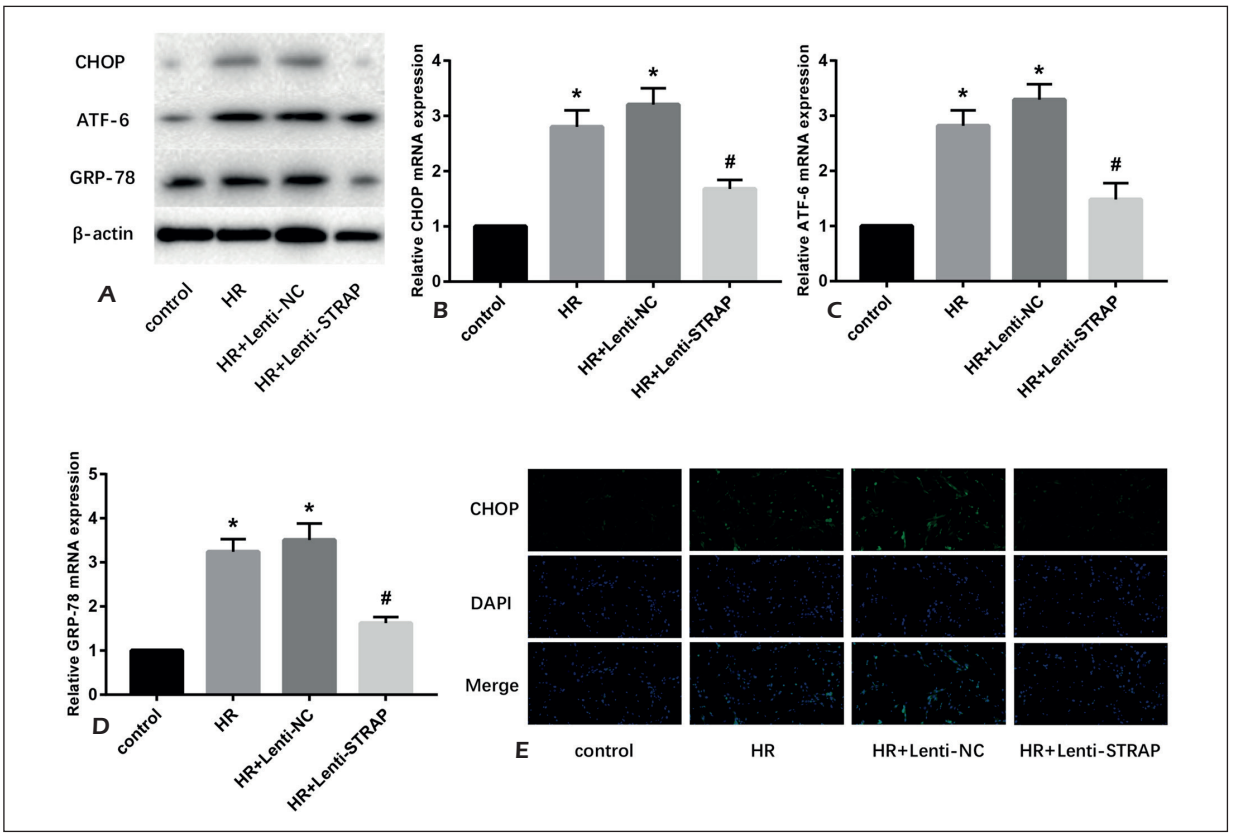

Figure 2. Overexpression of STRAP significantly reduces ERS levels in H9c2 cells. **A-D**, Expressions of CHOP, ATF-6 and GRP-78 in four groups determined by Western blot (**A**) and RT-PCR (**B-D**). **E**, Expression of CHOP detected via IF staining (magnification: 40×). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with HR+Lenti-NC group)

taining calcium homeostasis and functional protein stability in cardiomyocytes[11]. ERS refers to a subcellular organ pathological state caused by a variety of factors that disrupt the physiological function of the endoplasmic reticulum. Appropriate and non-overreactive ERS will increase the expression of calreticulin and glucose-regulated proteins (GRPs), thereby enhancing the ability of the endoplasmic reticulum to treat unfolded proteins[12]. However, severe and persistent ERS can activate the endoplasmic reticulum apoptosis signaling pathway, which leads to tissue damage and apoptosis. There are two important molecules in the downstream signaling pathway leading to excessive apoptosis induced by endoplasmic reticulum, namely CHOP and caspase-12. CHOP is known as the DNA damage and growth arrest gene. Excessive expression of CHOP causes apoptosis and regulates the expression of the two a subcellular organator molecules Bcl-2 (inhibiting apoptosis) and Bax (promoting apoptosis). Caspase-12 is activated during severe ERS, which in turn activates caspase-3 and caspase-9 by a chain reaction to promote apoptosis[13]. At present, inhibition of ERS-related apoptosis is a new target for improving MIRI. In our study, the expression of CHOP, ATF-6, and GRP-78 was significantly increased in H9c2 cells treated with HR, indicating that the level of ERS in cells treated with HR was significantly increased. The expression of the corresponding index in H9c2 cells overexpressed by STRAP was significantly decreased, indicating that STRAP effectively reduced the level of ERS in H9c2 cells. In addition, STRAP also effectively reduced the level of apoptosis in H9c2 cells, the expression of caspase-3, caspase-8, and caspase-9 was significantly decreased, and the ratio of Bcl-2/Bax was increased, indicating that STRAP reduced ERS in H9c2 cardiomyocytes and reduced levels of apoptosis. This may be the mechanism of action of STRAP to protect cardiomyocytes.

PDK1 is a serine threonine kinase that phosphorylates many signal transduction proteins, such

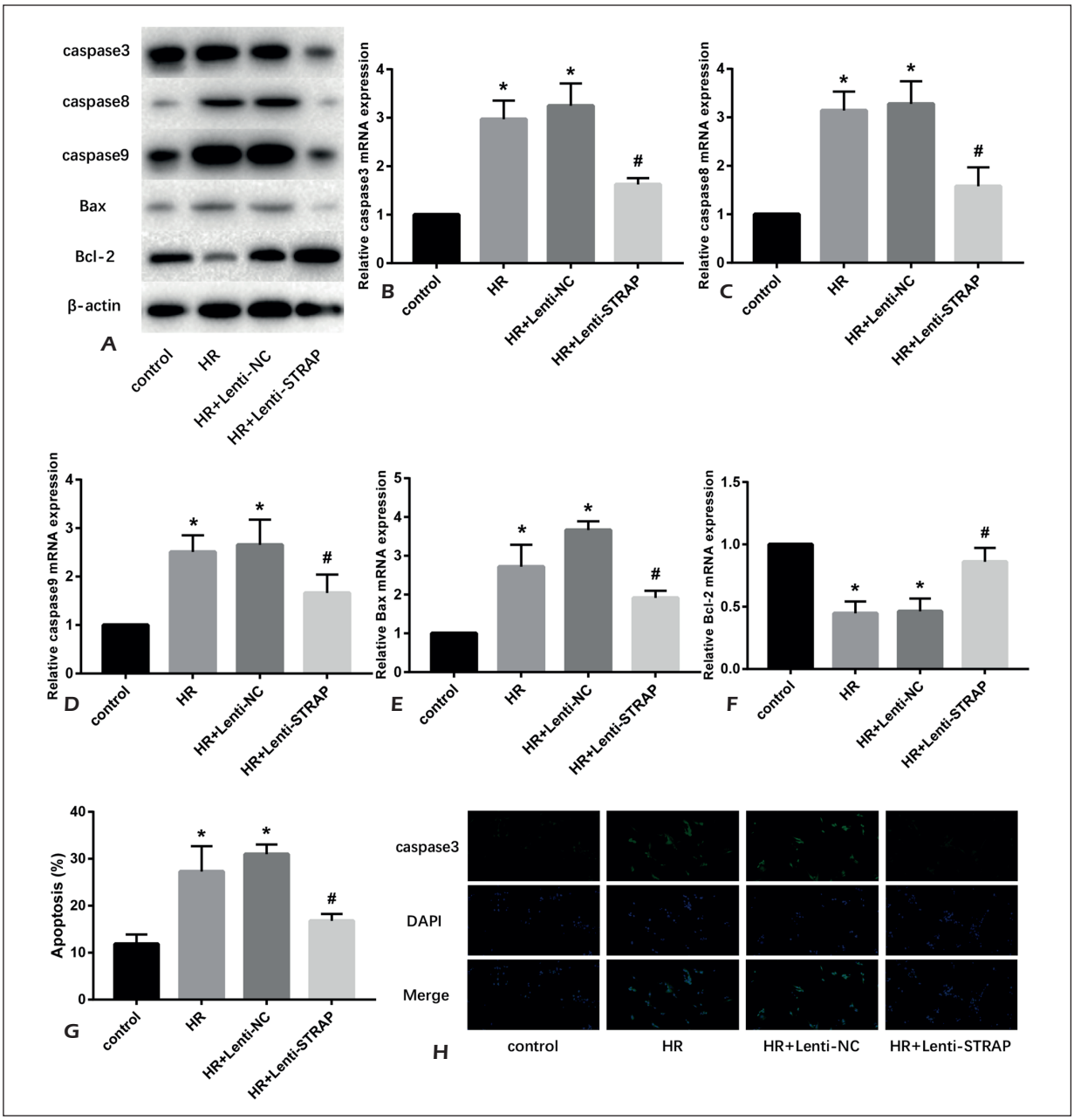

Figure 3. Overexpression of STRAP significantly reduced the apoptosis level in H9c2 cells. **A-F**, Expressions of caspase-3, caspase-8, caspase-9, Bax, and Bcl-2 in four groups determined by Western blot (**A**) and RT-PCR (**B-F**). **G**, Cell apoptosis rate detected by flow cytometry (**G**). **H**, Expression of caspase-3 detected via IF staining (magnification: 40×). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with the HR+Lenti-NC group).

as PKC, S6K, PAK1, and Akt. The PDK1 signaling pathway has been extensively studied in tumors in the past, and is closely related to tumor cell proliferation, migration and migration, and survival[14]. For this reason, PDK 1 inhibition is being evaluated to be effective against tumors. Seong et al[15] have shown that STRAP can directly bind to PDK1 and regulate the phosphorylation of PDK1 substrates S6K, Akt, and BAD. In addition, Seong et al[15] found that STRAP and PDK 1 interactions promotes Smad-7 binding to activated TBRI mutants and decreases TGF-β signaling. Subsequent studies further showed that PDK 1 interacts with Smad-2, Smad-3, Smad-4, and Smad-7 in the absence of exogenous TGF-β stimulation. Overexpression of STRAP promotes the formation of a complex between PDK 1 and

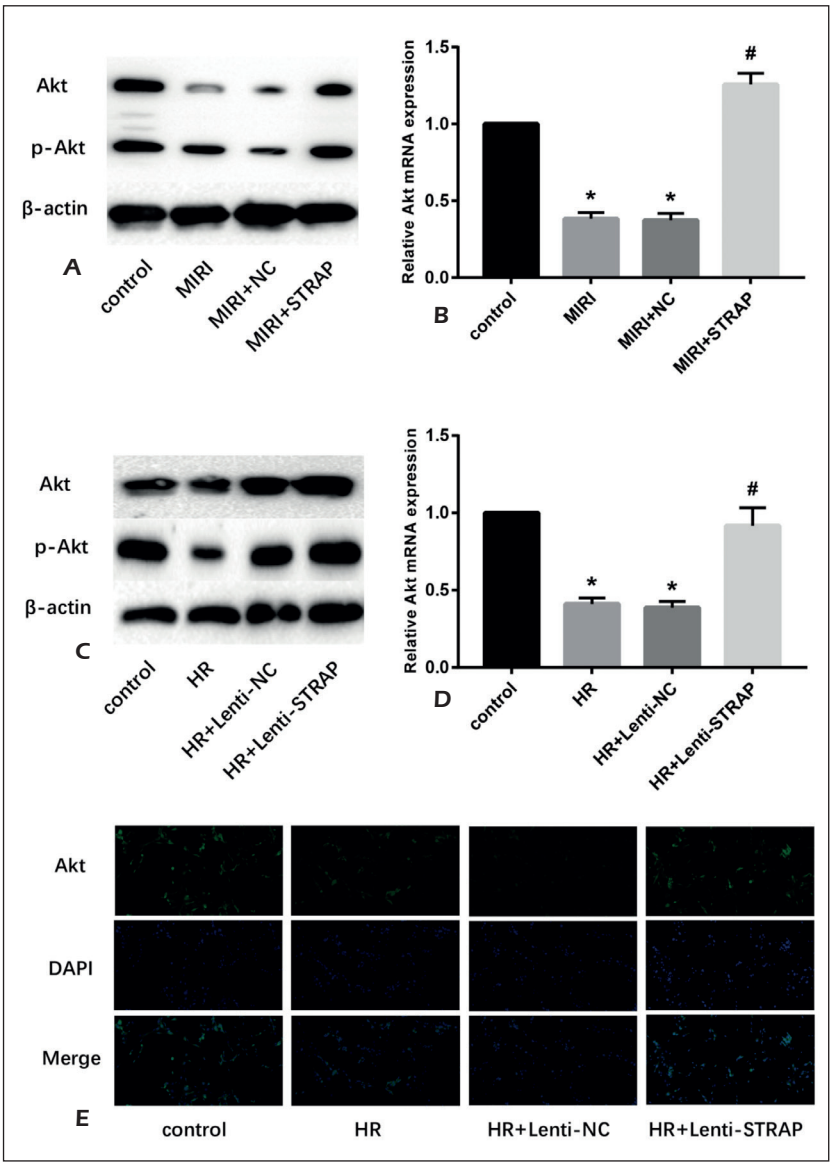

Figure 4. STRAP increases the activity of the PI3K/PDK1/Akt signaling pathway. **A-D**, Expressions of Akt and p-Akt in rat myocardial tissue (**A-B**) and H9c2 cells (**C-D**) determined by Western blot and RT-PCR. **E**, Expression of Akt in H9c2 cells detected via IF staining (magnification: 40×). ("*" means there is a statistical difference with the control group and "#" means there is a statistical difference with HR+Lenti-NC group).

these Smads[16]. In conclusion, the binding of STRAP and PDK 1 enhances the interaction of PDK 1 with its endogenous binding partner. Therefore, based on this research basis, we suspected that STRAP may be a therapeutic target for MIRI. STRAP may play a role in protecting cardiomyocytes by activating the PI3K/PDK1/Akt signaling pathway. Thus, we constructed a rat model of MIRI and H9c2 cell HR model, and increased the expression of STRAP in cardiomyocytes by *in vivo* transfection and cell transfection. It was found that overexpression of STRAP reduced oxidative stress levels and myocardial injury in rat myocardial tissue. In addition, overexpression of STRAP increased Akt levels and phosphorylation in both rat myocardial tissue and H9c2 cells, suggesting that STRAP effectively increases the activity of the PI3K/PDK1/Akt signaling pathway in cardiomyocytes.

To sum up, this is the first study to report that STRAP protects against MIRI. MIRI has been difficult to solve as a clinical problem. We suggest that this study can provide a new direction and theoretical basis for clinical prevention and treatment of MIRI.

Conclusions

Both animal and cellular experiments have shown that STRAP can reduce MIRI. STRAP

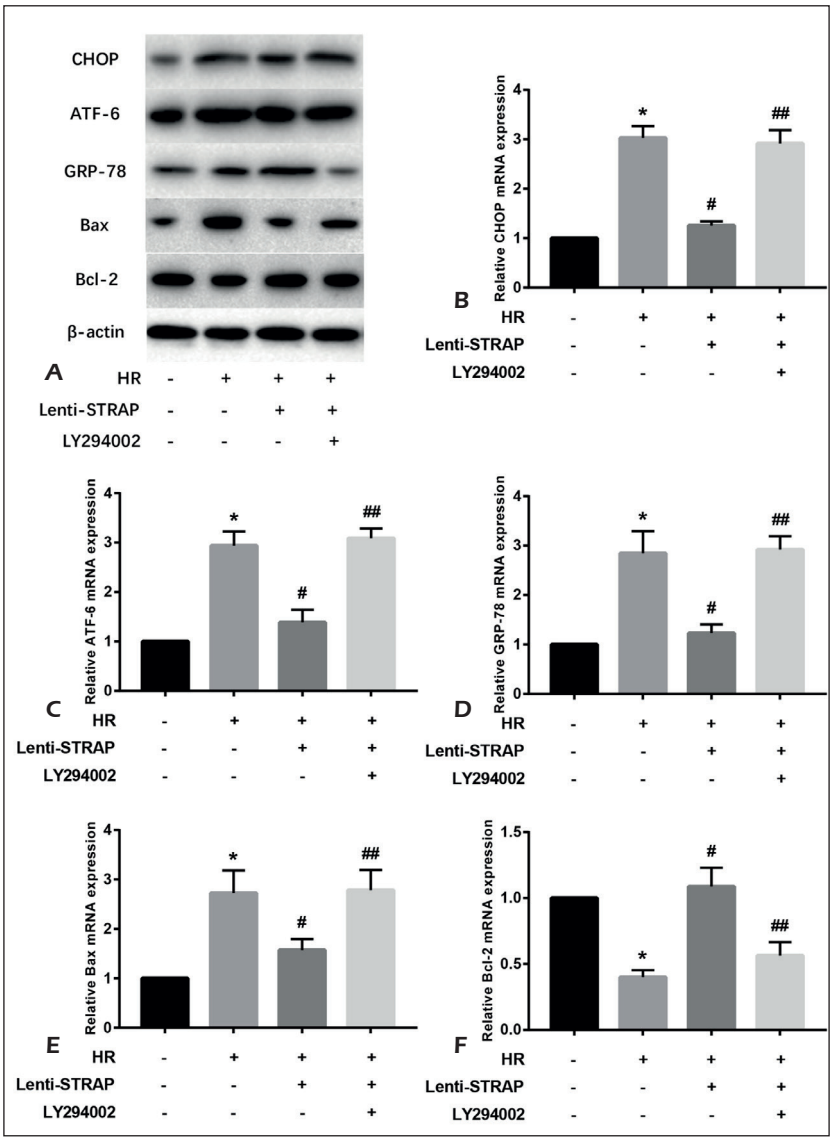

Figure 5. Inhibition of the PI3K/PDK1/Akt signaling pathway attenuates the protective effect of STRAP on H9c2 cells. **A-F**, Expressions of CHOP, ATF-6, GRP-78, Bax, and Bcl-2 in four groups determined by Western blot (**A**) and RT-PCR (**B-F**). ("*" means there is a statistical difference with the control group, "#" means there is a statistical difference with HR+Lenti-NC group and "##" means there is a statistical difference with HR+Lenti-STRAP+LY294002 group).

significantly reduces ERS and apoptosis in cardiomyocytes. The protective effect of STRAP on cardiomyocytes may be obtained by activating the PI3K/PDK1/Akt signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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