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Overexpression of DJ-1 expression protects cardiomyocyte apoptosis induced by ischemia reperfusion

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Abstract. – OBJECTIVE: Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway plays an important role in regulating cell survival, apoptosis and oxidative stress (OS). Phosphatase and tensin homolog deleted on chromosome ten (PTEN) can negatively regulate PI3K/AKT signaling pathway. DJ-1 is also a key negative regulator of PTEN. DJ-1-PTEN/PI3K/AKT signaling pathway regulates ischemia reperfusion (I-R). This study investigated the role of DJ-1 in affecting myocardial I-R injury.

MATERIALS AND METHODS: The rat myocardial I-R injury model was established. Expression of DJ-1 and PTEN in myocardial tissue was detected. The reactive oxidative species (ROS) content was detected using flow cytometry. Caspase-3 activity, malondialdehyde (MDA) content, and superoxide dismutase (SOD) activities were determined by ultraviolet spectrophotometry. Rat cardiomyocytes H9C2 were cultured *in vitro* and divided into control group, I-R group, I-R+pIRES2-NC group, and I-R+pIRES2-DJ-1 group. Levels of DJ-1, PTEN and phosphorylated AKT (p-AKT) were detected. Cell apoptosis and ROS content were evaluated using flow cytometry.

RESULTS: Compared with sham group, caspase-3 activity, MDA content, and PTEN expression were significantly increased, while SOD activity and DJ-1 levels were significantly reduced in myocardial tissue of I-R group ($p<0.05$). Compared with the control, I-R treatment markedly induced H9C2 cell apoptosis, decreased DJ-1 and p-AKT expression, and enhanced ROS production and PTEN expression. DJ-1 overexpression apparently down-regulated PTEN expression, elevated p-AKT level, and attenuated apoptosis and ROS production in H9C2 cells ($p<0.05$).

CONCLUSIONS: Abnormal expression of DJ-1 plays a regulatory role in the process of myocardial I-R injury. Over-expression of DJ-1 can reduce myocardial cell I-R damage sensitivity by inhibiting PTEN expression, enhancing the activity of PI3K/AKT signaling pathway, reducing ROS production, and alleviating apoptosis.

Key Words
DJ-1, PTEN, PI3K/AKT, Cardiomyocyte, I-R.

Introduction

Acute myocardial infarction (AMI) usually occurs on the basis of coronary atherosclerosis (AS) and is triggered by various causes, such as overwork, extreme stress, overeating, smoking, and heavy drinking[1-3]. The coronary artery reperfusion therapy after AMI is the most effective method to save the ischemic myocardium, protect the heart function, and save the patient's life[4,5]. However, blood recirculation inevitably causes ischemia reperfusion (I-R) injury in the infarcted myocardium and causes more severe damage[6,7]. Phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is widely expressed in a variety of tissue cells and is involved in cell growth, survival, and apoptosis[8-10]. It was showed that the reduction of PI3K/AKT pathway activity is related to the IR damage of various cells, such as cardiomyocytes[11], hepatocytes[12], and brain neurons[13], while enhanced PI3K/AKT pathway plays a role in alleviating I-R injury, indicating that PI3K/AKT has a regulatory role in the process of cellular I-R injury. Phosphatase and tensin homologue deleted on chromosome ten (PTEN) gene is the only tumor suppressor gene with the dual activity of protein esterase and phosphatase that can negatively regulate PI3K/AKT signaling pathway activity[14]. DJ-1 can increase the activity of PI3K/AKT signaling pathway by inhibiting the expression and function of PTEN to antagonize apoptosis, promote cell survival, and accelerate proliferation[15]. This study investigated the role of DJ-1 in affecting myocardial I-R injury via establishing rat myocardial I-R injury model and cardiomyocytes.

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Materials and Methods

Main Reagents and Materials

Healthy adult male Sprague Dawley (SD) rats (6 weeks, weight 250 ± 20 g) were purchased from Shanghai Slake Experimental Animal Co., Ltd. (Shanghai, China). Dulbecco's modified Eagle' medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NJ, USA). Rabbit anti-rat DJ-1 and PTEN polyclonal antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). Rabbit anti-rat β-actin polyclonal antibody was purchased from GeneTex Inc. (Irvine, CA, USA). Collagenase II was purchased from Gibco (Grand Island, NJ, USA). PrimeScript™ RT reagent kit was purchased from TaKaRa (Dalian, China). Malondialdehyde (MDA), superoxide dismutase (SOD), cell apoptosis detection kit, DCFH-DA, and horseradish peroxidase (HRP) goat anti-rabbit secondary antibody were purchased from Beyotime Biotech. (Shanghai, China). 371 gas Nested CO_2 cell incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). FC500MCL flow cytometry was purchased from Beckman Coulter Inc. (Brea, CA, USA). H9C2 cells were purchased from Shanghai Gefan Biological Cell Bank (Shanghai, China).

Rat Myocardial I-R Model Establishment

The rat was anesthetized by 10% chloral hydrate (Beyotime Biotech., Shanghai, China) by intraperitoneal injection. The chest was opened on the left 4th intercostal space and the left anterior descending coronary artery was identified between pulmonary arterial cone and aorta. Next, the artery was ligated by 6-0 suture. AMI modeling success was judged as ST segment arch lift 0.1 mV on Q lead or T wave highamplitude, myocardial color wanning, and pulse weakened. The blood supply was restored after blocking for 60 min and the cardiac apex became red was considered as successful reperfusion. The sham group was selected as control. The rats were killed at 24 h and 48 h post modeling to test the related levels, respectively.

Caspase-3 Activity Detection

According to the instructions of the kit, the pNA standard product was diluted in concentration gradient to prepare standard products. The absorbance was measured at 405 nm to make a standard curve. The tissue was smashed to prepare homogenate. The homogenate was added with caspase lysis buffer on ice for 20 min and centrifuged at 12000 ×g and 4°C for 10-15 min. Next, the supernatant was taken to a new 1.5 ml centrifuge tube and quantified by the bincinchonic acid (BCA, Biyotime Biotech. Shanghai, China) kit. A total of 65 μl assay buffer, 25 μl lysate supernatant, and 10 μl Ac-DEVD-pNA (2 mM) were added to a 96-well plate and incubated for 2 h at 37°C. When the color change was obvious, the plate was measured at 405 nm on a micro-plate reader (Awareness, Laurinburg, NC, USA). The relative enzyme activity was calculated based on A405 in the experimental group/A405 in the control group × 100%.

MDA and SOD Detection

Rat myocardial homogenate was prepared and quantified. MDA and SOD contents were tested by using the commercial MDA enzyme linked immunosorbent assay (ELISA) detecting kit and SOD ELISA detection kit (Beyotime Biotech., Shanghai, China).

H9C2 Cell Transfection and I-R Treatment

For I-R treatment, the cells were cultured in low glucose serum free DMEM to simulate ischemic condition. Next, the cells were maintained in incubator with 5% CO_2 and 95% N_2 to simulate hypoxic condition. The cells were changed to routine medium after 12 h and further cultured in normal condition for 12 h. H9C2 cells in logarithmic phase were divided into four groups, including control group, I-R group, I-R+pIRES2-NC group, and I-R+pIRES2-DJ-1 group.

Flow Cytometry Detection of Cell Apoptosis

The cells were washed twice in phosphorylate buffered saline (PBS, Beyotime Biotech. Shanghai, China) and digested by 0.25% trypsin. After centrifuged at 300 ×g for 5 min, the cells were resuspended and added with 100 μl Binding Solution. After that the cells were added with 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) solution in sequence, and incubated avoiding light for 15 min. After supplementation with 400 μ binding solution, the cells were tested on a FC500MCL flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

Flow Cytometry Detection of Reactive Oxidative Species (ROS)

ROS detection in rat myocardial tissue: the rat tissues were collected and cut into pieces. The

tissues were digested with 0.1% collagenase II (Beyotime Biotech. Shanghai, China) and hyaluronidase-containing digestive solution (Beyotime Biotech. Shanghai, China) for 45 min. After centrifugation at 250 ×g for 5 min, the cells were incubated in 0.1% DCFH-DA probe at 37°C avoiding light for 30 min. After washed twice in PBS and resuspended in 500 µl PBS, the tissues were tested on Beckman FC500MCL flow cytometer to measure ROS contents. *In vitro* H9c2 intracellular ROS assay: cells were washed twice in PBS and digested with 0.25% trypsin. After centrifuged at 300 ×g for 5 min, the cells were incubated in 0.1% DCFH-DA probe at 37°C for 30 min. After resuspension in 500 µl PBS, the cells were tested using a Beckman FC500MCL flow cytometer (Brea, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) was used to reverse transcribe RNA to complementary DNA (cDNA) for qPCR reaction. qPCR reaction system contained 5.0 µl 2×SYBR Green Mixture, 0.5 µl forward primer (5 µM), 0.5 µl reverse primer (5 µM), 1.0 µl cDNA, and ddH_2O. The reverse transcribe conditions were 50°C for 15 min and 85°C for 5 min. qPCR reaction conditions were pre-denatured at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The primers were listed in Table I.

Western Blot

Cells and tissues were lysed by radioimmunoprecipitation assay (RIPA, Beyotime Biotech. Shanghai, China). 40 µg proteins were separated by 10% sodium dodecyl sulphate-polyAcrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotech. Shanghai, China) gel and 4% concentrated gel. Then, the protein was transferred to polyvinylidene difluoride (PVDF, Amersham Biosciences, Piscataway, NJ, USA) membrane at 300 mA for 90 min. Next, the membrane was blocked with 5% skim milk at room temperature for 60 min and incubated in primary antibody (DJ-1, PTEN, AKT, p-AKT, and β-actin at 1:1000, 1:1000, 1:1000, 1:500, 1:5000, respectively) at 4°C overnight. After that, the PVDF membrane was incubated with horseradish peroxide (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:10000, Santa Cruz Biotech., Santa Cruz, CA, USA) at room temperature for 60 min and finally detected by enhanced chemiluminescence (ECL) (Thermo Scientific Pierce, Rockford, IL, USA).

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Differences among groups were analyzed by using Tukey's post hoc test to validate the one-way analysis of variance (ANOVA). Student's *t*-test was used to compare the differences between two groups. $p<0.05$ was considered as statistically significant.

Results

Obvious Oxidative Stress Injury of Myocardial Tissue in I-R Rats

It was demonstrated that the MDA content in myocardial tissue of I-R model rats was markedly higher (Figure 1A), while SOD enzyme activity (Figure 1B) was apparently decreased than that in Sham group. Spectrophotometry revealed that the caspase-3 activity in the I-R rats was significantly enhanced compared with the Sham rats (Figure 1C). Flow cytometry showed that ROS content in myocardial tissue of I-R rats was significantly increased compared with Sham rats (Figure 1D, Table II).

Table I. Primers for the RT-PCR assay.

Genes		Primers
DJ-1	Forwards	5'-ACCGCGCAGGAAAAACACGC-3'
	Reverse	5'-CTGCCAGACGGCTCTGCAC-3'
PTEN	Forwards	5'-GAGCGTGCAGATAATGACAAGGAAT-3'
	Reverse	5'-GGATTTGACGGCTCCTCTACTGTTT-3'
GAPDH	Forwards	5'-GGTATCGTGGAAGGACTCATGAC-3'
	Reverse	5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'

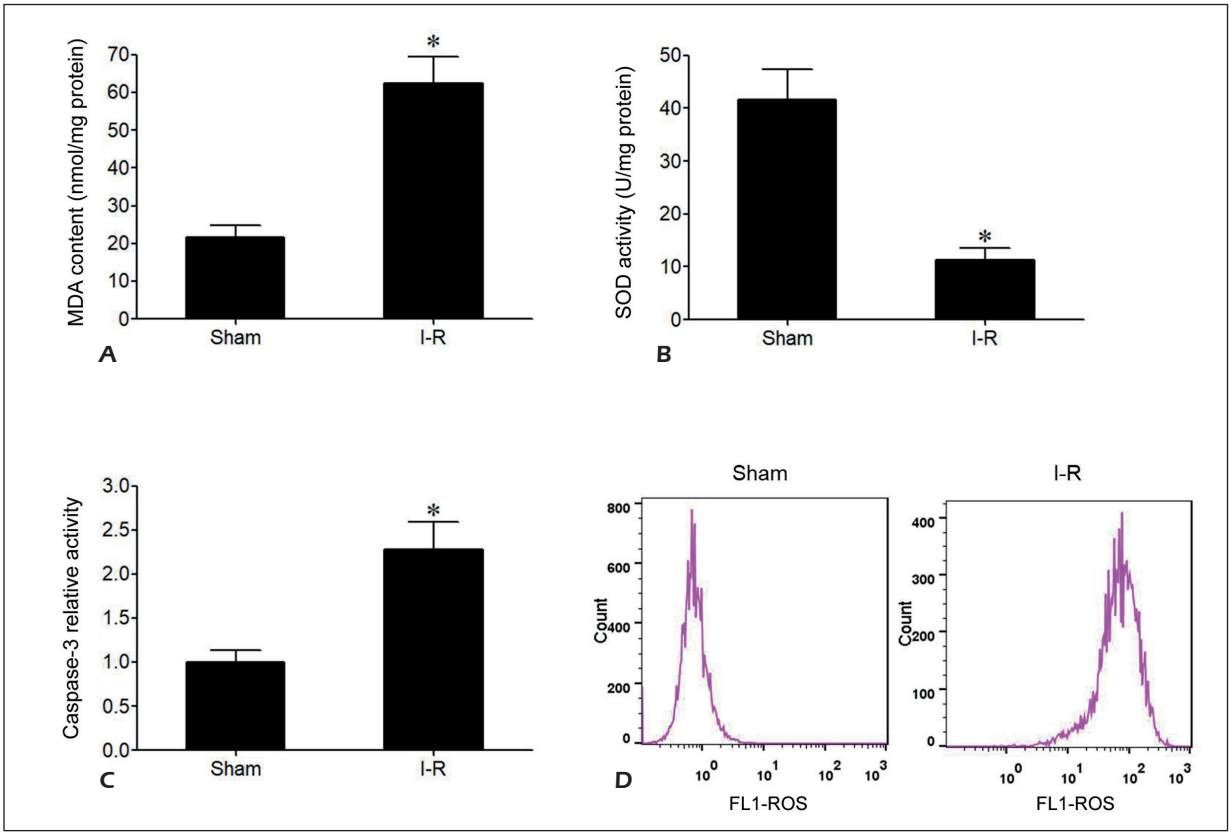

Figure 1. Significant oxidative stress injury of myocardial tissue in I-R rats. **A**, Spectrophotometry detection of MDA content. **B**, Spectrophotometry detection of SOD activity. **C**, Spectrophotometry detection of caspase-3 activity. **D**, Flow cytometry detection of ROS content. *p<0.05, compared with control.

DJ-1 Decreased, While PTEN Elevated in the Rat Myocardium

qRT-PCR showed that the expression of DJ-1 mRNA was significantly decreased, whereas PTEN mRNA was significantly up-regulated in myocardial tissue of I-R group than that of Sham group with time dependence (Figure 2A, B). Western blot revealed that the DJ-1 protein expression was markedly lower, while PTEN protein level was apparently higher in I-R group than that in Sham group (Figure 2C).

Elevated DJ-1 Expression Attenuated Myocardial Cell Oxidative Stress Injury and Apoptosis Induced by IR

qRT-PCR showed that compared with the control group, the expression of DJ-1 mRNA was significantly declined in H9C2 cells treated by IR (Figure 3A). Transfection of pIRES2-DJ-1 on the basis of I-R treatment apparently enhanced the expression of DJ-1 mRNA (Figure 3A). Western blot analysis exhibited that compared with the control group, I-R treatment significantly reduced the expression of DJ-1 protein, increased the expression of PTEN protein, and declined the expression of p-AKT protein in H9C2 cells. pIRES2-DJ-1 transfection significantly enhanced DJ-1 protein expression, reduced PTEN protein level, and up-regulated p-AKT protein expression (Figure 3B). pIRES2-DJ-1 transfection significantly reduced ROS production and cell apoptosis under I-R treatment (Figure 3C, D).

Table II. MFI of ROS in the myocardium.

Group	ROS MFI
Sham	0.86±0.11
I-R	71.53±6.69*

*p<0.05, compared with sham.

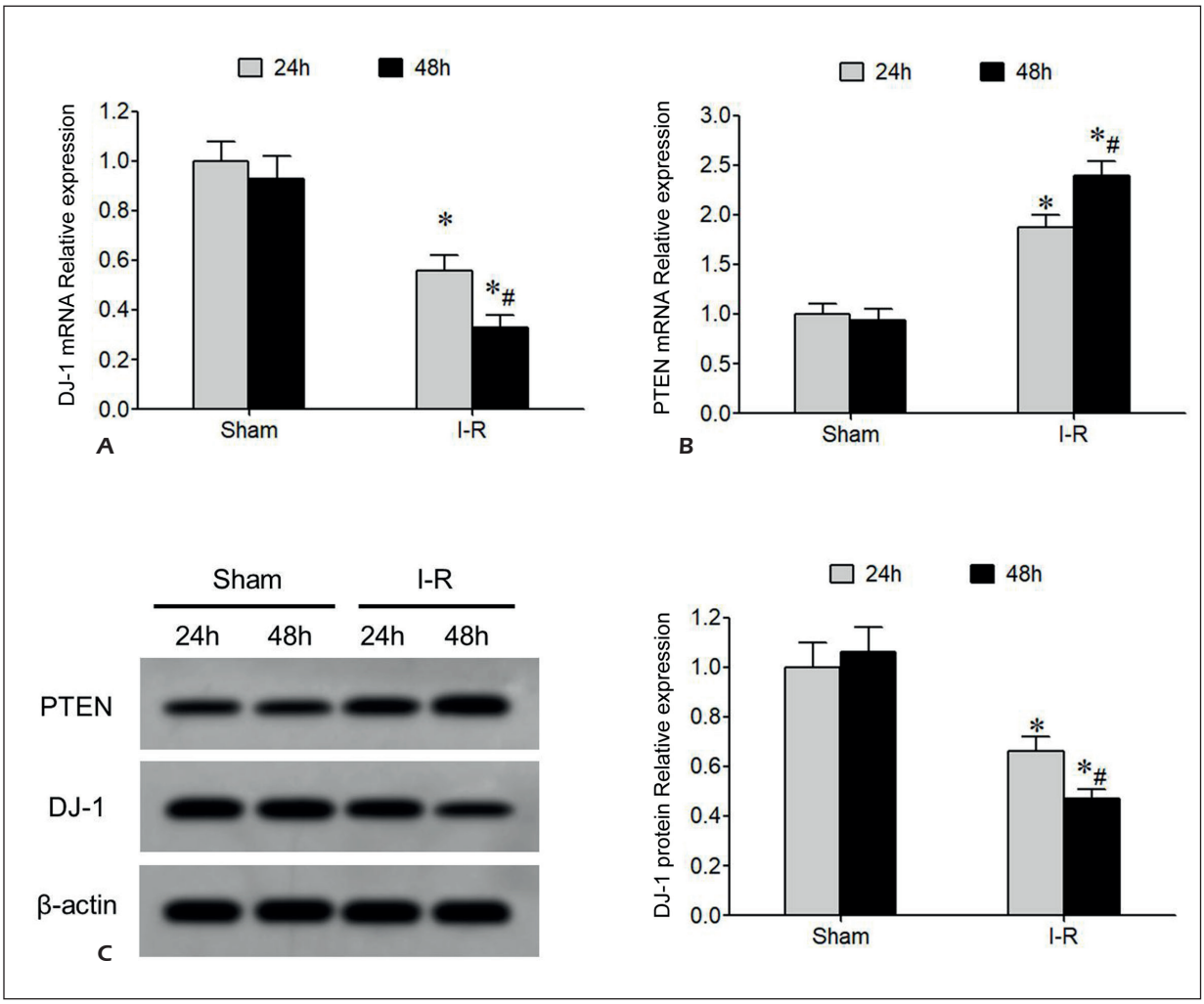

Figure 2. DJ-1 decreased, while PTEN elevated in the rat myocardium. **A**, qRT-PCR detection of DJ-1 mRNA expression. **B**, qRT-PCR detection of PTEN mRNA expression. **C**, Western blot detection of protein expression. *$p<0.05$, compared with sham. $^{\#}p<0.05$, compared with 24 h.

Discussion

PI3K/AKT is a signaling pathway that exists in a wide range of tissues and cells, and involves in the regulation of various biological processes, such as cell growth, proliferation and apoptosis[8,16,17]. Phosphatidylinositol-3 kinase (PI3K) auto-conformation change is activated under the stimulation of growth factor, mitogen, and other signaling molecules. Activated PI3K can promote phosphatidylinositol(4,5)-bisphosphate (PIP2) phosphorylation to phosphatidylinositol(3,4,5)-trisphosphate (PIP3) through its p110 catalytic subunit. PIP3 can act on the PH domain of Akt/PKB to change conformation. Moreover, it can phosphorylate Akt at Ser473 and Thr308 under the effect of phosphoinositide-dependent kinase 1 (PDK1) and phosphoinositide-dependent kinase 2 (PDK2). Phosphorylation-activated Akt regulates the transcription and translation of multiple target genes under the coordinated action of a variety of downstream signaling molecules, thereby achieving the regulation of biological effects, such as cell cycle, survival, proliferation, and apoptosis[18,19]. PTEN antagonizes the phosphorylation of phosphatidylinositol 3-kinase (PI3K) to PIP2 by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3) to maintain low levels of PIP3 kinase, PI3K), thereby inhibiting PI3K activation of AKT signaling molecules and downstream pathways through PIP3 phosphorylation[20,21]. This study investigated the role of DJ-1 in regulating PTEN-PI3K/AKT signaling pathway and myocardial I-R injury by establishing rat myocardial

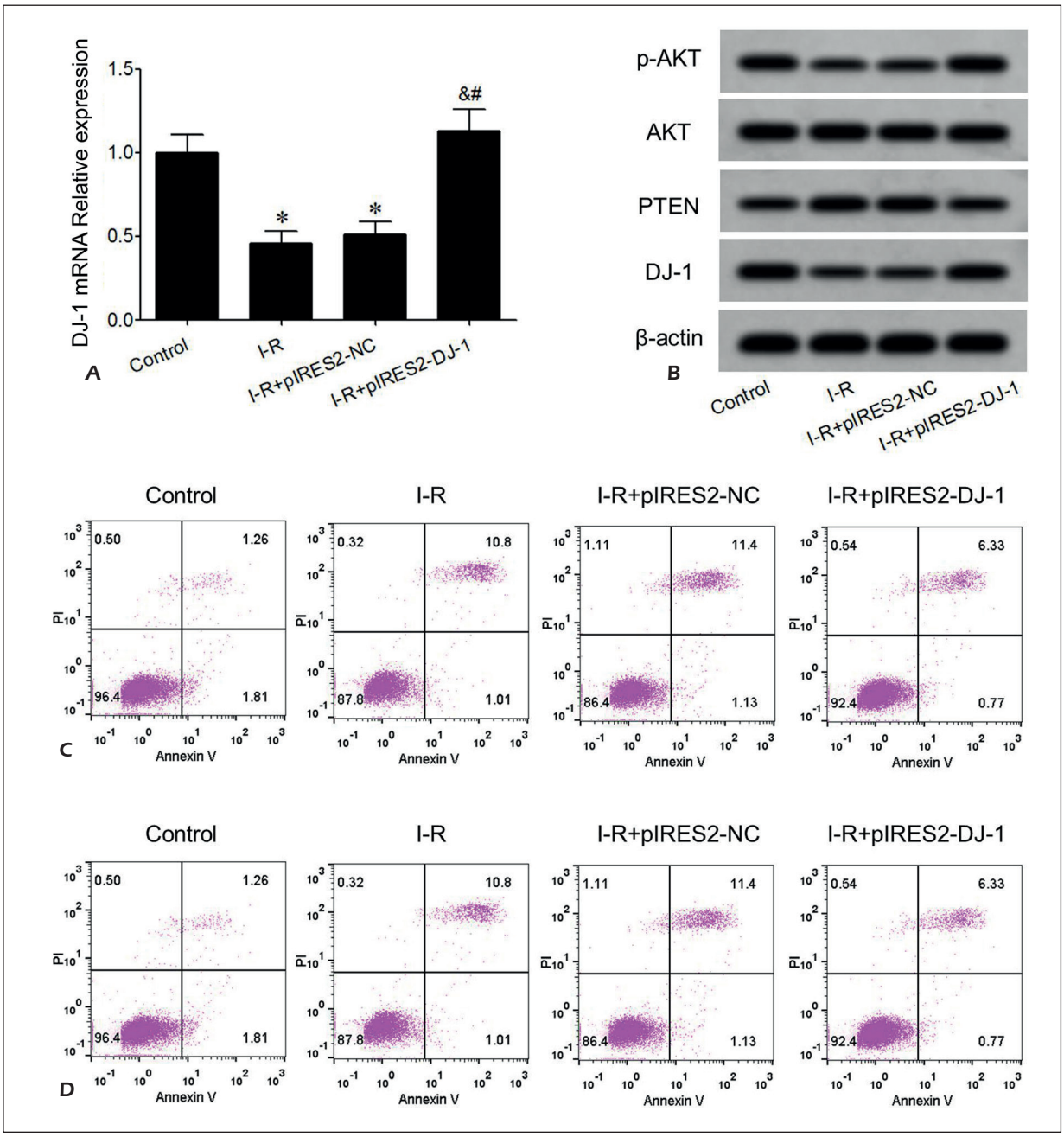

Figure 3. Elevated DJ-1 expression attenuated myocardial cell oxidative stress injury and apoptosis induced by IR. **A**, qRT-PCR detection of DJ-1 mRNA expression in H9C2 cells. **B**, Western blot detection of protein expression in H9C2 cells. **C**, Flow cytometry detection of ROS content in H9C2 cells. **D**, Flow cytometry detection of H9C2 cell apoptosis.

I-R injury model. Our results demonstrated that compared with the Sham group, the MDA contents, ROS contents, and caspase-3 activities were significantly increased, while SOD enzyme activities were significantly reduced in the myocardial tissue of I-R model group, indicating that I-R treatment aggravates the oxidative stress and enhances apoptosis. It was showed that during the course of I-R injury, DJ-1 mRNA and protein expression were significantly reduced, while PTEN mRNA and protein levels were significantly elevated with time dependence, suggesting that expression of DJ-1 was abnormally decreased, resulting in increased expression of PTEN, which may play a role in I-R injury. Shimizu et al[22] revealed that DJ-1 knockout mice exhibited increased sensi-

tivity to I-R injury, increased myocardial infarct size, and reduced cardiac function compared to wild-type mice. Dongworth et al[23] reported that the cardiac I-R injury in DJ-1 knockout mice was significantly higher than that in wild type mice, and the myocardial infarct size was obviously enlarged. Shimizu et al[22] and Dongworth et al[23] showed that the decreased expression of DJ-1 is an unfavorable factor in the process of myocardial I-R injury and can aggravate myocardial I-R injury, which was similar to our results. To further investigate whether the decreased expression of DJ-1 is a promoting factor of myocardial I-R injury, we over-expressed DJ-1 in rat cardiac myocytes H9c2 *in vitro* to observe whether it is possible to alleviate I-R injury. It was observed that after transfection of pIRES2-DJ-1 over-expression plasmid in H9c2 cells to increase the expression of DJ-1, PTEN levels were significantly decreased, p-AKT activity was markedly enhanced, and apoptosis and ROS production were apparently reduced under IR condition. About the relationship between DJ-1 and myocardial injury, Zhang et al[24] found that resveratrol treatment can reduce the effects of apoptosis, ROS production, and MDA content to alleviate I-R injury in rat myocardial cells. The injury and its function of reducing I-R damage are exerted by up-regulating the expression of DJ-1. Wang et al[25] exhibited that pre-ischemic preconditioning significantly up-regulated the expression of DJ-1 in myocardial tissue, reduced myocardial IR damage, and declined MDA, ROS, apoptosis, and myocardial infarct size, while elevated SOD, catalase (CAT), and glutathione peroxidase (GPx) activities. Lu et al[26] demonstrated that hypoxic preconditioning prior to ischemia-reperfusion could effectively modulate the endogenous protective mechanism to activate ERK1/2 and up-regulate the expression of DJ-1 in cardiomyocytes, thereby reducing the number of I-R injured myocardial cells. Dongworth et al[23] suggested that over-expression of DJ-1 significantly reduced the apoptosis of HL-1 in mouse cardiomyocytes under I-R conditions, which obviously reduced the sensitivity to I-R injury. The above studies indicated that elevated expression of DJ-1 is a protective factor in the process of myocardial I-R injury, and the over-expression of DJ-1 observed in this study can alleviate myocardial cell apoptosis and I-R injury. Shimizu et al[22] observed that the enhanced sensitivity of I-R damage to cardiomyocytes caused by the loss of DJ-1 expression was caused by abnormal small ubiquitin-like modifier (SUMO) modification, which was expressed as abnormal accumulation of SUMO-1 modified protein and decrease of SUMO-2/3 modified protein. Zhang et al[24] indicated that DJ-1 protects mitochondrial function and reduces ROS production to decrease cardiomyocyte apoptosis by binding to ND1 and NDUFS4. Our study revealed that DJ-1 regulates I-R injury to cardiomyocytes by affecting PTEN expression and PI3K/AKT signaling pathway activity. However, whether increasing the expression of DJ-1 may play a role in alleviating myocardial I-R injury in rats requires further animal studies and large-scale clinical validation.

Conclusions

We showed that abnormal expression of DJ-1 plays a regulatory role in the process of myocardial IR injury. Over-expression of DJ-1 can reduce myocardial cell I-R damage sensitivity by inhibiting PTEN expression, enhancing the activity of PI3K/AKT pathway, reducing ROS production, and alleviating apoptosis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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