Cellular Physiology and Biochemistry

Cell Physiol Biochem 2016;38:531-544
DOI: 10.1159/000438648
Published online: February 01, 2016
Accepted: December 16, 2015

1421-9778/16/0382-0531$39.50/0
www.karger.com/cpb

Original Paper

Spermine Inhibits Endoplasmic Reticulum Stress - Induced Apoptosis: a New Strategy to Prevent Cardiomyocyte Apoptosis

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Key Words
Acute myocardial infarction • Spermine • ERS • ROS • Apoptosis • PERK-eIF2α pathway

Abstract
Background/Aims: Endoplasmic reticulum stress (ERS) plays an important role in the progression of acute myocardial infarction (AMI), in part by mediating apoptosis. Polyamines, including putrescine, spermidine, and spermine, are polycations with anti-oxidative, anti-aging, and cell growth-promoting activities. This study aimed to determine the mechanisms by which spermine protects against ERS-induced apoptosis in rats following AMI. ***Methods and Results:*** AMI was established by ligation of the left anterior descending coronary artery (LAD) in rats, and exogenous spermine was administered by intraperitoneal injection (2.5 mg/ml daily for 7 days pre-AMI). Spermine treatment limited infarct size, attenuated cardiac troponin I and creatinine kinase-MB release, improved cardiac function, and decreased ERS and apoptosis related protein expression. Isolated cardiomyocytes subjected to hypoxia showed significant increase in reactive oxygen species (ROS) and the expression of apoptosis and ERS related proteins; these effects occurred through PERK and eIF2α phosphorylation. The addition of spermine attenuated cardiomyocyte apoptosis, suppressed the production of ROS, and inhibited ERS related pathways. ***Conclusions****:* Spermine was an effective pre-treatment strategy to attenuate cardiac ERS injury in rats, and the cardioprotective mechanism occurring through inhibition of ROS production and downregulation of the PERK-eIF2α pathway. These findings provide a novel target for the prevention of apoptosis in the setting of AMI.

Introduction

Acute myocardial infarction (AMI) is associated with a high mortality rate, in part due to frequent complications, including arrhythmias, shock, and heart failure. Cardiomyocyte apoptosis is a major mechanism of cardiac cell death that also contributes to MI-induced

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ventricular remodeling and heart failure [1, 2]. Endoplasmic reticulum stress (ERS) and the mitochondrial and death receptor pathway are two pathways that induce apoptosis and both can be triggered by a variety of factors, including ischemia [3, 4]. Increasing evidence indicates that inhibition of excessive ERS could be a critical intervention target in the protection of cardiomyocytes from injury [5-7].

Polyamines, such as putrescine, spermidine and spermine, play many roles in a wide variety of organisms and can also regulate cell-death processes *in vitro* [8-10]. In addition, the natural concentrations of polyamines responsible for polyamine metabolism are critical to cell survival, and have an effect on various aspects of cardiac disease [11, 12]. Out of the polyamines mentioned spermine has the highest biological activity [13]. Spermine carries four positive charges, which can function as scavengers of oxygen free radicals and protect nucleic acids and other cellular components from oxidative damage [14, 15]. However, the full cardioprotective mechanism by which spermine acts in AMI has yet to be elucidated.

Materials and Methods

Animal use and experimental design

Eight week male wistar rats weighing 250 ± 10 g were supplied by the animal house at Second affiliated hospital of Harbin medical university. All experiments were approved by the Animal care committee of the Harbin medical university for the Use of experimental animals. Rats were housed in a controlled environment (24 ± 1°C; 60 ± 10% relative humidity; fixed 12/12 h light/dark cycle) with food and water ad libitum, and were randomized into four groups (n = 8 each): (1) Control group (Control): rats not exposed to any surgical procedure; (2) Sham operated group (Sham): rats who underwent the surgical procedure used to ligate the left anterior descending (LAD) coronary artery, except the silk suture was placed around the left coronary artery without being tied; (3) Acute myocardial infarction group (AMI): rats underwent LAD ligation for 6 h; (4) Spermine treated group (Sp): rats were pre-treated with spermine (Sigma Chemical, St. Louis, MO, USA, 2.5 mg/kg daily, intraperitoneal injection) for seven consecutive days and then underwent LAD ligation. Spermine dissolved in dd-H_2O to the desired final volume used in the procedure.

AMI was induced as previously described [16]. Rats were anaesthetized by intraperitoneal injection of a 10% solution of chloral hydrate in saline (Sigma; 300 mg/kg). Electrocardiogram (ECG), heart rate, and respiratory rate were continuously monitored throughout the surgery. Following endotracheal intubation and mechanical ventilation, left thoracotomy was performed to expose the heart. MI was induced by permanent ligation of the LAD coronary artery at the location between the pulmonary cone and the left atrial appendage, using a 5-0 polypropylene suture. ECG ST-segment elevation was used to confirm AMI. Thorax was closed using 7-0 sutures. Rats were given intramuscular penicillin (Sigma, 4×10^5 units) and subcutaneous buprenorphine (Suboxone®; 0.1 mg/kg) to reduce post-operative infection and pain.

At 6 h post-AMI, cardiac function was measured by ultrasound (FUJIFILM VisualSonics, Inc., Canada), and the rats were sacrificed. Blood was collected by puncture of the LV for serum measurements of cardiac enzymes. The left ventricle free wall was collected and sliced into 3 mm^2 samples. One sample was used for histology and the remaining samples were snap frozen at -80°C for biochemical assessment.

Myocardial polyamine content detection by RP-HPLC

Spermine, spermidine and putrescine were obtained from Sigma (St. Louis, MO, USA). Myocardial polyamine content was determined by Reversed-phase high performance liquid chromatography (RP-HPLC) using ODS-C18 columns, as previously described [17]. Putrescine, spermidine and spermine were used as positive controls, and benzoyl chloride used as a derivative reagent, to compare the content of polyamines among the different groups. Derivatization was conducted at 50°C for 8 h, mobile phase as methanol-water (45:55), flow rate as 1.0 ml/min and detection wavelength 234 nm. Each peak of the derivatives was separated in HPLC. Content was determined by the size of the peak area.

Cardiac Toponin I (cTnI) and Creatinine Kinase-MB (CK-MB) assays

We measured myocardial enzyme levels in serum, which accompanied the electrocardiogram changes to confirm AMI diagnosis. cTnI and CK-MB were measured in serum using commercial kits (Jian Cheng Bioengineering Institute, Nanjing, China) and values were read on a microplate spectrophotometer.

Histopathological examination by Hematoxylin and Eosin (HE) staining

LV samples were fixed in 10% formalin solution, paraffin-embedded, and sectioned (3 μm). The sections were stained with hematoxylin and eosin for routine histopathology examination.

In situ apoptosis assay

Paraffin-embedded, 4-5 μm-thick myocardial sections were cut as described previously [18]. Apoptotic myocytes were stained by the TdT mediated dUTP nick end-labeling (TUNEL) assay using a Cell death detection kit (Roche, Mannheim, Germany). Three sections from each myocardial sample were randomly selected, and ten microscopic fields per section were evaluated. Apoptotic index was determined by dividing the cell number of TUNEL-positive nuclei by the total number of cells across the viewed areas, and multiplied by 100.

Neonatal cardiomyocytes hypoxia/ischemia treatment and experimental protocols

Newborn Wistar rats, 1-3 days-old and 5-8 g-weight, were used for this study. Primary cultures of neonatal cardiomyocytes were prepared as previously described [19]. The cardiomyocytes were plated in collagen-coated well plates and maintained at 37°C in a 5% CO_2 humidified incubator in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin or streptomycin. Cardiomyocytes spontaneously beating for 72 h after plating were put into a hypoxic incubator that was equilibrated with 1% O_2/5% CO_2/94% N_2 for 24 h. Ischemia was induced by incubating the cells in Hank's balanced salt solution (Gibco, USA) saturated with 95% N_2 and 5% CO_2. The pH was regulated to 6.8 with lactate to mimic ischemic conditions.

The cells were randomly divided into one of six groups: (1) Normoxia control group (Control): cardiomyocytes cultured with DMEM in 10% FBS for 24 h; (2) Hypoxia/Ischemia group (H/I): cardiomyocytes cultured in ischemic medium and exposed to hypoxia for 24 h; (3) Hypoxia/Ischemia+Spermine group (H/I+Sp): 5 μM spermine was added to the ischemic medium at the beginning and the cells were incubated under hypoxia for 24 h; (4) Hypoxia/Ischemia+4-Pheylbutyric acid group (H/I+4-PBA): 0.5 mM 4-PBA (Sigma, St. Louis, MO, USA) was added to the medium 30 min before hypoxia. In a second set of experiments, groups 1 and 2 above were compared to group 2 conditions. The two new groups were: (5) Hypoxia/Ischemia+n-acetyl cysteine (NAC) group (H/I+NAC): 5 mM NAC was added to the medium 30 min before H/I; and (6) Hypoxia/Ischemia+GSK2656157 (H/I+GSK): 1 mM GSK2656157, a PERK inhibitor (Selleck Chemicals, Houston, TX, USA) was added to the medium 30 min before H/I. Both group 1 and group 2 were four group comparisons.

Cell viability assay

Cell viability was measured using the cell counting kit-8 (CCK-8; Dojindo, WTS, Japan). Cells were seeded in 96-well plates at 3×10^3 cells/well. CCK-8 (10 μl) was added to each well immediately after 24 h of each treatment and incubated for 2 h at 37°C. The wells were read for OD at 570 nm (A570) using a microplate spectrophotometer.

Apoptosis assay by Annexin V/ Propidium iodide (PI) staining

The apoptotic rate was detected by flow cytometry using the FITC Annexin V apoptosis detection kit I (BD Biosciences, San Jose, CA, USA). Cells were washed with cold PBS and re-suspended in binding buffer at a concentration of 1×10^6 cells/ml. A total of 100 μl of the solution was transferred to a 5 ml culture tube and 5 μl of FITC-Annexin V and 5 μl PI were added. The cells were incubated for 15 min at room temperature in the dark, and 400 μl of binding buffer was added to each tube. The fluorescence was analyzed by flow cytometry. The percentage of apoptotic cells was determined using the Mod Fit LT software (Verity Software House Inc., Topsham, ME, USA).

Morphological analysis of apoptotic nuclei

Apoptotic cells were analyzed after visualization of nuclei morphology with the fluorescent DNA-binding dye Hoechst 33342. Cells were rinsed with PBS and incubated with 5 mg/ml Hoechst 33342 for 10 min. Nuclei were visualized at 400× magnification using fluorescent microscopy (Nikon Corporation, Tokyo, Japan) at an excitation wavelength of 330-380 nm. Apoptotic nuclei of cells were assessed by counting the number of cells that displayed nuclear morphological changes such as chromatin condensation and fragmentation.

Measurement of intracellular Reactive oxygen species (ROS)

ROS generation was estimated using a reactive oxygen species assay kit (Beyotime, China). Cells were seeded in 12-well plate (1×10^6 cells/sample) and exposed to different treatments. Intracellular ROS oxidizes non-fluorescent DCFH into fluorescent DCF, and then observed by a fluorescence microscope. The ROS level was reflected by the fluorescence intensity.

Immunoblotting assay

Myocardial samples and harvested cells were homogenized and lysed in RIPA buffer. Protein concentration was measured using a BCA protein assay kit. Equal amounts of proteins (80 g) were boiled and separated by SDS-PAGE and transferred using electrophoresis to a PVDF membrane. Membranes were blocked with Tris-buffered saline containing 5% non-fat milk at room temperature for 1 h, and then incubated overnight at 4°C with specific primary antibody. All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA) or Santa Cruz (Dallas, TX, USA). The membrane was washed three times with 1×Tris-buffer saline-Tween 20 (TBST) buffer and incubated in TBST solution with alkaline phosphatase-labeled secondary antibody for 2 h at room temperature on a shaker. Antibody-antigen complexes were detected using Western blue stabilized substrate for alkaline phosphatase. The intensities of the protein bands were quantified by a ChemiDoc™ EQ densitometer and Quantity one software (Bio-Rad Laboratories, USA). Protein loading was normalized to GAPDH levels. GAPDH is not a good loading marker for long-term MI but is suitable for < 24 h of ischemia [20, 21].

Statistical Analysis

All data were expressed as the mean±SE for n = 8 rats per group, and each measurement represented at least three independent experiments. Statistical comparisons were made using paired or unpaired t-tests or one-way ANOVA followed by Bonferroni post-test. Significance level was set at $p < 0.05$.

Results

AMI altered in vivo polyamine metabolism in rats

The peak of the three derivations for putrescine, spermidine, and spermine were obtained at approximately 8 min, 17 min, and 37 min. Compared to control and sham groups, the peak area of putrescine increased while both spermidine and spermine peaks decreased post-AMI (all $p < 0.05$). Administration of exogenous spermine restored polyamine metabolism balance. This result demonstrated that AMI decreases endogenous spermine, which feeds forward to alter putrescine and spermidine metabolism profiles (Fig. 1).

Effects of spermine on AMI related indicators

AMI induced robust ST-segment elevation and a visible pathologic Q wave in the rats who underwent LAD ligation. Spermine pre-treatment reduced the extent of these changes (Fig. 2A).

Using echocardiography, contraction of the left ventricle anterior walls was impaired in the AMI animals compared to both control and sham groups (both $p < 0.05$), and spermine treatment attenuated this impairment (Table 1).

Both cTnI and CK-MB concentrations in the serum were low in both the control (0.3 ± 0.1 and 16.2 ± 1.1 U/l) and sham groups (0.3 ± 0.1 and 17.5 ± 1.5 U/l, all $p > 0.05$). AMI elevated concentrations of markers (3.9 ± 0.2 U/l for cTnI and 95.5 ± 3.0 U/l for CK-MB, both $p < 0.05$), and spermine significantly reduced the increase of markers compared to controls to 2.1 ± 0.2 U/l for cTnI and 47.8 ± 2.2 U/l for CK-MI; $p < 0.05$; Fig. 2B).

Exogenous spermine suppressed the apoptosis of AMI animals

HE staining revealed extensive and obvious bleeding and leukocyte infiltration, with obvious an obvious region of coagulation band necrosis and interstitial loose wave structure in the AMI group compared to both control and sham groups (Fig. 3A). Spermine treatment improved fiber structure compared to the AMI group.

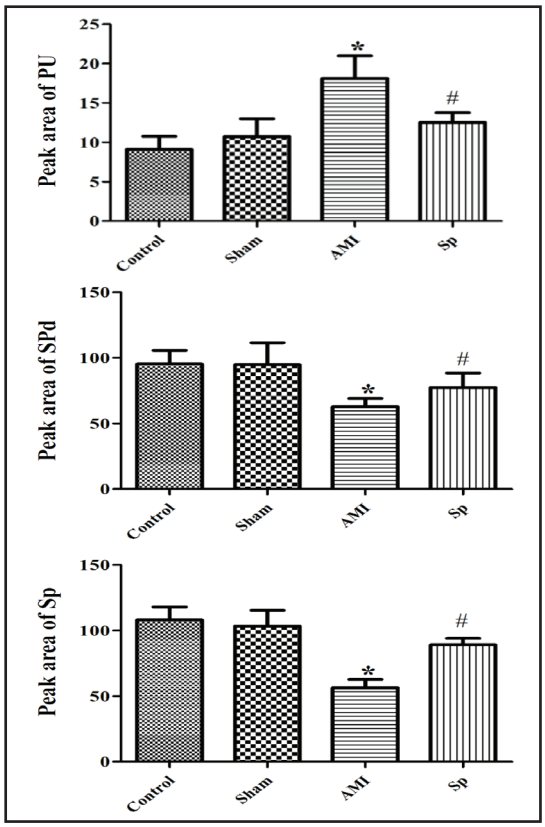

Fig. 1. AMI disrupted polyamine metabolism *in vivo* in rats. HPLC chromatogram of polyamines after derivation, indicating the peak areas of putrescine, spermidine, and spermine. Data are mean ± SE for n = 8 per group; * p < 0.05 vs. Control or Sham group; # p < 0.05 vs. AMI group.

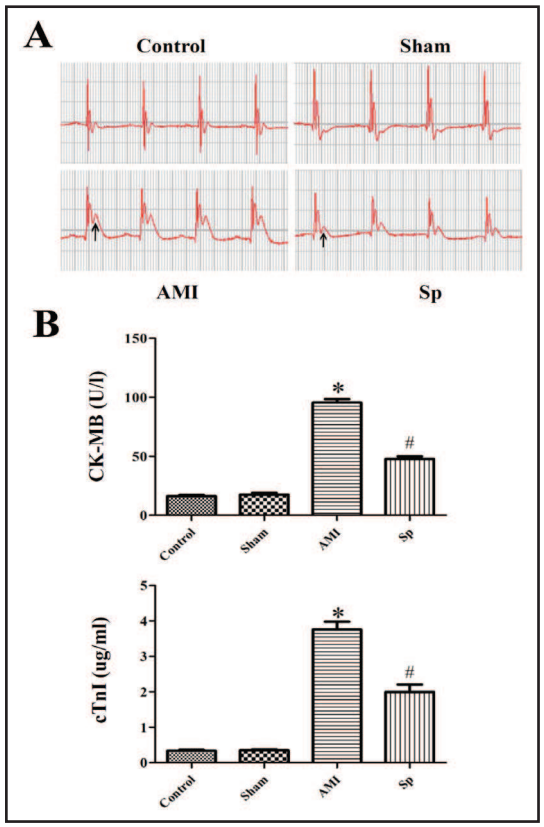

Fig. 2. Effects of spermine on AMI related indicators. (A) Electrocardiogram recordings in rats from the different groups. (B) Myocardial enzyme detection of cardiac troponin I (cTnI) and creatine kinase (CK)-MB. Data are mean ± SE for n = 8 per group; * p <0.05 vs. Control or Sham group; # p < 0.05 vs. AMI group.

AMI significantly increased the percentage of apoptotic cells ($p < 0.05$ vs. both control and sham groups; Fig. 3B). Compared to AMI, spermine reduced the extent of apoptosis ($p < 0.05$).

The expression of Bcl-2 was decreased and cleaved-Caspase-3 and -9 were increased in the AMI group ($p < 0.05$ vs. both control and sham groups; Fig. 3C). Spermine treatment reduced the AMI effect ($p < 0.05$).

Exogenous spermine decreased the expression of ERS-related protein

To elucidate whether ER stress was inhibited through the protective effect of spermine against LAD ligation or H/I-induced injury, ER stress associated proteins were analyzed by immunoblotting. *In vivo*, the expression of ER related proteins (GRP78, C/EBP homology protein- CHOP, Caspase-12 and activating transcription factor 6- ATF-6) increased with MI and this increase was attenuated by treatment with spermine (all $p<0.05$; Fig. 4). *In vitro*, spermine inhibited the expression of ER related proteins induced by H/I ($p<0.05$; Fig. 6). Similar results were observed when cells were treated with 4-PBA (p = not significant).

Exogenous spermine protected H/I-induced cell viability decreasing and apoptosis

Cell viability was reduced to 50.4±0.1% in the H/I group compared to the control group (100%, $p < 0.05$; Fig. 5A). Spermine or 4-PBA pre-treatment increased cell viability to 80.3

Table 1. Cardiac function detected by echocardiography. Data are mean±SE for n = 8 per group. * p < 0.05 vs. Control or Sham group; #p < 0.05 vs. AMI group. LVEDV- Left ventricular end diastolic volume; LVESV- Left ventricular end systolic volume; EF- Ejection fraction; FS- Fractional shortening

n=8	LVEDV(μl)	LVESV(μl)	EF%	FS%
Control	405±16.1	93±8.6	80.3±4.0	41.9±1.7
Sham	410±17.0	96±8.4	79.6±4.0	41.9±2.4
AMI	618±22.7*	246±11.5*	49.1±6.9*	24.4±3.3*
Sp	466±28.0#	163±17.4#	66.6±9.3#	34.2±4.4#

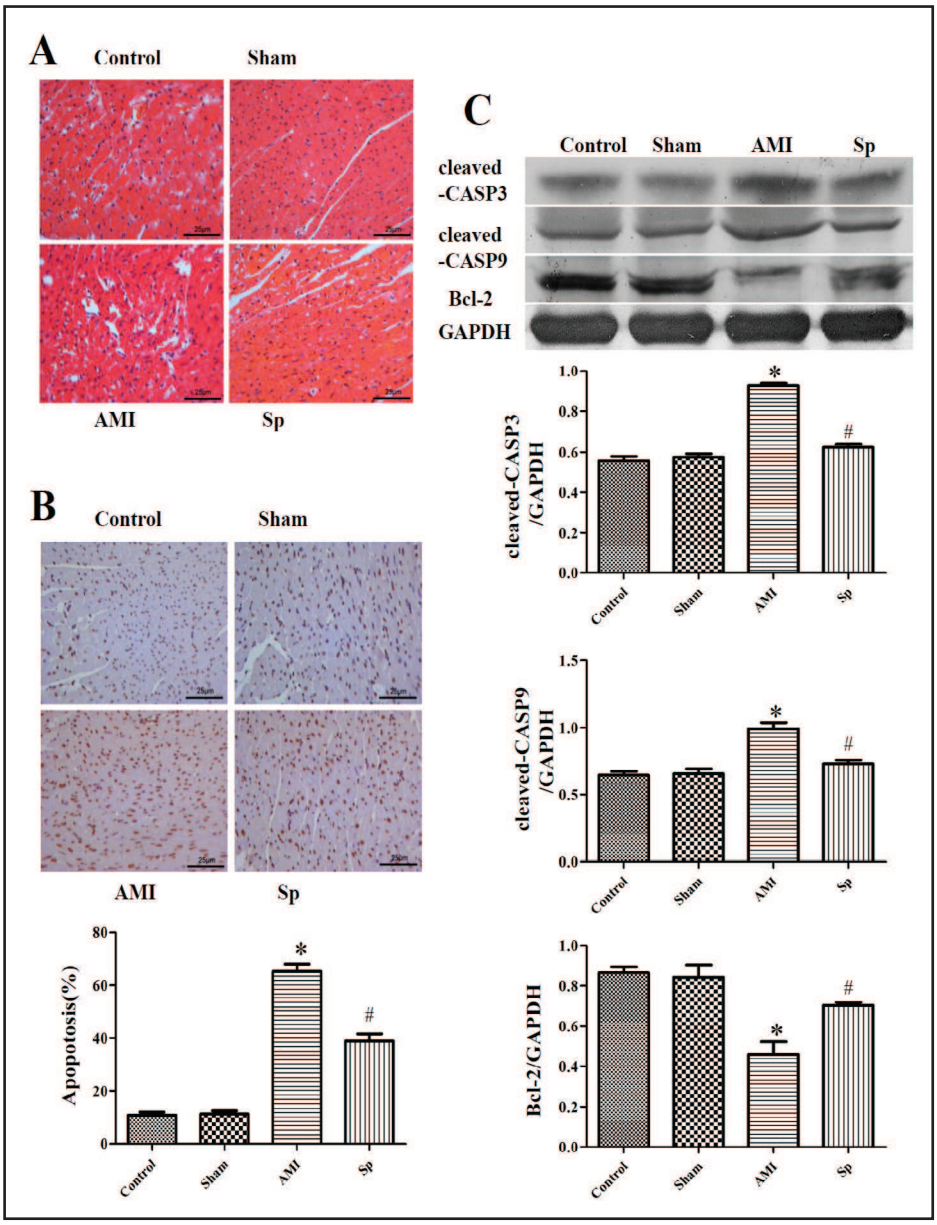

Fig. 3. Spermine suppressed the apoptosis of AMI animals. (A) Representative hematoxylin and eosin (HE) stained sections showing myocardial morphology is disrupted with AMI, an effect prevented by spermine pre-treatment. (B) Cardiomyocyte apoptosis detection by TUNEL. The apoptotic index was determined by dividing the cell number of TUNEL-positive nuclei by the total number of cells x 100. (C) Cleaved-Caspase-3 and-9 and Bcl-2 detected by immunoblotting. The intensity of each band was quantified by densitometry, and data were normalized to the GAPDH signal. Data are mean ± SE for n=8 per group; * p < 0.05 vs. Control or Sham group; # p < 0.05 vs. AMI group.

± 0.2% and 83.4 ± 0.3% vs. H/I group (p < 0.05), and there was no significant difference between the two treatment groups.

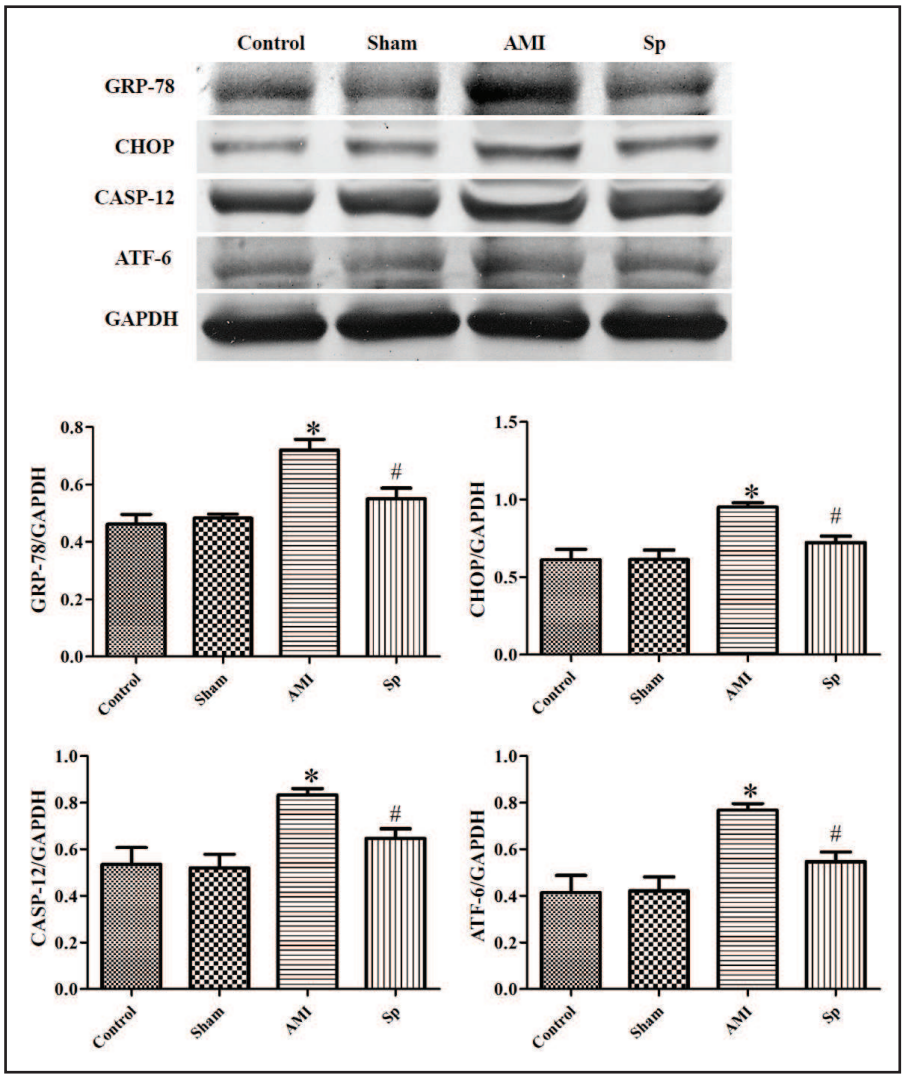

Fig. 4. Spermine decreased the expression of ERS-related proteins. Protein expression of GRP78, CHOP, Caspase-12 and ATF-6 were detected by immunoblot. The intensity of each band was quantified by densitometry, and data were normalized to the GAPDH signal. All data were from four independent experiments and are reported as mean ± SE; * $p < 0.05$ vs. Control or Sham group; # $p < 0.05$ vs. AMI group.

Flow cytometry was used to detect cardiac apoptosis caused by H/I, as well as the effect of spermine and 4-PBA on apoptosis. H/I significantly increased apoptosis to 43.8±2.1% ($p < 0.05$ vs. control group value of 3.6 ± 0.9%; Fig. 5B). Compared with the H/I group, spermine and 4-PBA reduced the apoptotic rate to 24.7 ± 1.6% and 25.3 ± 2.0% (both $p < 0.05$). The apoptotic rate of the H/I+4-PBA group was similar to the H/I+Sp group.

Hoechst 33342 was used to confirm the morphological changes in nuclei. Compared to a control group value of 9.7 ± 0.8% (Fig. 5C), the percentage of apoptotic nuclei with typical features of fragmentation and condensation in the H/I group was significantly increased to 59.6 ± 2.1% ($p < 0.05$). Spermine and 4-PBA reduced the incidence of observed change percentages to (33.6 ± 1.7% and 32.9 ± 1.1%, both $p < 0.05$).

The expression of cleaved-caspase-3 and -9 were increased and Bcl-2 expression decreased in the H/I group ($p < 0.05$ vs. control group; Fig. 5D). Spermine and 4-PBA treatment reversed the effect of H/I (both $p < 0.05$), with no significant difference between the two groups.

The effect of NAC on ROS production and the expression of ER-related proteins

As shown in Fig. 7A, the quantity of ROS determined by DCFH-DA in cardiomyocytes cultured under H/I condition was significant increased ($p < 0.05$). Treatment with exogenous spermine or n-acetylcysteine (NAC), a ROS scavenger, significantly reduced the intracellular ROS level induced by H/I (both $p < 0.05$). Likewise, the expression of ER-related proteins

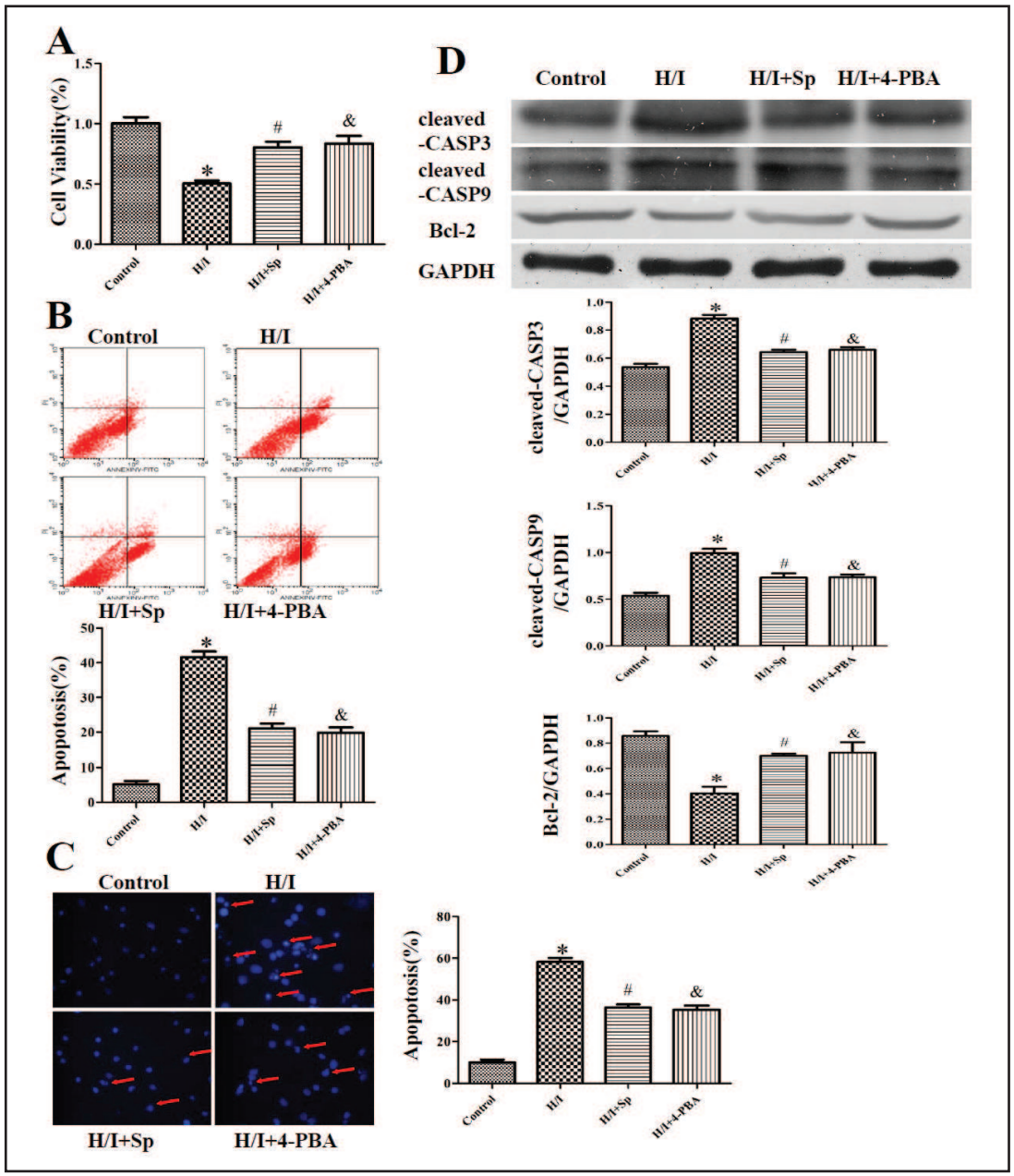

Fig. 5. Spermine protected H/I-induced cell viability decreasing and apoptosis. (A) Cell viability was measured by CCK-8 assay. Cells incubated with control medium were considered 100% viable. (B) Apoptosis detection by flow cytometry. (C) Nuclear morphology in apoptotic cells analyzed by Hoechst 33342 staining. Apoptotic cells were marked as cells with condensed, disrupted nuclei (arrow, × 400). Apoptotic cells were counted in at least ten random fields. (D) Western blot detected protein expressions of Cleaved-Caspase-3and-9 and Bcl-2. The intensity of each band was quantified by densitometry, and data were normalized to the GAPDH signal. All data were from 4-8 independent experiments and are reported as mean ± SE; * $p < 0.05$ vs. Control group; # $p < 0.05$ vs. H/I group.

increased in the H/I group (all $p < 0.05$ vs. control group; Fig. 7B). Spermine and NAC treatment prevented the effects of hypoxia (both $p < 0.05$).

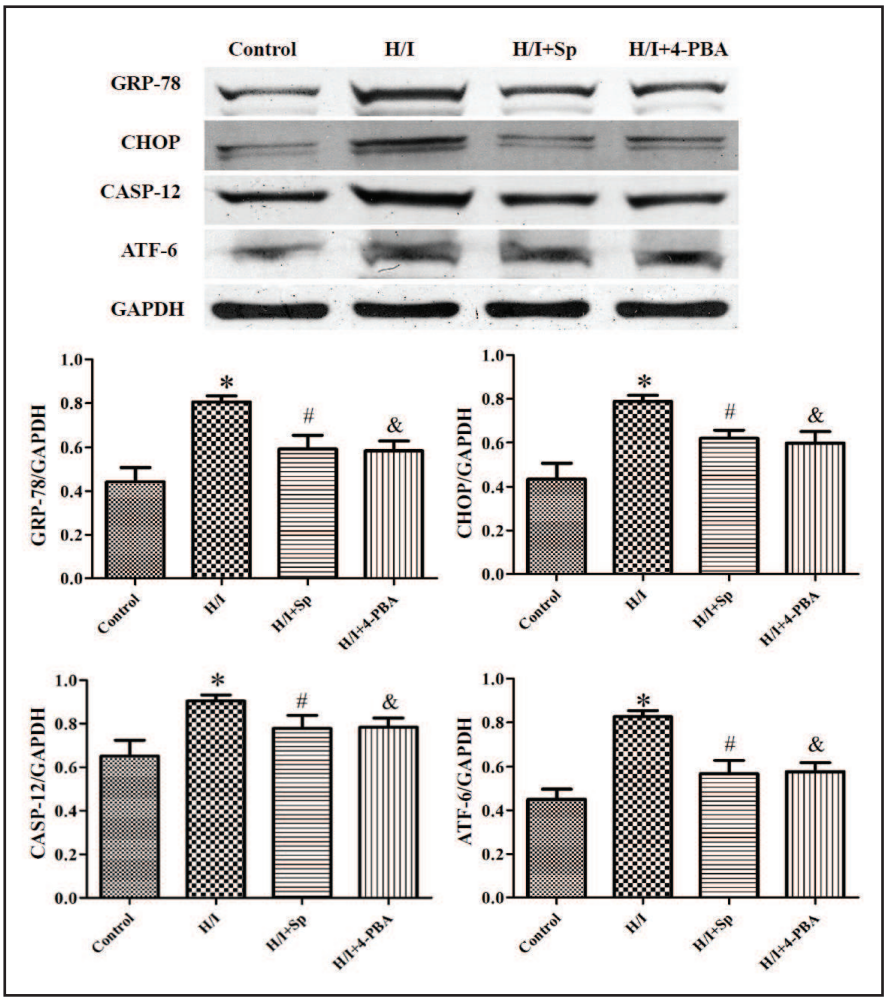

Fig. 6. Spermine decreased ERS-related protein expressions *in vitro*. Protein expressions of GRP78, CHOP, Caspase-12 and ATF-6 for each group were detected by immunoblot. The intensity of each band was quantified by densitometry, and data were normalized to the GAPDH signal. All data were from four independent experiments and are reported as mean ± SE; * $p < 0.05$ vs. Control group; # $p < 0.05$ vs. H/I group.

Exogenous spermine involved in the regulation of PERK- eIF2α pathway

To detect whether spermine could inhibit PERK- eIF2α pathway activated by H/I, we cultured cardiomyocytes in hypoxia/ischemia conditioned media with or without spermine. The levels of phosphorylated PERK and eIF2α increased in the H/I group compared to the control group ($p < 0.05$). Spermine prevented H/I-induced stimulation of the PERK- eIF2α pathway ($p < 0.05$). The total amount of PERK and eIF2α protein remained unchanged among the different stimulations (Fig. 8).

To determine whether PERK- eIF2α pathways were involved in Sp-inhibited ERs and apoptosis induced by H/I, GSK2656257 (a PERK inhibitor) was used. We found that the inhibition of PERK and eIF2α significantly suppressed H/I- induced phosphorylation of PERK and eIF2α (Fig. 8).

Discussion

The goal of this study was to evaluate the ability of spermine to prevent effects of *in vivo* ischemia and *in vitro* hypoxia on cardiomyocyte structure and function. The most significant findings of this study were that spermine pre-treatment prevented AMI-induced pathology, dysfunction, and cardiomyocyte apoptosis and prevented H/I-induced pathology and apoptosis in isolated cardiomyocytes. Furthermore, pre-treatment with spermine reduced ER stress, thereby increasing cell viability and decreasing the rate of apoptosis. These results

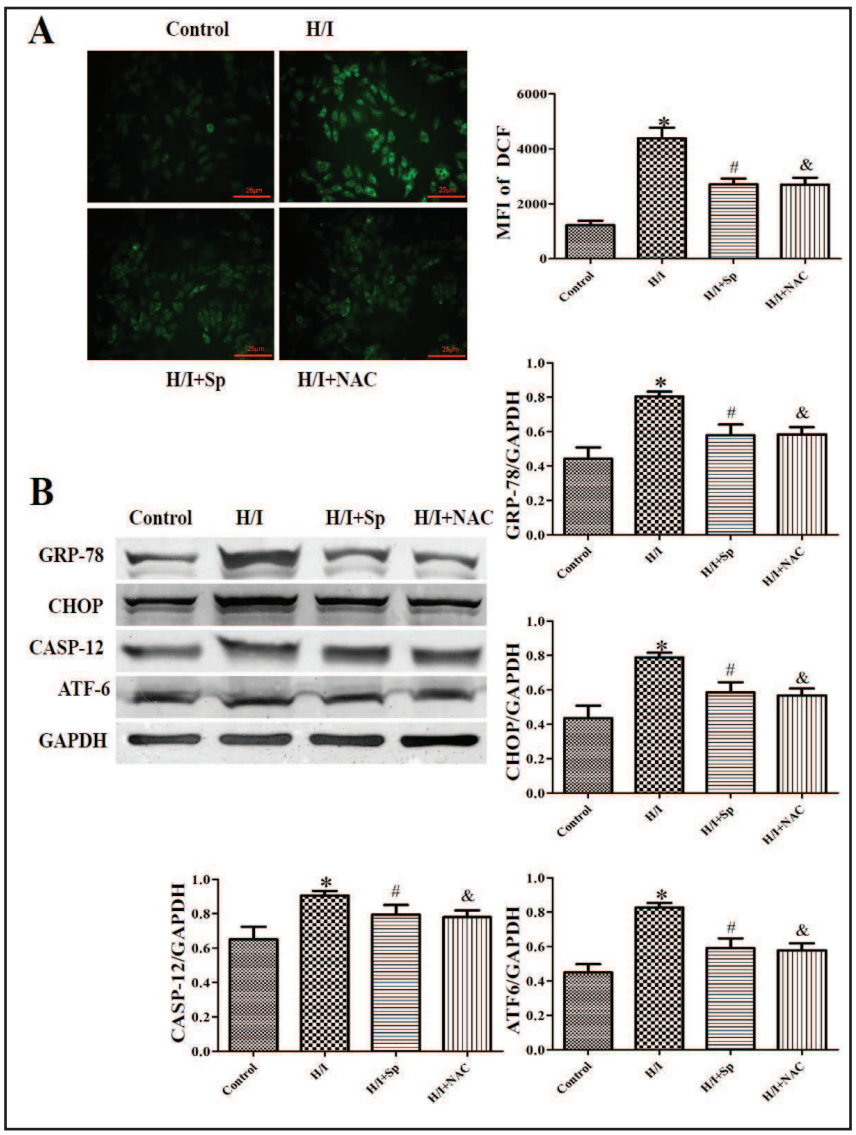

Fig. 7. Effect of n-acetyl cysteine (NAC) on reactive oxygen species (ROS) production and the expression of Endoplasmic reticulum (ER)-related proteins. (A) ROS production determined by Dichloro-dihydro-fluorescein diacetate (DCFH-DA). Cells were stained with DCFH-DA and imaged by fluorescent microscope (×400). (B) Both Spermine and NAC decreased ER-related protein expression *in vitro*. The intensity of each band was quantified by densitometry, and data were normalized to the GAPDH signal. All data were from four independent experiments and are reported as mean ± SE; * $p < 0.05$ vs. Control group; # $p < 0.05$ vs. H/I group.

indicate that pre-treatment of rats with spermine protects polyamine metabolism thereby protecting myocytes and maintaining cardiac function in the setting of AMI.

As expected, LAD ligation induced ST-segment elevation with pathologic Q-waves, cardiac dysfunction indicated by reduced Ejection fraction (EF), Fractional shortening (FS), increased Left ventricular end-diastolic volume (LVEDV) and Left ventricular end-systolic volume (LVESV), similar to previous studies [22]. HE staining showed myocyte karyopyknosis and fragmentation, interstitial edema with inflammatory cell infiltration, and myocardial fiber disarray in the infarcted regions of the AMI group. In addition, apoptotic myocytes were distributed around the infarct zone, as displayed by positive TUNEL staining, indicative of cardiomyocyte apoptosis.

Polyamines, especially spermine, have a variety of biological functions, including antioxidative properties, free radical scavenging, inhibition of mitochondrial swelling and mitochondrial permeability transition pore (mPTP) opening, and regulation of intracellular calcium. Polyamines can exert anti-apoptotic effects by maintaining the integrity of cell and mitochondria membranes [23-25]. Our previous study found that ischemia/reperfusion (I/R) injury could induce polyamine metabolism imbalance in rat myocardium, resulting in increased concentrations of putrescine and decreased spermidine and spermine concentrations [23, 24]. In our current study, we detected polyamine metabolic changes in

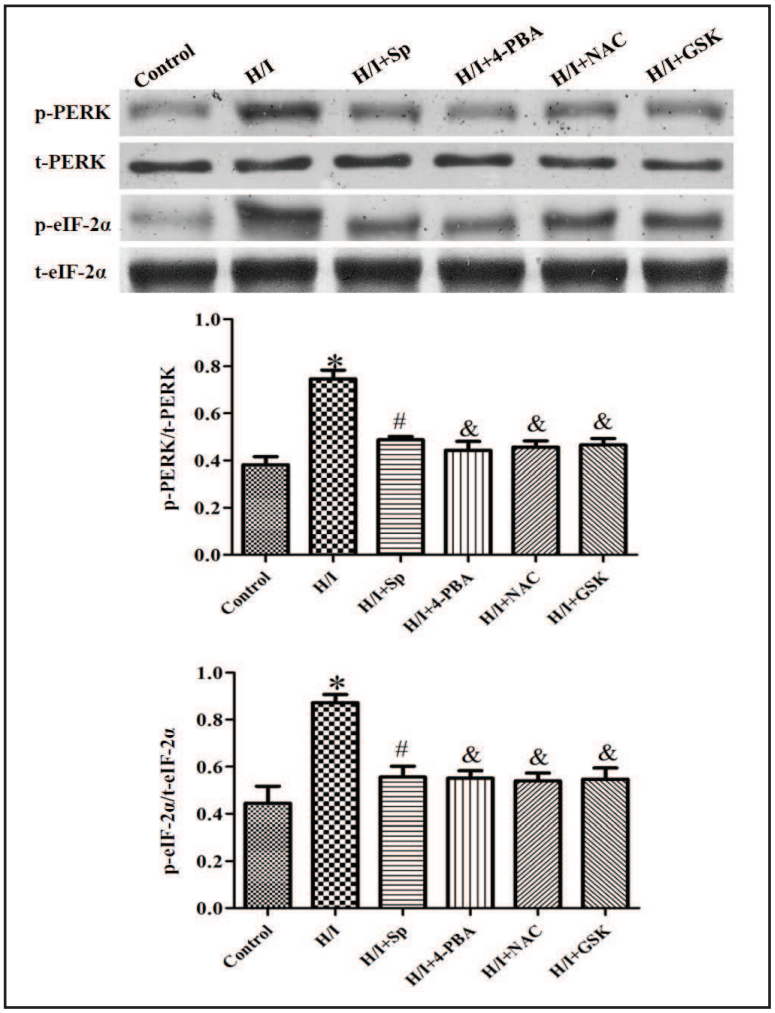

Fig. 8. Spermine involved in the regulation of PERK-eIF2α pathway. Cells were collected and subjected to western blot. The graphs represent the optical density of the band of phosphorylated PERK and eIF2α normalized with that of total PERK and eIF2α, respectively. All data were from four independent experiments and are reported as mean ± SE; * $p < 0.05$ vs. Control group; # $p < 0.05$ vs. H/I group.

AMI rat hearts by RP-HPLC and observed decreasing levels of endogenous spermine as a result of the AMI.

The endoplasmic reticulum (ER) is involved in the intrinsic pathway of apoptosis, and its function can be disturbed by various conditions that result in ER stress, including ischemia, hypoxia, exposure to free radicals and elevated protein synthesis [26, 27]. As a main signaling pathway, the unfolded protein response is initiated to cope with ERS. The unfolded protein response is mediated by Glucose-regulated protein-78 (GRP78, an ER chaperone) and response proteins, including Protein kinase-like ER kinase (PERK) and Activating transcription factor-6 (ATF-6), which can be activated by dissociation [27-29]. When the unfolded protein response is compromised or ERS is elevated, cell apoptosis can be stimulated by activating Caspase-12, which is localized on the ER membrane and specifically activated by ERS. Activated Caspase-12 is released into the cytoplasm, where it activates the final apoptotic pathway to sequentially activate Caspase-9 and then Capase-3 [30-32]. Another important component of the ERS-mediated apoptosis pathway is CHOP, which decreases Bcl-2 expression and stimulates ROS production through glutathione depletion in the cell. Evidence suggests that CHOP is the primary regulator of ERS-induced apoptosis, as the number of apoptotic cells was significantly reduced in CHOP null mice [33, 34].

Pretreatment with exogenous spermine significantly reversed ER stress and apoptosis in both *in vivo* and *in vitro* models. To further determine whether ERS was involved in H/I-induced apoptosis, we treated cardiomyocytes with 0.5 mM 4-PBA, a selective inhibitor of ERS, as previously described [35]. Treatment with 4-PBA substantially reduced the frequency

of apoptotic cells, which confirmed the effect of spermine occurred through ER-induced cell apoptosis.

In order to investigate the potential mechanism of cardioprotective effects of spermine, we further examined the generation of ROS and ERS-related pathways at the cellular level. Under physiological conditions ROS are generated at low levels and play important roles in signaling and metabolic pathways [36]. Hypoxia and other stimuli, however, can result in the overproduction of ROS and lead to oxidative stress [37]. Previous studies have suggested that the PERK-eIF2α pathway is involved in the regulation of ERS. Activation of PERK induced eIF2α phosphorylation, further promoted Caspase-12 and CHOP over-expression that can lead ERS towards cell apoptosis [31, 32, 38, 39]. Our results suggested that H/I increased ROS levels and activated the phosphorylation of PERK and eIF2α. Spermine pretreatment prevented both activation of PERK and eIF2α, and the addition of NAC (a ROS scavenger) or GSK2656257 (a PERK inhibitor) confirmed that reducing ROS production or preventing the activation of the PERK-eIF2α pathway activation could inhibit ERS and cardiomyocyte apoptosis.

In conclusion, we found that LAD ligation disrupted polyamine metabolism in rat cardiomyocytes, such that putrescine increased while both spermidine and spermine decreased post-MI (all $p < 0.05$). The decrease in spermine reduced its ability to block apoptosis, resulting in increased apoptosis through upregulation of ERS. The mechanisms by which administration of exogenous spermine to effectively prevent apoptosis have been described in detail. We further explored the cardioprotective mechanisms of spermine at the cellular level and demonstrated that spermine inhibited ERS-induced cardiomyocyte apoptosis by reducing ROS generation and suppressing PERK-eIF2α pathway activation. These findings may provide novel targets for early post-AMI treatments or during surgical cardiac procedures.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (No. 81270311, 81070123).

Disclosure Statement

The authors declare that they have no conflict of interest.

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