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Role of polyamines in myocardial ischemia/reperfusion injury and their interactions with nitric oxide

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Abstract

Polyamines (putrescine, spermidine, and spermine) are present in all higher eukaryotic cells and are essential for cell growth, differentiation and apoptosis. Sharing common precursor with polyamines, nitric oxide (NO) is associated with myocardial ischemia/reperfusion injury by the generation of peroxynitrite. Although polyamines have been implicated in tissue ischemia injury, their metabolism and interactions with NO in myocardial ischemia/reperfusion injury have not been fully understood. In our experiment, when Langendorff perfused rat hearts were subjected to 40 min ischemia without reperfusion, both ornithine decarboxylase (ODC) and Spermidine/spermine N^1-acetyltransferase (SSAT) activities were up-regulated and putrescine accumulated. While after reperfusion, ODC activity decreased and SSAT activity increased, resulting in putrescine accumulation and decreased spermidine and spermine. Meanwhile NO content was increased. In addition, sodium nitroprusside (SNP, a NO donor) decreased ODC activity in cardiac tissue homogenate but increased SSAT activity in a dose-dependent manner. Pre-treatment of isolated heart with N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, an inhibitor of NO synthase) increased ODC activity. Exogenous spermine (1 mM) administration prior to ischemia prevented spermine decrease, reduced cardiac myocyte necrosis and apoptosis, and promoted the recovery of cardiac function after ischemia/reperfusion. These results suggest that acute heart ischemia activates myocardial polyamine stress response characterized by increased ODC and SSAT activities and accumulation of putrescine. Ischemia/reperfusion disturbs polyamine metabolism, and the loss of spermine might be associated with NO increase and thereby influences myocardial cell viability. Exogenous spermine may protect the hearts from myocardial ischemia/reperfusion injury.

Keywords: Polyamines; Ornithine decarboxylase (ODC); Spermidine/spermine N^1-acetyltransferase (SSAT); Nitric oxide (NO); Ischemia–reperfusion injury

1. Introduction

Myocardial ischemia results in ATP depletion and accumulation of toxic metabolites, whereas reperfusion leads to the production of reactive oxygen intermediates and calcium overload. The alterations in cellular metabolism and generation of toxic molecules contribute to myocardial ischemia/reperfusion injury (Marczin et al., 2003).

Polyamines (spermine, spermidine, and putrescine) are intrinsic constituents of all eukaryotic cells. They play essential

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roles in programmed cell death, cell growth and differentiation, membrane permeability transition of mitochondria and cytosolic Ca^{2+} homeostasis (Thomas and Thomas, 2001; Salvi and Toninello, 2004)

Ornithine decarboxylase (ODC) and spermidine/spermine N^1-acetyltransferase (SSAT) are the key enzymes in polyamine biosynthesis and degradation, respectively. ODC transforms ornithine into putrescine and the latter is metabolized to spermidine and spermine. SSAT acetylates both spermine and spermidine and forms acetylated polyamines. Acetylated polyamines are converted to putrescine by polyamine oxidase (PAO). In this process, hydrogen peroxide (H_2O_2) and aminopropionaldehyde are generated (Niiranen et al., 2002).

Recently, it is reported that polyamine system is involved in tissue ischemia injury, such as cerebral ischemia, kidney ischemia/reperfusion injury (Adibhatla et al., 2002; Wang et al., 2004). Polyamines have been implicated in the cardiovascular diseases such as cardiac hypertrophy (Bordallo et al., 2001; Schlüter et al., 2000). In our study, we showed that spermine 1–10 mM) induced a concentration-dependent increase in $[Ca^{2+}]_i$ in isolated ventricular myocytes from rats (Wang et al., 2003). But little is known about their metabolism and function in myocardial ischemia/reperfusion injury.

Nitric oxide (NO) is both a cytostatic and cytotoxic free radical. It reacts with superoxide to generate peroxynitrite anion ($ONOO^-$), a stronger oxidant that damages myocardium in myocardial ischemia/reperfusion injury (Schulz et al., 2004). Polyamines share common precursor with NO. Previous studies have suggested that polyamine and NO pathways are interregulated. For example, NO can inhibit the proliferation of vascular smooth muscle cells through inhibiting ODC activity in atherosclerosis (Wallace et al., 2002). NO and polyamine-dependent pathways are involved in signal transmission of free radical molecule in neutrophils (Mühling et al., 2006). Whether polyamine metabolism is influenced by NO in ischemia/reperfusion heart, however, is not clear. Therefore, in the present study we investigated the role of polyamines in myocardial ischemia/reperfusion injury and their interactions with NO.

2. Materials and methods

2.1. Materials

DL-[1-^{14}C]ornithine hydrochloride (56 mCi/mmol), [1-^{14}C] acetyl-coenzyme A (56 mCi/mmol) were purchased from Amersham (UK). Collagenase I was provided by Wako (Japan). Other major chemicals were obtained from Sigma (USA).

2.2. Isolated rat heart preparation and experimental protocol

Male Wistar rats, weighting 250±20 g, were purchased from the Animal Center of Harbin Medical University, and treated in accordance with the Guide for Care and Use of Laboratory Animals published by the China National Institutes of Health. Rats were anesthetized with 20% pentobarbital sodium (50 mg/kg) administered intraperitoneally (i.p.) and heparinized (500 U/kg). Rat hearts were excised rapidly and the aorta was cannulated. The heart mounted in Langendorff apparatus and perfused with standard Krebs–Henseleit buffer (KHB) containing (mM): NaCl 119, KCl 4.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, CaCl 2.5, $NaHCO_3$ 25, and glucose 11.0 at pH 7.4. The KHB was equilibrated with 95% O_2 and 5% CO_2 at 37 °C. The organ chamber temperature was maintained at 37 °C. The KHB was equilibrated with 95% O_2 and 5% CO_2 at 37 °C.

All rat hearts underwent 20 min of stabilization perfusion with KHB, and then subjected to the next treatment. In protocol A, only 40 min global ischemia (I_{40}) was conducted. Protocol B was composed of 40 min of global ischemia followed by 40 min, 2 h, or 3 h of reperfusion ($I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$). In protocol C, during stabilization perfusion the hearts were continuously given spermine (1 mM) and then treated in the same way as in ischemia/reperfusion (IR) group (spermine). Control hearts were only perfused with KHB.

At the 5th min after the onset of reperfusion, coronary effluent was collected for LDH assay. At the end of reperfusion, the left ventricle was quickly removed and immediately frozen in liquid N_2 and stored at −80 °C for different assays.

In some isolated perfused rat hearts, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (0.2, 20, 80 μM) was used to pre-treat the hearts before the ischemia/reperfusion protocols. In addition, rat cardiomyocytes prepared from control heart were incubated in some experiments with sodium nitroprusside (SNP) (0.1, 10, 100 μM).

2.3. Cardiac function measurement

A water-filled latex balloon was inserted into the left ventricle through left atrium and adjusted to a left ventricular end-diastolic pressure of 5 to 7 mmHg during initial equilibrium. The distal end of the catheter was connected to a PowerLab 8/SP TM data acquisition system (Chart 5.0 software, AD Instruments Inc., MA, Australia) via a pressure transducer for continuous recording of cardiac function. Cardiac function was evaluated based on left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), positive and negative maximum rate of left ventricular pressure development ($+dp/dt$ and $-dp/dt$).

2.4. Determination of polyamines

Polyamine contents in cardiac tissues were measured by high-performance liquid chromatography (LC-6A, Shimadzu, Japan). Briefly, heart samples were homogenized with 0.3 M ice-cold perchloric acid and centrifuged at 4000 ×g for 10 min. The supernatants were added to 10 nM internal standard (1,6-hexanediamine), derivatization with benzoyl chloride, and extracted with chloroform. The derivatives were separated on Hypersil ODS C_{18} column (250 mm×4.6 mm, 5 μm, Waters, USA). The column eluate was monitored by ultraviolet detector at 229 nm (SPD-66A, Shimadzu, Japan). The polyamine concentration was expressed in nanomoles of amines per gram of wet tissue.

2.5. Measurement of ODC and SSAT activities

Frozen heart tissues were homogenized in buffer (15 mM Tris–HCl, 2.5 mM DTT, 40 μM pyridoxal-5-phosphate, 100 μM EDTA, pH 7.5) at 4 °C. The aliquots of the homogenate were centrifuged at 30,000 ×*g* for 20 min. The supernatants were used to assay ODC and SSAT activities respectively as described by Fogel-Petrovic et al. (1996).

ODC activity was measured by determining the amount of $^{14}CO_2$ released from 0.5 μCi of L-[1-^{14}C]ornithine at 37 °C during 1 h incubation. The mixture was incubated for 60 min at 37 °C. The assay was stopped by the addition of 0.8 ml of 1 mM citric acid and the incubation was maintained for another 20 min. The $^{14}CO_2$ evolved was collected on P81 Watman discs pre-spotted with 20 μl of 20 μM hydroxylamine hydrochloride. The radioactivity was measured with a liquid scintillation counter (LS-6500 Beckman, USA). ODC activity was expressed as pmol/mg protein per hour.

In measurement of SSAT activity, the resultant supernatant of 86 μl was added into a reaction mixture containing 100 mM Tris–HCl (pH 7.8), 3 mM spermidine (pH 7.0), 1 mM dithiothreitol, and 50 μCi [^{14}C] acetyl-coenzyme A. This mixture was then incubated at 37 °C for 10 min. The assay was stopped by the addition of 20 μM hydroxylamine hydrochloride, and centrifuged at 22,000 ×*g* for 5 min. The 50 μl supernatant was spotted on P81 Watman discs. The radioactivity was

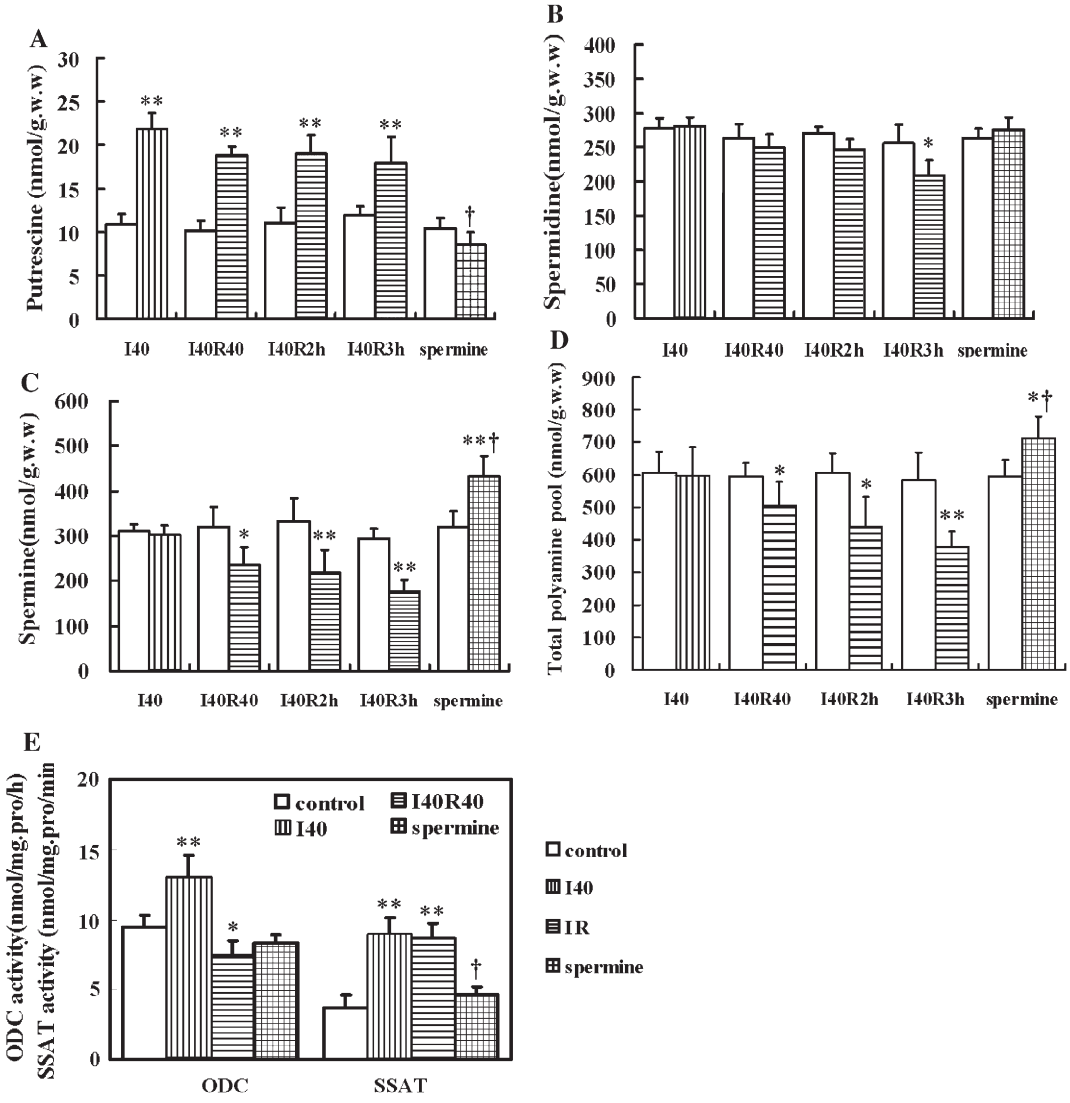

Fig. 1. Changes of polyamine metabolism. Changes in the myocardial contents of putrescine (A), spermidine (B), spermine (C), and total polyamine (D) from isolated hearts subjected to 40 min of ischemia (I_{40}), 40 min global ischemia followed by 40 min, 2 h, 3 h reperfusion ($I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$) and spermine (1 mM) treatment during stabilization perfusion. Putrescine content was significantly increased in I_{40}, $I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$ group; spermidine content was slightly decreased in $I_{40}R_{3h}$ group; both spermine and total polyamine levels were decreased in $I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$ group but increased in spermine pre-treatment group (*$P<0.05$ and **$P<0.01$ vs. control). Compared with $I_{40}R_{40}$ group, putrescine content was decreased, spermidine and total polyamine increased in spermine-treatment group ($^{\dagger}P<0.01$ vs. $I_{40}R_{40}$ group). Changes in ODC and SSAT activities were shown in E. Compared with control group, the activities of both ODC and SSAT were significantly increased in I_{40} group (**$P<0.01$). After reperfusion, ODC activity decreased, but SSAT activity still remained in high levels (*$P<0.05$ and **$P<0.01$). Pre-treated with spermine before ischemia, SSAT activity was lower than that in $I_{40}R_{40}$ group ($^{\dagger}P<0.01$). Data are mean±S.E.M., $n=8$ per group.

measured with a liquid scintillation counter. Enzyme activity is expressed as pmol [^{14}C] acetyl-spermidine formed per mg protein per min.

2.6. NO measurement

The NO production in cardiac tissue was determined by measuring the concentration of nitrite from nitrates, a stable metabolite of NO, with a modified Griess reaction method. Briefly, heart tissue was homogenized and centrifuged at 10,000 ×*g* for 10 min. Supernatant was taken and mixed with equal volume of Griess regent (Nanjing Jiancheng Bioengineering Institute, NJBI, China). After incubation for 15 min at room temperature, the concentration of the resultant chromophore was determined at 550 nm using a spectrophotometer (Beckman, US-640 UV, USA). The nitrite concentrations were calculated with reference to a standard curve constructed using sodium nitrite in sodium phosphate buffer.

2.7. Determination of lactate dehydrogenase and reactive oxygen species

Lactate dehydrogenase (LDH) in coronary effluent and reactive oxygen species in cardiac tissue were determined colorimetrically using a spectrophotometer with the associated detection kits (NJBI, China). According to Fenton-type reaction, the amount of OH^- generated is in proportion with H_2O_2. Here reactive oxygen species was measured as OH^- generation according to the kit manufacturer's protocol. Briefly, working solution containing electron receptor was added to the supernatants of cardiac homogenate and the mixture was incubated for 1 min at 37 °C. The assay was stopped promptly by the addition of Gress agent. After 20 min incubation at room temperature, the red resultants were generated. As an index of OH^- concentration, the resultant chromophore was determined colorimetrically at 550 nm. Data were expressed as units of activity per liter coronary effluent and activity unit (U) per mg protein respectively.

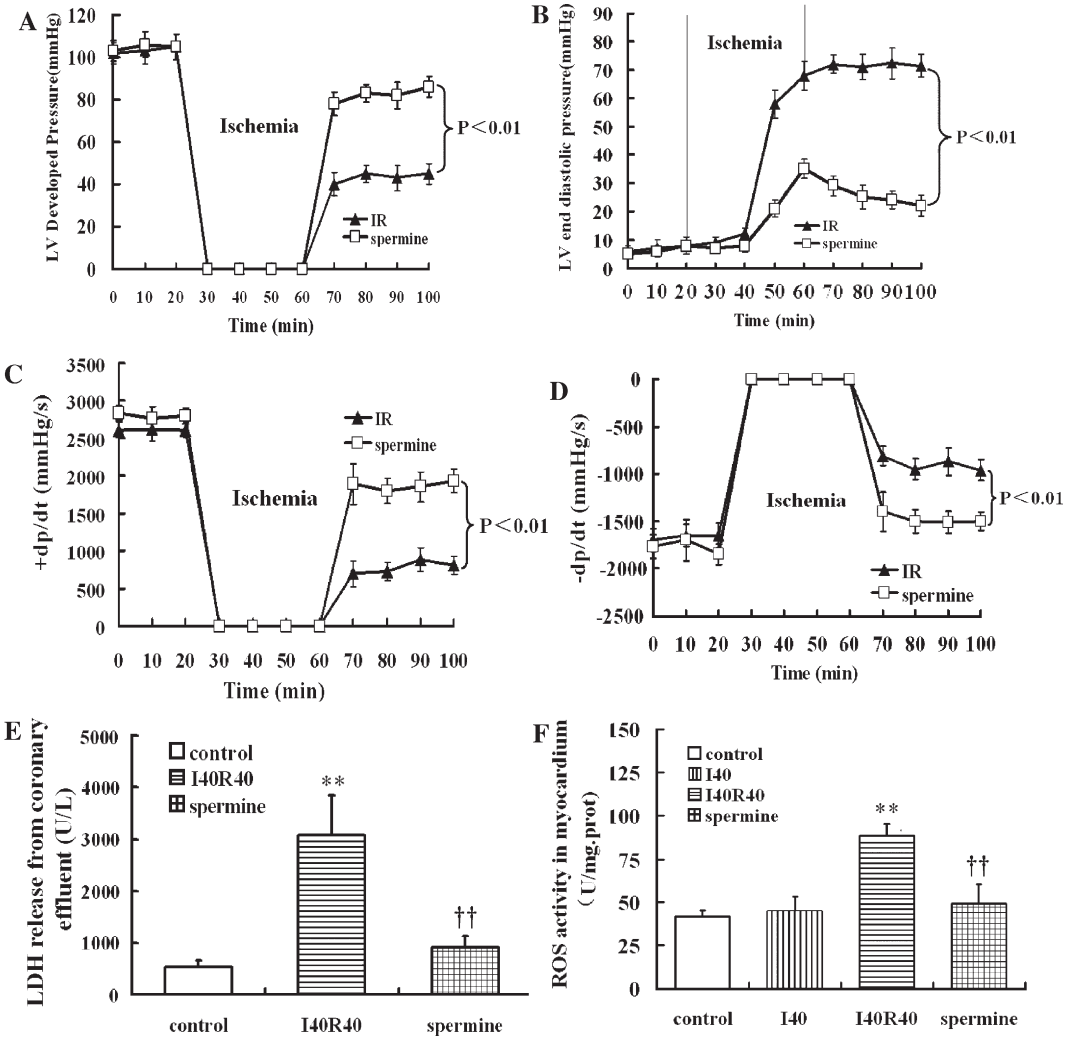

Fig. 2. Hemodynamic parameters of hearts were measured through the experiment, including left ventricular developed pressure (LVDP) (A), left ventricular end-diastolic pressure (LVEDP) (B), positive and negative maximum rate of LV pressure development (+d*p*/d*t*, −d*p*/d*t*) (C, D). Lactate dehydrogenase (LDH) released from coronary effluent (E) and reactive oxygen species (ROS) activity in myocardium (F) in different groups. The isolated rat hearts were subjected to 40 min global ischemia (I_{40}) and 40 min global ischemia followed by 40 min reperfusion ($I_{40}R_{40}$). Spermine (1 mM) was administered during 20 min stabilization period. Data are mean±S.E.M., $n=8$ per group. **$P<0.01$ vs. control; $^{\dagger\dagger}P<0.01$ vs. $I_{40}R_{40}$ group.

2.8. Protein determination

Protein content in various assays was determined with the method of Lowry (Lowry et al., 1951).

2.9. Transmission electron microscopy

Heart tissue (2–3 mm) was immersed immediately in fixative (3.0% glutaraldehyde buffered in 0.1 M sodium cacodylate, pH 7.2). Following 2–3 days storage, specimens were rinsed in PBS, postfixed in cacodylate-buffered 1% osmium tetroxide, dehydrated in an ethanol series, embedded in Polybed 812. Ultra-thin (90 nm) sections were made and examined in JEM-2000EX, Japan, electron microscope.

2.10. In situ apoptosis assay

The apoptotic myocytes were detected by TdT mediated dUTP nick end-labeling (TUNEL) assay using a Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instructions. Briefly, hearts were sectioned and embedded in paraffin, and then were incubated with TUNEL reaction mixture containing TdT and fluorescein-dUTP after permeability treatment. The TUNEL signal was then detected by an anti-fluorescein antibody conjugated with alkaline phosphatase, a reporter enzyme, which catalytically generates a coloured product. Three slides from each block were evaluated for percentage of apoptotic cells. Four slide fields were randomly examined with magnification 200×. One hundred cells were counted in each field.

2.11. Experimental protocol with isolated ventricular myocytes and $[Ca^{2+}]_i$ determination

The hearts were removed and perfused on the Langendorff apparatus with standard Tyrodes solution at 37 °C for 5 min, then switched to a Ca^{2+}-free Tyrode solution for 6 min and a Ca^{2+}-free Tyrode solution containing 0.03% collagenase for 30 min. The cells released from the left ventricle were settled for 1 h in KB solution (in mM: KOH 85, KCl 30, KH_2PO_4 30, EGTA 0.5, HEPES 10, L-glutamic acid 50, and taurine 20, pH 7.4). These cells were firstly loaded with Fluo-3/AM, then treated with anoxia/reoxgenation ($n=8$) or with spermine treatment ($n=6$).

In the former treatment, the cells were first superfused with oxygen-saturated Tyrode solution for 16 min, then with an anoxic glucose-free and nitrogen-saturated Tyrode solution (pH at 6.8) for 32 min. The last step was the same as the first one for 8 min. In the latter, 1 mM spermine replaced Tyrode solution at the 8–16 min stage while other steps remained unchanged. Fluorescence intensity of Fluo-3 in cardiomyocytes was recorded to reflect the changes of $[Ca^{2+}]_i$ using a laser scanning confocal microscope (MRC-1024 MP, BIO-RAD, American) with 488 nm for excitation from an argon ion laser and 530 nm for emission.

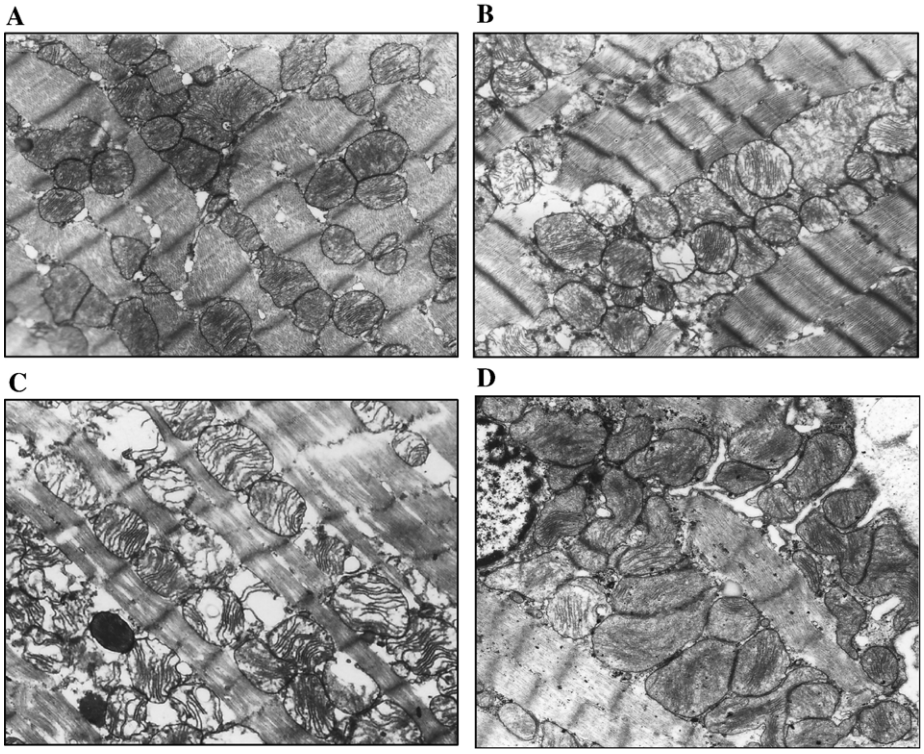

Fig. 3. Transmission electron microscope observation: control group (A), ischemia group (B), $I_{40}R_{40}$ group (C), spermine-treatment group (D) (14,000×). In control group, mitochondrial alignment and myofibrillary banding appeared normal. In $I_{40}R_{40}$ group, mitochondrial swelling and damage with breakage and deterioration of cristae resulted in vaculation. Myofibrillary degeneration and Z-band disalignment occurred. In ischemia group, a few mitochondrials showed swelling and vaculation, but myofibrillary alignment was observed. There were no significant changes of mitochondria and myofibrillary in spermine-treatment group.

2.12. Statistical analysis

All the data are presented as mean ± standard error (S.E.M.). Either one-way or two-way ANOVA was used to compare differences among groups where appropriate. In some cases, Bonferroni test was used. Statistical comparison was performed by paired or unpaired Student's *t*-test. Significance level was set up at $P<0.05$. The linear regression analysis was used to determine the correlation between different variables.

3. Results

3.1. Polyamine contents

Compared with control group, the content of putrescine was significantly increased ($P<0.01$), but spermidine and spermine levels were unchanged in hearts only exposed to 40 min ischemia. In hearts subjected to 40 min ischemia and different periods of reperfusion (40 min, 2 h, and 3 h), putrescine level was still high ($P<0.01$), but with reperfusion time prolonged, spermidine, spermine and total polyamine were lower ($P<0.05$ or $P<0.01$) with the exception of spermidine in $I_{40}R_{40}$ and $I_{40}R_{2h}$ groups. Exogenous spermine pre-treatment prior to ischemia replenished polyamine pool of ischemia/reperfusion heart. Spermine and total polyamine in spermine group were higher than those in control and $I_{40}R_{40}$ group ($P<0.01$) (Fig. 1A–D).

3.2. Activities of ODC and SSAT

At the end of the 40 min ischemia, the activities of both ODC and SSAT were significantly increased by 37% and 122% respectively, compared with control group ($P<0.01$). After reperfusion, ODC activity decreased, but SSAT activity stayed the same. ODC was lower than that in control group ($P<0.05$), but SSAT activity was higher ($P<0.01$). When rat hearts were pre-treated with spermine before ischemia, SSAT activity was lower than that in $I_{40}R_{40}$ group ($P<0.01$) (Fig. 1E). The results suggested that heart ischemia stress induced ODC and SSAT

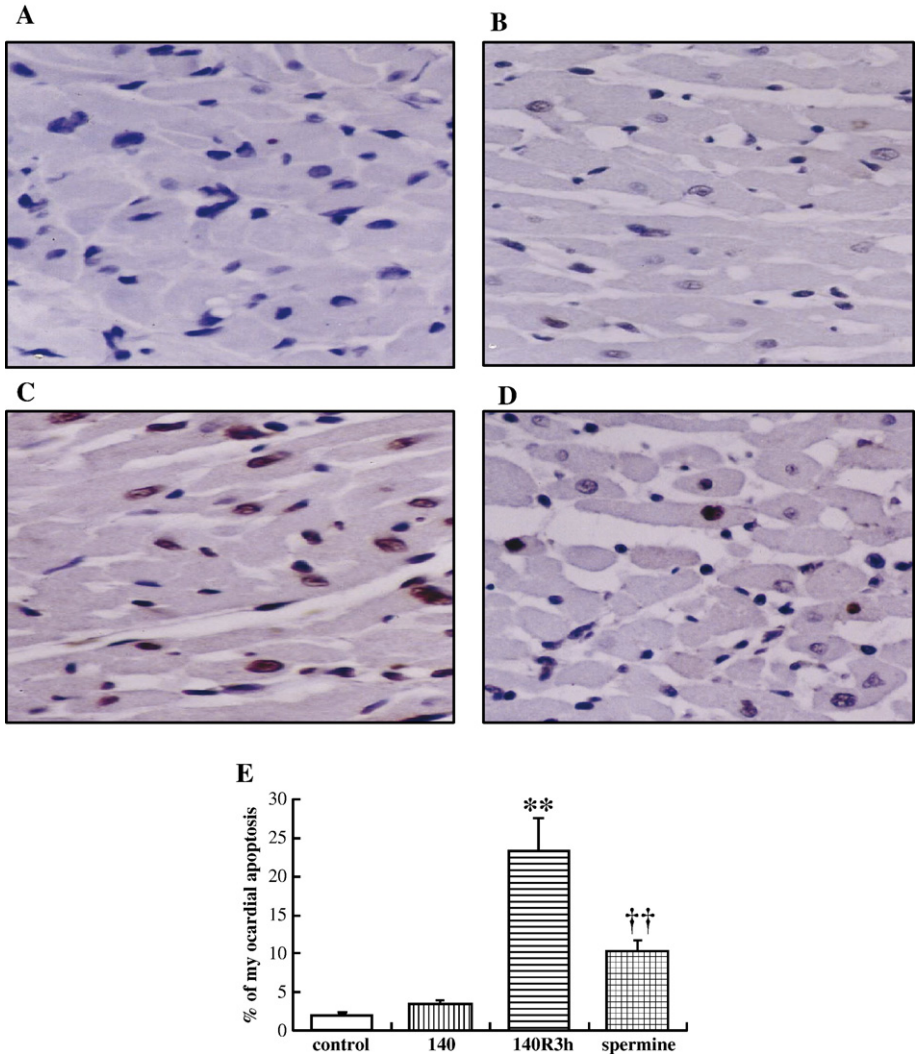

Fig. 4. Cardiac myocyte apoptosis was determined by the TUNEL assay. A, control group; B, 40 min of ischemia group (I_{40}); C, 40 min of ischemia and 3 h of reperfusion group ($I_{40}R_{3h}$); D, spermine-treatment group (400fsmagnification). TUNEL-positive nuclei stain brown and TUNEL-negative nuclei stain blue. E, apoptotic index expressed as percentage of nuclei staining positive for TUNEL in control, ischemia, IR and spermine-treatment group. $^{**}P<0.01$ vs. control. $^{††}P<0.01$ vs. $I_{40}R_{3h}$ group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activities, and reperfusion of ischemia heart down-regulated polyamine synthesis metabolism and up-regulated polyamine degradation metabolism. Although exogenous spermine restored intracellular polyamine pool, it did not depend on the alterations of ODC and SSAT activities.

3.3. Cardiac function changes

In our research, we found that during 20 min of stabilization perfusion with spermine (1 mM), heart rate remained unchanged compared with heart stabilized with KH buffer for 20 min. Spermine treatment significantly improved the recovery of cardiac function during reperfusion after ischemia. Post-ischemia recovery values of LVDP (expressed as percentage of pre-ischemic function) in spermine-treatment group (82.2 ±0.04%) were much higher than those in ischemia/reperfusion (IR) group (42.3±0.05%) ($P<0.01$) (Fig. 2A). And $+dp/dt$ and $-dp/dt$ were also remarkably improved in spermine-treatment group (63.9%±0.03 and 83.4%±0.06), compared with IR group (29.7%±0.02 and 49.9%±0.04) ($P<0.01$) (Fig. 2C and D). Spermine supplementation also greatly restored LVEDP during reperfusion ($P<0.01$ vs. IR) (Fig. 2B).

3.4. Measurement of LDH and reactive oxygen species levels

In IR group, LDH released was 5.3 times that of control ($P<0.01$), and reactive oxygen species increased by 52% compared with control ($P<0.01$). Exogenous spermine reduced the levels of LDH and reactive oxygen species by 70% and 45% in comparison with those of $I_{40}R_{40}$ group, respectively (Fig. 2E and F). The data indicate spermine attenuated heart oxidant injury caused by ischemia/reperfusion.

3.5. Cardiac ultrastructural characterization

Electron microscopical examination showed that in ischemia group only, mitochondria swelling and vaculation were observed occasionally (Fig. 3B). In IR group, the alterations in mitochondria and myofibrilla are prominent, including mitochondrion swelling and vaculation, mitochondrial cristae disintegration and disappearance; myofibrilla degeneration and Z-banding misalignments (Fig. 3C). But the changes did not appear in control group (Fig. 3A). In spermine-treatment group, relative normality of mitochondrial arrays and ultrastructure of organelles was observed (Fig. 3D).

3.6. Myocardial apoptosis

Heart tissue from control and 40 min of ischemia group exhibited very low levels of staining for TUNEL (Fig. 4A and B). In contrast, a significant number of TUNEL-positive myocytes were detected in myocardial tissue from the hearts subjected to 40 min ischemia and 3 h reperfusion (Fig. 4C).

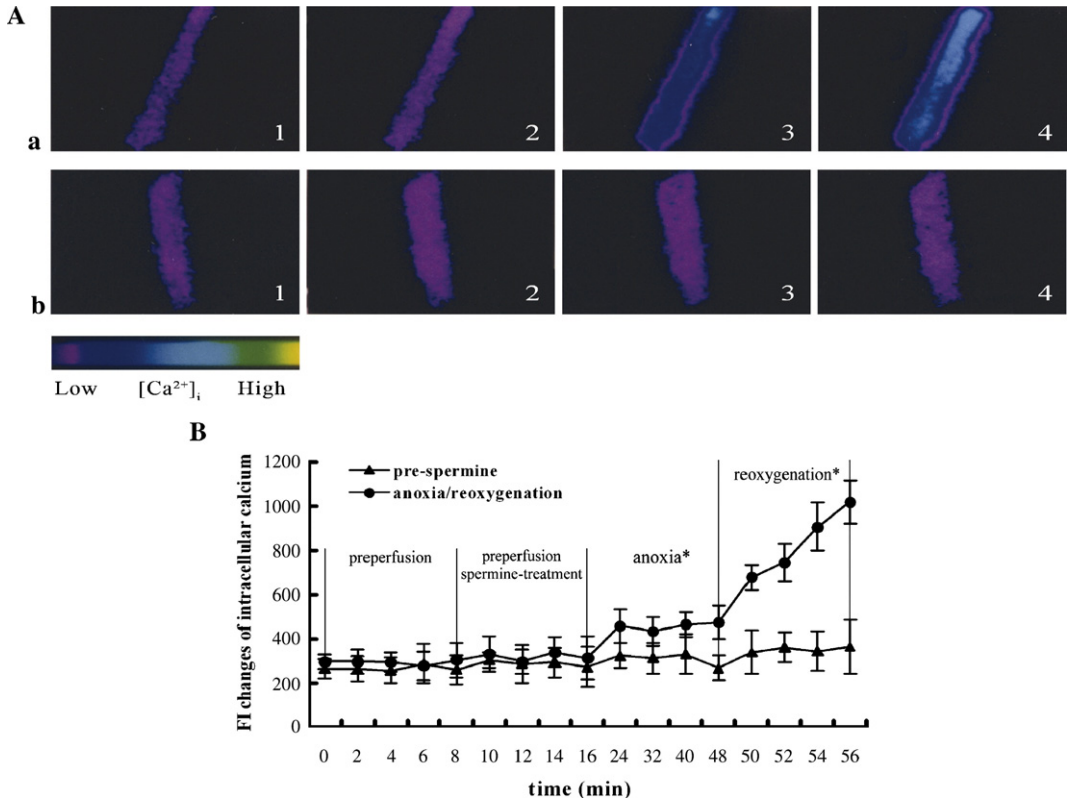

Fig. 5. Effect of spermine on $[Ca^{2+}]_i$ in rat ventricular myocytes. A: Fluorescent image in cell after anoxia/reoxygenation (a) and pre-spermine (b) treatment. Images 1, 2, 3, and 4 represent pre-perfusion (a) or spermine-treatment (b), anoxia and reoxygenation treatment respectively. B: The changes of fluorescent intensities in intracellular Ca^{2+} were recorded continuously with laser scanning confocal microscope in different treatments. Intracellular Ca^{2+} was recorded for 56 min in all, except for 32 min in anoxia period. Each period was 8 min. *$P<0.01$ vs. pre-perfusion period.

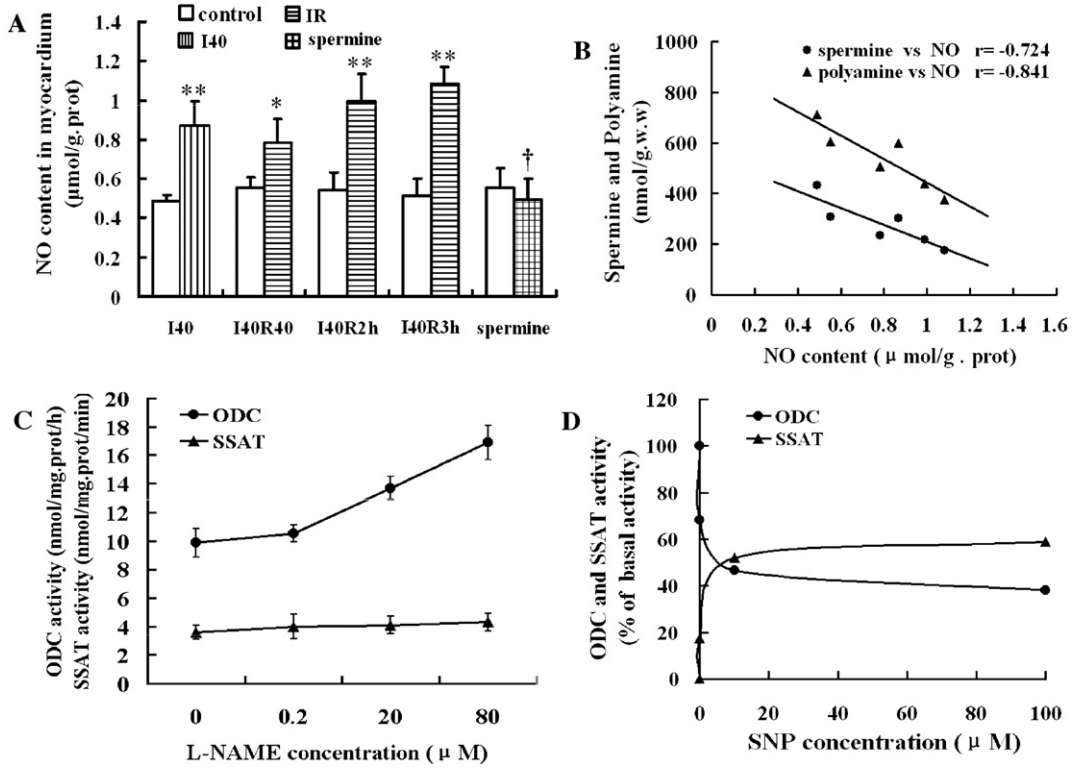

Fig. 6. (A) NO contents in cardiac tissue in different groups. Isolated hearts were subjected to 40 min of ischemia (I_{40}), 40 min of ischemia followed by 40 min, 2 h, 3 h of reperfusion ($I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$) and spermine (1 mM) pre-treatment during stabilization perfusion (spermine). Data are mean±S.E.M., $n=8$ per group. $^{**}P<0.01$ vs. control group; $^{\dagger}P<0.01$ vs. $I_{40}R_{40}$ group. (B) Negative correlation was showed between spermine and NO, and polyamines and NO in all experiment groups including control, ischemia, IR ($I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$) and spermine-treatment group. R value was displayed on the plot. (C) Dose–response curve of ODC and SSAT induction produced by L-NAME (an inhibitor of NOS). L-NAME was used to treat some isolated rat hearts prior to the IR injury. (D) Dose–response curve of ODC and SSAT induction produced by SNP (a NO donor). SNP was incubated with rat cardiomyocyte cytosol prepared from control heart.

Most notably, the number of TUNEL-positive myocytes in the spermine-treatment group significantly decreased (Fig. 4D). Quantitative measurement depicted a 55% reduction in TUNEL-positive myocytes in spermine-treatment group compared with $I_{40}R_{3h}$ group ($P<0.01$, $n=6$).

3.7. Intacellular Ca^{2+} concentration

Fig. 5 represents confocal images of cardiomyocytes loaded with calcium indicator Fluo-3/Am. Anoxia and anoxia/reoxygenation induced a marked increase in the fluorescent intensity (FI) for $[Ca^{2+}]_i$, reaching 497±43 and 878±100 respectively, higher than that in pre-perfusion periods (287±18) ($P<0.01$). Spermine (1 mM) pre-treatment abolished the increase in $[Ca^{2+}]_i$. These results indicated that exogenous spermine of low concentration significantly prevented calcium overload caused by anoxia/reoxygenation in isolated rat myocytes.

3.8. NO concentration determination and its interactions with polyamine pathway

In rat hearts subjected to 40 min of ischemia, NO levels were elevated from 0.49±0.06 (control) to 0.87±0.12 μmol/g prot ($P<0.01$ vs. control), and remained at high levels (0.78±0.10 μmol/g prot, 0.99±0.21 μmol/g prot and 1.08±0.14 μmol/g prot) after 40 min, 2 h, 3 h of reperfusion ($P<0.01$ vs. control). Most notably, NO contents were decreased by 38% in spermine-treatment group compared with $I_{40}R_{40}$ group ($P<0.01$) (Fig. 6A).

Regression analysis was made between the levels of NO and spermine, and the levels of NO and total polyamine in control, ischemia, IR ($I_{40}R_{40}$, $I_{40}R_{2h}$, $I_{40}R_{3h}$) and spermine-treatment hearts. An excellent negative correlation relationship was shown. The correlation coefficients (r) were −0.724 and −0.841, respectively ($P<0.01$) (Fig. 6B).

The effect of NOS inhibitor (L-NAME) and NO donor (SNP) on ODC and SSAT activities was observed. In isolated perfused rat hearts, L-NAME (0.2, 20, 80 μM) increased ODC activity in a dose-dependent manner, when L-NAME pre-treated isolated perfused rat hearts prior to the ischemia/reperfusion injury (Fig. 6C). SNP (0.1, 10, 100 μM) inhibited ODC activity, and triggered SSAT activity in the same manner, when cardiomyocyte cytosol prepared from control heart was incubated with SNP (Fig. 6D).

4. Discussion

Our results demonstrate that when rat hearts suffered from 40 min of ischemia, activities of ODC and SSAT, the key enzymes in polyamine synthesis and degradation, were both induced. Putrescine level was increased, but no significant changes were found in spermidine and spermine levels. Similar findings were observed in cerebral ischemia model (Coert et al.,

2000). Intracellular polyamines in the brain were very sensitive to various pathological states. Cerebral ischemia and traumatic brain injury disturbed polyamine metabolism and resulted in increased ODC and SSAT activities and putrescine accumulation. Activation of ODC and SSAT and the subsequently produced putrescine increased, dependent on magnitude of pathological stimuli. These processes were termed as polyamine stress response, and considered to be integral components of cellular stress program. ODC activity and tissue polyamine concentration served as markers of polyamine stress response (Hayashi et al., 2004). The polyamine metabolism changes as observed in the heart in the present study might be reminiscent of polyamine stress response in brain. These pathological changes may be implicated in ischemia-related cell injury in heart. However, the exact role of increase of ODC and SSAT activities has not been examined in this research, which merits future studies in this regard.

Free radical production and calcium overload are two major pathophysiological events implicated in the development of myocardial ischemia/reperfusion injury (Carcia-Dorado, 2004). Our data revealed that myocardial ischemia/reperfusion injury occurred when rat hearts were subjected to 40 min of ischemia followed by 40 min of reperfusion. Reperfusion decreased ODC activity and induced SSAT activity. As a result, spermidine and spermine levels were decreased but putrescine level increased, indicating that polyamine synthesis pathway was down-regulated and polyamine degradation pathway was up-regulated. ODC is the first rate-limiting enzyme in polyamine synthesis, and it catalyzes the decarboxylation of ornithine to produce putrescine. ODC is sensitive to direct oxidation (Dypbukt et al., 1994) and is *S*-nitrosylated on its Cys_{360} (a critical part of active site) by NO (Hillary and Pegg, 2003), which may contribute to the inactivation of the enzyme and to its proteolytic cleavage in this reperfusion stage. In the previous study, difluoromethylornithine (DFMO), a specific irreversible inhibitor of ODC, can deplete putrescine and spermidine and induce growth inhibition and apoptosis in variety of cell types (normal and malignant) (Marton and Pegg, 1995). We inferred that the down-regulation of ODC activities may imply in heart ischemia/reperfusion injury herein. But the exact role of ODC activity decrease in reperfusion heart has not been examined in this research.

SSAT is the key enzyme in polyamine degradation that acetylates the primary amines of aminopropyl group of spermidine and spermine. SSAT can be induced by various toxic agents and other stress stimuli (Casero and Pegg, 1993). According to several studies, toxic metabolic products are generated from the acetylation and subsequent oxidation of spermine and spermidine, and increase the extent of cell death (Schipper et al., 2000). Rao et al. reported that conversion of spermine and spermidine via SSAT/PAO resulted in the decrease of spermine and production of H_2O_2 and aldehyde, which might induce apoptosis after ischemia/reperfusion in the brain (Rao et al., 2000). It is also reported that SSAT up-regulation accompanied by polyamine pool decrease may mediate the majority of the oxidative stress in kidney ischemia/reperfusion injury (Zahedi et al., 2003). Accumulated evidence shows that polyamines play beneficial roles, such as regulating Ca^{2+} homeostasis (Nilsson et al., 2002), scavenging free radicals and reducing lipid peroxidation (Marzabadi and Løvaas, 1996; Bellé et al., 2004). In this regard, the up-regulation of SSAT activity could lead to the formation of H_2O_2 and toxic aldehydes in the ischemia/reperfusion heart and spermine depletion could decrease antioxidant effect and Ca^{2+} buffering capacity in cell. So, we proposed that the up-regulation of SSAT, together with spermine depletion during reperfusion may involve in the pathophysiology in heart injury.

It is known that the effects of polyamines are mostly related to the number of positive charges they bear. Spermine bears more positive charges than other polyamines (Das and Misra, 2004). According to Hegardt, exogenous spermine prevented glucocorticoid mediated apoptosis by preventing cytochrome *c* release and attenuating the mitochondrial membrane potential ($\Delta\psi_m$) loss (Hegardt et al., 2003). The protective effects of spermine may be due to DNA stabilization (Bash et al., 1997), protection of DNA from oxidative stress (Muscari et al., 1995), or inhibition of endonucleases (Ribeiro and Carson, 1993). However, the opposite was found, where the treatment of ICE-6 cells with difluoromethlornithine (DFMO) depleted polyamine pool, and delayed the onset of apoptosis by tumor necrosis factor-α (Pfeffer et al., 2001). One possible explanation for this controversy is that the response of the cell depends on multiple signals for survival or death, and one signal can produce either response, depending on the environment (Wallace et al., 2003). In our study, exogenous spermine (1 mM) given before ischemia not only restored intracellular polyamine pool, but also reduced cardiomyocyte necrosis and apoptosis. It improved the recovery of cardiac contraction function and decreased ultrastructure injury through stabilizing cell membrane (decrease LDH), scavenging free radicals (decreased reactive oxygen species and NO content) and preventing increase in the intracellular free calcium. Hence, exogenous spermine may be considered as a possible approach to protecting the heart from ischemia/reperfusion injury.

NO is an important intracellular signal molecule, and its role in myocardial ischemia/reperfusion injury has gained considerable attention. NO may protect hearts against reperfusion injury by augmenting coronary dilation, reducing leukocyte and platelet interactions with vascular endothelium. On the other hand, increased myocardial injury during reperfusion after ischemia may occur due to the formation of cytotoxic free radical species if NO combines with superoxide to generate $ONOO^-$. The conflicting results of NO effects on the myocardium may arise in the different models (Schulz et al., 2004). L-arginine is a common precursor of polyamines and NO, and polyamine and NO pathways are reported to interact with each other (Wallace et al., 2002; Mühling et al., 2006). Our results indicated that ischemia/reperfusion caused decrease of polyamine pool and increase of NO contents. Exogenous spermine administration replenished polyamine pool and decreased NO contents in the isolate perfusion rat model. The levels of spermine and total polyamine were negatively correlated with NO levels in all experimental groups. In addition, we found that ODC activity was elevated by L-NAME pre-treatment in ischemia/reperfusion

injury hearts and ODC activity was reduced, but SSAT activity was induced by SNP in a dose-dependent manner in cardiac tissue homogenate. Taken together, our results suggested that polyamine metabolism pathway could be affected by NO in this ischemia/reperfusion mode, and down-regulation of polyamine pathway may be associated with the increase of NO levels. Our results are in line with the similar findings by other groups. Satriano (2004) reported that L-arginine/NO and L-arginine/polyamine pathways were regulated in inflammatory models. Singh et al. found that N^{ω}-hydroxy-L-arginine, an intermediate in the NO synthetic pathway, significantly reduced intracellular polyamines, activated caspase-3 and induced apoptosis in human breast cancer cell line. Meanwhile, they pointed out that decreased polyamine might be responsible for apoptosis (Singh et al., 2001). Hu et al. found that spermine, spermidine and putrescine all inhibited NOS from conversing L-arginine to NO in cytosolic preparations of rat cerebellum and cerebellar granule neurons, with the following rank order of potency: spermine > spermidine > putrescine (Hu et al., 1994).

In conclusion, our results suggest that both cardiac ischemia and ischemia/reperfusion cause alteration in polyamine metabolism. Acute cardiac ischemia may induce polyamine stress response, characterized by increased ODC and SSAT activity and accumulated putrescine. Ischemia/reperfusion leads to depletion of myocardial polyamine pool, which may be associated with the increase in the NO levels, and the loss of spermine might get involved in the cardio-injury of reperfusion. Exogenous spermine restores intracellular polyamine pool and confers cardio-productive effect of ischemia/reperfusion.

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