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**Research Paper**

# Insulin protects isolated hearts from ischemia/reperfusion injury: cross-talk between PI3-K/Akt and JNKs

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**Abstract:** Our previous results have demonstrated that insulin reduces myocardial ischemia/reperfusion (MI/R) injury and increases the postischemic myocardial functions via activating the cellular survival signaling, i.e., phosphatidylinositol 3-kinase (PI3-K)-Akt-endothelial nitric oxide synthase (eNOS)-nitric oxide (NO) cascade. However, it remains largely controversial whether c-Jun $NH_2$-terminal kinase (JNK) is involved in the effects of insulin on MI/R injury. Therefore, the aims of the present study were to investigate the role of JNK, especially the cross-talk between JNK and previously expatiated Akt signaling, in the protective effect of insulin on I/R myocardium. Isolated hearts from adult Sprague-Dawley rats were subjected to 30 min of regional ischemia and followed by 2 or 4 h of reperfusion ($n$=6). The hearts were pretreated with PI3-K inhibitor LY294002, or phosphorylated-JNK inhibitor SP600125, respectively, then perfused retrogradely with insulin, and the mechanical functions of hearts, including the heart rate (HR), left ventricular developed pressure (LVDP) and instantaneous first derivation of left ventricular pressure ($\pm LVdp/dt_{max}$) were measured. At the end of reperfusion, the infarct size (IS) and apoptotic index (AI) were examined. MI/R caused significant cardiac dysfunction and myocardial apoptosis (strong TUNEL-positive staining). Compared with the control group, insulin treatment in MI/R rats exerted protective effects as evidenced by reduced myocardial IS [(28.9±2.0)% *vs* (45.0±4.0)%, $n$=6, $P$<0.01], inhibited cardiomyocyte apoptosis [decreased AI: (16.0±0.7)% *vs* (27.6±1.3)%, $n$=6, $P$<0.01] and improved recovery of cardiac systolic/diastolic function (including LVDP and $\pm LVdp/dt_{max}$) at the end of reperfusion. Moreover, insulin resulted in 1.7-fold and 1.5-fold increases in Akt and JNK phosphorylation in I/R myocardium, respectively ($n$=6, $P$<0.05). Inhibition of Akt activation with LY294002 abolished, and inhibition of JNK activation with SP600125 enhanced the cardioprotection by insulin, respectively. And the abolishment by LY294002 could be partly converted by SP600125 pretreatment. In addition, SP600125 also decreased the Akt phosphorylation ($n$=6, $P$<0.05). These results demonstrate that insulin simultaneously activates both Akt and JNK, and the latter further increases the phosphorylation of Akt which attenuates MI/R injury and improves heart function; this cross-talk between Akt and JNK in the insulin signaling is involved in insulin-induced cardioprotective effect.

**Key words:** ischemia/reperfusion injury; insulin; apoptosis; Akt; JNK; cross-talk

# 胰岛素保护缺血 / 再灌注心脏：PI3-K/Akt 和 JNKs 信号通路间的交互作用

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**摘 要：**我们前期研究表明胰岛素可激活细胞内信号转导机制如磷脂酰肌醇 3- 激酶 - 蛋白激酶 B- 内皮型一氧化氮合酶 - 一氧化氮(PI3-K-Akt-eNOS-NO)信号通路，减轻心肌缺血 / 再灌注(ischemia/reperfusion, I/R)损伤，改善缺血后心肌功能恢复。然而 c-Jun 氨基末端激酶(c-Jun $NH_2$-terminal kinase, JNK)信号通路在胰岛素保护 I/R 心肌中的作用尚不清楚，本研究旨在探讨 JNK

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信号通路在胰岛素保护 I/R 心肌中的作用及其与 PI3-K/Akt 信号通路间的相互关系。离体 Sprague-Dawley 大鼠心脏缺血 30 min 后施行 2 h 或 4 h 的再灌注，缺血前用 LY294002 (15 mmol/L)和 SP600125 (10 mmol/L)灌注 15 min，分别阻断 PI3-K/Akt 和磷酸化 JNK (phosphorylated-JNK, p-JNK)活化，观测心脏功能、心肌梗死、细胞凋亡和蛋白磷酸化水平。与对照组相比，胰岛素再灌注 2 h 后，心率、左心室发展压和左心室收缩 / 舒张最大速率均明显增加，梗死面积减少约 16.1% [(28.9±2.0)% *vs* (45.0±4.0)%, $n$=6, $P$<0.01]，细胞凋亡指数从(27.6±1.3)% 减少到(16.0±0.7)% ($n$=6, $P$<0.01)，Akt 的活性增加 1.7 倍($n$=6, $P$<0.05)，同时 JNK 活性增加 1.5 倍($n$=6, $P$<0.05)。用 LY294002 处理后，胰岛素对 I/R 心肌的保护作用消失；而用 SP600125 处理可增强胰岛素的保护作用，且可部分逆转 LY294002 的抑制作用。进一步观察发现 SP600125 减弱了 Akt 的磷酸化($n$=6, $P$<0.05)。上述结果表明，在 I/R 心肌中，胰岛素可同时激活 PI3-K/Akt 及 JNK 信号通路，且通过后者进一步增加 Akt 活化，从而减轻 I/R 损伤，改善心肌功能。这种 PI3-K/Akt 与 JNK 信号通路交互机制对胰岛素保护 I/R 心肌有重要意义。

**关键词**：缺血 / 再灌注损伤；胰岛素；凋亡；蛋白激酶 B；c-Jun 氨基末端激酶；交互机制
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Reperfusion therapy has become a practical and effective strategy in the salvage of ischemic myocardium. The direct enhancement of cardiac cellular tolerance against myocardial ischemia/reperfusion (MI/R) injury should further improve patient outcome in acute coronary syndromes (ACS). Our previous studies demonstrated that insulin might play an important role in attenuating MI/R injury *in vivo* when it was administered during reperfusion and activation of the cellular survival signaling, that is, phosphatidylinositol 3-kinase (PI3-K)-Akt-endothelial nitric oxide synthase (eNOS)-nitric oxide (NO) cascade, is one of the central mechanisms underlying the protective effect of insulin against MI/R injury[1-5]. Besides the PI3-K/Akt pathway, the mitogen-activated protein kinase (MAPK) cascades also are the downstream molecules of insulin in the heart[6,7], which have been shown to play an important regulatory role in a variety of cellular processes[8,9]. MAPKs phosphorylate selected intracellular proteins, including transcription factors, which subsequently regulate gene expression by transcriptional and post-transcriptional mechanisms[10]. MAPKs are, in turn, activated by phosphorylation at conserved threonine and tyrosine residues by upstream dual-specific MAPK kinases (MAPKKs), which themselves are activated by MAPKK kinases (MAPKKKs)[11]. In particular, the c-Jun $NH_2$-terminal kinases (JNKs) are a group of MAPKs that play a role in apoptosis, proliferation, and embryonic morphogenesis in both transcription-dependent and -independent mechanisms[10,12,13]. However, regarding the MAPK signaling cascades are one of the main signaling pathways of insulin, it remains largely elusive whether JNK is also involved in insulin-induced anti-apoptotic effects on I/R myocardium.

In addition, JNK activities were shown to be antagonized by Akt kinase activity in numerous cell systems, and this cross-talk may underlie many of the prosurvival effects of Akt[14]. It has been demonstrated that Akt regulates several proteins to suppress the JNK pathway, such as extracellular regulated kinases (ERKs), JNK-interacting protein (JIP) 1[15-18], and MAPK/ERK kinase (MEK)[15,19]. Indeed, the role of Akt-JNK cross-talk in the cardioprotective effects of insulin against MI/R is unclear so far.

Therefore, the aims of the present study were: (1) to investigate whether the JNK signaling pathway participates in the protective effect of insulin, and if so, (2) to further explore the possible role of Akt-JNK cross-talk in insulin-induced anti-apoptosis and subsequent attenuation of MI/R injury.

## 1 MATERIALS AND METHODS

### 1.1 *Experimental protocols*

The experiments performed were in adherence with Guidelines on the Use of Laboratory Animals in the National Institutes of Health and were approved by the Fourth Military Medical University Committee on Animal Care.

The experimental protocol was shown in Fig.1. Isolated hearts from adult male Sprague-Dawley rats [(200±50) g] were randomly assigned to the following groups. (1) Sham group: the hearts were subjected to the MI/R procedure (30 min of regional ischemia and followed by 2 or 4 h of reperfusion) except that the suture that was passed under the left coronary artery was left untied ($n$=6); (2) Control (Vehicle) group: the hearts were subjected to 30 min of regional ischemia and followed by 2 or 4 h of reperfusion with vehicle ($n$=6); (3) Insulin group: insulin (Novo Nordisk, 60 U/L) was administered 15 min before MI/R procedure ($n$=6); (4) Insulin + LY294002 (a specific inhibitor for PI3-K) group: the hearts were pretreated with LY294002 (15 mmol/L)[19] for 15 min before insulin administration ($n$=6); (5) Insulin + SP600125 (a specific inhibitor for JNK) group:

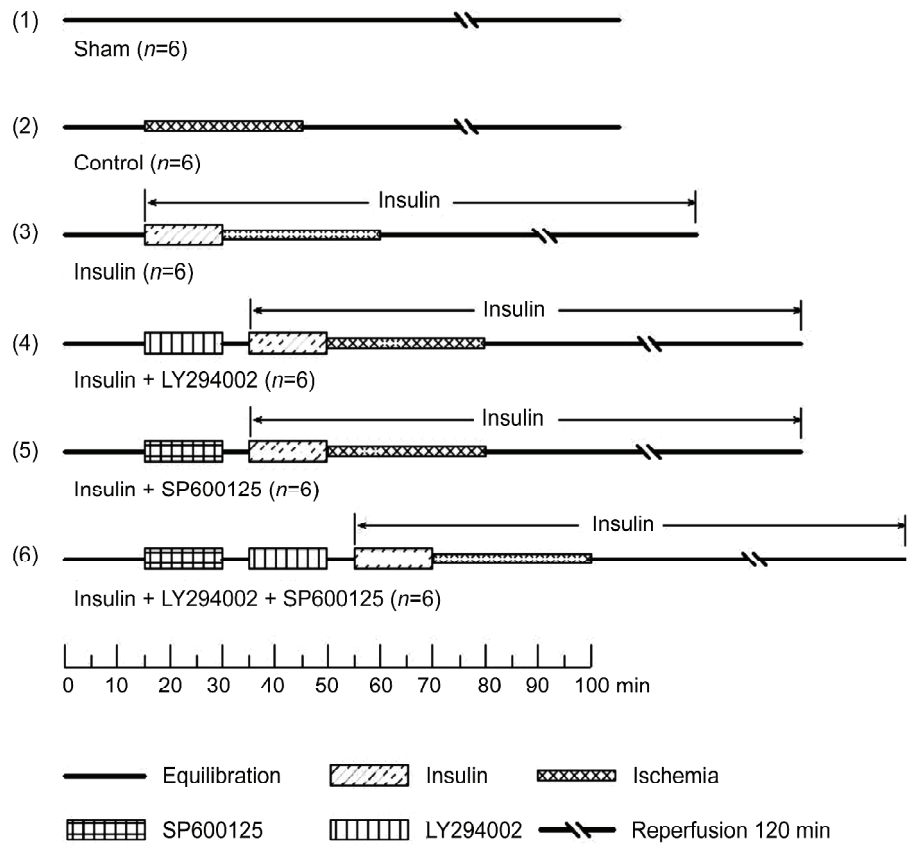


Fig. 1. Protocol chart. Isolated hearts from adult male Sprague-Dawley rats [(200±50) g] were randomly assigned to the following groups. (1) Sham group: the hearts were subjected to the MI/R procedure (30 min of regional ischemia and followed by 2 or 4 h of reperfusion) except that the suture that was passed under the left coronary artery was left untied ($n$=6); (2) Control (Vehicle) group: the hearts were subjected to 30 min of regional ischemia and followed by 2 or 4 h of reperfusion with vehicle ($n$=6); (3) Insulin group: insulin (Novo Nordisk, 60 U/L) was administered 15 min before MI/R procedure ($n$=6); (4) Insulin + LY294002 (a specific inhibitor for PI3-K) group: the hearts were pretreated with LY294002 (15 mmol/L) for 15 min before insulin administration ($n$=6); (5) Insulin + SP600125 (a specific inhibitor for JNK) group: the hearts were pretreated with SP600125 (10 mmol /L) for 15 min before insulin administration ($n$=6); (6) Insulin + LY294002 + SP600125 group: the hearts were pretreated with SP600125 and LY294002 for 15 min before insulin administration ($n$=6).

the hearts were pretreated with SP600125 (10 mmol /L)[12,20,21] for 15 min before insulin administration ($n$=6); (6) Insulin + LY294002 + SP600125 group: the hearts were pretreated with SP600125 and LY294002 for 15 min before insulin administration ($n$=6). The doses of the reagents were based on our previous experiments in rat hearts.

1.2 *Isolated heart preparation and MI/R*

After exteriorized through a left thoracic incision, the hearts were placed rapidly in ice-cold Krebs-Henseleit buffer to arrest and prevent them from ischemic preconditioning.

The hearts were then perfused retrogradely through the ascending aorta by a peristaltic pump at a constant flow rate of 10 mL/min, with perfusion medium containing (in mmol/L): NaCl 11.9, $NaHCO_3$ 24.9, KCl 4.74, $KH_2PO_4$ 1.19, $MgSO_4$ 1.2, $CaCl_2$ 1.8 and glucose 11, oxygenated and kept at pH 7.4 by gassing with the mixture of 95% $O_2$ and 5% $CO_2$[21,22]. The heart was kept throughout at constant temperature of 36.5 °C by being placed in a recirculation glass jacket. The initial perfusion pressure was 60 mmHg. When the heart began contracting spontaneously, a latex balloon, connected to a pressure transducer, was inserted into the left ventricle via the left atrium. The balloon was then filled with saline buffer at a steady diastolic pressure of 4-8 mmHg to measure left ventricular pressure. Two silver electrodes were fixed, one at the apex and another at the atria, for electrocardiogram recording of a bipolar derivative. The efficiency of the peristaltic pump in maintaining a stable coronary flow was determined by collecting the effluent

from the heart in a graduated glass container at fixed intervals. All variables were recorded using a computer acquisition data system[23].

Making a slipknot with a 4-0 silk around the left anterior descending (LAD) coronary artery produced myocardial ischemia. After 30 min of ischemia, the slipknot was released and the heart was reperfused for 2 h to measure infarct size (IS) or 4 h for terminal deoxynucleotidyl nick-end labeling (TUNEL) and Western blot assays[24].

1.3 *Determination of myocardial infarction*

To determine myocardial infarct, the LAD coronary artery was ligated again after 2 h of reperfusion, and perfused with 3 mL/kg of 1% Evans blue to stain the area at risk (AAR). The heart was then removed and divided into 4-5 slices of 1-2 mm width transversally. The Evans blue solution stained the perfused myocardium leaving the occluded vascular bed uncolored. All the colored non-ischemic tissue and the non-colored AAR were weighed to calculate the percentage of AAR with respect to the whole left ventricle. To distinguish between viable ischemic and infarcted tissue, the slices were incubated with TTC (10 mg/mL, 20 min, at 37 °C). In the presence of intact dehydrogenase enzyme systems (normal myocardium), TTC formed dark red formation, while areas of necrosis lacking dehydrogenase activity were not stained, and TTC-stained tissue was weighed. IS was then calculated and expressed as percentage of AAR[25].

1.4 *Western blot analysis*

Heart tissue samples were lysed with lysis buffer. After sonication, the lysates were centrifuged; proteins were separated by electrophoresis on SDS-PAGE and transferred onto a polyvinylidene difluoride-plus membrane. After being blocked with 5% fat-free milk, the immunoblots were probed with anti-p-JNK and anti-p-Akt antibodies overnight at 4 °C followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. The blots were visualized with ECL-plus reagent. p-JNK and p-Akt immunoblots were then stripped with strip buffer at 50 °C for 30 min and reblotted for total JNK and Akt[26].

1.5 *TUNEL assay*

Hearts were washed by perfusion with ice-cold PBS for 1 min followed by a fixation step with ice-cold 4% paraformaldehyde in phosphate buffer (peristaltic pump flow: 50 mL/min for 10 min). After 24 h in fixation solution, hearts were cryosectioned at a thickness of 10 μm and serial sections were stained with TUNEL reagents (Roche Co., USA), according to the manufacturer's instructions. The digoxigenin-conjugated dUTP was incorporated to the ends of DNA fragments by terminal deoxynucleotidyl transferase (TdT). The signal of TdT-mediated dUTP nick-end labeling was then detected by an anti-fluorescein antibody conjugation with alkaline phosphatase, a reporter enzyme that catalytically generates a red-colored product from vector red substrate. For each slide, 10 fields were randomly chosen and a defined rectangular field area (40 × objective) was used, a total of 200 cells per field were counted. The apoptotic index (AI) was determined (i.e., number of positively stained apoptotic myocytes/total number of myocytes counted ×100%) from a total of 80 fields per heart. The assays were performed in a blinded manner[26].

1.6 *Statistical analysis*

All values were presented as means±SEM. Differences were compared by ANOVA or Student's *t*-test, when appropriate. $P<0.05$ was considered to be statistically significant. All of the statistical tests were performed with the GraphPad Prism software version 4.0 (GraphPad Software, Inc., San Diego, CA).

## 2 RESULTS

### 2.1 Cardiac hemodynamics in isolated hearts subjected to MI/R

Baseline values for functional parameters were obtained after stabilization. In sham group, the left ventricular developed pressure (LVDP), heart rate (HR) and instantaneous first derivation of left ventricular pressure ($\pm LVdp/dt_{max}$) remained at a relatively stable level throughout the whole MI/R procedure. In the control group, the left ventricular pressure showed a large scatter, which was frequently found despite a large sample. Coronary occlusion caused a significant decline in LVDP, HR and $\pm LVdp/dt_{max}$. However, compared with the control, administration of insulin at 60 U/L induced a significant increase in LVDP, HR and $\pm LVdp/dt_{max}$ by 26%, 25%, 29% and 28% ($n$=6, all $P<0.05$) (Fig.2), respectively. In insulin + LY294002 group, the values of LVDP, HR, $\pm LVdp/dt_{max}$ decreased by 34%, 23%, 43% and 42% ($n$=6, $P<0.05$ *vs* insulin group), respectively. All values between the control and insulin + LY294002 groups had no statistical significance. Nevertheless, SP600125 converted the abolishment caused by LY294002 when it was administered together with LY294002, that is, compared with those in insulin + LY294002 group, LVDP, $+LVdp/dt_{max}$ and $-LVdp/dt_{max}$ in insulin + LY294002 + SP600125 group increased by 30%, 24% and 33%, respectively ($n$=6, $P<0.05$) at 1 h after reperfusion, while HR was not significantly altered ($P>0.05$) (Fig.2). These results indicate that

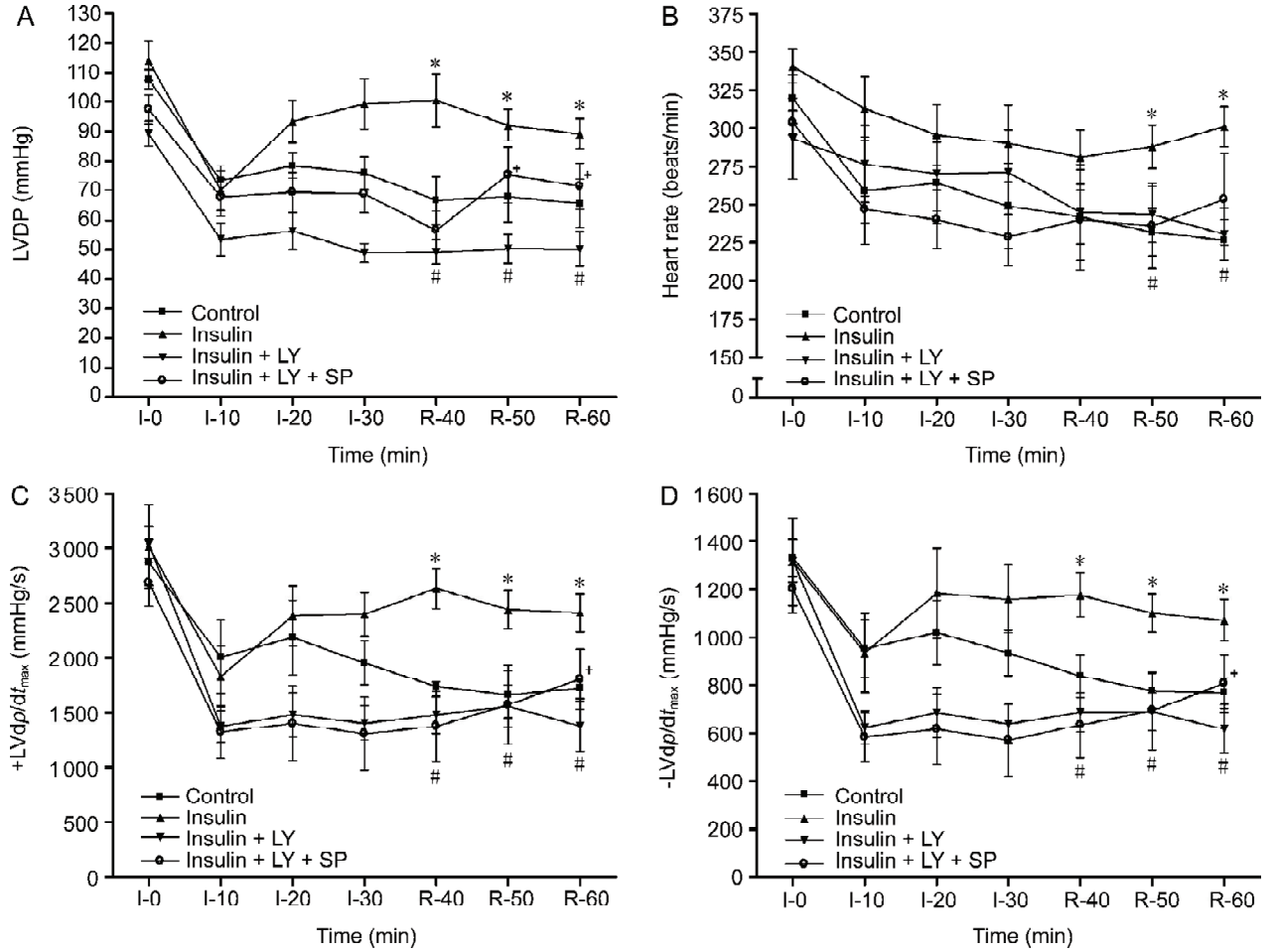


Fig. 2. Cardiac hemodynamics in isolated hearts subjected to ischemia/reperfusion and receiving different treatments. *A*: Left ventricular developed pressure (LVDP). *B*: Heart rate (HR). *C* and *D*: The instantaneous first derivation of left ventricular pressure ($\pm LVdp/dt_{max}$). I, ischemia; R, reperfusion; LY, LY294002; SP, SP600125. means±SEM, $n$=6. $^{*}P<0.05$ *vs* control, $^{\#}P<0.05$ *vs* insulin, $^{+}P<0.05$ *vs* insulin + LY.

both Akt and JNK play roles in the effects of insulin on MI/R hearts.

### 2.2 Myocardial IS in isolated hearts subjected to MI/R

To determine myocardial injury, the effects of insulin, insulin + LY294002, insulin + SP600125, and insulin + LY294002 + SP600125 on myocardial IS were determined. There were no significant differences in AAR/LV among all groups (data not shown). Occlusion of the LAD coronary artery for 30 min followed by reperfusion with vehicle for 2 h resulted in an IS of (45.0±4.0)%. Compared with the control, administration of insulin at 60 U/L caused a significant reduction in IS by approximately 16.1% ($n$=6, $P$<0.01) (Fig.3). Pretreatment of the isolated heart with the PI3-K inhibitor LY294002 abolished the reduction of IS by insulin [(43.5±5.0)% *vs* (28.9±2.0)%, $n$=6, $P$< 0.05]. While hearts in insulin + SP600125 group showed a smaller IS compared with that in insulin group ($P$<0.05). Interestingly, cotreatment with LY294002 and SP600125 attenuated the inhibition of LY294002 on insulin-induced reduction of myocardial infarction ($n$=6, $P$<0.05) (Fig.3). These data suggest that insulin exerts cardioprotection via a PI3-K/Akt-dependent mechanism and that JNK activation plays a role in this action of insulin.

### 2.3 Cardiomyocyte apoptosis in isolated hearts subjected to MI/R

In sham I/R hearts, few nuclei were stained TUNEL positively. In contrast, TUNEL-positive nuclei were prevalent in the control I/R hearts (Fig.4). Administration of insulin reduced the percentage of TUNEL-positive nuclei from (27.6±1.3)% to (16.0±0.7)% ($n$=6, $P$<0.01 *vs* control) (Fig.4). However, after the hearts were treated with insulin + LY294002, TUNEL-positive cells increased to (21.9± 0.8)% ($n$=6, $P$<0.01 *vs* insulin) (Fig.4). These results

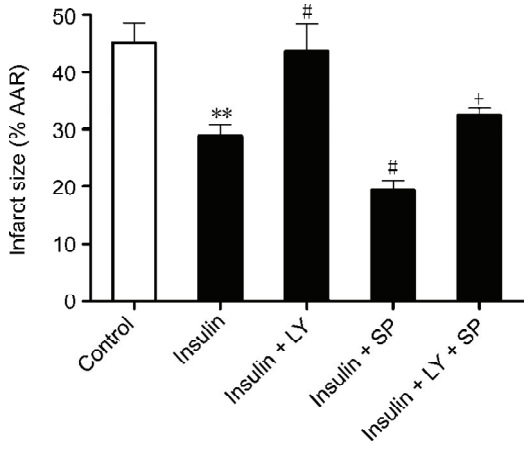


Fig. 3. Infarct size in isolated hearts subjected to ischemia/reperfusion and receiving different treatments. TTC-negative staining portions (infarct) and TTC-positive staining portions within ischemic/reperfused regions (area at risk, AAR) were weighed and infarct size was expressed as percent of infarct area relative to AAR. LY, LY294002; SP, SP600125. means±SEM, $n$=6. $^{**}P$<0.01 *vs* control, $^{\#}P$<0.05 *vs* insulin, $^{+}P$<0.05 *vs* insulin + LY.

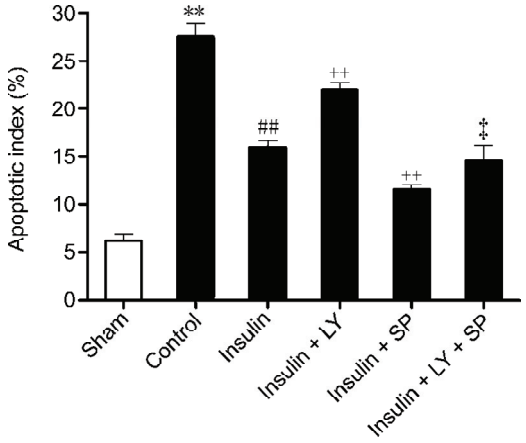


Fig. 4. Apoptosis in isolated hearts subjected to ischemia/reperfusion and receiving different treatments. Data were expressed as percentage of nuclei staining positive for TUNEL. LY, LY294002; SP, SP600125. means±SEM, $n$=6. $^{**}P$<0.01 *vs* sham, $^{\#\#}P$<0.01 *vs* control, $^{++}P$<0.01 *vs* insulin, $^{\ddagger}P$<0.05 *vs* insulin + LY.

indicate that insulin exerts an anti-apoptotic effect on I/R heart via a PI3-K/Akt-dependent pathway.

Insulin plus SP600125 reduced the apoptotic cells compared with insulin alone [(11.6±0.5)% *vs* (16.0±0.7)%, $n$=6, $P$<0.01]. In another group, in which hearts were pretreated with SP600125 plus LY294002 15 min before ischemia and reperfusion with insulin during reperfusion, it was found that the number of TUNEL-positive nuclei in I/R hearts declined to (15.0±1.6)%, less than that in insulin + LY294002 group ($n$=6, $P$<0.05) (Fig.4). These data demonstrate that administration of SP600125 plus LY294002 could abate the effect caused by LY294002, suggesting

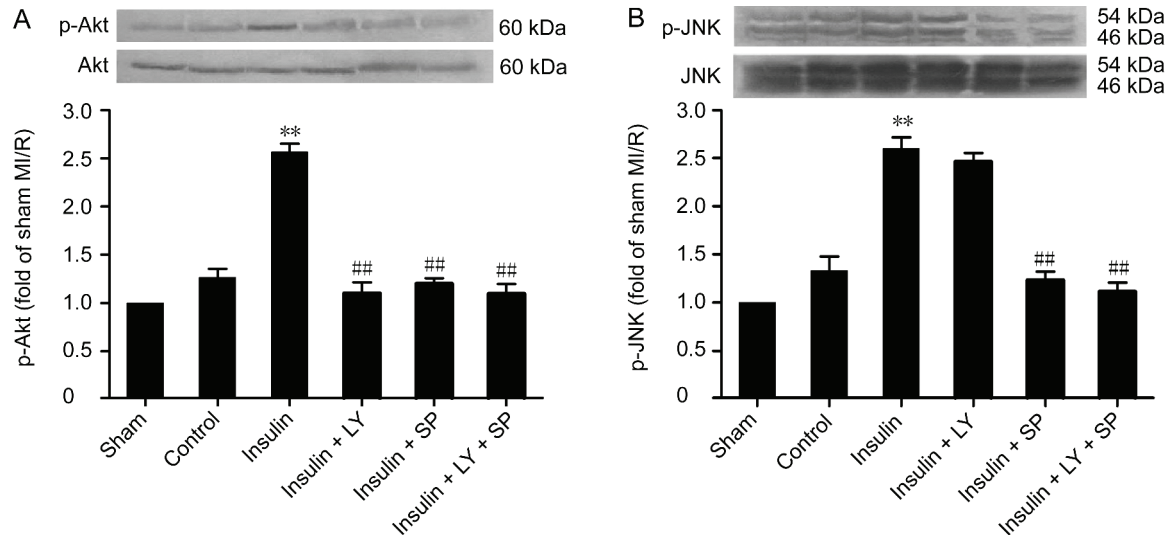


Fig. 5. Total and phosphorylated Akt and JNK in isolated hearts subjected to ischemia/reperfusion and receiving different treatments. *A*: Phosphorylated Akt. Insets: Representative blots of phosphorylated and total Akt. $^{**}P$<0.01 *vs* control, $^{\#\#}P$<0.01 *vs* insulin. *B*: Phosphorylated JNK. Insets: Representative blots of phosphorylated and total JNK. $^{**}P$<0.01 *vs* control, $^{\#\#}P$<0.01 *vs* insulin. Data obtained from quantitative densitometry were presented as means±SEM of at least six independent experiments. LY, LY294002; SP, SP600125.

that JNK may act as a proapoptotic factor in MI/R hearts.

### 2.4 Akt and JNK activation in isolated hearts subjected to MI/R

Using Western blot analysis, the levels of JNK and Akt phosphorylation were examined after 4 h of reperfusion (Fig.5). It was observed that after 4 h of reperfusion with vehicle, Akt and JNK phosphorylation levels slightly increased in I/R myocardium and there was no statistical significance in the expressions. However, treatment with insulin markedly increased both Akt and JNK phosphorylation in I/R hearts ($n$=3, $P$<0.01 *vs* control), suggesting the activation of insulin signaling transduction pathway in MI/R (Fig.5).

Previously we have demonstrated that LY294002 treatment inhibited the anti-apoptotic effects of insulin on MI/R rat hearts[1]. Here we examined the possibility that JNK inhibition leads to convert the action of LY294002 on cell apoptosis. Rat hearts were pretreated with a selective JNK inhibitor, SP600125, for 15 min, before LY294002 treatment, then Akt phosphorylation was assessed after 4-hour reperfusion. As anticipated, LY294002 and SP600125 did inhibit insulin-induced Akt and JNK phosphorylation, respectively ($n$=6, $P$<0.01). Cotreatment with LY294002 and SP600125 almost completely abolished both Akt and JNK activation induced by insulin ($n$=6, $P$<0.01). Interestingly, it was found that inhibition of JNK activity by SP600125 simultaneously decreased phosphorylation of Akt ($n$=6, $P$< 0.01), which seemed different from that by LY294002. The tight temporal correlation between these two events supported the notion that PI3-K/Akt activation in isolated hearts might be related to JNK activation.

## 3 DISCUSSION

In the present study, it was observed that, consistent with our previous results, administration of insulin at the onset of reperfusion exerted cardioprotective effects on MI/R hearts as evidenced by improved cardiac functions, reduced IS and cell apoptosis via activation of the PI3-K/Akt pathway, i.e., the survival signaling. In addition, along with Akt activation, there was also increased phosphorylation of JNK when insulin was administered, which aggravated MI/R injury. The main findings of this study are that insulin-induced JNK activation could further increase Akt phosphorylation and this cross-talk between the Akt and JNK signaling seems to play an important role in the cardioprotection exerted by insulin.

The "decision" of a cell to live or to die is derived from the dominance of both prodeath or prosurvival proteins and cellular factors. Our previous study showed that *in vivo* treatment with insulin reduced postischemic apoptotic death in both cardiomyocytes and coronary endothelial cells by activating the PI3-K-Akt-eNOS pathway (the survival signaling pathway), which might further contribute to the prolonged improvement of cardiac performance following MI/R[1-5]. Consistent with those results, inhibition of Akt activation by LY294002 in the present study almost completely abolished the protective effects of insulin on isolated I/R hearts, indicating that the prosurvival factor Akt plays a key role in this beneficial action of insulin.

In a variety of cultured cells, JNK has been implicated in the cell death pathway, and several mechanisms have been proposed[11-13]. Previous studies have shown that JNK activation could be detected in myocardium within minutes after the onset of reperfusion[12]. Here we also found JNK activation was accompanied with reperfusion in the isolated rat hearts. These data suggest a possible role for JNK in the death of cardiomyocytes following MI/R, but do not provide the causality. It was reported that insulin induced stimulation of JNK[27], and in rat hippocampus, the neuroprotection of insulin against ischemic brain injury was via negative regulation of the JNK signaling pathway[28]. In the present study, we treated the isolated MI/R hearts with a JNK inhibitor, SP600125, and found that there was not only an inhibition of JNK phosphorylation, but also a reduction in IS and TUNEL-positive nuclei, and an improvement in the heart functions. Interestingly, although LY294002 abolished the reduction of MI/R injury and the improvement of heart function caused by insulin, this abolishment could be partly converted by SP600125 pretreatment possibly due to the inhibition of the proapoptotic effects of JNK. In addition, previous studies showed that SP600125 could not only inhibit JNK itself, but also suppress the activation of JNK downstream members, for example, C-JUN, ATF-2, ELK-1, p53, and so on, thus induces anti-apoptotic effect. These data suggest that activation of JNK induced by insulin aggravates myocardial injury and decreases the mechanical work of isolated hearts after regional MI/R.

Although insulin treatment protects the hearts from MI/R injury and improves the prolonged myocardial performance, whether there exists a cross-talk between insulin-induced Akt and JNK remains largely unclear. In the present study, we found that the activity of Akt could be regulated by JNK. Specifically, reperfusion with insulin resulted in both increased Akt and JNK phosphorylation. Pretreatment with LY294002, the PI3-K inhibitor, inhibited phosphorylation of Akt but not JNK. SP600125 pretreatment inhibited the

activation of JNK, and the Akt activation induced by insulin was simultaneously decreased, although SP600125 itself had no effect on Akt activation[15]. These data suggest that JNK activation induced by insulin increases Akt phosphorylation. The attenuation of Akt phosphorylation by the inhibition of JNK by SP600125 might be due to direct JNK-mediated phosphorylation of Akt, or change of upstream of PI3-K/Akt due to the regulation of PTEN (3'-phosphoinositide phosphatase) by JNK[29,30]. Further studies are expected to elucidate this issue. It was reported that the dissociation of Akt from its negative regulator JIP1 is regulated by JNK[17]. Moreover, Kilter *et al.*[31] reported that JNKs are necessary for the reactivation of Akt after ischemic injury. Inhibition of JNK phosphorylation induced reduction in Akt activity. They further identified Thr450 of Akt as a residue that is phosphorylated by JNKs, and the phosphorylation status of Thr450 regulates reactivation of Akt after hypoxia. Taken together, it seems that more phosphorylated JNK induced by insulin, more phosphorylated Akt is produced, which is involved in insulin-induced cardioprotective effects on MI/R hearts, although JNK itself to some extent increases MI/R injury after activation. Future investigations are proposed to observe whether Akt activation activated by JNK could reversely inhibit phosphorylation of JNK and exert impelling anti-apoptotic effect, and whether the balance between Akt and JNK may play a role in the beneficial effects of insulin on I/R myocardium.

In summary, the present study demonstrates for the first time that insulin simultaneously actives both Akt and JNK, and the latter further increases the phosphorylation of Akt which attenuates MI/R injury and improves heart function. This cross-talk between Akt and JNK in the insulin signaling is involved in insulin-induced cardioprotective effect.

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