Original Article
JNK/PI3K/Akt signaling pathway is involved in myocardial ischemia/reperfusion injury in diabetic rats: effects of salvianolic acid A intervention

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Abstract: Recent studies have demonstrated that diabetes impairs the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway, while insulin resistance syndrome has been associated with alterations of this pathway in diabetic rats after ischemia/reperfusion (I/R), and activation of C-jun N-terminal kinase (JNK) is involved. The present study was designed to investigate whether inhibiting JNK activity would partially restore the PI3K/Akt signaling pathway and protect against myocardial I/R injury in diabetic rats, and to explore the effect of intervention with salvianolic acid A (Sal A). The inhibitor of JNK (SP600125) and Sal A were used in type 2 diabetic (T2D) rats, outcome measures included heart hemodynamic data, myocardial infarct size, the release of lactate dehydrogenase (LDH), SERCA2a activity, cardiomyocyte apotosis, expression levels of Bcl-2, Bax and cleaved caspase-3, and the phosphorylation status of Akt and JNK. The p-Akt levels were increased after myocardial I/R in non-diabetic rats, while there was no change in diabetic rats. Pretreatment with the SP600125 and Sal A decreased the p-JNK levels and increased the p-Akt levels in diabetic rats with I/R, and heart hemodynamic data improved, infarct size and LDH release decreased, SERCA2a activity increased, Bax and cleaved caspase-3 expression levels decreased, and the expression of Bcl-2 and the Bcl-2/Bax ratio increased. Our results suggest that the JNK/PI3K/Akt signaling pathway is involved in myocardial I/R injury in diabetic rats and Sal A exerts an anti-apoptotic effect and improves cardiac function following I/R injury through the JNK/PI3K/Akt signaling pathway in this model.

Keywords: Diabetic rats, ischemia/reperfusion, JNK, PI3K/Akt signaling pathway, Sal A

Introduction

Epidemiological data and clinical studies have demonstrated that diabetes is a major risk factor for cardiovascular events. Statistically, ischemic heart disease is twice as common in diabetic as in non-diabetic patients [1]. Diabetic patients are more liable to myocardial ischemia/reperfusion (I/R) injury than non-diabetic patients [2, 3]. Therefore, prevention and treatment of myocardial I/R injury in diabetes has become a global challenge. Animal experiments have confirmed that myocardial apoptosis is higher in diabetic rats than in non-diabetic rats after I/R injury [4, 5].

The PI3K/Akt pathway has important biological functions in cell proliferation, survival and apoptosis. Previous studies in our laboratory have revealed that activation of this pathway improves cardiac contractility and decreases cardiomyocyte apoptosis, thus ameliorating I/R injury in non-diabetic rats [6]. It is well known that diabetes could impair PI3K/Akt pathway, and some pharmacological agents and ischemic preconditioning could decrease I/R injury. But they appear to be less effective in diabetes [7-9]. Insulin resistance syndrome has been associated with alterations in Akt phosphorylation in diabetic rats after I/R [10]. Insulin resistance enhances the production of inflammatory factors, endoplasmic reticulum stress and oxidative stress, Both conditions involve in activation of C-jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family in the myocardium and other tissues. A

large body of literature indicates that JNK is involved in the formation of insulin resistance [11, 12]. Activation of JNK increases serine phosphorylation of insulin receptor substrate 1 (IRS-1), decreases tyrosine phosphorylation, and decreases Akt phosphorylation in liver [13, 14]. Shah et al. have reported that the novel agent osmotin attenuated glutamate-induced synaptic dysfunction and neurodegeneration via the JNK/PI3K/Akt pathway in postnatal rat brain [15]. The heart is an also insulin-sensitive organ, and its failure to receive normal insulin signals is deleterious. In this context, it is uncertain whether activated JNK impairs the PI3K/Akt pathway after myocardial I/R injury in diabetic rats.

A large body of evidence demonstrate that traditional Chinese medicines rich in salvianolic acid are effective in protecting the myocardium against I/R injury [16-18]. Salvianolic acid A (Sal A, (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-[2-[(E)-2-(3,4-dihydroxyphenyl) ethenyl]-3,4-dihydroxyphenyl] prop-2-enoyl] oxypropanoic acid) is a water-soluble ingredient of *Salvia miltiorrhiza*, which has been reported to exert a wide range of pharmacological effects, including anti-inflammatory, anti-platelet aggregation, antioxidant, anticarcinogenic, and anti-fibrotic [19, 20]. Investigations in our laboratory have demonstrated that Sal A displays cardioprotective effects in non-diabetic rat hearts following myocardial I/R injury [21]. However, to date, little is known about the effects of Sal A on diabetic rats after myocardial I/R injury.

Therefore, the aims of the current study were (1) to determine whether inhibiting the activity of JNK partially could restore the PI3K/Akt signaling pathway and act against myocardial I/R injury in diabetic rats, and if so, (2) to elucidate whether Sal A intervention exerts cardioprotection against myocardial I/R injury via JNK/PI3K/Akt signaling pathway.

Materials and methods

Experimental animals

This experimental design was reviewed and approved by the Animal Ethics Committee of Xuzhou Medical University. Male Sprague-Dawley rats (160-180 g) were provided by the Experimental Animal Center in Xuzhou Medical University.

Induction of type 2 diabetes (T2D) in rats

T2D was induced by a high-fat diet combined low-dose streptozotocin as previously described [13]. Briefly, the rats were randomly allocated to two dietary regimens by feeding either normal pellet diet (NPD) or high-fat diet (HFD 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) for two weeks. After this dietary manipulation, the HFD rats were injected intraperitoneally with a single dose of STZ (35 mg kg^{-1}), while the normal group rats received an equal volume of the vehicle, citrate buffer (pH 4.4). Seven days after the STZ injection, the animals with non-fasting plasma glucose (PGL)$\geq$16.7 mmol/L were considered to be T2D rats. In order to maintain the stability of the model, the rats were allowed to continue to feed on their respective diets for two weeks prior to use in the following experiments.

Isolated heart perfusion

The hearts of all the rats were isolated and perfused as previously described [22, 23]. Briefly, after 20 min of heparinization, the rats were anesthetized with sodium pentobarbital (150 mg/kg) by intraperitoneal injection. The hearts were rapidly excised and immediately mounted and perfused on a Langendorff device. The perfusate was a modified Krebs-Henseleit buffer containing (in mM): 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose and 1.25 $CaCl_2$, pH 7.4, saturated with 95% O_2/5% CO_2 at 37°C. Perfusion was maintained at a constant pressure of 80 mmHg. When all the hearts including those of the control group had equilibrated, they were subjected to 30 min global ischemia and 120 min reperfusion, and then allocated as follows: (1) Ctrl-Sham (n=6); (2) Ctrl-I/R (n=6); (3) Ctrl-Sal A+I/R (n=6); (4) Diab-Sham (n=6); (5) Diab-I/R (n=6); (6) Diab-Sal A+I/R (n=6). In the groups receiving Sal A (groups 3 and 6), Sal A (20 μM) was injected via a side pipe just proximal to the heart cannula, as previously described [21].

To test whether Sal A inhibited JNK phosphorylation and protected the myocardium in diabetic rats hearts, the JNK inhibitor (SP600125) was added to perfusate prior to I/R procedure described above and the rat hearts were divided into five groups: (1) Diab-Sham (n=6); (2) Diab-I/R (n=6); (3) Diab-Sal A+I/R (n=6); (4) Diab-SP+I/R (n=6), (5) Diab-SP+Sal A+I/R

(n=6). The hearts were perfused with SP at the concentration of 10 µM for 30 min before administration of Sal A, then subjected to the same course as the Diab-Sal A+I/R group.

Hemodynamic parameters

To determine left ventricular systolic and diastolic function, a fluid-filled latex balloon connected to a Millar transducer (pressure sensor) was inserted into the left ventricle as described previously [24]. The parameters of cardiac function were continuously simultaneously monitored and the entire I/R procedure was recorded using a Biopac system. During the procedure, heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and ±dp/dtmax were recorded. The left ventricular function parameters were calculated using a hemodynamic analysis system (Chengdu, China).

LDH activity in coronary effluent

Samples were collected from the coronary effluent after 10 min reperfusion. LDH activity was measured spectrophotometrically and calculated using a commercially available assay kit (Jiancheng Bioengineering Institute; Nanjing, China).

Infarct size

Myocardial infarct size was measured by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as previously described [25]. After 2 h of reperfusion, the hearts were removed from the apparatus and the left ventricles were sectioned from apex to base into five to six 1-mm sections. The sections were incubated in 1% TTC in phosphate buffer, pH 7.4, at 37°C for 20 min and then fixed in 10% formalin for 1 h. The normal zone was stained red by TTC while the infarct region (which did not take up the TTC stain) remained gray. The myocardial tissue was weighed and the percentage ratios of infarcted regions in the left ventricles were calculated.

Myocardial apoptosis

A terminal deoxynucleotidyl nick-end labeling (TUNEL) kit (Roche; Switzerland) was used to detect myocardial apoptosis, using at least three hearts in each group. In brief, after the 2 h reperfusion, LV tissues were fixed in formalin

and stored at 4°C. The tissues were then embedded in paraffin and cut into 5 µm sections, which were TUNEL-stained according to the manufacturer's instructions. Then 4',6-diamidino-2-pheny-lindole (DAPI, Sigma-Aldrich, USA) was used to stain all the cardiomyocyte nuclei. The cells were examined by light microscopy; 10 fields containing at least 50 cells were viewed at ×400 magnification. In each field the nuclei were counted, and the percentage of apoptotic nuclei stained dark brown (TUNEL-positive nuclei) was calculated.

Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) activity

SERCA2a activities were measured using an ELISA kit (Jiancheng Bioengineering Institute; Nanjing, China), in which sarcoplasmic reticulum calcium pump activity was measured colorimetrically as the amount of inorganic phosphate released from ATP. Myocardial sarcoplasmic reticula were prepared by a modified differential centrifugation method previously described by Kodavanti et al. [26-28]. The sarcoplasmic reticula were separated from the homogenates and the protein concentrations were determined by the Bradford method. Each sarcoplasmic reticulum preparation was suspended in (each component in mmol/L): 50.0 histidine, 3.0 $MgCl_2$, 100.0 KCl, 5.0 NaN_3, 3.0 ATP and 50 $CaCl_2$, pH 7.0, and incubated for 10 min at 37°C. Tris-ATP (3 mmol/L) was added to initiate the reaction and the suspension was incubated at 4°C in a water bath for 20 min. The data from three replicate measurements of SERCA2a activity were expressed as µmol inorganic phosphate released per h per mg protein.

Western blotting

Hearts were collected at the end of the 2 h of reperfusion and the left ventricular tissue was frozen in liquid nitrogen before being stored at -80°C. Approximately 100 mg of ventricular tissue was used for protein extraction. After centrifugation at 20,000 g for 20 min at 4°C, the supernatant was collected and protein was quantified using a modified Bradford assay (Bio-Rad, CA, USA). Aliquots of the supernatants containing equal amounts of protein (40 µg) were electrophoresed on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were treated with primary antibodies against Bcl-2

Table 1. Effect of body weight, plasma glucose and plasma insulin in rats

Parameters	Weight body (g)	PGL (mmol/L)	PI (ng/ml)
NPD (n=36)	247.00±5.36	7.95±0.30	2.33±0.25
HFD+STZ (n=60)	255.00±4.36	23.50±0.51***	2.29±0.11

mean±S.E.M. ***P<0.001 versus NPD. NPD: normal pellet diet; HFD: high-fat diet; STZ: streptozotocin; PGL: plasma glucose; PI: plasma insulin.

and Bax (1:500, Santa Cruz, USA), cleaved caspase-3, phospho-Akt, Akt, phospho-JNK, JNK (1:1000; Cell Signaling Technology, MA, USA), and β-actin (1:1000; Zhongshan; Beijing, China). This step was followed by incubation with the corresponding secondary antibodies (1:1000; Zhongshan; Beijing, China). Protein bands were visualized by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The band densities were quantified by the Image J 3.0 system (National Institutes of Health; MD, USA).

Statistical analysis

All values are expressed as means ± SEM. GraphPad Prism 5 software was used for statistical analysis. One-way and two-way analysis of variance (ANOVA) was conducted across all groups, followed by a Bonferroni post-hoc correction for all group comparisons. For the Langendorff data, ANOVA for repeated measurements was used. Values of *p*<0.05 were considered statistically significant.

Results

Characteristics of the fat-fed/STZ-induced T2D rats

The success of the fat-fed, STZ-induced T2D rats model was confirmed by checking for PGL and plasma insulin (PI) as previously described [13]. PGL was markedly higher in the HFD+STZ-treated group than that of the NPD-treated group (p<0.001). Body weights and PI levels were not significantly different between the groups (**Table 1**).

Effects of Sal A pretreatment on diabetic and non-diabetic rats

Hemodynamic data: There was no significant difference in HR, LVSP, LVEDP or ±dp/dt max between the Diab and Ctrl groups at baseline (**Figure 1**). However, after I/R, HR, LVSP and

±dp/dtmax were decreased and LVEDP was increased significantly in both groups. The hemodynamic values were significantly higher in the Diab than those of the Ctrl group (p<0.05). Pretreatment with Sal A significantly improved cardiac systolic/diastolic function and increased HR before I/R in both groups.

LDH activity in coronary effluent and Infract size

LDH activity was significantly higher in the Diab than that of the Ctrl group (p<0.05). I/R significantly increased the release of LDH in the Ctrl group (222.00±9.07 i.u.l⁻¹ in Ctrl-I/R vs 51.51± 5.56 i.u.l⁻¹ in Ctrl-Sham; p<0.01) and in the Diab group (281.40±7.01 i.u.l⁻¹ in Diab-I/R vs 94.46±3.468 i.u.l⁻¹ Diab-Sham; p<0.01). Sal A significantly decreased LDH activity in the Ctrl group (158.80±5.18 in Ctrl-Sal A+I/R vs 222.00±9.07 in Ctrl-I/R; p<0.05) and the Diab group (224.1±4.47 i.u.l⁻¹ in Diab-Sal A+I/R vs 281.40±7.01 i.u.l⁻¹ in Diab-I/R; p<0.05) (**Figure 2A**).

Infarct size was expressed as the percentage ratio of infarct area/total area. As illustrated in **Figure 2B** and **2C**, there was a significant difference between the Ctrl and Diab groups (p<0.05). I/R significantly increased infarct size in both the Ctrl (44.96±2.16% in Ctrl-I/R vs 0.00±0.00% in Ctrl-Sham; p<0.01) and Diab (55.42±1.81% in Diab-I/R vs 0.00±0.00% in Diab-Sham; p<0.001) groups. Sal A administration significantly decreased infarct size at 2 h (25.36±0.74% in Ctrl-Sal A+I/R vs 44.96± 2.16% in Ctrl-I/R; p<0.05; 35.06±2.02% in Diab-Sal A+I/R vs 55.42±1.81% in Diab-I/R; p<0.05) after I/R.

Apoptosis: The apoptosis rates were significantly higher in the Diab group than those of the Ctrl group (p<0.05). Representative photomicrographs revealed TUNEL-positive cardiomyocytes more frequently in the I/R groups (21.67±2.18% in Ctrl-I/R vs 4.33±2.33% in Ctrl-Sham, 33.67±1.20% in Diab-I/R vs 9.00± 1.58% in Diab-Sham; p<0.01). Sal A decreased the number of TUNEL-positive cells (13.67± 1.88% in Ctrl-Sal A+I/R vs 21.67±2.82% in Ctrl-I/R; 22.33±3.33% in Diab-Sal A+I/R vs 33.67±3.20% in Diab-I/R; p<0.01) after I/R (**Figure 3A** and **3B**).
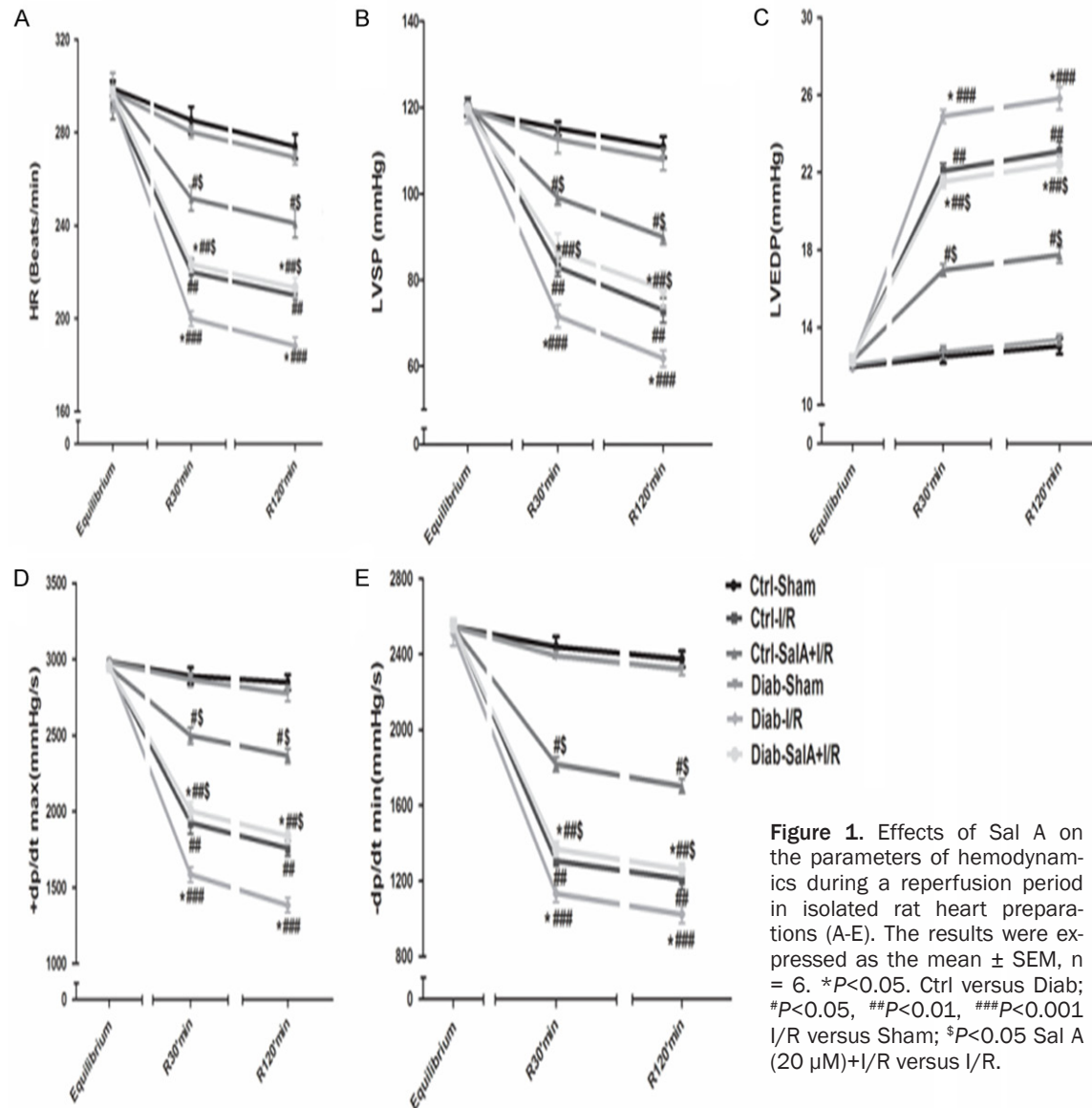
Figure 1. Effects of Sal A on the parameters of hemodynamics during a reperfusion period in isolated rat heart preparations (A-E). The results were expressed as the mean ± SEM, n = 6. *$P<0.05$. Ctrl versus Diab; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ I/R versus Sham; $$P<0.05$ Sal A (20 μM)+I/R versus I/R.

Akt and JNK phosphorylation: There were no differences in total Akt and JNK levels among the study groups. Therefore, p-Akt and p-JNK levels were expressed as densitometric levels normalized to total protein. The expression level of Akt phosphorylation in the Ctrl group was increased after I/R (p<0.05), and the value of p-Akt was significantly higher after pretreatment with Sal A than that of the I/R group (p<0.05). In contrast, compared with Diab-Sham, I/R had no effect on the expression of Akt. However, pretreatment with Sal A increased the p-Akt levels after I/R (p<0.05) (**Figure 3E** and **3F**). At the same time, the p-JNK levels in the diabetic group were higher than those in the Ctrl group (p<0.05). I/R significantly acti-

vated p-JNK expression in both the Ctrl and Diab groups (p<0.01), but this trend was partially reversed by Sal A (p<0.05) (**Figure 3C** and **3D**).

Effects of SP600125 and Sal A on the function of isolated hearts and SERCA2a activity in diabetic rats subjected to I/R

As **Figure 4A-E** indicated, HR, LVSP and ±dp/dt*max* were significantly lower and LVEDP was higher in the I/R than those of the sham group (p<0.001). Consistently, pretreatment with either SP600125 or Sal A before I/R improved myocardial function as evidenced by higher HR, LVSP and ±dp/dt*max* and lower LVEDP than
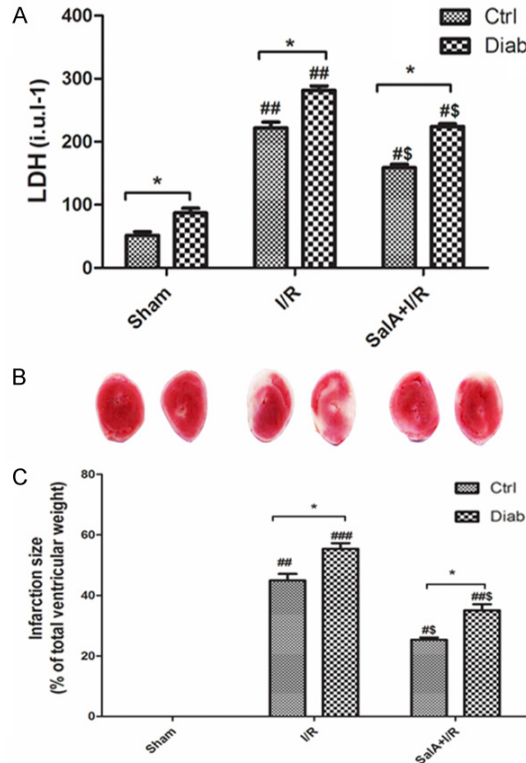
Figure 2. LDH release and Infarct size quantity in I/R rat hearts. Effects of SalA on release of LDH (A) and Infarct size (B, C) in isolated I/R rat hearts. The results were expressed as the mean ± SEM, n=6. *P<0.05 Ctrl versus Diab; #P<0.05,##P<0.01, ###P<0.001 I/R versus Sham; $P<0.05 SalA (20 μM)+I/R versus I/R.

those in the I/R group (p<0.05). In addition, administration of SP600125 before Sal A markedly improved these parameters of myocardial function in diabetic rats (p<0.05).

SERCA2a activity (**Figure 4F**) was significantly lower in the I/R group than that of the Sham group (5.62±0.16 vs 1.77±0.15; p<0.001). Pretreatment with either SP600125 or Sal A before I/R led to higher SERCA2a activities than in the I/R group (3.47±0.14, 3.49±0.18 vs 1.77±0.15 μmol Pi/mgprot/h; p<0.05). The protective effect of combining Sal A with SP600125 was markedly enhanced, as demonstrated by the comparison between the Sal A+I/R and SP+I/R groups (4.38±0.135 vs 3.49±0.18, 3.47±0.14; p<0.05).

Effects of SP600125 and Sal A on LDH activity in coronary effluent and infarct size in diabetic rats

After pretreatment with SP600125 or Sal A, LDH release was significant lower in the I/R

group (210.2±4.17, 224.1±4.47 vs 281.40± 7.01; p<0.05). However, co-administration of SP600125 and Sal A before I/R led to a statistically lower release of LDH than in the SP+I/R and Sal A+I/R groups (161.80±5.39vs 210.2± 4.17, 224.1±4.47; p<0.05) (**Figure 5A**).

Representative images of infarct size as revealed by TTC staining are presented in **Figure 5B** and **5C**. SP600125 or Sal A administration decreased significantly infarct size (31.26± 1.53%, 35.06±2.02% vs 55.42±1.81%; p< 0.05) after I/R than that in the I/R group, and the infarct size was smaller in the SP+Sal A+I/R group than that of the SP+I/R or Sal A+I/R groups respectively (22.33±1.45% vs 31.26±1.53%, 35.06±2.02%; p<0.05).

Effects of SP600125 and Sal A on cell apoptosis, p-Akt and p-JNK protein expression in diabetic rats

As illustrated in **Figure 6A** and **6B**, there were much more TUNEL-positive cardiomyocytes after I/R (33.67±1.20% vs 9.00±0.58%; p<0.01) than those in the Sham group. However, SP600125 or Sal A decreased I/R-induced cardiomyocyte apoptosis (21.67±1.45%, 22.33± 0.33% vs 33.67±1.20%; p<0.05). The combination of SP600125 and Sal A led to a further significant reduction in cardiomyocyte apoptosis (p<0.01). The results of Western blotting demonstrated that the p-JNK levels were increased after I/R (p<0.01), while the p-Akt level had no significant difference. After pretreatment with Sal A, the p-JNK level was lower (p<0.05) and the p-Akt level higher (P<0.05) than those in the I/R group. The p-JNK levels in the SP+I/R group were higher than those in the Sal A+I/R group (p<0.05). However, there was no difference for p-Akt levels between the Sal A+I/R and SP+I/R groups. Pretreatment with both SP600125 and Sal A had no significant effect on the p-JNK levels but p-Akt expression was significantly higher than in the SP+I/R group (p<0.05) (**Figure 6C-F**).

Effects of SP600125 and Sal A on Bcl-2, Bax and cleaved caspase-3

We measured the expression levels of the anti-apoptotic protein Bcl-2 and the apoptotic proteins Bax and cleaved caspase-3. As demonstrated in **Figure 7A-E**, the results of Western blotting also revealed that SP600125 or Sal A pretreatment decreased Bax and cleaved cas-
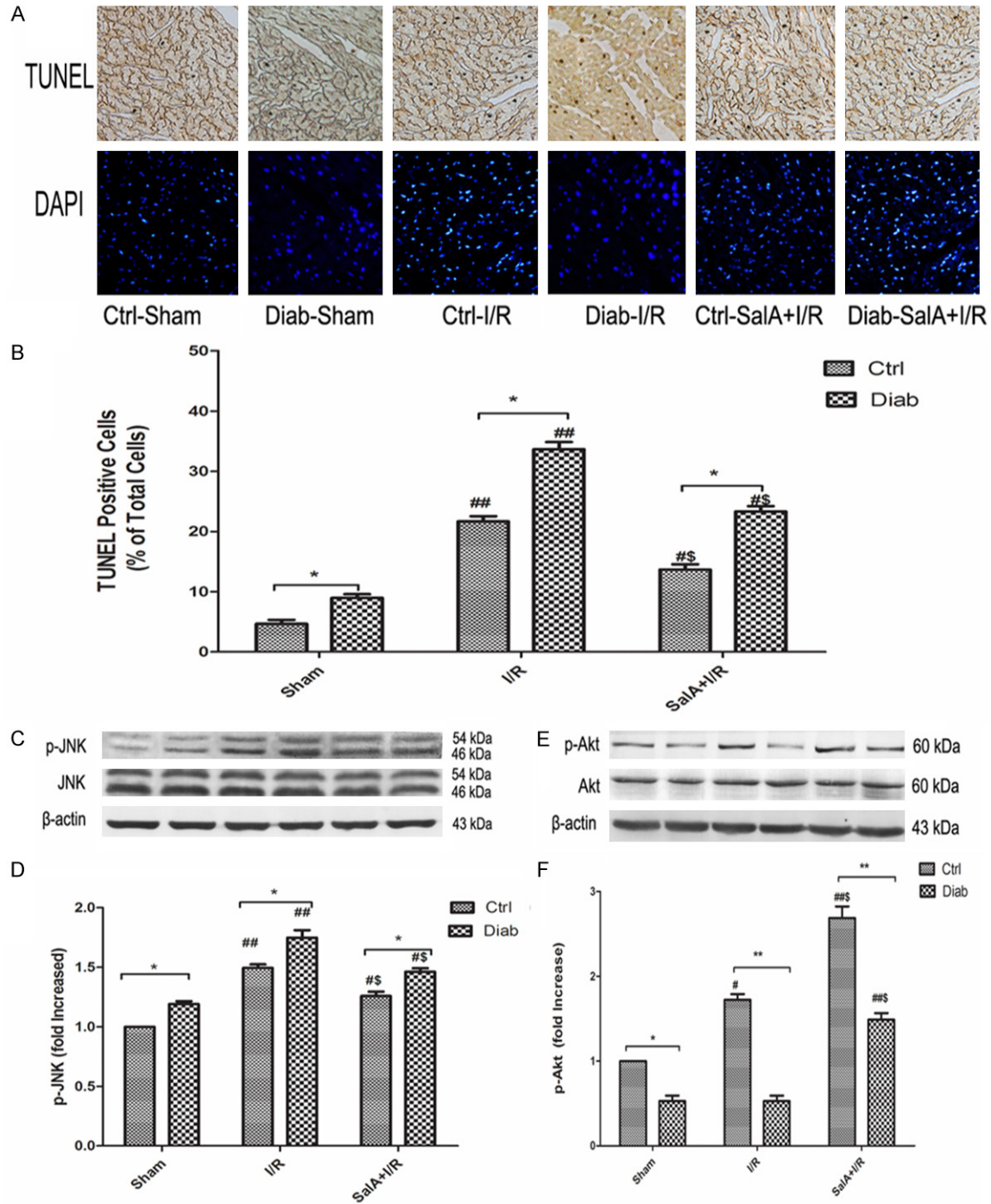
Figure 3. Cell apoptosis rate and the expression of p-Akt, p-JNK. A. TUNEL assay. Total nuclei labeled by DAPI (blue). Apoptotic nuclei detected by TUNEL staining (brown). B. The rate of apoptosis (%) of each group (n = 6 sections/group). C, E. Representative Western blot for p-JNK and p-Akt. D, F. Bar diagrams showing the increased fold in p-JNK and p-Akt. Data represent the mean ± SEM, n=6. $^{*}P<0.05$, $^{**}P<0.01$ Ctrl versus Diab; $^{#}P<0.05$, $^{##}P<0.01$ I/R versus Sham; $^{\$}P<0.05$ SalA+I/R versus I/R.

pase-3 expression, while Bcl-2 expression was increased, resulting in an increased Bcl-2/Bax ratio in cardiac tissue exposed to I/R injury (P<0.05). Pretreatment with SP600125 and Sal A led to a further significant reduction in Bax and cleaved caspase-3 expression, while Bcl-2 expression and the Bcl-2/Bax ratio were increased (p<0.05).
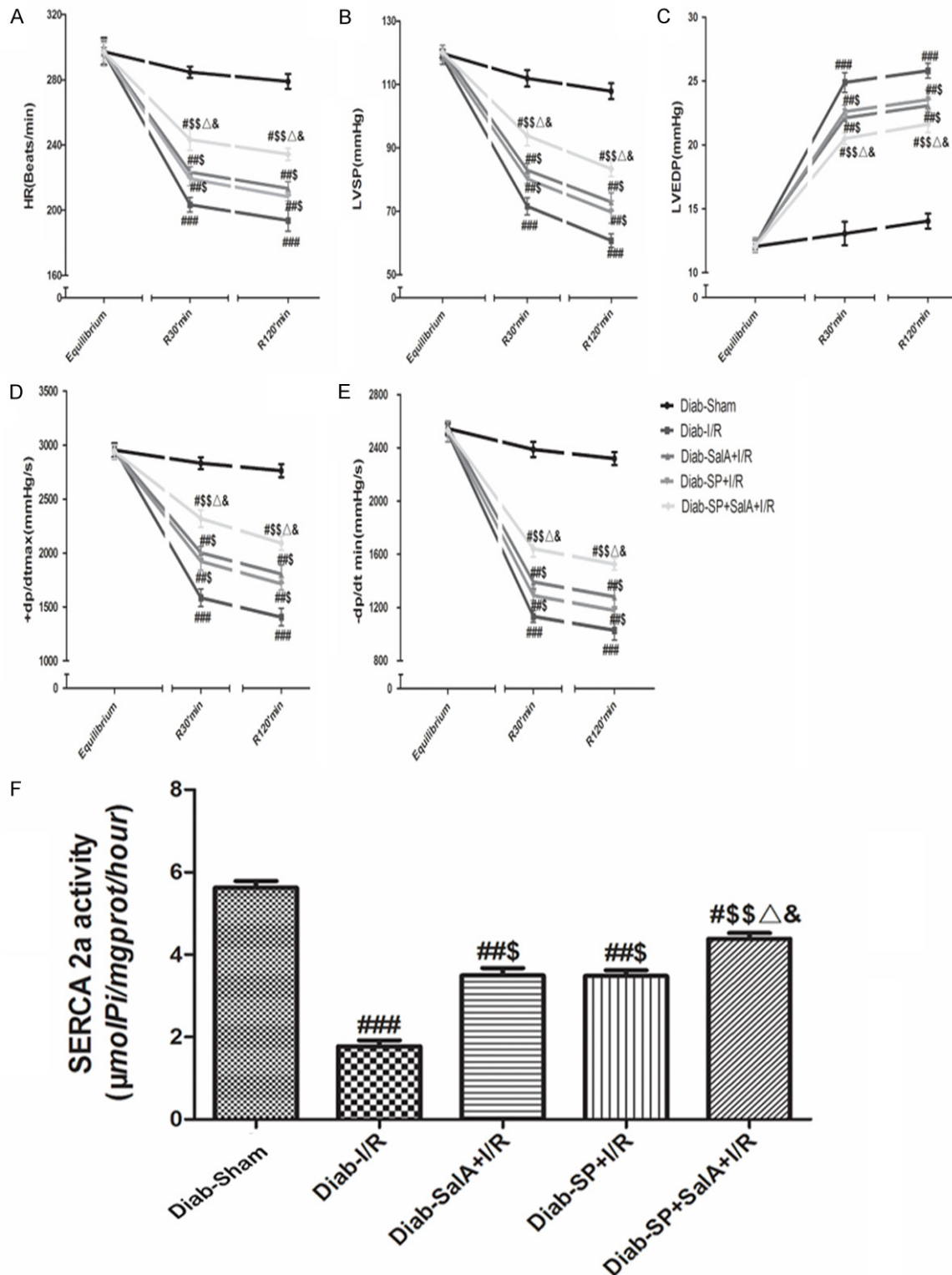
Figure 4. SP600125 and Sal A improved Myocardial Function and SERCA2a activity after I/R in diabetic rats. A-E. Effects of SP600125 and Sal A on various parameters of hemodynamics during a reperfusion period in isolated rat heart preparations. F. Effects of SP600125 and Sal A on SERCA2a activity after ischemia/reperfused myocardium in diabetic rats. The results were expressed as the mean ± SEM, n=3. #P<0.05, ##P<0.01, ###P<0.001 versus Diab-Sham; $P<0.05, $$P<0.01 versus Diab-I/R; ΔP<0.05 versus Diab-Sal A (20 μM)+I/R; &P<0.05 versus Diab-SP (10 μM)+I/R.
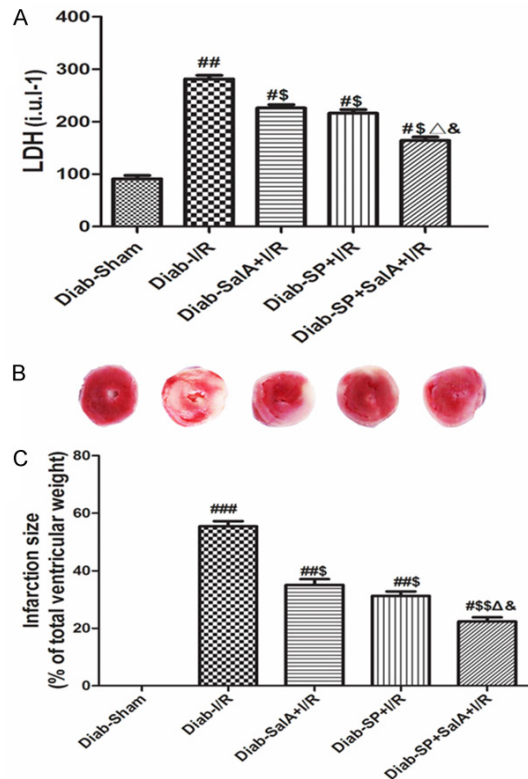
Figure 5. SP600125 and Sal A decreases the LDH release and Infarct size in diabetic rats. Sal A and SP600125 reduced the LDH release (A) and Infarct size (B, C) compared with the I/R group, Administration of SP600125 before SalA markedly alleviated the LDH release and Infarct size compared with Sal A+I/R and SP+I/R. The results were expressed as the mean ± SEM, n=6. #P<0.05, ##P<0.01, ###P<0.001 versus Diab-Sham; $P<0.05 $$P<0.01 versus Diab-I/R; ΔP<0.05 versus Diab-SalA (20 μM)+I/R; &P<0.05 versus Diab-SP (10 μM)+I/R.

Discussion

The reperfusion injury salvage kinase pathway (RISK) includes the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and extracellular signal regulated kinases (ERK 1/2), both of which confer protection against I/R injury [29, 30]. Several studies have revealed that the PI3K/Akt pathway is impaired in diabetic rats after myocardial I/R. Yin et al. demonstrated that diabetes inhibited the myocardial protective effects mediated by preconditioning and post-conditioning during I/R [31]. Miki et al. reported that Akt phosphorylation by EPO and phosphorylation of GSK-3β by ERK were impaired in diabetes [32]. Ghaboura et al. demonstrated that the PI3K/Akt pathway in diabetic rats was altered in insulin resistance and hyperglycemia

[8]. Our results demonstrated that the p-Akt level was increased after myocardial I/R in non-diabetic rats, while there was no difference in diabetic rats. Meanwhile, cardiomyocyte apoptosis, LDH leakage, contractile function and infarction size were worser significantly in diabetic than those of non-diabetic rats. These results suggest that an impaired PI3K/Akt signaling pathway made the diabetic myocardium more vulnerable to I/R injury.

Furthermore, the p-JNK expression levels were significantly increased in diabetic cardiomyopathy, especially in myocardial injury induced by I/R. Insulin resistance and hyperglycemia are closely associated with type 2 diabetes. While the molecular mechanisms of insulin resistance are multiple, recent evidence suggests that attenuation of insulin signaling by JNK could be a central part of the pathobiology of insulin resistance [33]. Taniguchi et al. have demonstrated that endoplasmic reticulum stress could inhibit the PI3K/Akt signaling pathway by increasing the activity of JNK in insulin resistance in liver cells [34]. Masharani et al. found that JNK activation could lower the PI3K/Akt activity during insulin resistance in skeletal muscle [35]. Jia et al. have demonstrated that (-)-epigallocatechin-3-gallate (EGCG) inhibited TNF-α/JNK signaling and increased Akt phosphorylation in the hippocampus of APP/PS1 mice [36]. Therefore, we speculates that JNK activation impairs PI3K/Akt signaling in the diabetic myocardium after I/R. To confirm this hypothesis, the JNK inhibitor SP600125 was utilized in our study. The expression of p-JNK was significantly lower and that of p-Akt significantly higher in diabetic rats than those in the I/R group. We also found that SP600125 effectively attenuated I/R-induced cardiomyocyte apoptosis, LDH leakage and infarct size, increased the Bcl-2/Bax ratio and decreased cleaved caspase-3 expression. It also improved the systolic/diastolic function and SERCA2a activity in diabetic rats, which suggested that inhibiting the activation of JNK led to a partial recovery of PI3K/Akt activity to improve cell apoptosis and myocardial contractility in diabetic rats.

Currently there is a lack of effective drugs to inhibit myocardial I/R injury in clinical practice, so the search for a drug conferring resistance to I/R injury is a top priority. Sal A is one of
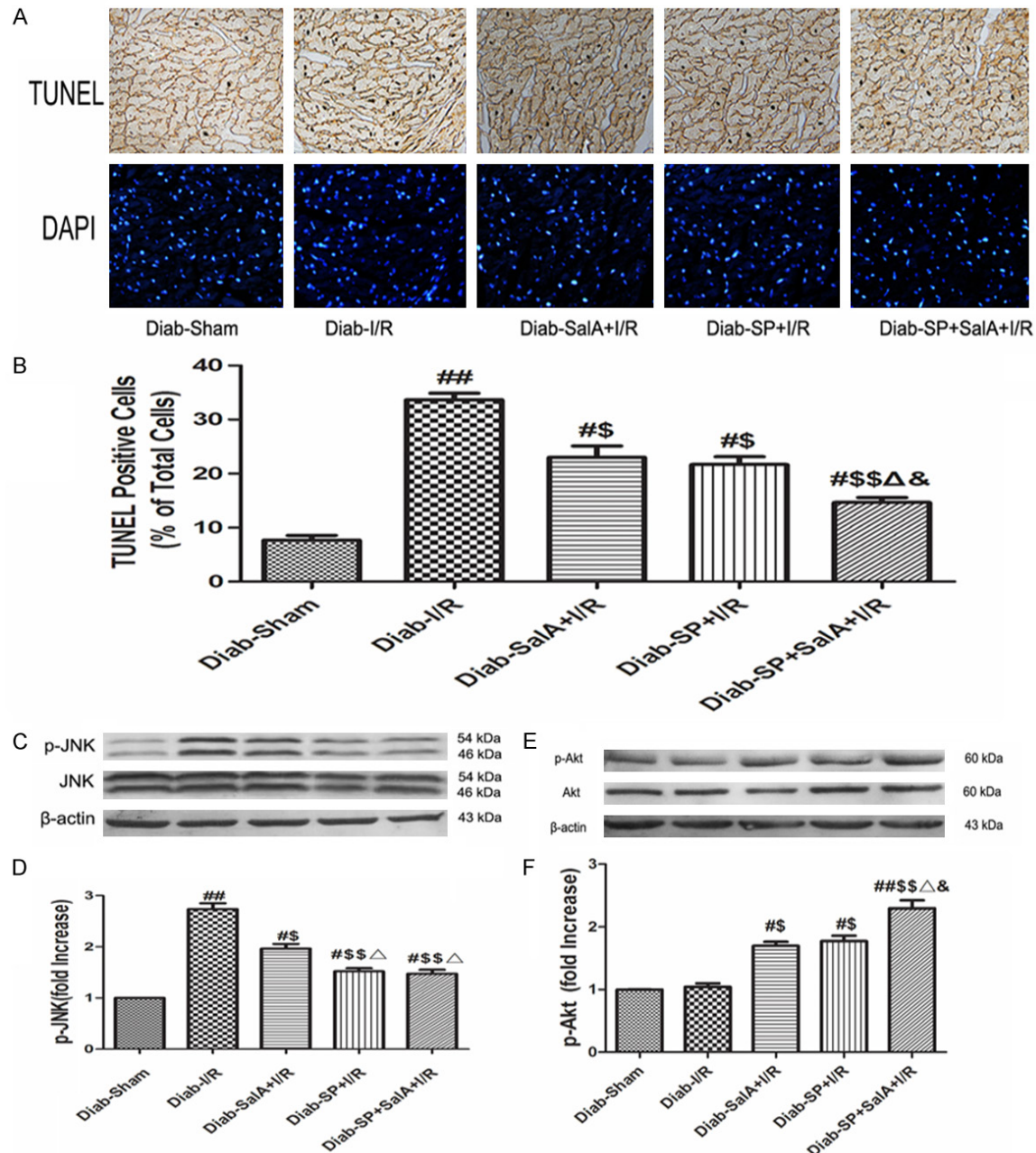
Figure 6. SP600125 and Sal A decreases apoptosis and the expression of p-Akt, p-JNK in diabetic rats with I/R. A, B. Pretreatment with SalA or SP600125 reduced the percentage of apoptotic cells, Administration of SP600125 before SalA markedly alleviated the cell apoptosis compared with Sal A (20 µM)+I/R and SP (10 µM)+I/R. C, E. Representative Western blot for p-JNK and p-Akt. D, F. Bar diagrams showing the increased fold in p-JNK and p-Akt. The results were expressed as the mean ± SEM, n=6. #P<0.05,##P<0.01 versus Diab-Sham; $P<0.05, $$P<0.01 versus Diab-I/R; ΔP<0.05 versus Diab-SalA (20 µM)+I/R; &P<0.05 versus Diab-SP (10 µM)+I/R.

the main active, water-soluble components in *Salvia miltiorrhizae*, which has been widely used in Asian countries for treating various conditions including cerebrovascular disease, coronary artery disease, and myocardial infarction.

Previous work in our laboratory demonstrated that pretreatment with Sal A could confer protective effects on the myocardium during I/R in non-diabetic rats. To the best of our knowledge, Sal A-induced cardioprotection in diabetic I/R injury has never been tested. Our results dem-
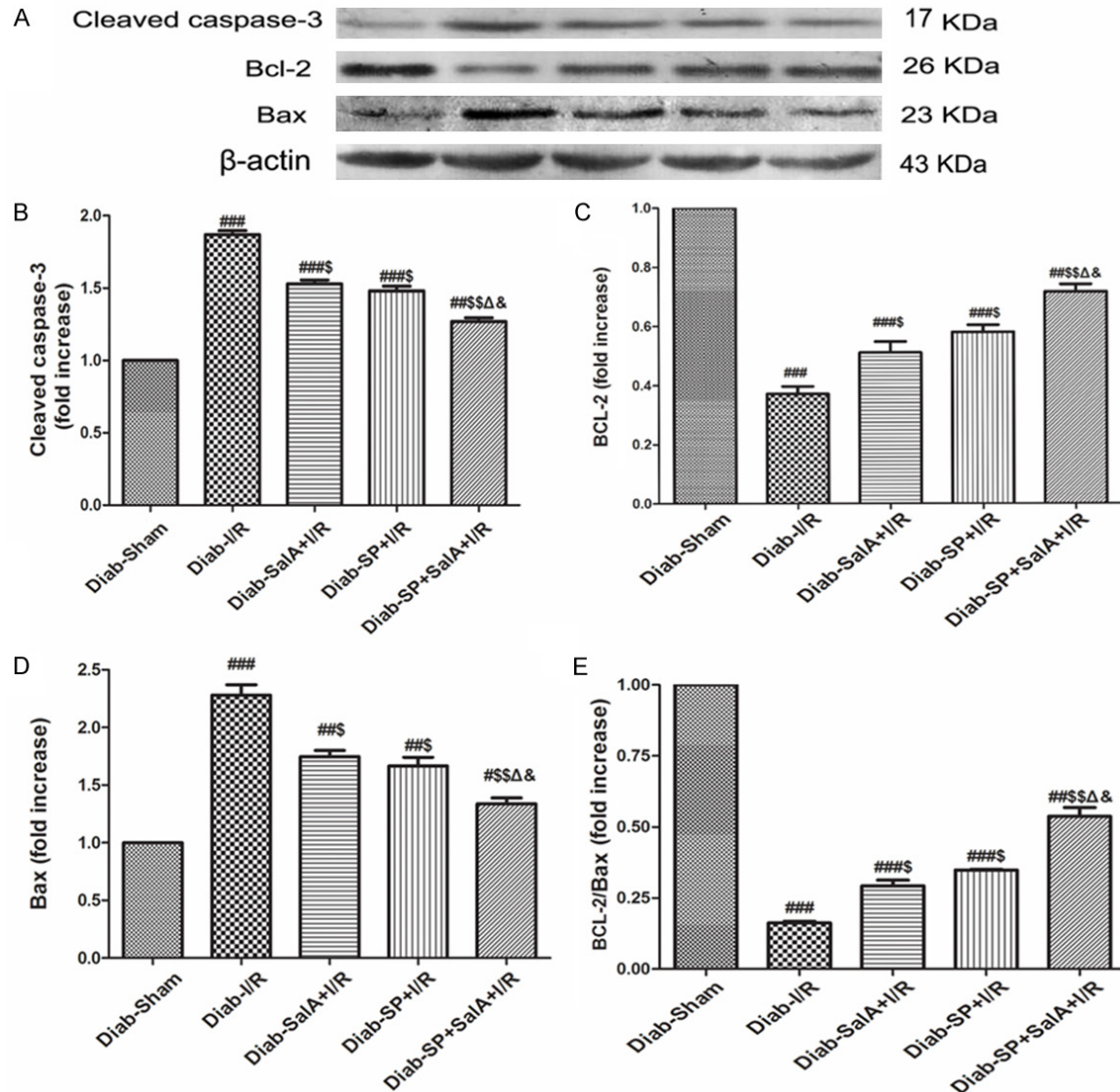
Figure 7. Sal A decreases the expression of pro-apoptotic proteins and increases the antiapoptotic proteins in diabetic rats with I/R. (A) Western blot analysis of activated Cleaved caspase-3, Bcl-2, and Bax protein in myocardial tissues. Quantitative analysis of (B) Cleaved caspase-3, (C) Bcl-2, and (D) Bax Western blots. (E) The ratio of Bcl-2/Bax. The results were expressed as the mean ± SEM, n=3. #P<0.05, ##P<0.01, ###P<0.001 versus Diab-Sham; $P<0.05, $$P<0.01 versus Diab-I/R; ΔP<0.05 versus Diab-SalA (20 μM)+I/R; &P<0.05 versus Diab-SP (10 μM)+I/R.

onstrated that the heart hemodynamic data and LDH activity were lower in the diabetic group than those of the Ctrl group after I/R. Infarct size and myocardial apoptosis rate were higher in the Diab group than those of the Ctrl group, while these indexes were partially reversed by Sal A pretreatment in both groups. This suggests that Sal A provides effective cardioprotection against myocardial I/R injury in diabetic rats. As with SP600125, Sal A effectively attenuated I/R-induced cardiomyocyte apoptosis, LDH leakage and infarct size, increased the SERCA2a activity and Bcl-2/Bax

ratio, and decreased cleaved caspase-3 expression in diabetic rats. However, the combination of Sal A and SP600125 was more effective than either agent alone on the diabetic myocardium after I/R injury. These data indicated that other signaling molecules could be involved in the protection against myocardial I/R injury by Sal A and additional investigations are required.

Among the numerous signaling pathways involved in regulating cell survival, the PI3K/Akt pathway is crucial for protecting the myocardi-

um from I/R injury [37, 38]. SERCA2a is a central regulator of cardiac function, and its overexpression can enhance cardiac contraction and relaxation [39]. Fang et al. demonstrated that luteolin increased the expressions of phospho-Akt, phospho-PLB and SERCA2a, but all three were markedly decreased by pretreatment with the Akt inhibitor LY294002 [6]. Chen et al. demonstrated that endogenous H_2S transiently and reversibly inhibits SR Ca^{2+} uptake in rat heart SR because of the downregulated SERCA2a activity associated with PLB phosphorylation by the PI3K/Akt pathway [40]. Rodent models of both type 1 and type 2 diabetes display reduced SERCA2a function, lengthened relaxation times, and impaired contractility [41], while SERCA2a activity was markedly decreased after myocardial I/R injury [42]. In line with our results concerning the diabetic myocardium, Sal A markedly increased the SERCA 2a activity via JNK/PI3K/Akt signaling and improved systolic/diastolic function in diabetic rats.

The pathogenesis of I/R injury is apparently multifactorial, and myocardial apoptosis is one of the major underlying pathogenic mechanisms [43, 44]. Most studies have suggested that Bcl-2 family members are key regulators of physiological and pathological apoptosis. It has been demonstrated that a high Bax/Bcl-2 ratio is associated with greater vulnerability to apoptosis [45, 46]. Shi et al. demonstrated that Bilobalide prevents apoptosis through activation of the PI3K/Akt pathway in SH-SY5Y cells [47]. Sun et al. indicated that by suppressing cardiomyocyte apoptosis through Akt-dependent mechanisms, acute ischemic injury in the heart in diabetic rats could be ameliorated [48]. Our present results clearly demonstrate that pretreatment with Sal A, via the JNK/Akt signaling pathway, significantly increased the levels of the anti-apoptotic protein Bcl-2, decreased the pro-apoptotic proteins Bax and cleaved caspase-3, and increased the Bcl-2/Bax ratio against myocardial I/R injury in diabetic rats.

In the present study, we provided direct ex vivo evidence that SP600125 inhibits JNK activation and partly restores PI3K/Akt signaling against I/R injury in T2D rats, as evidenced by the significantly improved cardiac function and reduced myocardial apoptosis. Our findings also demonstrated that Sal A attenuates myocardial I/R injury in diabetic rats, possibly through the JNK/PI3K/Akt signaling pathway.

Our study has some limitations. First, we employed a high-fat diet combined with low-dose streptozotocin as a model of T2D in rats to investigate the protective effects of Sal A against myocardial I/R injury. The model mimics the metabolic phenotype in patients, but extrapolation to humans requires further studies. Secondly, we employed SP600125 to inhibit p-JNK expression, but the agent not only inhibits this signaling pathway incompletely but also influences the other signaling molecules. Gene-knockout seems to act directly on potential therapeutic targets in animal models, providing the possibility of a direct relationship to human disease.

In conclusion, these results demonstrate that the JNK/PI3K/Akt signaling pathway is involved in myocardial I/R injury in diabetic rats, and Sal A exerts an anti-apoptotic action against myocardial I/R injury and improves cardiac functional recovery following reperfusion through JNK/PI3K/Akt signaling in this model. The present study can provide important insights into the molecular mechanisms involved in cardioprotective effects in diabetic rats that have undergone I/R injury.

Disclosure of conflict of interest

None.

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