TOTAL SALVIANOLIC ACID INJECTION PREVENTS ISCHEMIA/REPERFUSION-INDUCED MYOCARDIAL INJURY VIA ANTIOXIDANT MECHANISM INVOLVING MITOCHONDRIAL RESPIRATORY CHAIN THROUGH THE UPREGULATION OF SIRTUIN1 AND SIRTUIN3

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ABSTRACT—Sirtuin1 (Sirt1) and Sirtuin3 (Sirt3) are known to participate in regulating mitochondrial function. However, whether Total Salvianolic Acid Injection (TSI) protects against myocardial ischemia/reperfusion (I/R) injury through regulating Sirt1, Sirt3, and mitochondrial respiratory chain complexes is unclear. The aim of this study was to explore the effects of TSI on I/R-induced myocardial injury and the underlying mechanism. Male Sprague–Dawley rats were subjected to 30 min occlusion of the left anterior descending coronary artery followed by 90 min reperfusion with or without TSI treatment (8 mg/kg/h). The results demonstrated that TSI attenuated I/R-induced myocardial injury by the reduced infarct size, recovery of myocardial blood flow, and decreased cardiac apoptosis. Moreover, TSI protected heart from oxidative insults, such as elevation of myeloperoxidase, malondialdehyde, hydrogen peroxide, ROS, as well as attenuated I/R-elicited downregulation of Sirt1, Sirt3, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 10 (NDUFA10), succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA), and restoring mitochondrial respiratory chain complexes activity. The *in vitro* study in H9c2 cells using siRNA transfection further confirmed the critical role of Sirt1 and Sirt3 in the effect of TSI on the expression of NDUFA10 and SDHA. These results demonstrated that TSI attenuated I/R-induced myocardial injury via inhibition of oxidative stress, which was related to the activation of NDUFA10 and SDHA through the upregulation of Sirt1 and Sirt3.

KEYWORDS—Apoptosis, flavoprotein variant, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, Salvia miltiorrhiza Bunge, Sirtuin family, subunit A, succinate dehydrogenase complex

ABBREVIATIONS—AAR—the area at risk; ADP—adenosine diphosphate; AMP—adenosine monophosphate; ATP 5D—adenosine triphosphate synthase δ-subunit; ATP—adenosine triphosphate; Bax—Bcl-2-associated X protein; Bcl-2—B-cell lymphoma 2; ELISA—enzyme-linked immunosorbent assay; ETC—electron transport chain; GAPDH—glyceraldehyde-3-phosphatedehydrogenase; H/R—hypoxia/reoxygenation; H2O2—hydrogen peroxide; I/R—ischemia/reperfusion; LADCA—left anterior descending coronary artery; LV—left ventricle; MBF—myocardial blood flow; MDA—methylenedioxyamphetamine; MPO—myeloperoxidase; MRC—mitochondrial respiratory chain; NAD+—nicotinamide adenine dinucleotide; NDUFA—NADH dehydrogenase [ubiquinone] 1 alpha subcomplex; ROS—reactive oxygen species; Sal B—salvianolic acid B; SDHA—succinate dehydrogenase complex; siRNA—small interfering RNA; Sirt—Sirtuin; subunit A—flavoprotein variant; TSI—Total Salvianolic Acid Injection; TTC—2,3,5-triphenyltetrazolium chloride; TUNEL—terminal-deoxynucleoitidyl transferase mediated nick end labeling

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INTRODUCTION

Coronary heart disease remains a serious threat for the public health worldwide (1), particularly for the people older than 35 years, which accounts for the major cause of death and disability (2). The currently applied percutaneous coronary intervention is effective to recover myocardial perfusion, though, but cannot reduce mortality due to ischemia/reperfusion (I/R) injury (3, 4).

I/R injury is known as a complex disease mediated by a diversity of signaling pathways, among which oxidative stress plays a critical role. Reactive oxygen species (ROS) provokes a variety of insults, including mitochondrial permeability transition pore opening, endoplasmic reticulum stress, calcium
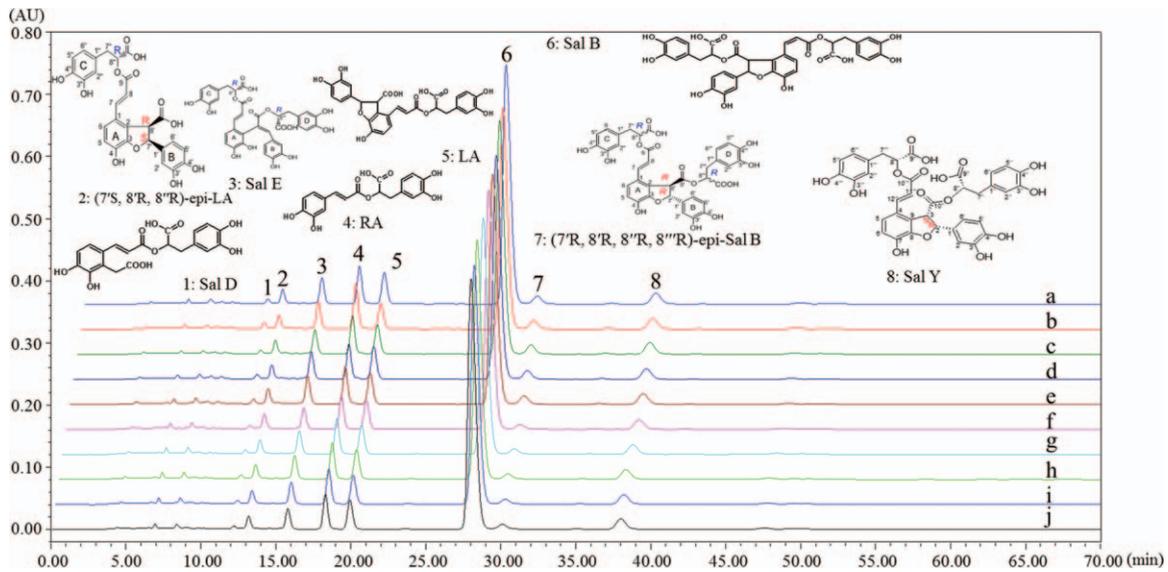
Fig. 1. **The batch numbers and fingerprint of TSI.** (a–j) Represent different batch numbers of TSI from 2015 to 2016. (a) 20151001, (b) 20151002, (c) 20151003, (d) 20151004, (e) 20160202, (f) 20160701, (g) 20160702, (h) 20160703, (i) 20160704, (j) 20160705. Sal D, salvianolic acid D; (7′S, 8′R, 8″R)-epi-LA, (7′S, 8′R, 8″R)-epi-lithospermic acid; Sal E, salvianolic acid; RA, rosmarinic acid; LA, lithospermic acid; Sal B, salvianolic acid B; (7′R, 8′R, 8″R, 8‴R)-epi-Sal B, (7′R, 8′R, 8″R, 8‴R)-epi-salvianolic acid B; Sal Y, salvianolic acid Y.

overload, and DNA damage leading to cardiomyocyte apoptosis (5, 6). In myocardium tissue subjected to I/R challenge, mitochondria are the major source of ROS (7). Electrons escape from complex I and complex III in the dysfunctional mitochondrial respiratory chain (MRC) and react with molecular oxygen generating about 90% of cellular ROS (8). In view of the central role of MRC in I/R damage, protecting mitochondria MRC from dysfunction may be a therapeutic strategy for ameliorating I/R-evoked injury.

Sirtuin1 (Sirt1) is a nicotinamide adenine dinucleotide (NAD$^+$)-dependent deacetylases. Recent studies reveal that changes in Sirt1 expression bring about alternations in mitochondrial genes expression and enzymes activity (9). NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 10 (NDUFA10) is one of the accessory subunits of complex I. Our previous study has proved that the activation of Sirt1 increases NDUFA10 expression, complex I activity, and cardiac mitochondrial function after I/R (10). Sirtuin3 (Sirt3), another member of the NAD$^+$-dependent deacetylases, has been reported to contribute to cardiac dysfunction (11) through multiple mechanisms (12, 13). Our previous study showed that Sirt3 regulates the acetylation of succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA) and NDUFA9, affecting the activity of complex I and complex II, and thus inhibiting the mitochondrial oxidative stress in liver after I/R (14). However, the role of Sirt3 in myocardial I/R injury remains unknown.

Total Salvianolic Acid Injection (TSI) is a preparation of active components of Salvia miltiorrhiza Bunge, which was approved by the Chinese State Food and Drug Administration (Z20110011) in 2011 for the prevention and treatment of ischemic stroke (15). TSI is produced by standardized procedures with each batch of products having quality assurance (Fig. 1 for further details). Our previous study has demonstrated

the ability of TSI to improve I/R-induced microcirculation disturbance in rat mesentery, which was found to correlate with inhibiting the production of oxygen free radicals and the expression of adhesion molecules CD11b/CD18 (16). TSI was also reported to attenuate I/R-induced brain injury in rats, which was attributable to its antioxidant activity via inactivation of nicotinamide adenine dinucleotide phosphate-oxidase through adenosine 5′-monophosphate activated protein kinase/protein kinase B/protein kinase C signaling pathway (17). However, the effects of TSI on I/R-induced myocardial injury were so far unknown.

Sal B, the major ingredient of TSI, was reported to protect inflammation and apoptosis through activation of Sirt1, thus attenuate liver injury (18) and brain injury (19). However, whether Sal B can regulate the expression of Sirt1 to improve the cardiac MRC complexes activity after I/R has never been reported. Besides, no study is available to date concerning the effect of TSI or Sal B on Sirt3. The purpose of this study was to explore the effects of TSI on I/R-induced myocardial injury and the underlying mechanism, with emphasis on its regulation of Sirt1 and Sirt3 expression and MRC complexes activity.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (250 ± 10 g) were provided by the Animal Center of Peking University with certificate number of SCXK (Jing) 2006-0008. The condition for housing the animals was kept at temperature $22 \pm 2°$C and humidity $40 \pm 5\%$ with a 12-h light/dark cycle. The animals were allowed to access standard diet and water *ad libitum* and fasted for 12 h before experiment. All animal operations conformed to Guide of Peking University Animal Research Committee. All experimental procedures involving animals were approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2010-001), complying with the Guide for the Care and Use of Laboratory Animals (NIH Publication 85–23, 1996).

Table 1. **Number of animals in each group for different experiments**

	Sham	Sham + TSI	I/R	I/R + TSI	Total
Myocardial infarct size and MBF	6	6	6	6	24
Confocal microscopy	3	3	3	3	12
Electron microscopy	2	2	2	2	8
ELISA assay	6	6	6	6	24
Western blotting assay	6	6	6	6	24
Total	23	23	23	23	92

The same animals were used for detection of myocardial infarct size and MBF.
Sham, Sham group; Sham + TSI, Sham plus TSI treatment group; I/R, I/R group; I/R + TSI, I/R plus TSI treatment group.

Acute myocardial I/R model and experiment group

After being anesthetized with 2% pentobarbital sodium (60 mg/kg) via peritoneal injection, the animals were placed in a supine position. Before operation, a tracheal cannula was inserted via mouth, which was connected to an animal breathing apparatus (ALC-V8, Shanghai Alcott Biotech Co., China). The breathing apparatus was set at the breathing ratio 1:1, the frequency 75/min, and tidal volume 12 mL/kg. The animals maintained on ventilation throughout the ischemia and reperfusion. The heart was exposed by thoracotomy, myocardial I/R was achieved by ligation of the proximal left anterior descending coronary artery (LADCA) with a 5/0 silk for 30 min and followed by reperfusion for 90 min. The animals in sham groups underwent the same procedure except for the ligation of LADCA.

A total of 92 rats were enrolled in this study, which were randomly assigned to four groups: Sham group, Sham + TSI group, I/R group, and I/R + TSI group, 23 animals in each (Table 1). The animals in TSI-treated groups received TSI (Tasly Pharmaceutical Co Ltd, Tianjin, China, dissolved in saline making a solution of 1 mg/mL before use) at 8 mg/kg 2 min before the initiation of reperfusion via femoral vein at a speed of 2 mL/h. In Sham group and I/R group, rats were given equivalent volume of saline in the same way. All indicators were examined at 90 min after reperfusion in this study.

Myocardial infarct size

LADCA was ligated again 90 min after reperfusion, 2 mL of 4% Evans blue (Sigma, St. Louis, Mo) was injected via femoral vein for assessment of myocardial infarct size. Hearts were resected and cut into five slices of 1 mm thick from the apex cordis to the ligation site parallel to the atrioventricular groove. The slices were immersed in a 0.375% solution of 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) at 37°C for 5 min. A stereomicroscope connected with Digital Sight (DS-5M-U; Nikon, Nanjing, Jiangsu, China) was applied to evaluate and photograph the slices, wherein infarct area was stained white, the area at risk (AAR) was pink, and normal area was blue. The areas of infarct, AAR, and left ventricle (LV) on each slice were measured by Image-Pro Plus 6.0 (Media Cybernetic, Bethesda, Md) (n = 6). The percentage of AAR/LV (%) and infarct area/AAR (%) from five slices were averaged and used to assess the degree of myocardial infarction (20).

Myocardial blood flow

Determination of myocardial blood flow (MBF) was undertaken on rat's open chest using a Laser-Doppler Perfusion Imager (PeriScan PIM3 System, Perimed, Stockholm, Sweden) at baseline, 0, 30, 60, and 90 min after reperfusion (21). Briefly, the scanner head was kept in parallel to the surface of exposed heart at a distance of 18 cm and directed low-powered He−Ne laser beam to irradiate the tissue to a depth of 0.5 mm, successively. The room temperature was kept at 28°C; each scan took about 10 s. The acquired color-coded images were displayed on a video monitor and evaluated with the software LDPIwin 3.1 (PeriScan PIM3 System, Perimed, Stockholm, Sweden), in which blue to red denoted low to high MBF, respectively. Results were expressed as percentages of the baseline MBF.

Immunohistochemistry

Hearts were excised at 90 min of reperfusion, fixed in 4% paraformaldehyde for 48 h, and then further prepared for paraffin sectioning. The area of left ventricle was selected for immunohistochemistry. Rhodamine phalloidine (R415) (Life Technologies, Carlsbad, Calif) and cell death detection kit (Fluorescein dUTP Kit) (Roche, Indianapolis, Ind) were applied to stain F-actin and terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) positive cells, respectively, whereas Hoechst 33342

(Invitrogen, St. Louis, Mo) was used to stain the nuclei (22), according to the manufacturer's instructions. Five fields were selected randomly in each section and observed with a laser scanning confocal microscope (TCS SP5, Leica, Mannheim, Germany). The numbers of the TUNEL-positive cardiac myocytes in five fields were counted and expressed as mean positive cell number per field. The TUNEL-positive leukocytes were not counted (23).

Electron microscopy

Hearts were infused with 3% glutaraldehyde for 40 min. Left ventricles were excised and trimmed into blocks less than 1 mm^3, and further fixed overnight in the same fixative at 4°C. Following washing 3 times with 0.1 mol/L phosphate-buffered solution, the samples were postfixed with 1% osmium tetraoxide for 2 h, and prepared for ultrathin sections. A transmission electron microscope (JEM 1400 plus, JEOL, Tokyo, Japan) was applied to observe the ultrastructure of myocardium (14).

Enzyme-linked immunosorbent assay

The heart tissue from left ventricle was sampled at about 2 mm under ligature at 90 min of reperfusion, quickly frozen in liquid nitrogen, and then stored at −80°C. Total protein was extracted using a protein extraction kit (Applygen Technologies, Beijing, China). Enzyme-linked immunosorbent assay (ELISA) was undertaken using a specific kit indicated, respectively, to determine the levels of cardiac tissue myeloperoxidase (MPO) (Hycult Biotech, Plymouth, Pa), malondialdehyde (MDA) (Abcam, Cambridge, Mass), hydrogen peroxide (H_2O_2) (Cell Biolabs, San Diego, Caif), ROS (Cell Biolabs), complex I, II, IV, V activity (Abcam), adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) (BD, Tianjin, China), by microplate reader (Multiskan MK3, Thermo, USA). Each test was performed according to the manufacturer's instructions (14).

Western blot assay

Using a protein extraction kit (Applygen Technologies), total protein was extracted and the concentration of protein was determined by a BCA protein assay kit (Applygen Technologies). Western blot analysis was performed routinely (10), with primary antibodies against NDUFA10 (Santa Cruz, Santa Cruz, Calif), adenosine triphosphate synthase δ-subunit (ATP 5D) (Abcam), Sirt1, Sirt3, SDHA, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), cleaved caspase-3, and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, Mass). Enhanced chemiluminescence detection kit (Applygen Technologies, Beijing, China) was used to detect the bands. Band intensity was expressed as mean area density using Quantity One image analyzer software (Bio-Rad, Richmond, Calif) for quantification.

Cell culture and hypoxia/reoxygenation model

H9c2, a rat cardiac myoblast cell line (ATCC, Manassas, Va), was cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, Calif) containing 4 mM L-glutamine, 4.5 g/L glucose, and 10% fetal bovine serum (Invitrogen) at 37°C in a humidified incubator with 95% air and 5% CO_2.

The cells were subjected to hypoxia/reoxygenation (H/R) by incubation in a microaerophilic system (Thermo Scientific, Waltham, Mass) at 5% CO_2 and 1% O_2 for 6 h followed by transferring to and incubation in a normoxic condition (95% air and 5% CO_2) for 18 h with or without 0.013 mg/mL TSI (10). The concentration of TSI used in vitro study was determined based on a preliminary study (Supplement Figure 2, http://links.lww.com/SHK/A756).
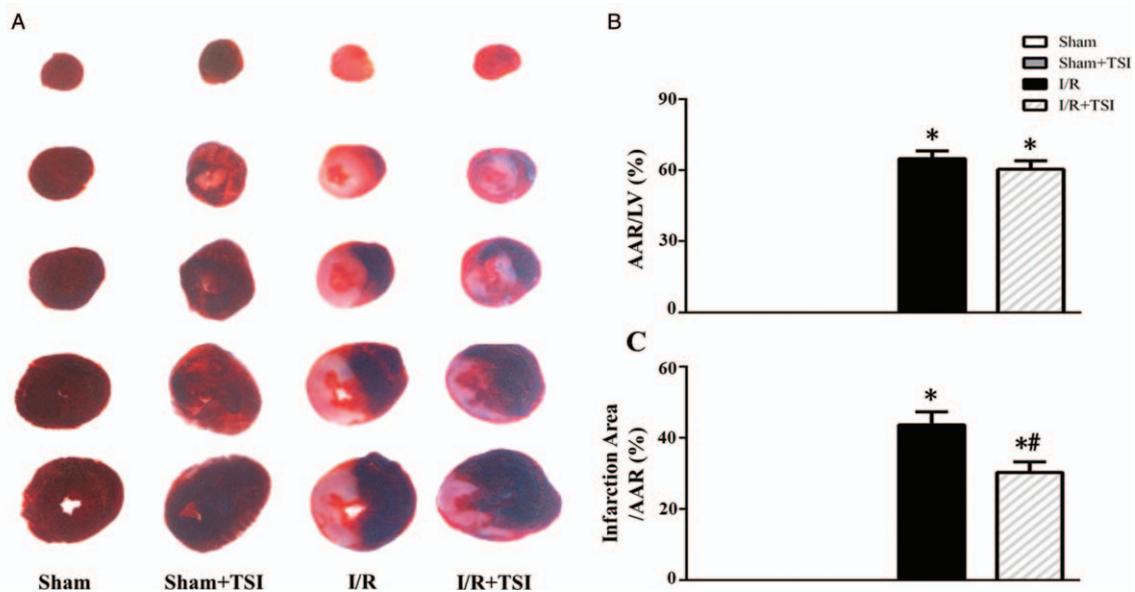
Fig. 2. **Effect of TSI on rat myocardial infarct size after I/R.** (A) Representative images of heart slices stained with Evans blue-TTC in Sham group, Sham + TSI group, I/R group, and I/R + TSI group at 90 min after reperfusion. The infarct zone is white, the normal zone is blue, and the AAR is pink. (B, C) Quantitative evaluation of AAR/LV and infarct area/AAR at 90 min after I/R in various groups. Data are presented as mean ± SEM (n = 6). $^*P < 0.05$ vs. Sham group; $^\#P < 0.05$ vs. I/R group.

Small interfering RNA transfection

H9c2 cells were transfected with Sirt1 siRNA (sc-108043) or control siRNA with Sirt1 siRNA Transfection Reagent (Santa Cruz) when cells proliferated to 50% to 60% confluence, according to manufacturer's instructions. Similarly, H9c2 cells were transfected with Sirt3 siRNA or control siRNA with Lipofectamine 2000 (Invitrogen). The sense sequences of Sirt3 siRNA was as follows: 5′-UUC UCC GAA CGU GUC ACG UTT-3′ (Genepharma, Shanghai, China) (10).

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls test or using two-way ANOVA (MBF) followed by Bonferroni for multiple comparisons. Data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc.) and expressed as mean ± SEM. A P value less than 0.05 was considered to be statistically significant.

RESULTS

TSI reduces I/R-induced myocardial infarct size

After 90 min of reperfusion, myocardial infarct was assessed by Evans blue-TTC staining. Displayed in Figure 2A are the representative images from various groups. Apparently, no infarct was visible in myocardial tissues from Sham group and Sham + TSI group. In contrast, infarct areas were obvious in myocardial tissue in I/R group, whereas the heart slices from TSI treatment groups had smaller infarct areas but similar ischemic areas, as compared with I/R group. This impression was proved by quantitative analysis of AAR/LV and infarct area/AAR (Fig. 2, B and C), showing that AAR/LV significantly increased in I/R group compared with Sham group, whereas no significant difference in AAR/LV was detected between I/R and I/R + TSI groups. Infarct area/AAR in the I/R group increased pronouncedly alike compared with Sham group, which, however, was attenuated significantly by treatment with TSI, demonstrating the protection of TSI on I/R-induced myocardium infarct. The beneficial role of TSI on I/R-

induced myocardium infarct was also observed after reperfusion for 24 h (Supplement Figure 1, http://links.lww.com/SHK/A756).

TSI attenuates I/R-induced myocardial apoptosis

Double staining of F-actin and TUNEL was applied to assess the effect of TSI on the I/R-induced alteration in myocardial structure and apoptosis, and the results are displayed in Figure 3. As expected, a large number of TUNEL-positive cardiomyocytes (green) were detected at 90 min after reperfusion in I/R group, which were decreased considerably in TSI treatment group. However, F-actin stained with rhodamine phalloidine in I/R group showed severe rupture and abundant actin bundle formation, which was not apparently alleviated by TSI treatment (Fig. 3A). The statistical result of the percentage of TUNEL-positive cardiomyocytes in infarction surrounding areas validated the qualitative survey (Fig. 3B).

To verify the results acquired by TUNEL staining, apoptosis regulating proteins Bcl-2, Bax and cleaved caspase-3 were assessed in each group by Western blot (Fig. 3, C and D). As an antiapoptosis factor, Bcl-2 decreased significantly after I/R, whereas, the levels of proapoptotic molecules Bax and cleaved caspase-3 increased in response to I/R challenge. Noticeably, TSI significantly inhibited I/R-induced the changes in these proteins, demonstrating the potential of TSI to attenuate the I/R-induced apoptosis in myocardium.

TSI reduces I/R-induced oxidative stress

ELISA test revealed that I/R exposure evoked an obvious oxidative stress in rat myocardial tissue, as indicated by the significant increase in the level of MPO, ROS, and relative less, but still significant increase in the level of MDA, H_2O_2 in I/R group, compared with Sham groups. All the alterations above were attenuated by TSI treatment after I/R, maintained the level
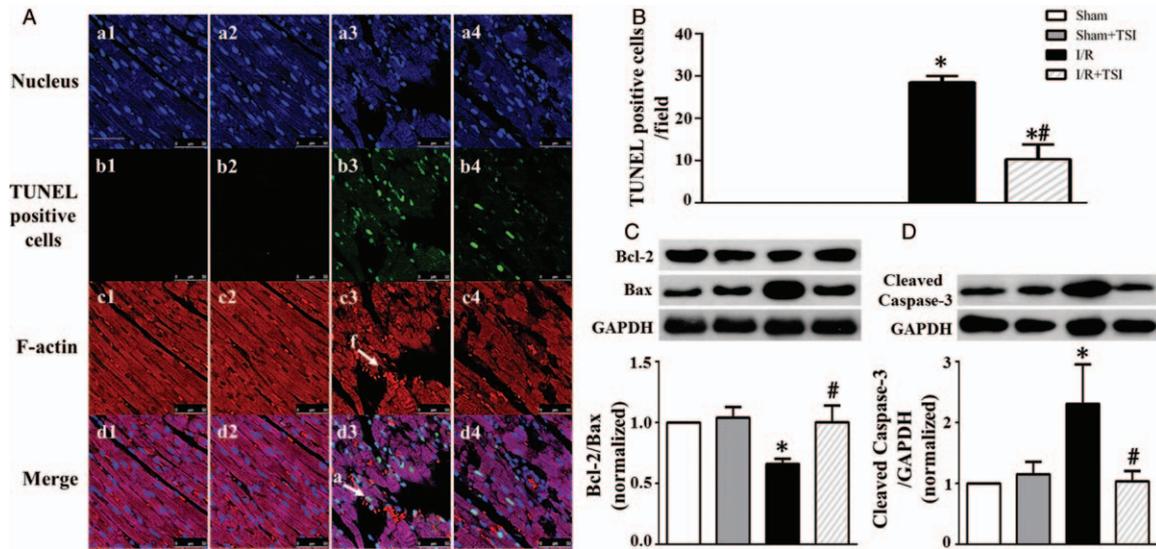
FIG. 3. **Effect of TSI on myocardial apoptosis and apoptosis-related protein expression after I/R.** (A) The representative photographs of rat myocardium of double stained with F-actin and TUNEL. Nuclei are stained blue, F-actin red, and TUNEL-positive cells green. (a1, b1, c1, d1), Sham group; (a2, b2, c2, d2), Sham + TSI group; (a3, b3, c3, d3), I/R group; (a4, b4, c4, d4), I/R + TSI group. a: TUNEL-positive apoptotic cell, f: disrupted myocardial fiber. Bar = 50 μm. (B) Quantification of myocardial apoptosis in different groups, n = 3. (C) The expression of Bcl-2 and Bax determined by Western blot and respective quantification in different groups. (D) The expression of cleaved caspase-3 determined by Western blot and respective quantification in different groups. All the quantifications were undertaken based on the data of six animals and normalized to GAPDH. All band intensities were calculated based on the results from three independent experiments. Data are expressed as mean ± SEM. n = 3 in (A and B), n = 6 in (C and D). $^*P < 0.05$ vs. Sham group; $^\#P < 0.05$ vs. I/R group.

of MDA, H_2O_2 and ROS at that of Sham group, and reducing the level of MPO to 60% of that in I/R group (Fig. 4). Nevertheless, the clinical significance of the effect of TSI on MDA and H_2O_2 remains ambiguous because only a small degree of increase in these two variables were observed in response to I/R challenge.

TSI improves I/R-induced ultrastructure injury of mitochondria in myocardium

Mitochondrial ultrastructure in myocardium was observed by transmission electron microscopy. As shown in Figure 5, I/R challenge caused a dramatic injury in mitochondrial ultrastructure, such as disordered mitochondrion distribution, disarranged and obscure crista, and vacuoles within the matrix. Noticeably, these changes were markedly improved by TSI treatment.

TSI mitigates I/R-induced changes in the activity of MRC complexes

We tested by ELISA the activity of complex I, II, IV, and V (ATP synthase) in myocardial tissues of different groups, and found that the activity of complex I, II, IV, and V were suppressed to various degrees in I/R group compared with Sham groups. Interestingly, TSI treatment significantly restored the activity of complex I, II, and IV (Fig. 6, A–C), but not Complex V (Fig. 6D), after I/R.

TSI increases the expression of Sirt1, NDUFA10, Sirt3, and SDHA

To further gain insight into the mechanism responsible for the beneficial role of TSI in the dysfunctional complex I and II after I/R, the expression of NDUFA10, SDHA, Sirt1, and Sirt3 was assessed in different groups by western blot, among which

NDUFA10 and SDHA each is one of the subunits of complex I and complex II, respectively, whereas latter two are the upstream enzyme of each. As shown in Figure 7, the expression of NDUFA10 and SDHA decreased significantly after I/R, accounting for the decreased activity of complex I and II in the circumstance. Parallel with this result, the expression of Sirt1 and Sirt3 significantly decreased in I/R group compared with Sham group, implying the involvement of the two enzymes in the I/R-elicited mitochondrial dysfunction. Noticeably, TSI treatment alleviated the downregulation of NDUFA10 and SDHA, as well as Sirt1 and Sirt3, after I/R, suggesting the latter two as the targets of TSI. The implication of Sirt1 and Sirt3 in TSI effect was further verified by in vitro study, showing that TSI treatment attenuated cell viability after H/R, which was abolished by Sirt1 siRNA or Sirt3 siRNA (Supplement Figure 3, http://links.lww.com/SHK/A756)

TSI has no effect on I/R-induced energy metabolism disorder

The ratios of ATP/ADP and ATP/AMP in cardiac tissue were determined by ELISA to assess the energy metabolism. Compared with Sham group, I/R challenge decreased ATP/ADP and ATP/AMP considerably, indicative of a disorder in the balance of energy metabolism. TSI treatment had no effect on either ATP/ADP or ATP/AMP after I/R (Fig. 8, A and B). The protein expression of ATP 5D in cardiac tissue from different groups were then determined. As shown in Figure 8C, ATP 5D expression reduced significantly after I/R in comparison with Sham group, which was not prevented by treatment with TSI.

TSI recovers MBF during I/R

Figure 9A presents the representative images of MBF in different groups assessed by the Laser Scanning Doppler at
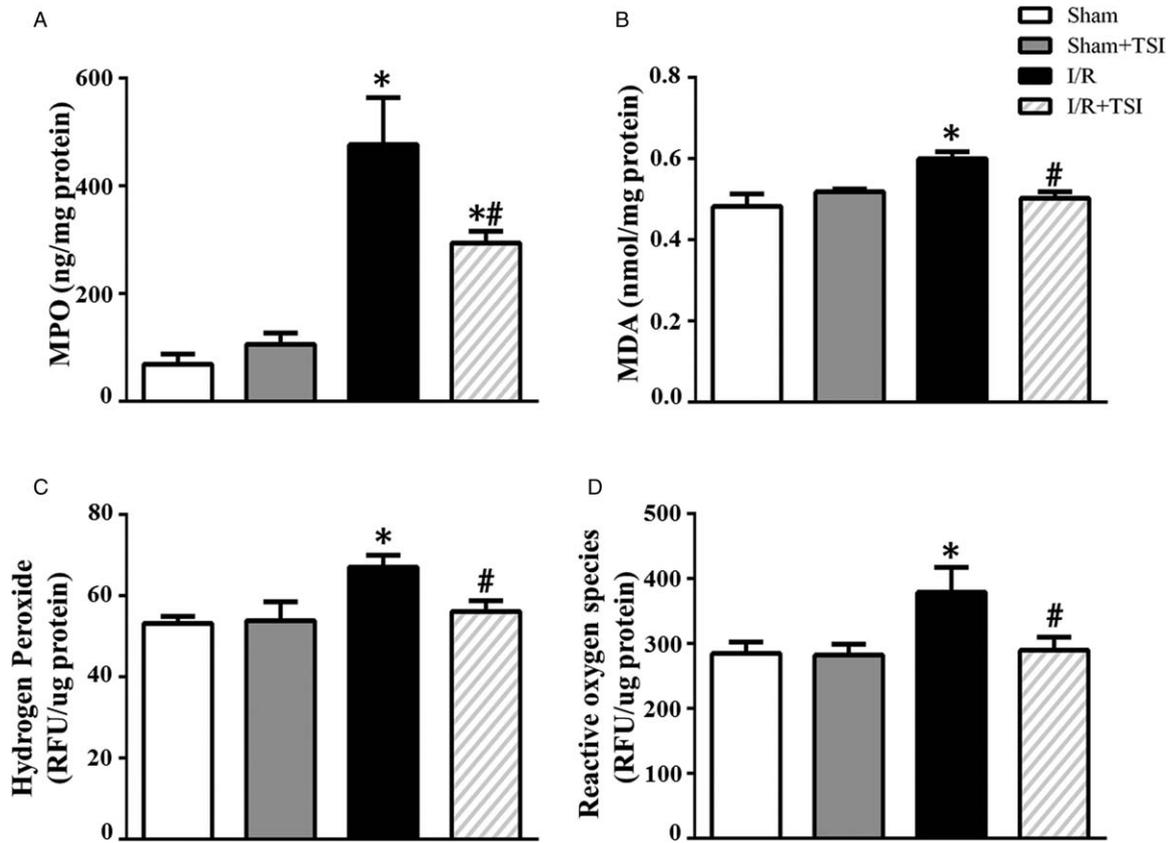
A

B

C

D

Fig. 4.   **Effects of TSI on MPO, MDA, H$_2$O$_2$, and ROS expression tested by ELISA in rat myocardial tissue after I/R.** (A) MPO expression. (B) MDA expression. (C) H$_2$O$_2$ production. (D) ROS production. Data are presented as mean ± SEM (n = 6). $^*P < 0.05$ vs. Sham group; $^\#P < 0.05$ vs. I/R group.

different time points. MBF in Sham group and Sham + TSI group did not vary significantly over I/R period. Obviously, ischemia induced a prominent decrease of MBF in I/R group, which sustained till the end of the observation. TSI prevented the I/R-induced decrease of MBF.

The time courses of MBF changes in different groups are plotted in Figure 9B, verifying the results from inspection of the images. MBF in I/R group decreased to nearly 50% of baseline immediately after ischemia, which did not recover completely at 90 min after reperfusion. TSI treatment significantly
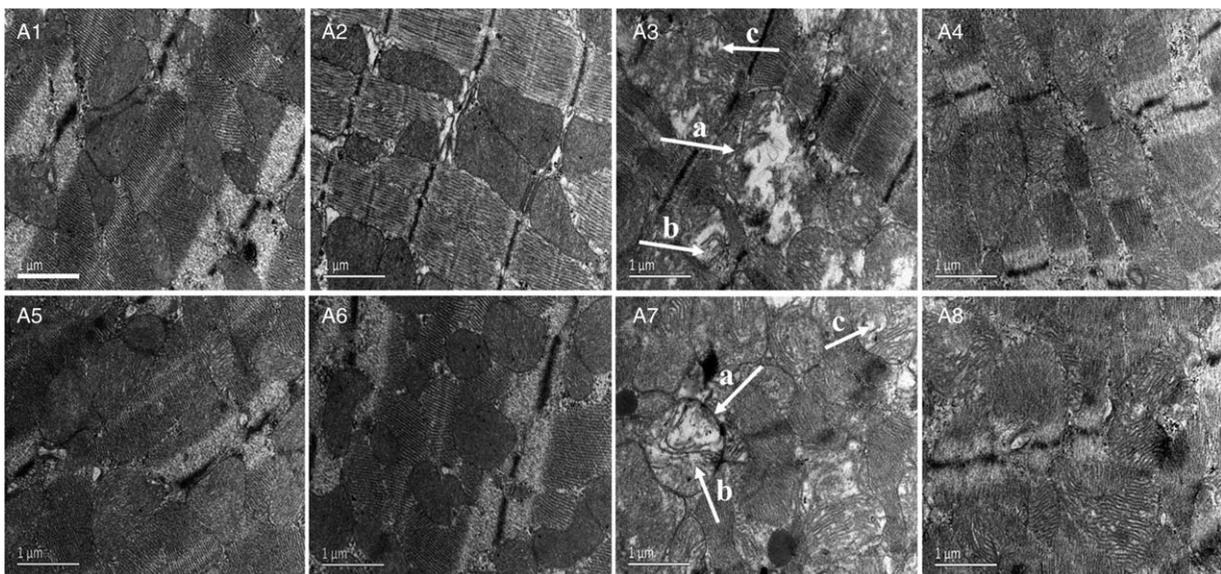

Fig. 5.   **Effect of TSI on myocardium ultrastructure after I/R.** Presented are the representative electron micrographs of myocardium from various groups. (A1, A5), Sham group; (A2, A6), Sham + TSI group; (A3, A7), I/R group; (A4, A8), I/R + TSI group. a: Swelling mitochondria, b: disarranged and obscure crista, c: vacuoles among crista. Bar = 1 μm. n = 2.
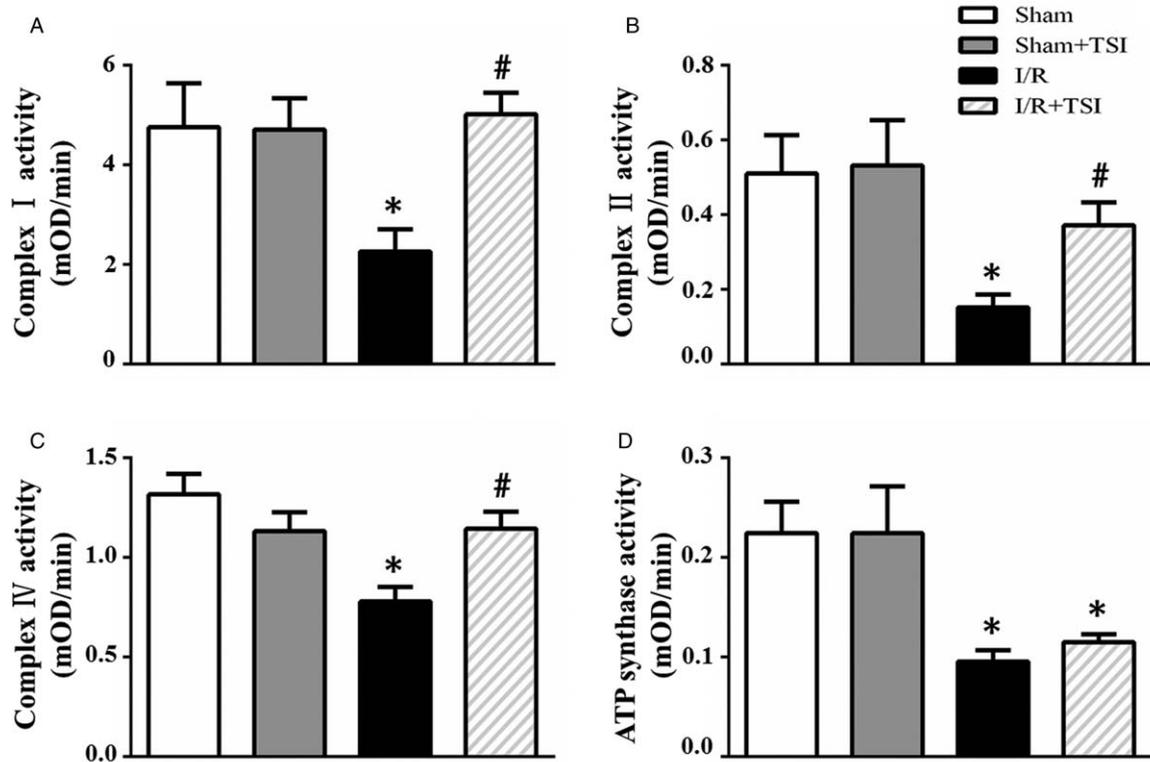
FIG. 6.   **Effects of TSI on the activity of MRC complexes in rat myocardial tissue tested by ELISA after I/R.** (A) Complex I activity, (B) complex II activity, (C) complex IV activity, and (D) ATP synthase activity. Data are presented as mean ± SEM (n = 6). *$P < 0.05$ vs. Sham group; #$P < 0.05$ vs. I/R group.
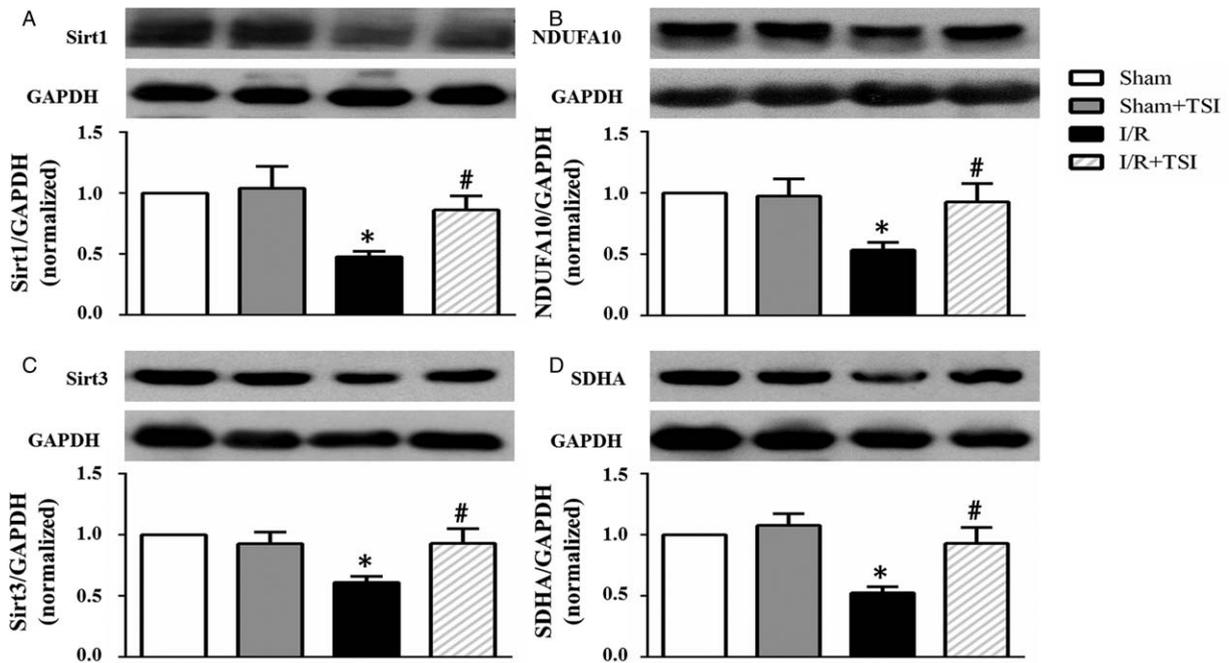

FIG. 7.   **Effects of TSI on the expression of Sirt1, NDUFA10, Sirt3, and SDHA in rat myocardium subjected to I/R.** (A–D) Representative western blot bands of Sirt1 (A), NDUFA10 (B), Sirt3 (C), and SDHA (D) in various groups shown above and quantitative results below. All the quantifications were undertaken based on the data of six animals and normalized to GAPDH. All band intensities were calculated based on the results from three independent experiments. Data are expressed as mean ± SEM (n = 6). *$P < 0.05$ vs. Sham group; #$P < 0.05$ vs. I/R group.
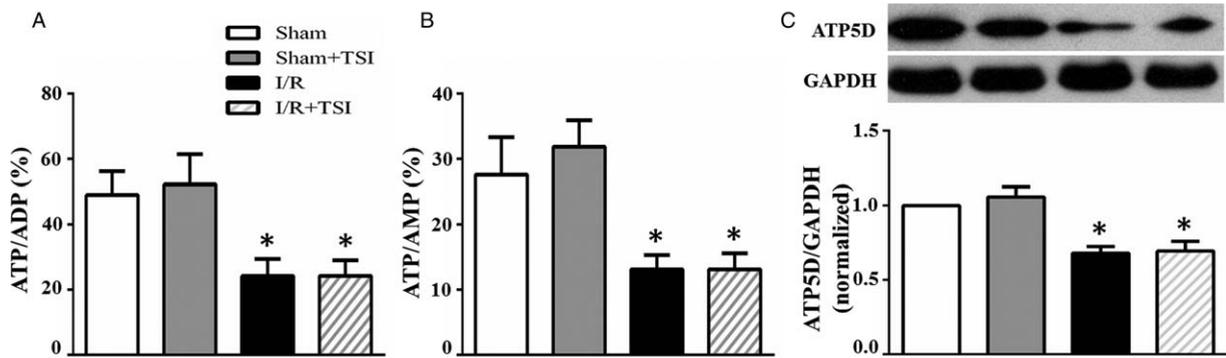
Fig. 8.   **Effect of TSI on energy metabolism after I/R.** (A, B) The effects of TSI on the ratios of ATP/ADP and ATP/AMP in myocardium in different groups tested by ELISA. (C) Western blot band of ATP 5D and respective quantification in different groups. All the quantifications were undertaken based on the data of six animals and normalized to GAPDH. All band intensities were calculated based on the results from three independent experiments. Data are expressed as mean $\pm$ SEM (n = 6). $^*P < 0.05$ vs. Sham group; $^\#P < 0.05$ vs. I/R group.

attenuated the decrease of MBF at 30 min after reperfusion; this effect persisted until 90 min after reperfusion. However, MBF in I/R + TSI group was still at least 20% less than Sham group, so the clinical significance needs to be validated by more research.

The protection of TSI against decreased NDUFA10 expression is mediated by Sirt1

Western blot showed that treatment with Sirt1 siRNA considerably reduced the protein level of Sirt1 in both normal H9c2 cells and the H9c2 cells exposed to H/R, demonstrating the effectiveness of transfection (Fig. 10, A and B). As expected, H/R led to a decrease in NDUFA10 expression, which was restrained by treatment with TSI (Fig. 10C). Impressively, this protective effect of TSI was abolished by Sirt1 siRNA transfection (Fig. 10C), indicating the critical role of Sirt1 in TSI action.

Sirt3 is required for the protection of TSI against decreased SDHA expression

Similarly, Sirt3 siRNA transfection considerably reduced Sirt3 expression in both normal H9c2 cells and that exposed to H/R, as demonstrated by western blot (Fig. 11, A and B). Using this approach, we tested the role of Sirt3 in the effect of TSI on the expression of SDHA, the subunit of complex II. The result showed that the protein level of SDHA (Fig. 11C) was considerably decreased after H/R compared with control cells. TSI treatment restored the expression of SDHA (Fig. 11C), whereas Sirt3 siRNA transfection abolished the protective role of TSI in both cases.

DISCUSSION

The present study confirmed the potential of TSI to attenuate the I/R-induced myocardial injury, as shown by the reduction of
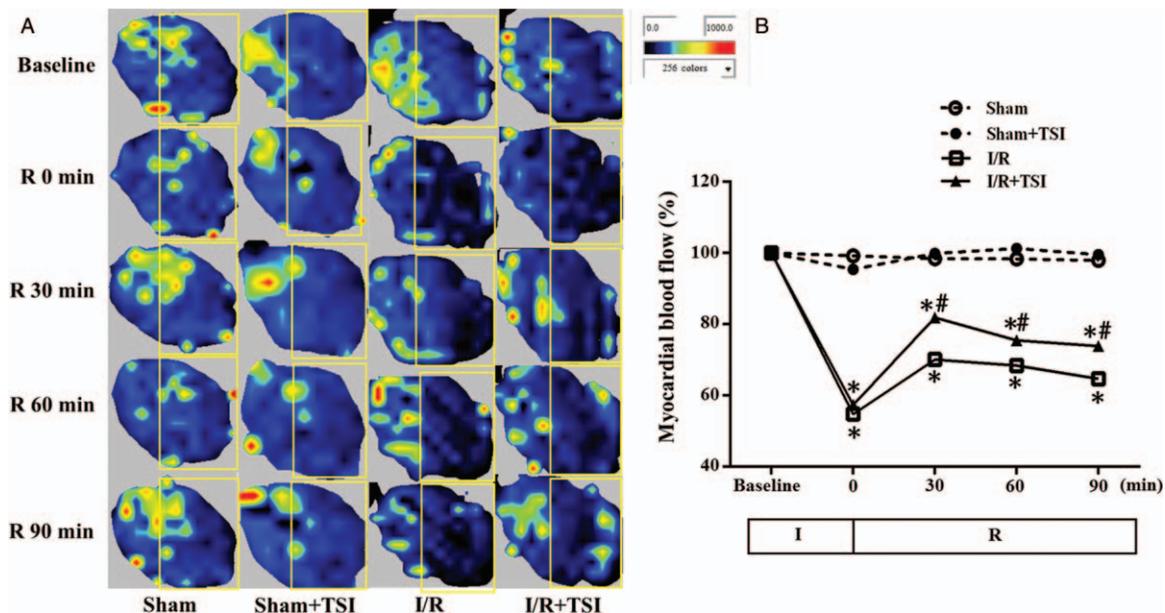

Fig. 9.   **Effect of TSI on MBF during I/R.** (A) MBF images of rats from Sham group, Sham + TSI group, I/R group, and I/R + TSI group acquired by Laser Scanning Doppler Perfusion Imager at indicated time points. The box on each figure defined the area within left ventricle for determination of MBF. The different colors represent the magnitude of MBF, with blue to red defining low to high. (B) Changes of MBF with time in rats from various groups. Data are expressed as mean $\pm$ SEM (n = 6). $^*P < 0.05$ vs. Sham group; $^\#P < 0.05$ vs. I/R group.

FIG. 10.   **Sirt1 siRNA transfection in H9c2 cells abolishes the protective role of TSI after H/R.** (A) The expression of Sirt1 in H9c2 cells treated with control siRNA or Sirt1 siRNA determined by Western blot and respective quantification in different groups. (B, C) Representative western blot bands of Sirt1 and NDUFA10 expression in various groups of H9c2 cells after TSI treatment with or without Sirt1 siRNA. The quantification was based on the data of three independent experiments and normalized to GAPDH. Data are presented as mean ± SEM (n = 4). $^*P < 0.05$ vs. control group; $^\#P < 0.05$ vs. H/R group; $^\&P < 0.05$ vs. H/R+TSI group.
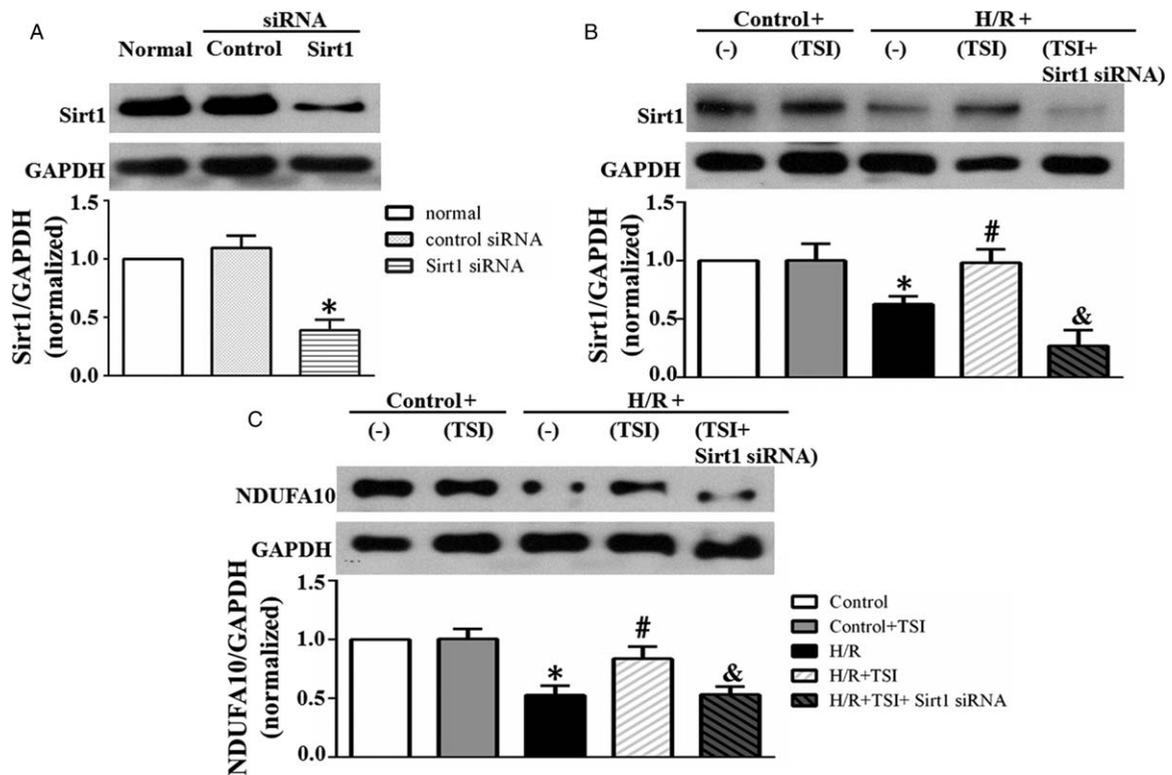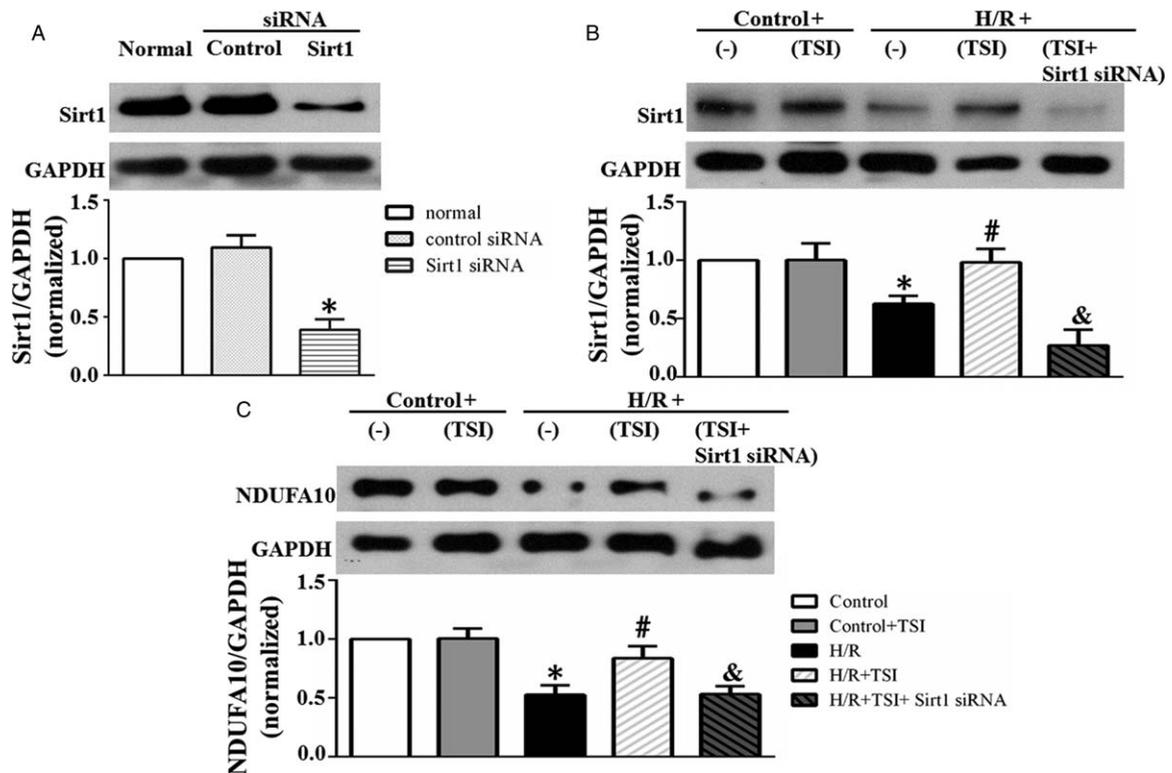

FIG. 11.   **Sirt3 siRNA transfection in H9c2 cells abolishes the protective role of TSI after H/R.** (A) The expression of Sirt3 in H9c2 cells treated with control siRNA or Sirt3 siRNA determined by Western blot and respective quantification in different groups. (B, C) Representative western blot bands of Sirt3 and SDHA expression in various groups of H9c2 cells after TSI treatment with or without Sirt3 siRNA. The quantification was based on the data of three independent experiments and normalized to GAPDH. Data are presented as mean ± SEM (n = 4). $^*P < 0.05$ vs. Control group; $^\#P < 0.05$ vs. H/R group; $^\&P < 0.05$ vs. H/R+TSI group.

infarct size, recovery of MBF, and decrease of myocardial apoptosis. Moreover, this potential of TSI was proven to attribute to protection of heart from oxidative insults, such as elevation of MPO, MDA, H_2O_2 and ROS, downregulation of Sirt1, Sirt3, NDUFA10, and SDHA expression, and decrease of MRC complexes activity. In addition, the *in vitro* study in H9c2 cells using siRNA transfection confirmed the effect of TSI on Sirt1, Sirt3, NDUFA10, and SDHA, and demonstrated the requirement of Sirt1 and Sirt3 for the effect of TSI on the expression of NDUFA10 and SDHA, respectively.

LADCA ligation followed by reperfusion remains to be the most preferred and acceptable I/R model in rodents (24), in which ischemia for more than 30 min may result in irreversible damage and necrosis of myocardial cells. We thus in the present study established the acute I/R rat model by ischemia for 30 min and reperfusion for 90 min, as we did in previous I/R model in our laboratory (20).

TSI is currently used in clinic in China for the treatment of cerebral stroke. Our previous study has verified the beneficial role of TSI in rat cerebral I/R injury (17). The present study provides further evidence in an animal model supporting the clinical use of TSI to fight against coronary heart disease. Consistent with the result from the rat cerebral I/R injury, we showed that the effect of TSI on I/R-induced myocardial injury is attributable to the antioxidant activity of TSI. This result is not a surprise given the fact that the major ingredient of TSI, Sal B, is the most active antioxidant in Salvia miltiorrhiza Bunge.

Oxidative stress plays a central role in I/R injury. The ROS is known to generate from NADPH oxidase in cell membrane, peroxisomes, endoplasmic reticulum, and mitochondria (25, 26). Studies from our laboratory and others have shown the implication of NADPH oxidase in the mechanism responsible for antioxidant activity of TSI (17) or Sal B (27, 28). However, the effect of TSI on the ROS generated from mitochondria has never been proved, despite the central role of mitochondria in generation of superoxide anions in postischemic heart has been widely recognized (29). Previous studies of other laboratories showed that Sal B markedly preserved redox status (30), elevated the level of mitochondria membrane potential (31), maintained the mitochondrial structure stabilization (32), and decreased the release of cytochrome C from the mitochondria into the cytosol (33). We also have known SMND-309, a novel derivative of Sal B, about its improvement of MRC complexes activity in cerebral ischemia rats (34), but no research went on with the underlying mechanism. Therefore, our present research was to focus on the effect of TSI on MRC complexes activity and the underlying mechanism in myocardial I/R injury rats. The results from the present study revealed that TSI significantly reduced the expression of MPO and elevated the activity of the complex I, II, and IV in myocardial tissue exposed to I/R, indicating the involvement of peroxisomes and mitochondria in the effect of TSI in the present circumstance, which suggests TSI as a novel option in this respect.

We next explored the signaling that mediated the effect of TSI on mitochondria. We speculated that this signaling involved Sirt1 and/or Sirt3, the two NAD^+-dependent enzymes. The reason for this speculation is as follows. Sal B has been reported to attenuate apoptosis and inflammation via Sirt1 activation (18, 19), suggesting the critical role of Sirt1 in the beneficial role of TSI; we have previously shown that caffeic acid attenuates rat liver reperfusion injury through Sirt3-dependent regulation of MRC (14), whereas Sal B contains three molecules of β-3,4-dihydroxyphenyl lactic acid and one molecule of caffeic acid in the structure. The results of the present *in vivo* and *in vitro* studies both validated this speculation, showing that TSI treatment significantly restored the expression of Sirt1 and Sirt3 in myocardial tissue and cultured myoblast cells after I/R, and more importantly, increased the expression of NDUFA10 and SDHA, which are the subunits of complex I and complex II, respectively. Particularly, the *in vitro* experiments using siRNA demonstrated the requirement of Sirt1 and Sirt3 for the effect of TSI on NDUFA10 and SDHA expression, highlighting the involvement of Sirt1 and Sirt3 in the signaling pathway that mediates TSI effect. Nevertheless, more works are required to clarify the detailed signaling that mediates the beneficial role of TSI in I/R-impaired mitochondria.

As expected, the present study revealed an increased level of MPO along with elevation of ROS and H_2O_2 in the myocardial tissue after I/R. MPO predominantly expresses in neutrophils and is thus extensively used as an indicator for neutrophil infiltration, which occurs in infection as well as in I/R injury. MPO participates in inflammatory reaction by producing hypochlorous acid using hydrogen peroxide and chloride anion, which not only kills the exogenous pathogens, but also damages the host tissue. TSI was observed in the present study to inhibit the increase in MPO by I/R, contributing to the beneficial role of this drug in the current setting.

Taken together, we show in the present study that TSI as a strong antioxidant exerts effect by acting at the mitochondrial ETC and, possibly, the MPO, in addition to the previously reported NADPH oxidase.

On the contrary, the present study revealed that TSI had no effect on energy metabolism disorder in myocardial tissue after I/R. This result is inconsistent with a previous report showing that Sal B, the major ingredient of TSI, protects rat brain from I/R injury by improving energy metabolism and scavenging free radicals (35).

TSI was approved by the Chinese State Food and Drug Administration (Z20110011) in 2011 for prevention and treatment of ischemic stroke (15); TSI also has therapeutic effects on I/R injury of other organs. Our research demonstrated that TSI as a potential option on acute myocardial infarction in clinic. As to the mechanisms implicated, this study mainly focused on the antioxidant effect of TSI and did not explore its anti-inflammatory effect. Besides, TSI administration could not restore the activity of ATP synthase and relieve myocardial energy metabolism disorder after I/R challenge, suggesting the need to combine TSI with other energy regulation drugs in clinical application. In addition, more studies are needed for translating TSI to clinic to combat with myocardial infarction, including study in large animal and human. Finally, a drug safety study is also required considering the ineffectiveness of TSI at high concentration on restoring the expression of Sirt1 (Supplement Fig. 2, http://links.lww.com/SHK/A756).
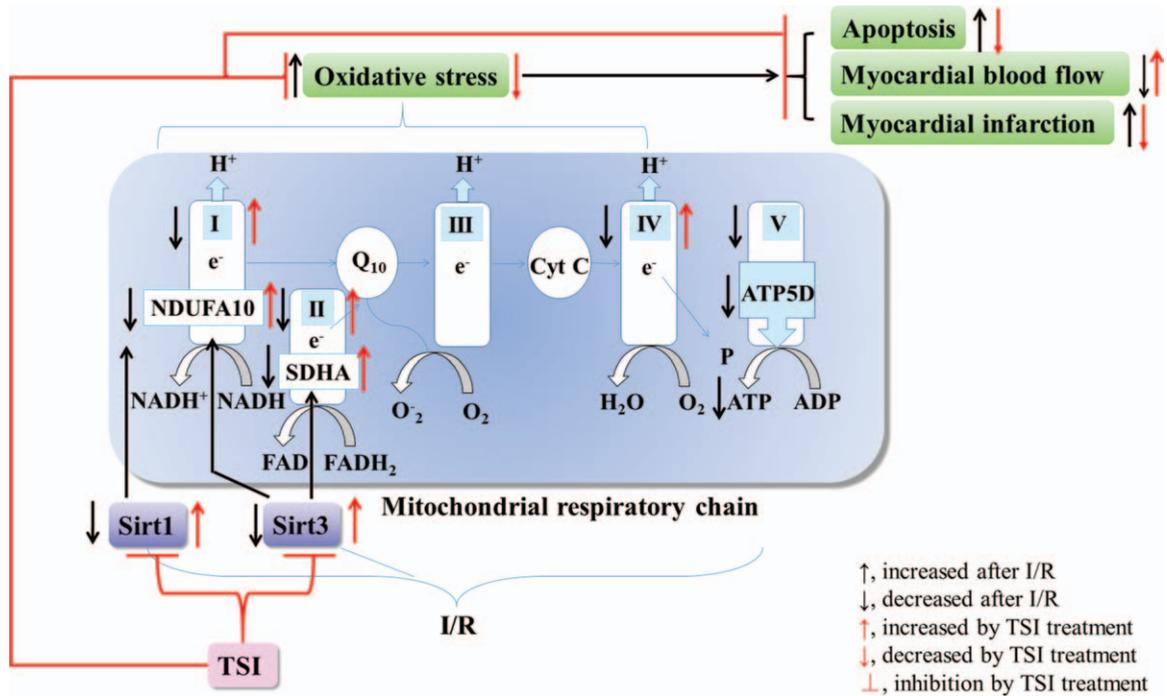
FIG. 12. **Summary of the ameliorating effects of TSI on myocardial I/R injury.** I, Mitochondrial complex I; II, mitochondrial complex II; III, mitochondrial complex III; IV, mitochondrial complex IV; V, mitochondrial complex V; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATP 5D, adenosine triphosphate synthase δ-subunit; Cyt C, cytochrome c; FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; NDUFA, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex; Q10, Coenzyme Q10; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein variant; Sirt, sirtuin; TSI, Total Salvianolic Acid Injection.

CONCLUSIONS

TSI attenuated I/R-induced myocardial injury, which was related to inhibition of oxidative stress, possibly by attenuating I/R-elicited downregulation of Sirt1 and Sirt3 expression, downregulation of NDUFA10 and SDHA expression, and decrease of MRC complexes activity. Moreover, the *in vitro* experiments using siRNA demonstrated the requirement of Sirt1 and Sirt3 for the effect of TSI on NDUFA10 and SDHA expression (Fig. 12). These results highlight TSI as a multiple target antioxidant, and suggest the potential efficiency of TSI in clinic to cope with myocardial I/R injury.

ACKNOWLEDGMENTS

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