Pharmacological postconditioning with atorvastatin calcium attenuates myocardial ischemia/reperfusion injury in diabetic rats by phosphorylating GSK3β

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Abstract. Diabetes is an independent risk factor for myocardial ischemia, and many epidemiological data and laboratory studies have revealed that diabetes significantly exacerbated myocardial ischemia/reperfusion injury and ameliorated protective effects. The present study aimed to determine whether pharmacological postconditioning with atorvastatin calcium lessened diabetic myocardial ischemia/reperfusion injury, and investigated the role of glycogen synthase kinase (GSK3β) in this. A total of 72 streptozotocin-induced diabetic rats were randomly divided into six groups, and 24 age-matched male non-diabetic Sprague-Dawley rats were randomly divided into two groups. Rats all received 40 min myocardial ischemia followed by 180 min reperfusion, except sham-operated groups. Compared with the non-diabetic ischemia/reperfusion model group, the diabetic ischemia/reperfusion group had a comparable myocardial infarct size, but a higher level of serum cardiac troponin I (cTnI) and morphological alterations to their myocardial cells. Compared with the diabetic ischemia/reperfusion group, the group that received pharmacological postconditioning with atorvastatin calcium had smaller myocardial infarct sizes, lower levels of cTnI, reduced morphological alterations to myocardial cells, higher levels of p-GSK3β, heat shock factor (HSF)-1 and heat shock protein (HSP)70. The cardioprotective effect conferred by atorvastatin calcium did not attenuate myocardial ischemia/reperfusion injury following application of TDZD-8, which phosphorylates and inactivates GSK3β. Pharmacological postconditioning with atorvastatin calcium may attenuate diabetic heart ischemia/reperfusion injury in the current context. The phosphorylation of GSK3β serves a critical role during the cardioprotection in diabetic rats, and p-GSK3β may accelerate HSP70 production partially by activating HSF-1 during myocardial ischemic/reperfusion injury.

Introduction

Previous laboratory and clinical studies suggest that reperfusion following myocardial infarction may induce ischemia/reperfusion injury (1-15). Lethal reperfusion injuries are hypothesized to account for up to half of the final infarction area (2). Myocardial ischemia/reperfusion injury is a frequent clinical problem (13-19). Despite optimal myocardial reperfusion, the mortality rate after acute myocardial infarction approaches 10% (19). This is the main pathophysiological basis for cardiac insufficiency and arrhythmia following angina and a number of clinical treatments (such as early reperfusion therapy of acute myocardial infarction and cardiac transplantation), and is the focus of much current research. Ischemic preconditioning and postconditioning have been therapeutically validated in various ischemia/reperfusion injury animal models, and several clinical trials (16-18). Currently, ischemic events cannot be predicted in clinical practice; hence, ischemic and pharmacological preconditioning is less implementable. However, ischemic postconditioning has its own pitfalls; repetitive inflations and deflations of the heart during percutaneous coronary angioplasty may lead to coronary endothelial damages, plaque rupture or dislodgement, coronary artery rupture and intervention complications (20). Pharmacological postconditioning via a simulative endogenous protective mechanism (following ischemia and prior to reperfusion) is more practicable due to its effectiveness, safety and easy manipulation (8,21).

Statins, inhibitors of reductase and hydroxymethylglutaryl-CoA (HMG-CoA) reductase, are frequently used to lower blood lipids, especially cholesterol, and have proven cardioprotective effects (22-26). Besides their lipid-lowering properties, statins have been demonstrated to exert extrahepatic, cholesterol-independent effects, or 'pleiotropic' effects, in previous animal studies (22,23,25,27), including improved endothelial function, altered inflammatory responses,

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Abbreviations: GSK3β, glycogen synthase kinase 3β

maintenance of plaque stability and prevention of thrombus formation. In animal models of ischemia/reperfusion injury, all members of the statin family including atorvastatin calcium, have been proven to reduce the myocardial infarction size (22,23,25,28,29).

Common cardioprotective strategies such as ischemic preconditioning (IPC) and ischemic postconditioning (Ipost) have also been reported to limit the size of myocardial infarction in young, healthy male animals (8,10,30-35). However, additional experiments have revealed that the protection may be impaired or removed in animals dependent on aging, hyperglycemic state, hypertension or hypercholesterolemia. The mortality of diabetic animals and patients following reperfusion was reported to be many times higher than that of non-diabetic animals or patients undergoing the same treatment; this may be partly caused by the inhibition of IPC- and Ipost-mediated protective mechanisms that occurs in diabetes (7,12,36,37). It was previously reported that IPC and Ipost mediate myocardial protection by stimulating phosphoinositide 3-kinase (PI3K)/Akt and the associated glycogen synthase kinase (GSK3β) pathway. Increasing evidence indicates that PI3K/Akt and the associated GSK-3β pathway is inhibited in diabetes, implying that diabetes attenuated IPC-and Ipost-mediated myocardial protection may occur via these pathways (36,38-40). Theoretically, any strategy to reactivate PI3K/Akt and the associated GSK-3β pathway may exert a protective effect against ischemia/reperfusion injuries in diabetes.

Therefore, the present study was designed to address the hypothesis that atorvastatin calcium may provide a protective effect against reperfusion injury in STZ-induced diabetes by phosphorylating GSK-3β.

Materials and methods

Animals and materials. A total of 96 male Sprague-Dawley (SD) rats (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China), 3-4 weeks of age, and weighing 50±5 g, were used in this study (n=12 per group) which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health guidelines regulating the care and use of laboratory animals and was approved by the Experimental Animal Care Committee of Fujian Medical University Union Hospital (Fuzhou, China). Atorvastatin calcium was obtained from Dailan Melone Biotechnology Co., Ltd. (Dalian, China). Primary antibodies against phospho-GSK3β (p-GSK3β; ab131097), total GSK3β (ab124661), and heat shock factor (HSF)-1 (ab131081) were purchased from Abcam (Cambridge, UK). Secondary antibodies (ZB-2301) and primary antibodies against GAPDH (YT5052) were purchased from Beijing Chinese Fir Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Serum total cholesterol (TC; 467852), triglyceride (TG; 445850), heat shock protein (HSP)70 (CSB-E08308r) and cardiac troponin I (cTnI; S795) were determined using commercially available ELISA kits (Stanbio Laboratory, Boerne, TX, USA), in accordance with the manufacturer's instructions. Evan's blue, triphenyltetrazolium chloride (TTC), dimethyl sulfoxide and streptozotocin, were purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany).

Induction of diabetes. Previous studies have reported different susceptibilities of the hearts of patients with diabetes to reperfusion injury compared with patients without diabetes (24,39). Prior to the development of a myocardial ischemia/reperfusion injury model, vulnerability to myocardial ischemia/reperfusion injury in the streptozotocin (STZ)-induced diabetic rat model was examined. The experimental SD rats were housed in standard polypropylene cages (4 rats/cage) under a 12/12-h light/dark cycle and an ambient temperature of 22-25°C. Animals were randomly divided into two groups, and fed either a regular chow, as a control, or a high-fat diet. Regular chow consisted of 60% carbohydrate, 12% fat, and 28% protein and the high-fat diet consisted of 41% carbohydrate, 40% fat, and 18% protein. After 4 weeks, 1% STZ (45 mg/kg, dissolved in 0.1 mmol/l citrate buffer pH 4.5-4.6) was injected into the abdominal cavity of high-fat diet group rats to create the SD rat model of diabetic mellitus, and the rats from the control group were injected with 0.1 mmol/l citrate buffer (4.5 ml/kg). STZ-injected animals were provided with access to food and water *ad libitum* following the STZ injection, and both STZ-injected and non-injected animals continued their individual diets. At 72 h after injection, the blood glucose levels were tested using a glucose meter (Optium Xceed; Abbott Pharmaceutical Co. Ltd., Lake Bluff, IL, USA) and rats with blood glucose levels>11 mmol/l were determined to be diabetic.

Measurements of general characteristics. Water intake and food consumption were evaluated daily, body weight was monitored weekly. The blood glucose levels were tested once a week using a glucose meter. At termination (4 weeks after injection), rats were weighed subsequent to an overnight fast of 8-10 h, and were anesthetized with 75 mg/kg ketamine and 7.5 mg/kg diazepam intraperitoneally. Blood samples were obtained from the abdominal aorta after 180 min reperfusion, and the separated plasma was stored at -80°C until assayed. Serum TC, TG, HSP70 and cTnI were determined using the aforementioned kits.

Animal grouping and treatments. Diabetic rats (DM) were randomly divided into six groups (n=12/group), and age-matched male non-diabetic SD rats (NDM) were randomly divided into two groups (n=12/group). Non-diabetic rats were randomly assigned to the sham or ischemia/reperfusion (I/R) groups. Diabetic rats were divided into groups as follows: i) Sham; ii) I/R; iii) TDZD-8 +optimal dose (2 mg/kg) of atorvastatin calcium (T+AP); iv) AP1, atorvastatin calcium postconditioning (1 mg/kg); v) AP2, atorvastatin calcium postconditioning (2 mg/kg); and vi) DMSO groups. The sham operation (Sham) group was treated with open chest operation but without myocardial ischemia/reperfusion. The study design is outlined in Fig. 1.

Myocardial ischemia reperfusion injury in vivo. The experimental rats' body temperature was maintained at 37°C during surgery, which was performed under anesthesia. Rats were placed in a supine position and mechanical ventilation was maintained with a rodent respirator (Jiangxi Teli Anaesthesia & Respiration Equipment Co., Ltd., Jiangxi, China) with a tidal volume of 1.0 ml/100 mg body weight (60 breaths/min) in
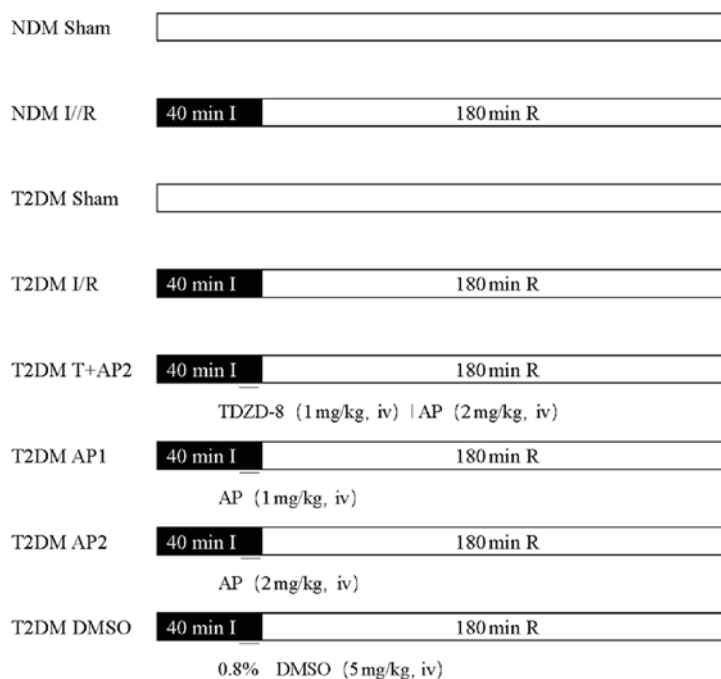
Figure 1. Animal grouping and treatments. Diabetic rats were randomly divided into six groups (n=12/group) and age-matched non-diabetic rats were randomly divided into two groups (n=12/group). Sham group were subjected to surgery but not ligation of the LCA. The I/R group received 40 min I and 180 min R. The T + AP2 group was subjected to intravenous injection of 1 mg/kg TDZD-8 5 min prior to R and 2 mg/kg atorvastatin calcium 3 min prior to R. The AP1 and AP2 groups were administered intravenous atorvastatin calcium (1 and 2 mg/kg, respectively). The DMSO received intravenous injection of 5 mg/kg TDZD-8 5 min prior to an additional dose 3 min prior to R. I, ischemia; R, reperfusion; LCA, left main cerebral artery; T, TDZD-8.

accordance with a previous study (10). An electrocardiogram (ECG) monitor was connected to provide long-term, continuous and real-time ECG information. A left thoracotomy was performed on the fourth intercostal space, and the thoracic cavity was exposed by blunt dissection. The pericardial tissue was removed, a single 5-0 Prolene suture was placed under the LCA, 1-2 mm from its origin. A small polyethylene tube was placed between the ends for reversible coronary artery occlusion. Rats received 40 min of LAD occlusion followed by 180 min of reperfusion. The sham group rats were treated in the same manner, with the exception that the suture was not ligated.

Blood collection and tissue harvest. Following reperfusion, blood samples were obtained immediately. The heart was removed and the myocardium was divided into two parts: One was fixed with 10% formalin, paraffin-embedded, made into slices for hematoxylin and eosin staining or immuno-histochemical analysis; the other was stored at -70˚C for western-blot analysis.

Determination of area at risk and infarct size. LAD was reoccluded in situ at the end of reperfusion, 1.5-2 ml of 3% Evan's blue dye was injected into the right ventricle via the venous system. Cardiac arrest in diastole was induced by an intravenous bolus injection of 10% KCl solution. The heart tissue was kept at -20˚C until it was in a semi-frozen state such that it was easily sliced. The frozen heart was transversely cut into slices with a thickness of 2 mm. The slices were then incubated in a 1% solution of TTC and agitated at a temperature of 37˚C for 10 to 15 min. Following staining

and fixation (in 10% formalin for 24 h), these were arranged sequentially, imaged by digital camera and quantified using ImageJ v. 1.36 (imagej.nih.gov/ij/). The area of necrosis (AN; uncolored), area at risk (AAR; including the uncolored and red area) and the left ventricular area (LV) were measured. The infarct size was expressed by AN/AAR and the risk area was expressed by AAR/LV.

Histochemical analysis. Pathological characterization of the heart was performed by histology and immunohistochemical staining. After reperfusion, the heart was removed, and the ventricular wall was removed from the site of ligation (about 3x5 mm in size) for HE staining. For immunohistochemical staining, the myocardial specimens were harvested after 5 min reperfusion, and deparaffinized tissue sections were treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were then incubated in a protein-free blocking agent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 10 min to inhibit the nonspecific binding of primary antibodies. Sections were examined for the presence and distribution of p-GSK3β. The dilution ratios of the primary antibodies (anti-GSK3β, anti-p-GSK3β and anti-HSF-1) and the goat anti-rabbit secondary antibody were 1:900, 1:700, 1:1,200 and 1:1,000, respectively. Incubationw ith the primary antibodies was performed for 2 h at 37˚C and the secondary antibody was incubated for 30 min at 37˚C.

Immunostains were examined using a microscope (Leica, Munich, Germany), and digital images were analyzed using Image-Pro Plus software (version 6; Media Cybernetics Inc., Bethesda, MA, USA).

Table I. Baseline characteristics prior to ischemia/reperfusion.

Parameters	NDM	DM
Water intake, ml/kg/day	120.7±8.6	308.3±11.3[a]
Food consumption, g/kg/day	60.9±3.8	137.6±10.2[a]
Body weight, g	412.8±13.7	355.4±22.9[a]
Plasma glucose, mM	5.1±0.8	13.8±3.6[a]
Triglycerides, mg/dl	120.1±10.4	420.4±20.9[a]
Cholesterol, mg/dl	78.16±7.7	184.6±24.3[a]

All values are expressed as mean ± standard deviation. n=12 per group. Water intake and food consumption, body weight, plasma glucose, triglycerides and cholesterol were measured 4 weeks after STZ injection. [a]P<0.05 vs. NDM. NDM, non-diabetic rats; DM, diabetic rats.

cTnI and HSP70 levels. Expression levels of these proteins were measured using rat ELISA kits in accordance with the manufacturer's instructions.

Western blot analysis. After 15 min reperfusion, hearts were quickly obtained for subsequent protein immunoblot analysis. The samples (60 μg protein/lane) were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred from the gel onto Hybond-C Extra membranes (Amersham, Pittsburgh, PA, USA). To prevent the non-specific binding, the membrane was placed in a membrane block, such as 3-5% bovine serum albumin. Following blocking of non-specific binding, the membranes were sequentially incubated with primary antibodies (anti- GSK3β, anti-p-GSK3β and anti-HSF-1) and the secondary antibody (Sigma-Aldrich). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference. The immunoreactive bands were detected by Enhanced Chemiluminescence Plus reagent (GE Healthcare Life Sciences, Chalfont, UK) using an X-ray film (Kodak, Rochester, NY, USA). Target signals were assessed using ImageJ 1.36 software.

Statistical analysis. Data were analyzed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). All values were continuous variables with normal distribution and were expressed as the mean ± standard deviation (SD). One-way analysis of variance was used for statistical analyses of data obtained within the same group of rats and between groups of rats, and this was followed by Tukey's test for multiple comparisons of group means. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

General characteristics at termination. As presented in Table I, at 4 weeks after STZ injection, STZ-induced diabetic rats presented significantly increased water intake, food consumption, plasma glucose, triglycerides and cholesterol, compared with control group (all P<0.05). Furthermore, the body weight in the diabetic group was lower than non-diabetic group (P<0.05).
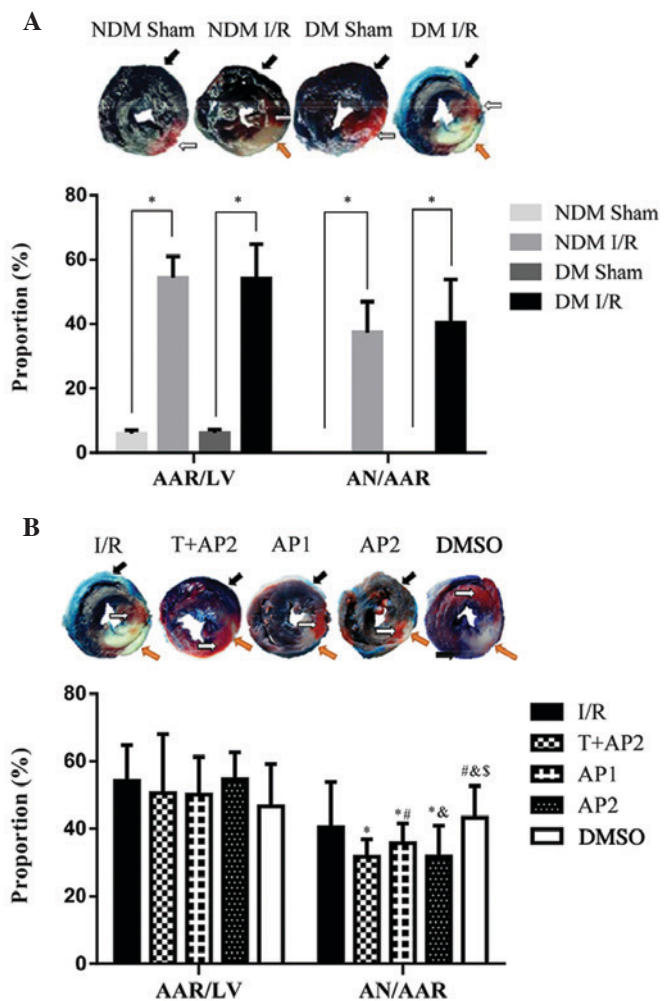

Figure 2. (A) Myocardial infarct size following myocardial ischemia reperfusion in non-diabetic and diabetic rats. The infarct size was expressed as AN/AAR and the risk area was expressed as AAR/LV. *P<0.05. (B) Effects of various treatments on infarct size in STZ-induced diabetic rats. Black arrows indicate non-ischemic areas, white arrows indicate AAR and red arrows indicate necrotic areas. *P<0.05 vs. I/R; #P<0.05 vs. T + AP2; &P<0.05 vs. AP1; $P<0.05 vs. AP2. NDM, non-diabetic rats; DM, diabetic rats; AAR, area at risk; LV, left ventricle; AN, area of necrosis; AP1, atorvastatin calcium postconditioning (1 mg/kg); AV2, atorvastatin calcium postconditioning (2 mg/kg).

Evans blue-TTC dyeing. As presented in Fig. 2, the black arrow pointed to the non-ischemic (various tones of blue), white arrow to AAR (various tones of red), and orange arrow to necrotic area (pale, uncolored). AAR is expressed as AAR/LV and the AN is expressed as AN/AAR. Myocardial infarct size was significantly increased in DM groups subjected to ischemia and postischemic reperfusion than that in the NDM groups (Fig. 2A). No necrotic areas were observed in the sham group. No significant difference was identified among the other groups in AAR/LV (P>0.05). Infarct sizes (AN/AAR) in the T+AP2, AP1 and AP2 groups were significantly decreased compared with the the I/R control group (31.71±5.20, 35.68±5.87 and 31.79±9.13 vs. 40.46±13.36%, respectively; P<0.05; Fig. 2B). As shown in Fig. 2B, compared with AP1 group, the AP2 treatment could further reduce myocardial infarct size (35.68±5.87 vs.31.79±9.13%, P<0.05). No significant differences were observed between the I/R and DMSO groups (40.46±13.36 vs. 43.27±9.40%, P>0.05), nor between the T+AP2 and AP2
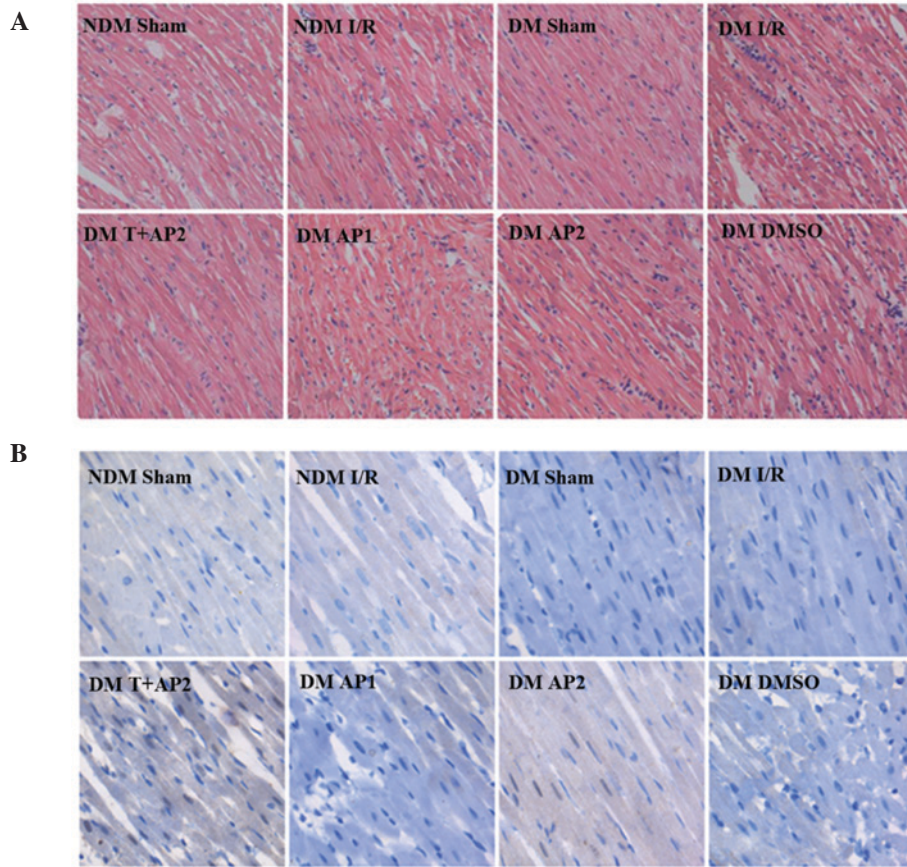
Figure 3. (A) Hematoxylin and eosin staining of cardiomyocytes in NDM and DM groups. (B) Expression of p-GSK3 in cardiomyocytes by immunohistochemistry. Magnification, x200. NDM, non-diabetic rats; DM, diabetic rats; I/R, ischemia/ reperfusion; T, TDZD-8; AP1, atorvastatin calcium postconditioning (1 mg/kg); AV2, atorvastatin calcium postconditioning (2 mg/kg).
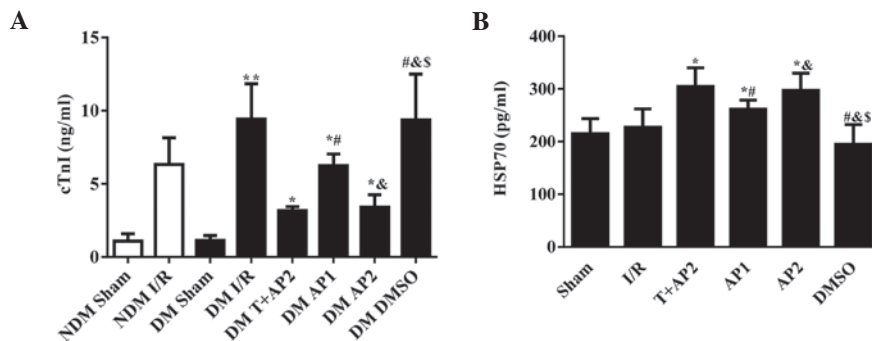

Figure 4. (A) Serum cTnI levels in NDM and DM groups. (B) Serum HSP70 levels in DM groups. **P<0.05 vs. NDM I/R; *P<0.05 vs. I/R; #P<0.05 vs. T + AP2; &P<0.05 vs. AP1; $P<0.05 vs. AP2. cTnI, cardiac troponin I; HSP70, heat shock protein 70; NDM, non-diabetic rats; DM, diabetic rats; I/R, ischemia/ reperfusion; T, TDZD-8; AP1, atorvastatin calcium postconditioning (1 mg/kg); AV2, atorvastatin calcium postconditioning (2 mg/kg).

groups (31.71±5.20 vs. 31.79±9.13%; P>0.05). Compared with the I/R group, the T+AP, AP1 and AP2 groups presented with significantly reduced infarct sizes (AN/AAR; all P<0.05). The reduction in infarct size induced by optima dose atorvastatin calcium postconditioning was not enhanced by TDZD-8 (T+AP2 group), a specific inhibitor of GSK3β.

Hematoxylin-eosin staining. To evaluate the severity of the cardiomyocyte injuries in the different groups, both in NDM and DM, morphologic changes were observed using hematoxylin-eosin staining (Fig. 3A). In the NDM sham group, myocardial fibers were regularly arranged in order, and no swelling, denaturation, necrosis, neutrophil infiltration, or other obvious pathological changes were observed. By contrast, inflammatory cells were observed in the DM Sham cytoplasm. Other groups, including NDM I/R and DM I/R, DM T+AP2, DM AP1, DM AP2 and DM DMSO, displayed varying degrees of morphological lesion. The order of the damage degree among the six groups, from most serious to least, was as follows: DM I/R, DMSO, NDM I/R, AP1, while histological changes in myocytes were least noticeable in the T+AP2 and AP2 groups (Fig. 3A).
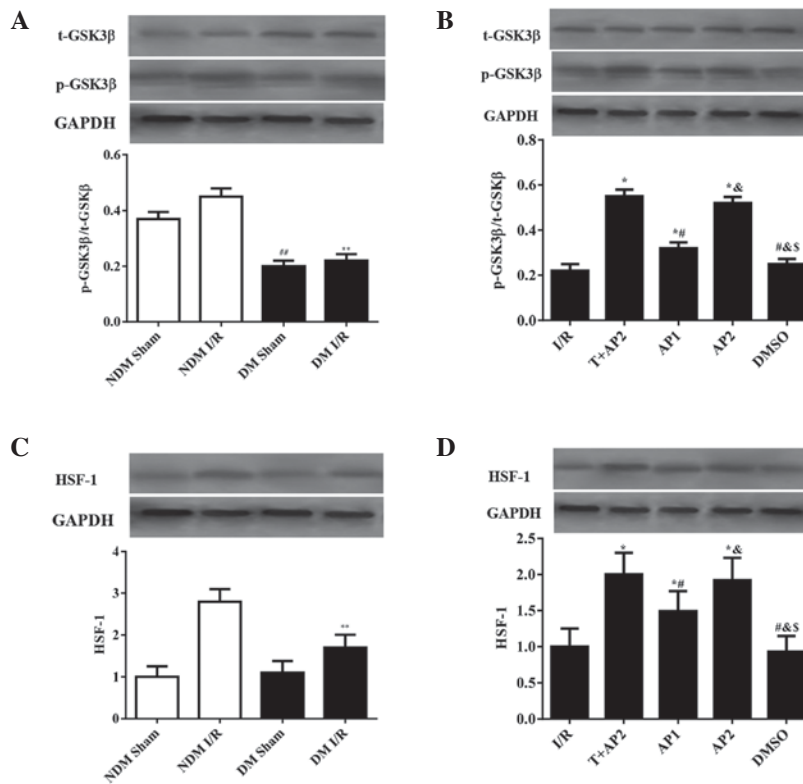
Figure 5. Western blotting analyses to indicate (A and B) t-GSK3β and p-GSK3β; and (C and D) HSF-1 in NDM and DM groups, or DM groups only, respectively. ##P<0.05 vs. NDM Sham; **P<0.05 vs. NDM I/R; *P<0.05 vs. I/R; #P<0.05 vs. T + AP2; &P<0.05 vs. AP1; $P<0.05 vs. AP2. t-GSK3β, total glycogen synthase kinase 3β; p-GSK3β, phospho-glycogen synthase kinase 3β; HSF-1, heat shock factor-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NDM, non-diabetic rats; DM, diabetic rats; T, TDZD-8; AP1, atorvastatin calcium postconditioning (1 mg/kg); AV2, atorvastatin calcium postconditioning (2 mg/kg).

Immunohistochemical staining of p-GSK3β. Immunohistochemical staining was used for the qualitative analysis and the spatial distribution of p-GSK3β. p-GSK3β positive expression present as brown coloration distributed in the cytoplasm and nucleus. p-GSK3β was not expressed or weakly expressed in the DM Sham and DM I/R groups. p-GSK3β was positively expressed in the NDM, T+AP2, AP1 and AP2 groups, in the cytoplasm and nucleus. The orders of staining intensities from strong to weak were as follows: T+AP, AP2, NDM and AP1. However, the expression levels of p-GSK3β in these groups were not marked (Fig. 3B). p-GSK3β quantitative analysis were attained by western blot analysis, as described below.

Cardiac troponin I levels in the NDM and DM groups. As displayed in Fig. 4A, significant differences were detected between the NDM sham and DM sham groups compared with the corresponding groups suffered from ischemia reperfusion injury (P<0.01). Following 40 min of LAD with 180 min reperfusion, the concentration of serum cTnI was significantly increased in the DM I/R relative to the NDM I/R group (9.43±2.41 vs. 6.33±1.83, P<0.05). By contrast, no significant differences were observed between the NDM sham and DM sham groups (1.09±0.50 vs. 1.13±0.34; P>0.05). DM I/R and DM DMSO groups (8.74±3.02 vs. 9.36±3.15, P>0.05), nor between the DM T+AP2 and DM AP2 groups (3.17±0.27 vs. 3.40±0.86, P>0.05). The serum cTnI concentrations in the DM T+AP2, DM AP1 and DM AP2 groups were significantly lower than compared with the DM I/R group

(3.17±0.27, 6.25±0.79 and 3.40±0.86 vs. 8.74±3.02, respectively; P<0.05). Compared with the AP1 group, the AP2 group presented with significantly decreased serum cTnI concentration (3.40±0.86 vs. 6.25±0.79, P<0.05; Fig. 4A).

Serum HSP70 levels in DM groups. As show in Fig. 4B, the differences in serum HSP70 concentration among all the DM groups appeared less marked than those in cTnI (Fig. 4A); however, a number of the inter-group differences reached statistical significance. No significant differences were observed between the I/R and DMSO groups (215.01±28.92 vs. 194.93±37.72, P>0.05), nor between the T+AP2 and AP2 groups (304.92±35.24 vs. 297.04±32.67, P>0.05). However, the serum HSP70 concentrations in the T+AP2, AP1 and AP2 groups were significantly higher than in the I/R group (304.92±35.24, 261.51±17.41 and 297.04±32.67 vs. 227.17±35.08, respectively; P<0.05). Compared the AP1 group, the AP2 group displayed significantly increased serum HSP70 levels (297.04±32.67 vs. 261.51±17.41, P<0.05; Fig. 4B).

Western blot analysis. Expression levels of p-GSK3β/t-GSK3β and HSF-1 in the myocardium were evaluated using western blot analysis (Fig. 5). No statistically significant difference was observed in HSF-1 levels between the NDM sham and DM sham groups (Fig. 5C), while the NDM sham had significantly higher p-GSK3β/t-GSK3β expression ratios compared with the DM sham (0.37±0.025 vs. 0.20±0.020; P<0.05, Fig. 5A). Compared with the DM I/R groups, the NDM I/R

group had higher p-GSK3β/t-GSK3β and HSF-1 expression ratios (p-GSK3β/t-GSK3β, 0.45±0.03 vs 0.23±0.024; HSF-1, 2.8±0.30 vs. 1.7±0.31, respectively; all P<0.05, Fig.5A and C).

As shown in Fig. 5B and D, the differences in HSF-1 expression among all the DM groups show similar patterns to the p-GSK3β/t-GSK3β expression ratios. No significant differences were observed between the I/R and DMSO groups (p-GSK3β/t-GSK3β, 0.22±0.03 vs. 0.25±0.022; HSF-1, 1.0±0.25 vs. 0.93±0.22, respectively; all P>0.05), nor between the T+AP2 and AP2 groups (p-GSK3β/t-GSK3β, 0.55±0.03 vs 0.52±0.027; HSF-1, 2.0±0.30 vs. 1.92±0.31, respectively; all P>0.05; Fig. 5B and D). The p-GSK3β/t-GSK3β expression ratios and HSF-1 levels in the T+AP2, AP1 and AP2 groups were higher compared with the I/R group (p-GSK3β/t-GSK3β, 0.55±0.03, 032±0.026 and 0.52±0.027 vs. 0.22±0.03; HSF-1, 1.92±0.31, 1.49±0.28 and 2.0±0.30 vs. 1.0±0.25, respectively; all P<0.05). In addition, compared with the AP1 group, the AP2 group presented with significantly increased p-GSK3β/t-GSK3β expression ratios and HSF-1 levels (p-GSK3β/t-GSK3β, 032±0.026 vs 0.52±0.027; HSF-1, 1.0±0.25 vs. 2.0±0.30, respectively; all P<0.05; Fig. 5B and D).

Discussion

Despite the ongoing development of therapies, the mortality rate of patients with ischemic heart disease remains high, particularly in cases of DM (7,36,37). Diabetes is an independent risk factor for myocardial ischemia, and numerous epidemiological data and laboratory studies have also revealed that diabetes significantly exacerbated myocardial ischemia/reperfusion injury and weakened the regular protective effects (9,12,36,37). The present study intended to develop a rat model of type 2 diabetes similar to that in clinical practice. However, in previous studies, the infarct size in animal models of DM has been reported to be both different to and similar in size to those of nondiabetic controls (7,9,11,12,30,41). Due to differences in experimental design, a single factor would be insufficient to explain the vast variance in the effects of diabetes on the infarct size. In the current study, the fasting blood glucose and the serum TG and TC of diabetic rats were increased compared with the non-diabetic rats, while the body weight in these rats did not fall significantly in the first three weeks after injection of STZ. Following 40 min ischemia and 180 min reperfusion, no significant difference of the myocardial infarction size was observed between the non-diabetic rats and diabetic rats, while in diabetic rats, the levels of serum cTnI, morphological lesions of myocardial cells, and the level of GSK3β were higher than in non-diabetic rats. This indicated that the diabetic heart may be more susceptible to ischemia/reperfusion injury.

Atorvastatin calcium, as the inhibitor of HMG-CoA reductase, is fat-soluble and is commonly used in the treatment of cardiovascular diseases. Its cardioprotective effects were confirmed in numerous previous studies (22,28,29). It was hypothesized in the current study that atorvastatin calcium postconditioning alleviates myocardial ischemia/reperfusion injury on STZ-induced diabetic rats by phosphorylating GSK3β.

In the present study, compared with DM I/R group, the myocardial infarction size and the levels of cTnI decreased, and the size of the morphological lesions were attenuated in the atorvastatin calcium postconditioning groups. Atorvastatin calcium postconditioning (1 or 2 mg/kg) therefore alleviated myocardial ischemia/reperfusion injury in STZ-induced diabetic rats. Compared with the 1 mg/kg atorvastatin calcium postconditioning group, the 2 mg/kg atorvastatin calcium postconditioning group further alleviated morphological lesions, reduced myocardial infarct size, reduced the level of cTnI and increased the level of p-GSK3β. It was therefore demonstrated that 2 mg/kg atorvastatin calcium was the optimal dose. No significant differences were reported in the myocardial infarction size, cTnI levels, morphological lesions of the myocardial cells or the level of p-GSK3β between the TDZD-8+optimal dose of atorvastatin calcium group and the 2 mg/kg atorvastatin calcium group. These data suggest no synergy between TDZD-8 and atorvastatin calcium, and that they may protect the myocardial cells by the same pathway. A previous study of TDZD-8, as the specific inhibitor of GSK3β, has demonstrated that GSK-3β inhibitors protect against myocardial ischemia/reperfusion injury via inhibition of inflammation and apoptosis (34). Therefore, GSK3β phosphorylation may serve a vital role during atorvastatin calcium postconditioning. The changes to HSF-1 and the serum HSP70 levels tend to follow a similar expression pattern as p-GSK3β in cardiomyocytes, and p-GSK3β was localized to the plasma and nuclei of cardiomyocytes, as determined by immunohistochemical analyses. Concordantly, p-GSK3β accelerated HSP70 production partially by activating HSF-1 during myocardial ischemia.

GSK3 proteins are serine/threonine kinases, first identified in 1980 (42), is highly conserved in evolution. In mammals, GSK-3 proteins include GSK-3α (51 kDa) and GSK3β (47 kDa), encoded by separate genes, and the kinase domain has up to 98% homology (39,43). Nearly 20 years after the discovery, researchers previously considered that the role of GSK3 was as its name indicates; a glycogen kinase involved in the phosphorylation of protein kinase regulation of glucose metabolism. However, an increasing number of studies have suggested that protein substrates of phosphorylated GSK3 included ~50 types, including as many as a dozen transcription factors (31,34,38-40,43-45). GSK3 serves an important function in the regulation of numerous cellular functions and activities, such as embryogenesis, cell proliferation, differentiation, apoptosis, signal transduction and microtubules movements. Furthermore, GSK3 is involved in the occurrence and development of a number of diseases, such as Alzheimer's disease, bipolar disorder, schizophrenia, cancer and diabetes (39).

GSK-3β is a constitutively active 47-a Ser/Thr protein kinase, which decreases glycogen synthase activity. However, GSK-3β is now known as a multifunctional kinase, serving functions in glycogen metabolism in addition to cell proliferation, growth and death (39,40,43). In the cardiovascular system, GSK-3β serves major roles in glucose metabolism (45), cardiomyocyte hypertrophy (46) and cell death. S9-phosphorylation of GSK3β is required for postconditioning, and is hypothesized to function by inhibiting the opening of the mitochondrial permeability transition pore (mPTP) (31). Prior studies have indicated that mPTP is involved in the development of

myocardial reperfusion injury. mPTP, which was first reported by Haworth and Hunter in 1979 (47), is a non-specific channel located in the mitochondrial membrane, and allows small molecules (<1.5 kDa) to pass though. mPTP is closed under normal physiological conditions in order to maintain the integrity of the structure and function of mitochondria, and remains closed following ischemia. However, as a result of reperfusion injury, oxygen free radical levels are increased and calcium overload activates the opening of mPTPs (32). A prior study indicated that mPTPs opened at 5-10 min after reperfusion (48), allowing water and solutes into the mitochondria non-selectively, resulting in mitochondrial membrane potential imbalance, uncoupling of oxidative phosphorylation, mitochondrial swelling and ultimately leading to cell death. It is now generally believed that mPTP is a key factor in myocardial reperfusion injury, and it could be an important target for alleviating reperfusion injury (2,21,31,32,38,44,47-49).

GSK3β serves a crucial function in the initial phase of mPTP opening, which was first reported by Juhaszova *et al* in 2004 (44). They found that inactivating cardiac myocyte GSK3β could increase the mPTP opening threshold caused by ROS *in vitro*. Gomez *et al* (31) provided further evidence that the mPTP opening caused by calcium overload *in vitro* was inhibited after ischemic postconditioning in wild-type mice, while in mutated mice whose GSK3β coudl not be phosphorylated and inactivated, ischemic postconditioning could not inhibit mPTP opening. Miki *et al* (38) suggested that the proportion of phosphorylated GSK3β relative to total GSK3β in mitochondria is associated with with the threshold of mPTP opening triggered by calcium ions. Theoretically, GSK3β inactivation inhibits ROS generation and mPTP opening induced by calcium overload, and thus reduces myocardial reperfusion injury. In basic research, the application of GSK3β pharmacological inhibitor (SB216763 and SB415286) before ischemia or after reperfusion has been shown to reduce infarct size, and furthermore ischemic preconditioning, ischemic postconditioning andcardioprotective drug application all led to the phosphorylation of GSK3β following reperfusion (31). Nishihara *et al* (33) indicated a negative correlation between tissue levels of phosphorylated GSK3β within 5 min after reperfusion, and of infarct size within 2 h after reperfusion. Collectively, these previous findings suggest that the phosphorylation state of GSK3β may be associated with the degree of myocardial reperfusion injury.

TDZD-8, a non-selective ATP-competitive inhibitor of GSK3β, phosphorylates GSK3β at Ser9 and thus decreases the activity and elevates the mPTP opening threshold in reperfusion injury (44). TDZD-8 does not inhibit the other series of kinases, such as protein kinase A and C, casein kinase II and cyclin-dependent kinase 1. Previous results have indicated the cardioprotective effect of TDZD-8 in myocardial ischemia/reperfusion injury, which resulted in the phosphorylation of GSK3βser9 (34). In the present study, pharmacological postconditioning with 2 mg/kg atorvastatin calcium did not further attenuate myocardial ischemia reperfusion injury after TDZD-8 phosphorylated and inactivated GSK3β. On the basis of these results we propose that atorvastatin has the same method of action as TDZD-8 in the reperfusion injury in rats with early stages of streptozotocin-induced diabetes.

It is also notable that GSK3β should be administered at an optimum concentration level. In the present experiment,

2 mg/kg atorvastatin calcium was considered to be a relatively optimal dose; however, more research is required, such as the optimal atorvastatin calcium dose in the progress of alleviating myocardial ischemia/reperfusion injury for the different species, or at different stages of diabetes. The activity of GSK3β is closely associated with I/R injury, and its inhibitors could reduce the injury. However, it should be noted that the effect of the phosphorylation of GSK3β may differ between species of organism, and GSK3β inhibitors have a number of side effects. For example, studies have indicated that long-term use of GSK3β inhibitors in patients with heart failure may cause ventricular hypertrophy and tumor growth (43). Therefore, more comprehensive and in-depth studies are required to investigate the most effective use of the phosphorylation or directly inhibition of GSK3β in the context of treating I/R injury.

In the present study, immunohistochemical methods were used to detect the distribution of p-GSK3β, and the results suggested that p-GSK3β was distributed in the cytoplasm and nucleus. Although p-GSK3β is inactivated, it may continue to phosphorylate numerous proteins, including HSF-1 (50). HSF-1 is an important pro-survival transcription factor which is induced to trimerization in the cytoplasm after being phosphorylated, and subsequently combines with the heat shock element HSP promoter region in the nucleus to synthesize HSP (4,6,51-53).

HSPs are a class of endogenous proteins that act against various types of cellular stress, including heat shock, ischemia and hypoxia, and are highly conserved evolutionarily. HSPs are named according to their molecular weight, including HSP90, HSP84, HSP70, HSP60, HSP27 and HSP20. HSP90, HSP84, HSP70, HSP27 and HSP20 have been associated with cardiovascular disease (54).

Xu *et al* (30) reported that the PI3K/AKt pathway could increase the level of HSP70, and resulted in a cytoprotective effect in human tissues. Furthermore, other studies have suggested that HSP70 exerts a protective effect in non-diabetic myocardium, including the protection of myocardial contractility (51,52), reduction of myocardial necrosis (6) and inducing an antiarrhythmic effect (4).

It has been reported that in the PI3K/Akt pathway, Stat3 could be the upstream signal of HSF-1, and thus promote the synthesis of HSP70 during myocardial reperfusion (50). In the present experiment, the changes of HSF-1 and serum HSP70 were comparable to those of p-GSK3β in cardiomyocytes. According to the results of this experiment and other experiments, HSF-1 could be phosphorylated by p-GSK3β, and p-GSK3β may accelerate HSP70 production partially by activating HSF-1 during the experimental conditions, thus serving a protective function.

DMSO is a colorless organosulfur liquid and an important polar aprotic solvent that dissolves both polar and nonpolar compounds. DMSO is known to possess antioxidative, anti-inflammatory and analgesic properties, and to promote blood circulation. Furthermore, DMSO has been applied clinically in the treatment of gastrointestinal diseases, rheumatism and amyloidosis disease. On the basis of its antioxidative and anti-inflammatory properties, numerous prior studies have observed the effects of DMSO in various organs during myocardial ischemia, including the heart (55), brain (56), liver (57), gastrointestinal (58), lung (59) and ovaries (60). The usage and dosage were different, and the results vary from against

reperfusion injury (3,55,61), no protect effect (3,5,62) to aggravate damage (63). In the present study, TDZD-8 and atorvastatin calcium were dissolved in 0.8% DMSO for administration to rats, and indexes of cardiac injury were compared between DM IR and DM DMSO groups. No significant differences in the level of myocardial infarction size, cTnI, morphological lesion of myocardial cell or the level of p-GSK3β were detected between the DM IR and DM DMSO group. The results of the present study therefore indicate that the 0.8% concentration of DMSO used had no influence in the result.

Nevertheless, the present study contained a number of limitations. First, the results of animal diabetic models in MIRI were incomparable from one to another, reduce infarct size, and enlarge infarct size or no change. Due to differences in experimental design details, a single factor is insufficient to explain the marked variance in the effects of diabetes on the infarct size. Therefore, we compared the tolerance against infarction between DM and NDM groups, and the model we used proved more sensitive to myocardial ischemia. Additional estrogen usage may result in decreased infarct size (35). Second, previous experiments suggest that the infarct area in females are smaller than in males relative to body mass (49,64,65). In prior experiments (64,65), it has been suggested that additional preconditioning and other cardioprotective approaches do not have a further protective effect in females. This may be due to the interactions between cardioprotective signaling pathways in female animals and pre- and post-conditioning signaling pathways. Considering the successful rate of diabetic model, we selected male SD rats. Moreover, our study didn't investigate the time-and dose-effect of atorvastatin calcium postconditioning on MIRI. On the basis of the above evidence, the relevance of the present results is limited to the specific settings, and cannot be generalized. Finally, numerous pharmacological agents that show promise in a laboratory-based animal model have not reduced the infarct size in clinical trials as expected. Thus, atorvastatin calcium postconditioning as presently described is far from clinical use, and more research is required.

Pharmacological postconditioning with atorvastatin calcium can attenuate diabetic diabetic heart ischemia/reperfusion injury in the present settings, and the protective effect of 2 mg/kg group was improved compared with the 1 mg/kg group. The phosphorylation of GSK3β may play a critical role in this protective process in diabetic rats, and p-GSK3β can may induce HSP70 expression partially by activating HSF-1 during MIRI.

Acknowledgements

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