Gaseous signalling molecule SO_2 via Hippo-MST pathway to improve myocardial fibrosis of diabetic rats

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Abstract. Recent studies have indicated the existence of an endogenous sulfur dioxide (SO_2)-generating system in the cardiovascular system. The present study aimed to discuss the function and regulatory mechanism of gaseous signal molecule SO_2 in inhibiting apoptosis and endoplasmic reticulum stress (ERS) via the Hippo-MST signaling pathway to improve myocardial fibrosis of diabetic rats. A total of 40 male Sprague-Dawley rats were randomly divided into four groups (10 rats per group): Normal control group (control group), diabetic rats group [streptozotocin (STZ) group], SO_2 intervention group (STZ+SO_2 group) and diabetes mellitus rats treated with L-Aspartic acid β-hydroxamate (HDX) group (HDX group). Diabetic rats models were established by intra-peritoneal injection of STZ (40 mg/kg) Following model establishment, intra-peritoneal injection of Na_2SO_3/$NaHSO_3$ solution (0.54 mmol/kg) was administered in the STZ+SO_2 group, and HDX solution (25 mg/kg/week) was administered in the HDX group. A total of 4 weeks later, echocardiography was performed to evaluate rats' cardiac function; Masson staining, terminal deoxynucleotidyl transferase dUTP nick end labeling staining and transmission electron microscopy examinations were performed to observe myocardial morphological changes. ELISA was employed to determine the SO_2 content. Western blot analysis was performed to detect the expression of proteins associated with apoptosis, ERS and the Hippo-MST signalling pathway. Compared with the control group, the STZ group and HDX group had a disordered arrangement of myocardial cells with apparent myocardial fibrosis, and echocardiography indicated that the cardiac function was lowered, there was an obvious increase of apoptosis in myocardial tissue, the expression levels of apoptosis-associated protein B-cell lymphoma associated protein X, caspase-3 and caspase-9 were upregulated, and Bcl-2 expression was downregulated. The expression of ERS and Hippo-MST pathway-associated proteins, including CHOP, GRP94, MST1 and MST2, were significantly upregulated. By contrast, these above-mentioned changes were reversed by SO_2 treatment. Compared with STZ group, the HDX group had a further increase of myocardial fibrosis and apoptosis, while there were no statistically significant differences in the expression of Bax/Bcl-2, caspase-3, caspase-9 and ERS and Hippo-MST pathway-associated proteins. The results of the present study demonstrated that the gaseous signal molecule SO_2 can effectively improve the myocardial fibrosis of diabetic rats, and its mechanism may be associated with reduced apoptosis and ERS by downregulated Hippo-MST pathway.

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Abbreviations: SO_2, sulfur dioxide; ERS, endoplasmic reticulum stress; HDX, L-Aspartic acid β-hydroxamate; TEM, transmission electron microscopy; DC, diabetic cardiomyopathy; DM, diabetic mellitus; H_2S, hydrogen sulfide; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic diameter; EF, ejection fraction; FS, fractional shortening

Key words: sulfur dioxide, myocardial fibrosis, Hippo-MST pathway, endoplasmic reticulum stress, apoptosis

Introduction

With the continuous change in modern lifestyles, the incidence rate of diabetes is on the rise (1). Diabetic cardiomyopathy (DC) is a chronic complication of diabetes, a specific myocardial lesion independent from valvular heart disease, coronary heart disease, congenital heart disease and other diseases. Such disease is one of the major causes of death among diabetic patients (2). The pathological changes of DC are mainly characterised by cardiomyocyte hypertrophy, apoptosis and necrosis as well as progressive myocardial interstitial fibrosis (3). Myocardial fibrosis is an important marker of the myocardial remodelling of DC and the major cause of left ventricular dysfunction. Moreover, the mechanism involved is considerably complicated. Evidence shows that the pathogenesis of myocardial fibrosis is associated with oxidative stress,

endoplasmic reticulum stress (ERS), apoptosis (4-8). However, the specific regulatory mechanism remains unclear.

Sulfur dioxide (SO_2) is a poisonous, small-molecule gas. However, recent studies have found the existence of an endogenous SO_2-generating system in the cardiovascular system. As a new gaseous signalling molecule following the discovery of NO, CO and hydrogen sulfide (H_2S), endogenous SO_2 performs the following functions: Vasodilation, suppression of inflammation and improvement of vascular collagen remodelling in the cardiovascular system (9). However, whether SO_2 takes part in the occurrence and development of DC and its internal mechanism remains an open issue.

As one of the prevalent cell signalling mechanisms of pathological and physiological processes in animals, the Hippo-MST signalling pathway participates in the regulation of cell proliferation and apoptosis (10-12). The components of the Hippo-MST pathway mainly include MST1/2, Sav, LATS1/2 and MOBI. MST1/2 is a protein kinase of the STE20 family and could phosphorylate the core members of the Hippo-MST pathway-Sav1, LATS1/2 and MOBI. At present, MST has been proven to play a role in regulating apoptosis in some animal models in the presence of cardiovascular diseases. It can also induce cell death by activating LATS. The upregulation of MST1 expression or its specific overexpression has been confirmed to induce the apoptosis of myocardial cells. The Hippo-MST pathway is also known to participate in the regulatory mechanism of myocardial fibrosis, and the overexpression of MST1 may result in myocardial fibrosis. However, the role of the Hippo-MST pathway in diabetic myocardial fibrosis and its mechanism of action remain unclear.

In the present research, we propose to establish an streptozotocin (STZ)-induced model of diabetic rats with intervention by a SO_2 donor and endogenous SO_2 synthase inhibitor L-Aspartic acid β-hydroxamate (HDX) so as to explore the role of the gaseous signalling molecule SO_2 in inhibiting apoptosis by regulating the Hippo-MST pathway and improving ERS and myocardial fibrosis of diabetic rats.

Materials and methods

Experimental animals. Adult male Sprague-Dawley (SD) rats (240±20 g) were obtained from the Animal Experiment Centre of South China University. Prior to the experiments, the SD rats were fed with standard rat chow and normal water *ad libitum*. The rats were housed and separated by treatment group under a 12 h light-dark cycle at a temperature of 23±1˚C and humidity of 60%. The rats were fed according to institutional policies, and all the experiments were carried out with the approval of the University Committee on the Use and Care of Animals of South China University.

Chemicals and reagents. Na_2SO_3, $NaHSO_3$ and HDX were purchased from Sigma-Aldrich (St. Louis, MO, USA). STZ was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). The antibodies for matrix metalloproteinase (MMP)9, MMP24, tissue inhibitor of metalloproteinase (TIMP)1 and GAPDH were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The dilution rate of these antibodies was 1:400. Furthermore, the antibodies for MST1, MST2, MOB1, LATS1, BIP, PDI and ERO1-L were purchased from Cell Signalling Technology (Danfoss, MA, USA). The antibodies for Bcl-2, Bax, caspase-3, caspase-9, CHOP, GRP94, eIF2α and GOT1 were purchased from Proteintech Group, Inc. (Chicago, IL, USA). The dilution rate of these antibodies was 1:1,000. Anti-rabbit secondary antibody and anti-rat secondary antibody were also purchased from Proteintech Group, Inc. The cell lysis buffer for western blot analysis, Bicinchoninic Acid (BCA) Protein Assay kit, Enhanced Chemiluminescence Reagent kit and SDS-PAGE Gel Preparation kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China). The ELISA kit of SO_2 was obtained from Mlbio Co. (Shanghai, China).

Model establishment and grouping. Forty experimental rats were randomly divided into four groups: Normal group (control group), diabetes mellitus rats (STZ 40 mg/kg) group (STZ group), diabetes mellitus rats (STZ 40 mg/kg) treated with SO_2 (STZ+SO_2 group) and diabetes mellitus rats (STZ 40 mg/kg) treated with HDX group (HDX group). Each rat in the STZ group, STZ+SO_2 group and HDX group were made diabetic via a single intraperitoneal injection of 40 mg/kg body weight STZ. At the same time, the rats of the control group were treated with saline once (i.p.). Blood glucose from the rat tail vein was detected with a blood glucose monitor. The rats were considered diabetic when the concentration of glucose was higher than 16.7 mmol/l 3 days after STZ injection. Then, SO_2 donor (Na_2SO_3/$NaHSO_3$) was administered to the rats of the STZ+SO_2 group. Na_2SO_3/$NaHSO_3$ was freshly dissolved in saline at 0.54:0.18 mmol/kg before injection. The HDX group was treated with HDX (25 mg/kg, i.p.) once a week. The control group and STZ group were intraperitoneally injected with physiological saline every day. The experiment lasted for four weeks.

Specimen collection and processing. After 4 weeks, the rats were weighed and subjected to echocardiography. They were then anesthetised with chloral hydrate (350 mg/kg). The hearts of the rats were lavaged with ice-cold normal saline before they were removed and weighed. Parts of the heart tissues were preserved at -80˚C for the experiment. The remaining heart tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline and then embedded in paraffin.

Echocardiography analysis. After 4 weeks, the mice were anesthetised with chloral hydrate (350 mg/kg, i.p.). We used transthoracic echocardiography to test left ventricular function. The left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were measured to evaluate heart function.

Histopathology analysis of myocardial fibres. After 4 weeks, the animals that survived were sacrificed. Their hearts were removed immediately and then fixed in 4% paraformaldehyde. Each heart was embedded in paraffin, dewatered with alcohol, embedded in paraffin again and sliced up in 5 μm sections. These sections were stained using a Masson staining kit and observed under an optical microscope.

SO_2 content assay. Myocardial SO_2 content was assayed by ELISA. The ELISA kit of SO_2 was obtained from Mlbio Co.

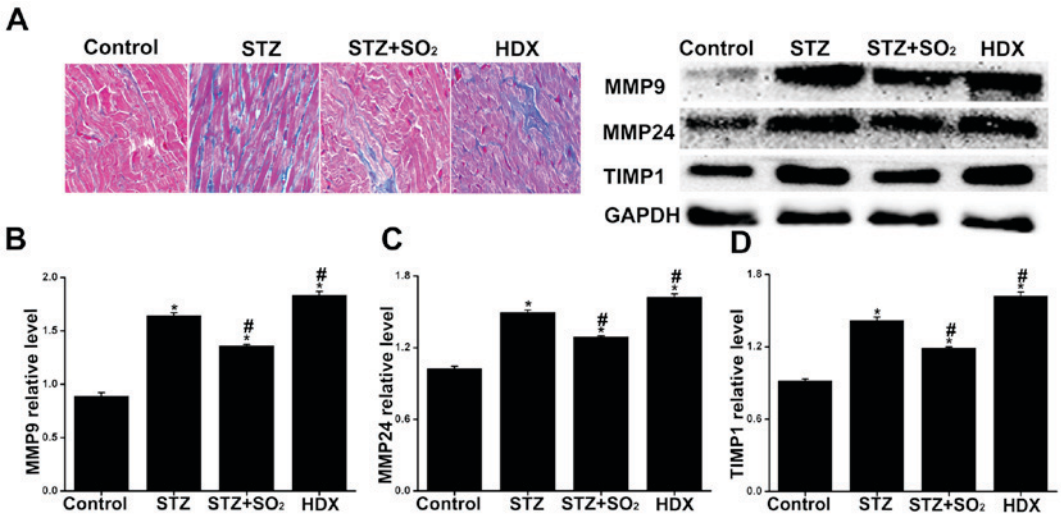

Figure 1. SO_2 improves myocardial fibrosis in diabetic rats. (A) Morphological changes in myocardium assessed by Masson staining. Images were acquired at x400 magnification. Expression levels of (B) MMP9, (C) MMP24 and (D) TIMP1 in each group. Date are expressed as mean ± standard deviation (n=3). *P<0.05 vs. control group; #P<0.05 vs. STZ group. SO_2, sulfur dioxide; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; STZ, streptozotocin; HDX, L-Aspartic acid β-hydroxamate.

The steps were taken following the manufacturer's instructions.

TUNEL assay. The myocardial tissue of each rat was fixed in 10% formalin and embedded in paraffin. It was then processed for TUNEL assay. The slides were treated with H_2O_2 and incubated with a reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37˚C. Labelled DNA was visualised with a peroxidase-conjugated anti-digoxigenin antibody using 3,3'-diaminobenzidine (DAB) as the chromogen. Rat testicular tissue was used as positive control in the TUNEL assay.

Transmission electron microscopy (TEM). The left ventricular tissues in each group were cut into small pieces on the ice and then immersion-fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and dehydrated in a series of graded ethanol solutions. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Ultrathin sections were observed via electron microscopy.

Western blot analysis. Total proteins were extracted in ice-cold radioimmunoprecipitation assay buffer containing protease inhibitors (Beyotime Institute of Biotechnology) and then quantified using a BCA protein assay kit. Proteins were denatured, separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane via wet transfer method. The membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween-20 (TBST) for 2 h at room temperature. The membranes were incubated with a blocking solution containing primary antibodies (anti-GAPDH, 1:400; anti-MMP9, 1:400; anti-MMP24, 1:400; anti-TIMP1, 1:400; anti-Bax, 1:1,000; anti-Bcl-2, 1:1,000; anti-caspase-3, 1:1,000; anti-caspase-9, 1:1,000; anti-GOT1, 1:1,000; anti-CHOP, 1:1,000; anti-GRP94, 1:1,000; anti-eIF2α, 1:1,000; anti-BIP, 1:1,000; anti-PDI, 1:1,000; anti-ERO1-L, 1:1,000; anti-MST1, 1:1,000; anti-MST2, 1:1,000; anti-MOB1, 1:1,000; anti-LATS1, 1:1,000) overnight at 4˚C. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 h at room temperature. Next, the membrane was washed in TBST buffer thrice. Finally, these membranes were subjected to chemiluminescence detection assay. The bands were analysed with a Molecular Imager VersaDoc MP 5000 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data were expressed as mean ± standard deviation. The statistical differences among the groups were assessed via one-way analysis of variance with SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between two groups were made using the Student-Newman-Keuls test. A P-value of <0.05 was considered statistically significant.

Results

SO_2 improves myocardial fibrosis in diabetic rats. Four weeks later, there were 10, 7, 9, 7 mice that had survived in the control group, STZ group, STZ+SO_2 and HDX group respectively. In evaluating the deposition of collagen fibres in the myocardium, Masson staining was carried out at the end of the experiment. The result revealed the deposition of the collagen fibres. Blue staining demonstrated the intensity of myocardial fibrosis. As indicated by the result of Masson staining (Fig. 1A), myocardial fibrosis was barely observed in the control group. In the STZ group, the myocardial cells showed a disorderly arrangement, and myocardial fibres significantly increased. Unlike in the STZ group, the myocardial fibrosis was obviously alleviated in the STZ+SO_2 group. Moreover, the myocardial fibrosis was more serious in the HDX group than in the STZ group. On the whole, these results above suggest that treatment with the SO_2, decreases diabetes-induced myocardial fibrosis. Therefore, it indicates that SO_2 improves myocardial fibrosis in diabetic rats.

Effects of SO_2 on MMP9, MMP24 and TIMP1 expression in diabetic rats. The balance of MMPs/TIMPs determines the ratio of collagen synthesis and degradation. Therefore, we

Table I. SO_2 improves cardiac function in diabetic rats. Effects of SO_2 on LVEDD, LVESD, EF and FS% in diabetic rats.

Group	control group	STZ group	STZ+SO_2 group	HDX group
LVEDD (mm)	4.77±0.59	6.27±0.32[a]	5.26±0.31[b]	6.50±0.42[a]
LVESD (mm)	2.50±0.18	4.17±0.21[a]	3.10±0.26[b]	4.56±0.71[a]
EF (%)	85.35±2.27	68.03±0.90[a]	79.40±1.56[b]	63.80±3.68[a]
FS (%)	46.27±2.73	32.77±1.46[a]	41.57±1.43[b]	30.35±2.62[a]

Values are expressed as mean ± standard deviation (n=7). [a]P<0.05 vs. control group; [b]P<0.05 vs. STZ group. SO_2, sulfur dioxide; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic diameter; EF, ejection fraction; FS, fractional shortening; STZ, streptozotocin; HDX, L-Aspartic acid β-hydroxamate.

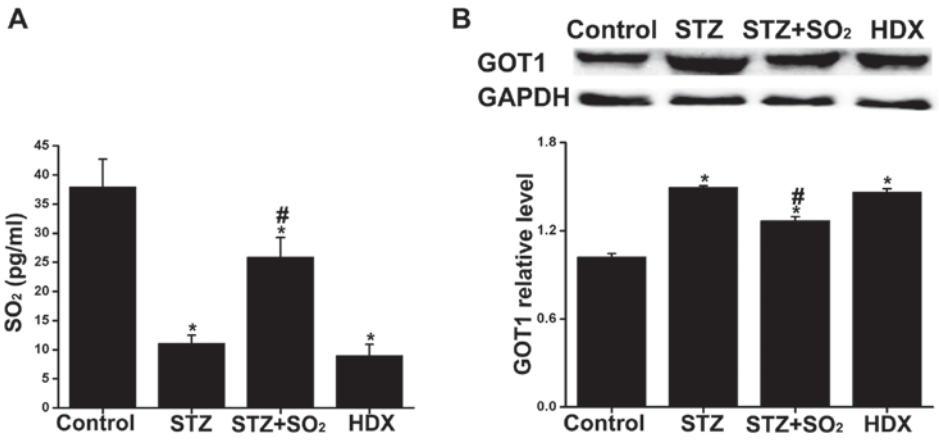

Figure 2. (A) Quantification of myocardial SO_2 content in rats from each group and SO_2 synthase aspartate aminotransferase (GOT1) protein expressions in each group (B) Data are expressed as mean ± standard deviation (n=3). *P<0.05 vs. control group; #P<0.05 vs. STZ group. SO_2, sulfur dioxide; STZ, streptozotocin; HDX, L-Aspartic acid β-hydroxamate.

determined the expressions of MMP9 (Fig. 1B), MMP24 (Fig. 1C) and TIMP1 (Fig. 1D) to reflect fibrosis to some degree. Compared with the control group, the expression levels of MMP9, MMP24 and TIMP1 in STZ group, STZ+SO_2 group and HDX group were significantly increased. Compared with STZ group, the myocardial expression levels of MMP9, MMP24 and TIMP1 were significantly reduced in STZ+SO_2 group, meanwhile, the expression levels of MMP9, MMP24 and TIMP1 were obviously increased in HDX group.

SO_2 improves cardiac function in diabetic rats. In this study, the parameters of cardiac function evaluated via echocardiography, namely, LVEDD, LVESD, ejection fraction (EF) and fractional shortening (FS), were measured to evaluate left ventricular function. Compared with the control group, LVEDD and LVESD increased more significantly in the STZ group and HDX group EF and FS decreased more significantly (by 17.32 and 13.5%, respectively) in the STZ group and (by 21.55 and 15.92%, respectively) HDX group. Compared with STZ group, the EF and FS increased more significantly in the STZ+SO_2 group, while the LVEDD and LVESD obviously decreased in the STZ+SO_2 group. Moreover, compared with STZ group, EF and FS slightly decreased in the HDX group, while the LVEDD and LVESD slightly increased in the HDX group, but there were not statistically significant (Table I). Taken together, our results indicate that SO_2 improves diabetic-induced the decrease of cardiac function.

SO_2 content in each group. To determine whether diabetes-induced myocardium damage was related to the decreased generation of endogenous SO_2, we assayed SO_2 content via ELISA. In comparison with those in the control group, the expression levels of SO_2 in the STZ group and HDX group were significantly decreased. In comparison with that in the STZ group, the myocardial expression of SO_2 was significantly increased in the STZ+SO_2 group. Nevertheless, no significant changes were observed in the HDX group in comparison with the STZ group (Fig. 2A). The results demonstrate that the content of SO_2 in diabetic rats were significantly decreased.

GOT1 protein expressions in each group. The expressions of SO_2 synthase aspartate aminotransferase (GOT1) protein in myocardium tissues were measured by western blot analysis. In comparison with that in the control group, the expression levels of GOT1 in the STZ group, STZ+SO_2 group, and HDX group were significantly increased. In comparison with the STZ group, the myocardial expression of GOT1 was significantly decreased in the STZ+SO_2 group. Nevertheless, no significant changes were observed in the HDX group in comparison with the STZ group (Fig. 2B).

SO_2 can reduce ERS in diabetic rats. By performing western blot analysis, we determined the expressions of CHOP (Fig. 3A), GRP94 (Fig. 3B), BIP (Fig. 3C), eIF2α (Fig. 3D), PDI (Fig. 3E) and ERO1-L (Fig. 3F) to observe ERS in the

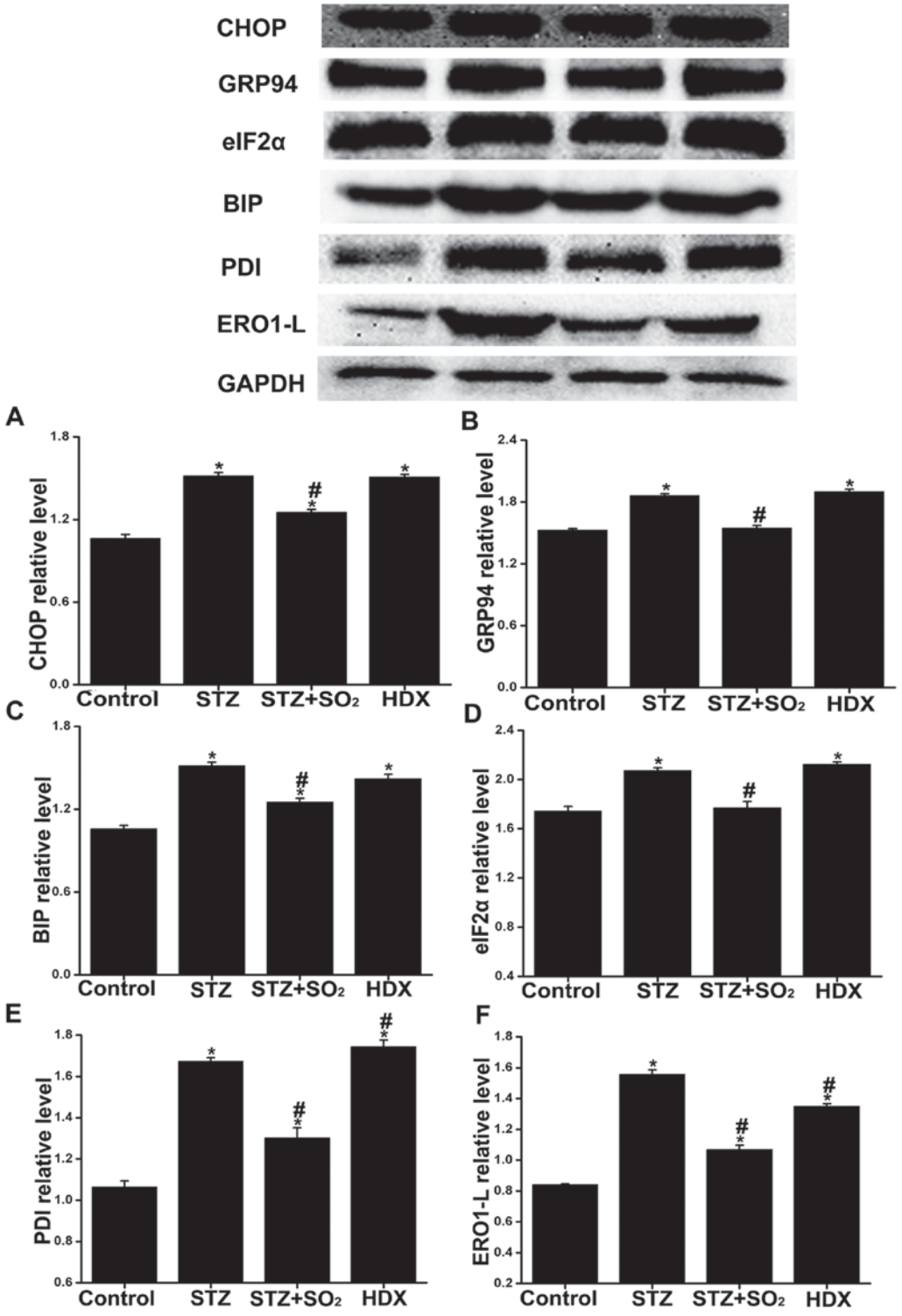

Figure 3. SO_2 can reduce endoplasmic reticulum stress in diabetic rats. Expression levels of (A) CHOP, (B) GRP94, (C) BIP, (D) eIF2α, (E) PDI and (F) ERO1-L in each group. Data are expressed as mean ± standard deviation (n=3). *P<0.05 vs. control group; #P<0.05 vs. STZ group. SO_2, sulfur dioxide; STZ, streptozotocin; HDX, L-Aspartic acid β-hydroxamate.

myocardium and to assess whether SO_2 protects against ERS. Unlike those in the control group, the expressions of CHOP, eIF2α, GRP94, BIP, PDI and ERO1-L were significantly increased in the STZ group and HDX group. In comparison with those in the STZ group, the expression levels of CHOP, eIF2α, GRP94, BIP, PDI and ERO1-L in the STZ+SO_2 group significantly decreased. The expression levels of some ERS protein markers slightly increased in the HDX group, but the change was not statistically significant. Our data thus indicate that SO_2 inhibits the expression of proteins associated with ERS in diabetic rats.

Effects of SO_2 on myocardial ultrastructure in diabetic rats. To investigate the internal mechanism underlying the SO_2-elicited beneficial effects against diabetes, we performed TEM in our observation of the endoplasmic reticulum and myocardial

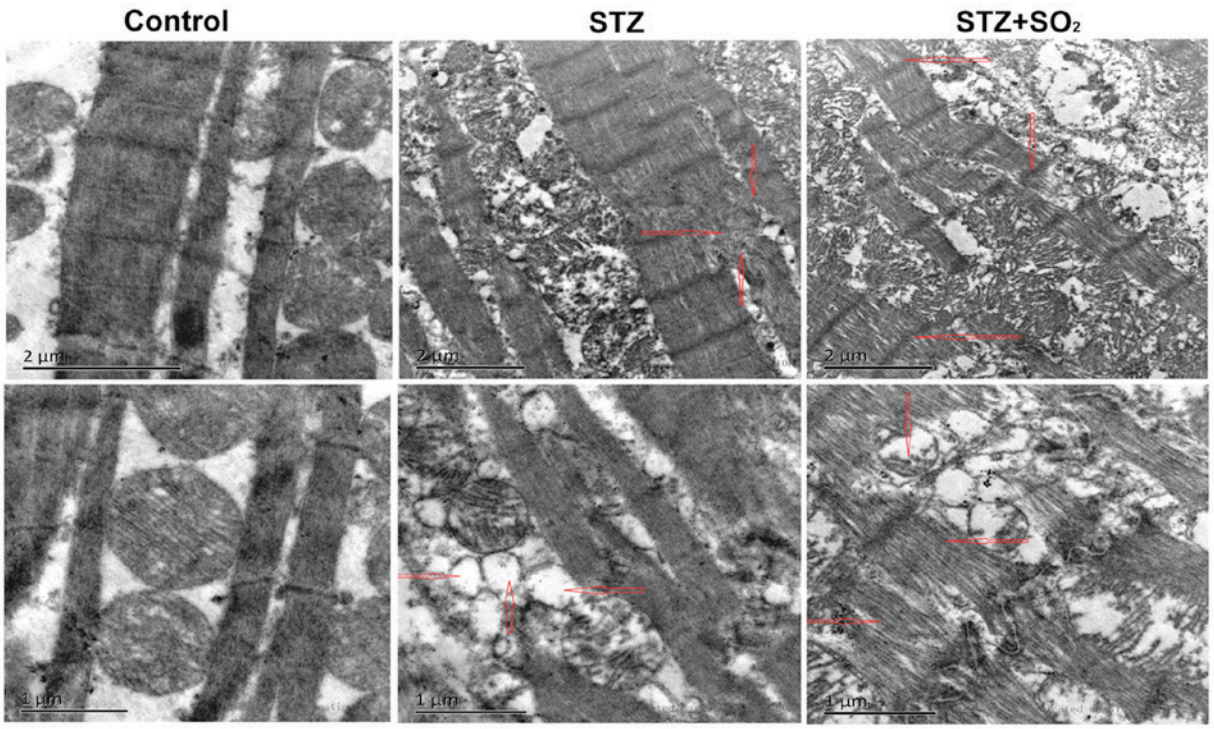

Figure 4. SO_2-alleviated myocardial ultrastructural disorder and swelling of the endoplasmic reticulum in diabetic rats. Transmission electron micrographs at bars of 1 and 2 μm. SO_2, sulfur dioxide; STZ, streptozotocin.

fibres. As indicated by the TEM results, the myocardial fibres were arranged normally, and the endoplasmic reticulum was normal and showed no swelling in the control group. In some myocardial tissues from the STZ group, the myocardial fibres were arranged disorderly and were dropsical, and focal cytolysis necrosis was observed. Swelling and vesiculation of the endoplasmic reticulum were also observed in the STZ group. These changes in the STZ group obviously improved in the STZ+SO_2 group. Additionally, the myocardial ultrastructural changes in the HDX group were similar to those in the STZ group (Fig. 4). These results above indicates that SO_2 improves the injury of myocardial ultrastructure in diabetic rats.

SO_2 can reduce cardiomyocyte apoptosis in diabetic rats. In this experiment, we used TUNEL assay to detect apoptosis in heart tissue. The number of apoptotic cells increased more obviously in the STZ group than in the control group. We also found that under SO_2 treatment, the number of TUNEL-positive cells decreased obviously in the STZ+SO_2 group. In comparison with those in the STZ group, the apoptotic cells in the HDX group obviously increased (Fig. 5A). These results indicate that SO_2 suppresses diabetic-induced cell apoptosis indiabetic rats.

SO_2 can reduce myocardial apoptosis in diabetic rats. To expound the molecular basis of the increased myocardial apoptosis in the diabetic rats, we examined the expression levels of Bax (Fig. 5B), Bcl-2 (Fig. 5C), caspase-3 (Fig. 5D) and caspase-9 (Fig. 5E) via western blot analysis. The expressions of Bax, caspase-3 and caspase-9 expression increased, and the expression of Bcl-2 decreased in the STZ group in comparison with the control group. However, under SO_2 treatment, the expression of Bcl-2 increased, and those of Bax, caspase-3 and caspase-9 decreased in the STZ+SO_2 group in comparison with the STZ group. These results indicated that diabetes-induced apoptosis was likely mediated, at least in part, by the Bcl-2/Bax-mediated caspase-3 and caspase-9 activation pathway in the heart. Our results thus indicate that SO_2 can reduce myocardial apoptosis in diabetic rats.

Effects of SO_2 on diabetes-induced change in the Hippo-MST signalling pathway. To demonstrate the Hippo-MST signalling pathway involved in the development of DC, we detected the expression of proteins related to the Hippo-MST signalling pathway via western blot analysis. In comparison with those in the control group, the expressions of MST1 (Fig. 6A), MST2 (Fig. 6B), MOB1 (Fig. 6C), and LATS1 (Fig. 6D) in the STZ group and HDX group significantly increased. In comparison with those in the STZ group, the expressions of MST1, MST2, MOB1 and LATS1 in STZ+SO_2 group significantly decreased. Taken together, our results indicate that high glucose induced the activation of Hippo-MST signalling pathway and SO_2 can downregulate the expression of proteins associated with Hippo-MST signalling pathway in diabetic rats.

Discussion

Diabetic mellitus (DM) is a global health issue that has attracted significant attention. As one of the major complications of DM, DC imposes a severe threat on human health (13). The crucial pathological changes of myocardial fibrosis in relation to the occurrence and development of DM are also an important cause of myocardial remodelling and heart failure. In this research, a model of diabetic rats was built with the intraperitoneal injection of STZ. Masson staining and TEM revealed that collagen deposition obviously increased in the myocardial

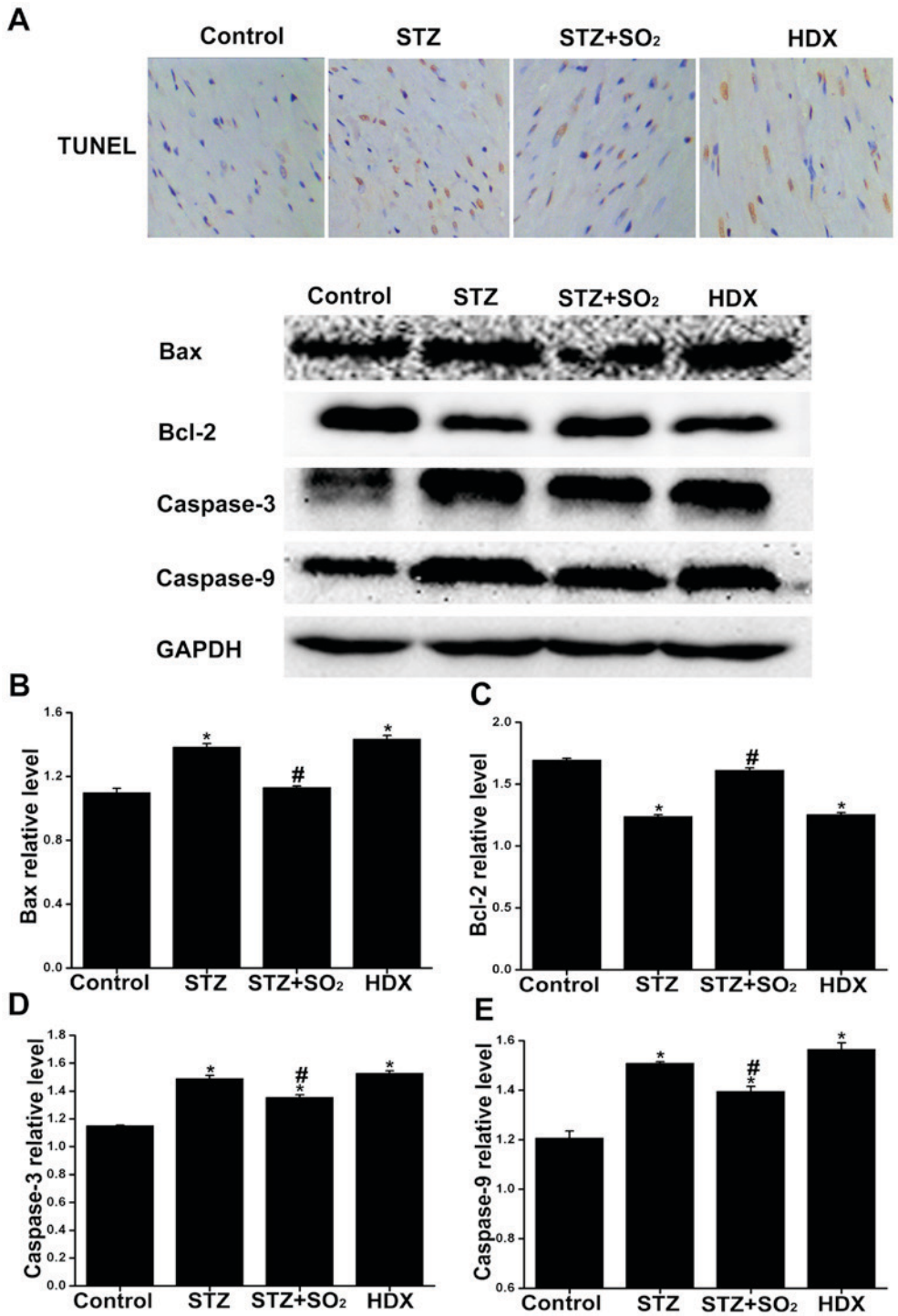

Figure 5. SO_2 reduces cardiomyocyte apoptosis in diabetic rats. (A) Representative photomicrographs of heart tissues showing TUNEL-positive (apoptotic) myocardiocytes. Images were acquired at x400 magnification. Expression levels of (B) Bax, (C) Bcl-2, (D) caspase-3 and (E) caspase-9 in each group. Data are expressed as mean ± standard deviation (n=3). *P<0.05 vs. control group; #P<0.05 vs. STZ group. SO_2, sulfur dioxide; STZ, streptozotocin; HDX, L-Aspartic acid β-hydroxamate.

tissue of the diabetic rats, and the western blot analysis indicated the significant maladjustment of MMPs/TIMPs in the myocardial tissue of the diabetic rats. The results suggest an obvious interstitial fibrosis in the myocardial tissue of the diabetic rats. Echocardiography also further confirmed the decline of cardiac function of the diabetic rats. Myocardial fibrosis participates in the mechanism of the occurrence and development of DC, and it is closely associated with the poor prognosis of diabetic patients. However, the mechanism of diabetic myocardial fibrosis has not been completely understood. Some studies have shown a close correlation between diabetic myocardial fibrosis and oxidative stress, ERS, apoptosis.

Currently, apoptosis is known to take part in the occurrence and development of DC (14,15). When the body is under common apoptosis-promoting stimulations of diabetes, such as high glucose, oxidative stress and metabolic disorders, the caspase cascade pathways in the myocardial tissue are

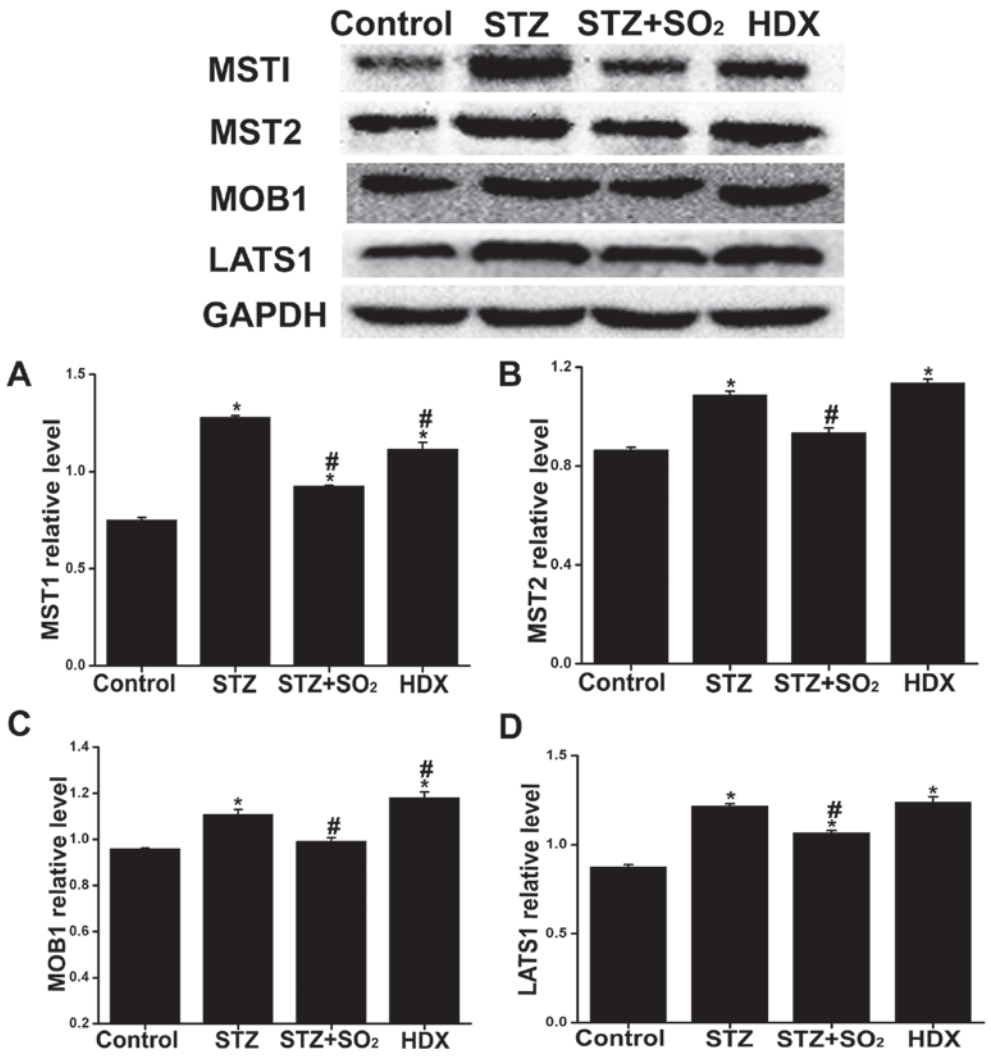

Figure 6. Effects of SO_2 on diabetes-induced change in Hippo-MST signalling pathway. Expression levels of (A) MST1, (B) MST2, (C) MOB1 and (D) LATS1 in each group. Data are expressed as mean ± standard deviation (n=3). *P<0.05 vs. control group; #P<0.05 vs. STZ group. SO_2, sulfur dioxide; STZ, streptozotocin; HDX, L-Aspartic acid β-hydroxamate.

activated one by one, thus inducing the occurrence of apoptosis. The activation of caspase-3 is a key link in the apoptosis signalling pathway (16), and Bcl-2 has been proven to block the activation of caspase and inhibit cell apoptosis (17). The result of TUNEL staining revealed that the number of apoptotic cells in the myocardial tissues of the diabetic rats increased more remarkably relative to the control group. At the same time, the expressions of the pro-apoptotic proteins caspase-3, caspase-9 and Bax in the myocardial tissue was significantly upregulated, and the expression of the anti-apoptotic protein Bcl-2 was obviously downregulated. ERS has been also found to take part in the occurrence mechanism of DC, and it serves as the initial common channel of oxidative stress and other cell stress reactions. However, sustained or extremely strong ERS may result in cell apoptosis. ERS in diabetics may be induced by numerous factors, including hyperglycemia, oxidative stress, activation of RAS system and lipid metabolism. Some studies have found that ERS in myocardial cells is excessively activated (18,19). The current research found that the ERS-associated proteins of DC, such as CHOP, GRP94, BIP and eIF2α, were obviously upregulated. Furthermore, TEM showed that swelling occurred in the endoplasmic reticulum of the myocardial cells and that the volume increased, thereby suggesting excessively activated ERS in the myocardial tissue as well as endoplasmic reticulum damage. ERS may activate CHOP, and the upregulation of CHOP may inhibit Bcl-2 expression, thus inducing ERS-associated apoptosis. In the present work, we found that the endoplasmic reticulum was obviously activated in the myocardial tissues of the diabetic rats and that CHOP expression was upregulated, thereby suggesting that ERS participated in the occurrence mechanism of apoptosis in the process of diabetic myocardial fibrosis.

As one of the prevalent cell signalling mechanisms of pathological and physiological processes in animals, the Hippo-MST signalling pathway participates in the regulation of cell proliferation and apoptosis, tissue repair and other processes (20). Such pathway mainly includes MST, MOB, LATS and other signalling molecules. As a key transcription factor of this pathway, MST1 plays an important role in promoting cell apoptosis and proliferation (21,22). Activated MST may enter the cell nucleus to promote apoptosis, inhibit cell growth and proliferation and associate with the phosphorylation of FOXO. LATS may be activated by the

phosphorylation of MST, enter its own phosphorylation cycle and become activated continuously. MST and LATS can also interact with each other to create a positive feedback circuit and thus promote the pro-apoptotic effect. Some studies have shown that MST1 overexpression may promote myocardial apoptosis and that the inhibition of MTS1 expression may improve apoptosis as well as myocardial remodelling and cardiac dysfunction caused by destructive stimuli, such as apoptosis and ischaemia-reperfusion injury (23-26). The latest research shows that the abnormal activation of MST1 is closely associated with the occurrence mechanism of diabetes and that it participates in the occurrence of myocardial fibrosis (27,28). In the current research, we found obvious myocardial fibrosis in the diabetic rats and a remarkably upregulated expression of the Hippo-MST pathway-associated proteins. Such finding suggests the possible participation of the Hippo-MST pathway in the occurrence of myocardial fibrosis in the diabetic rats. MST1/2 may phosphorylate the core members of Hippo signalling, namely, Sav1, LATS1/2 and MOB1. Therefore, the upregulation of MST1/2 may promote apoptosis. In the present research, we found that the expressions of MST1/2 and its downstream pathway proteins MOB and LATS were obviously upregulated. The expressions of MST1/2 and its downstream pathway proteins MOB and LATS may participate in apoptosis regulation under oxidative stress and ERS in diabetics. Hence, the Hippo-MST signalling pathway maintains the balance between tissue injury and repair and participates in the occurrence mechanism of diabetic myocardial fibrosis by regulating cell apoptosis and maintaining proliferation.

After the discovery of NO, CO and H_2S in recent years, some studies have shown that SO_2 may be a novel gaseous signalling molecule (29). As the research on sulfurous gases continue, an endogenous SO_2-generating system has been found in the cardiovascular system. Sulfur-containing amino acids may generate H_2S and SO_2 via in vivo metabolism (30,31). Thus far, H_2S is known to exhibit a myocardial protective effect in the presence of oxidative stress and hypoxic-ischaemic injury and improve myocardial fibrosis. Both H_2S and SO_2 are metabolites of sulfur-containing amino acids, and their pathological actions closely correlated with each other. Recent studies have found that SO_2 improved the ISO-induced myocardial apoptosis of the rats (32). Some studies have shown that SO_2 gas performs many biological functions, such as vasodilation, improvement of vascular and collagen remodelling; it also inhibits inflammation and autoxidation and exerts an important protective effect on vascular diseases (33-36), such as pulmonary arterial hypertension, myocardial ischaemia-reperfusion injury, myocardial hypertrophy, spontaneous hypertension and acute lung injury (37-40). However, the role of SO_2 in diabetic myocardial fibrosis and the relevant regulatory mechanism have not been studied yet. The result of the present research indicated that after the intervention with SO_2 donor, collagen deposition was obviously decreased in the myocardial interstitium of the diabetic rats and that the dysregulation of MMPs/TIMPs obviously improved, thus suggesting that exogenous SO_2 could improve the myocardial fibrosis of diabetic rats. The ERS level in the myocardial tissue of the diabetic rats obviously decreased after the intervention with SO_2. The result showed that the expressions of ERS-associated proteins CHOP, GRP94, eIF2α, BIP, PDI and ER01-L were obviously downregulated. Furthermore, after the intervention with SO_2, cell apoptosis obviously decreased in the myocardial cells of the diabetic rats. At the same time, the expressions of the pro-apoptotic proteins caspase-3 and caspase-9 were obviously downregulated, and the expression of the anti-apoptotic protein Bcl-2 was obviously upregulated. Such findings indicate that exogenous SO_2 can improve ERS and inhibit cell apoptosis, which may be associated with its role in improving myocardial fibrosis.

Existing studies have found that the role of SO_2 in inhibiting cell apoptosis may be correlated with its mechanism of regulating the Hippo-MST signalling pathway. The expressions of the Hippo-MST signalling pathway proteins (MST1, Sav1, LATS1 and MOB1) in the myocardial tissues of the rats in the SO_2 intervention group were obviously downregulated in comparison with those in the diabetic group. Hence, exogenous SO_2 may inhibit the Hippo-MST signalling pathway to reduce ERS and cell apoptosis as well as the resulting diabetic myocardial fibrosis. On the contrary, after the intervention with the SO_2-generating enzyme inhibitor HDX, the myocardial fibrosis of the diabetic rats became severe in Masson staining, but he myocardial ultrastructural changes in the HDX group were similar to those in the STZ group, It is likely to have some factors that was difficult to control, because we can not make sure the myocardial tissues were obtained from the identical parts in the heart and there may be some differences between Masson staining and TEM in the steps of making myocardium specimens, we need to evaluate the difference of myocardial fibrosis between STZ group and HDX group in multiple aspects. For example, compared with STZ group, the expression levels of MMP9, MMP24 and TIMP1 were obviously increased and the cardiac function was even worse in HDX group, these results remind us that the myocardial fibrosis may become severe in HDX group, thus suggesting that endogenous SO_2 may take part in the regulatory mechanism of myocardial fibrosis in diabetic rats. Unlike that in the diabetics group, the intervention with HDX led to no statistically significant changes in the Hippo-MST signalling pathway, ERS and apoptosis in the myocardial tissues of the diabetic rats. Hence, the aforementioned changes in diabetic myocardial tissue may be related to the downregulated generation of endogenous SO_2 and its signal regulation.

In the present research, the downregulated generation of endogenous SO_2 wasobserved in DC. This result corresponded to the compensatory upregulation of the expression of the endogenous SO_2-generating enzyme GOT1, thereby suggesting that the insufficient endogenous generation of the gaseous signalling molecule SO_2 may contribute to the occurrence mechanism of myocardial interstitial fibrosis in diabetic rats and that the internal mechanism by which SO_2 improves myocardial fibrosis may be associated with its inhibition of the Hippo-MST signalling pathway to improve cell apoptosis and ERS. The above results would provide new evidence for the further discussion on the occurrence mechanism of DC and offer a new target for the treatment and prevention of diabetic myocardial fibrosis. However, the specific molecular mechanism by which endogenous SO_2 regulates the Hippo-MST signalling pathway and improves

cell fate still need further discussion because of the limitations of present data, We planed to do further research about the specific molecular mechanism by which endogenous SO_2 regulates the Hippo-MST signalling pathway and improves cell fate on the basis of present results in the next research, such as adding the SO_2 treatment group on normal rats and MST inhibitor treatment group on STZ-induced diabetic rats and useing cell culture to explore the role of Hippo-MST signalling pathway in high glucose-induced cardiomyocyte injury and whether SO_2 protects cardiomyocytes against high glucose-induced injury by Hippo-MST signalling pathway by. In order to discuss the specific relation among ERS, apoptosis and Hippo-MST signalling pathway, it is important to conduct enzymatic activity assays and reporter gene assays. Meanwhile, we intended to use Mst1-knockout mice to investigate Mst1-knockout whether can alleviate the myocardial fibrosis in DC. We hope the further research to make more contributions to the treatment of DC.

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