

Original Article

Gaseous signal molecule SO₂ regulates autophagy through PI3K/AKT pathway inhibits cardiomyocyte apoptosis and improves myocardial fibrosis in rats with type II diabetes

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ABSTRACT Myocardial fibrosis is a key link in the occurrence and development of diabetic cardiomyopathy. Its etiology is complex, and the effect of drugs is not good. Cardiomyocyte apoptosis is an important cause of myocardial fibrosis. The purpose of this study was to investigate the effect of gaseous signal molecule sulfur dioxide (SO₂) on diabetic myocardial fibrosis and its internal regulatory mechanism. Masson and TUNEL staining, Western-blot, transmission electron microscopy, RT-qPCR, immunofluorescence staining, and flow cytometry were used in the study, and the interstitial collagen deposition, autophagy, apoptosis, and changes in phosphatidylinositol 3-kinase (PI3K)/AKT pathways were evaluated from *in vivo* and *in vitro* experiments. The results showed that diabetic myocardial fibrosis was accompanied by cardiomyocyte apoptosis and down-regulation of endogenous SO₂-producing enzyme aspartate aminotransferase (AAT)_{1/2}. However, exogenous SO₂ donors could up-regulate AAT_{1/2}, reduce apoptosis of cardiomyocytes induced by diabetic rats or high glucose, inhibit phosphorylation of PI3K/AKT protein, up-regulate autophagy, and reduce interstitial collagen deposition. In conclusion, the results of this study suggest that the gaseous signal molecule SO₂ can inhibit the PI3K/AKT pathway to promote cytoprotective autophagy and inhibit cardiomyocyte apoptosis to improve myocardial fibrosis in diabetic rats. The results of this study are expected to provide new targets and intervention strategies for the prevention and treatment of diabetic cardiomyopathy.

INTRODUCTION

The global prevalence of type 2 diabetes remains high, with the number of people projected to reach 693 million in 2045 [1]. Diabetes has become a serious burden on patients' families

and society due to its serious complications and poor prognosis. Diabetes can cause persistent damage to systemic organs [2], and cardiovascular complications are the leading cause of death in patients with diabetes, especially those with type 2 diabetes [3]. Diabetic cardiomyopathy, as one of the independent and important



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cardiovascular complications of diabetes, is also an important cause of death and disability in patients with type 2 diabetes [4-7]. Diabetic cardiomyopathy, which can lead to poor prognosis, such as heart failure and acute cardiovascular events [8,9], has become a major public health problem worldwide in the chronic disease management of diabetes.

The key link in the occurrence and development of diabetic cardiomyopathy is myocardial fibrosis. Myocardial fibrosis is pathological remodeling of the myocardium characterized by myocardial cell loss, excessive deposition of myocardial extracellular matrix, and changes in the phenotype of cardiac fibroblasts [10]. Various injury factors can promote the imbalance of myocardial cell homeostasis to induce myocardial cell apoptosis, resulting in pathological myocardial remodeling, reduced ventricular wall motility, and heart failure [11]. The pathogenesis of diabetic myocardial fibrosis is not very clear at present, and there is currently a lack of clear and effective clinical interventions for diabetic myocardial fibrosis.

Apoptosis, the most common way of programmed cell death, plays an important role in the pathological outcome of many diseases. Studies have shown that high glucose levels caused by diabetes can produce glucotoxicity and lead to cardiomyocyte apoptosis, and high glucose can cause mitochondrial damage and ROS accumulation in cardiomyocytes. Finally, the apoptosis program is initiated, which can lead to diabetic cardiomyopathy [12-14]. Moderate autophagy is critical for cellular homeostasis under stress, and glucotoxicity from high glucose levels inhibits cardiomyocyte autophagy [14-16]. Impairment of autophagy can lead to cardiomyocyte apoptosis, excessive repair of fibrosis, and pathological remodeling [17]. However, the role of autophagy in cardiomyocyte apoptosis and myocardial fibrosis induced by type 2 diabetes is still unclear and needs further study.

As an important pathway mediating autophagy, the phosphatidylinositol 3-kinase (PI3K)/AKT (AKTV-murine thymoma viral oncogene/protein kinase B) signaling pathway can regulate cell survival and homeostasis, and is an important target of cardiovascular disease [18,19]. Excessive activation of the PI3K/AKT signaling pathway induced by a high-glucose and high-fat environment can lead to abnormal organ function. A recent study found that the PI3K/AKT pathway was significantly up-regulated in the myocardial tissue of rats with diabetic cardiomyopathy, accompanied by impaired autophagosome formation and defective autophagy [20]. Previous studies have found that inhibition of PI3K/AKT can activate autophagy, thereby alleviating fibrosis and cell damage [21,22]. In the process of myocardial fibrosis, inhibiting the excessive activation of PI3K/AKT can inhibit the progression of myocardial fibrosis [23]. In addition, studies have shown that in the myocardial tissue of rats with diabetic cardiomyopathy, inhibiting the activation of the PI3K/AKT signaling pathway can reduce oxidative stress, thereby improving impaired cardiac function and reducing pathological changes such as fibrosis [24]. However, the role of PI3K/AKT-mediated autophagy

in the occurrence and development of diabetic cardiomyopathy is still unclear.

Sulfur dioxide (SO₂) is generally known as “a toxic gas.” But in recent years, it has been found that SO₂ is also an important endogenous gas signal molecule like NO and CO. SO₂ can be endogenously generated *in vivo* by an enzymatic reaction catalyzed by aspartate aminotransferase (AAT) in the metabolic pathway of sulfur-containing amino acids. It has been found to play an important role in cardiovascular homeostasis and collagen remodeling [25]. Studies have shown that the expression of SO₂-producing enzymes in the myocardial tissue of diabetic rats is low [26], and SO₂ can inhibit the proliferation and migration of fibroblasts and the progression of myocardial fibrosis [27]. However, it is unclear whether SO₂ can antagonize myocardial fibrosis in diabetic rats and its internal regulation mechanism. The effect of SO₂ on autophagy and apoptosis of myocardial cells in diabetic rats is also unclear.

Therefore, this study intends to construct the diabetic rat model and give exogenous SO₂ donors to observe the effect of gas signaling molecule SO₂ on myocardial cell apoptosis and myocardial fibrosis in rats with type 2 diabetes and to explore whether its mechanism is related to the regulation of autophagy by PI3K/AKT pathway.

METHODS

Materials

Streptozotocin (STZ), Na₂SO₃, NaHSO₃, D-glucose, and HDX (serine racemase inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PI3K/AKT activator 740 Y-P was provided by MedChemExpress (Shanghai, China). PI3K (67071-1-Ig), AKT (10176-2-AP), Beclin1 (11306-1-AP), LC3 (14600-1-AP), Atg5 (10181-2-AP), Atg16L1 (19812-1-AP), P62 (66184-1-Ig), AAT1 (14886-1-AP), AAT2 (14800-1-AP), Caspase3 (19677-1-AP), Caspase9 (10380-1-AP), Bax (50599-2-Ig), Bcl2 (26593-1-AP), MMP3 (66338-1-Ig), MMP8 (17874-1-AP), MMP13 (18165-1-AP), CollagenIII (22734-1-AP), and GAPDH (10494-1-AP) primary antibodies and mouse/rabbit secondary antibodies were purchased from Proteintech (Wuhan, China). P-PI3K (4228S) and P-AKT (9614T) primary antibodies were offered by Cell Signaling Technology (Beverly, MA, USA). Gel configuration kits and BCA protein quantification kits were purchased from Biyuntian Institute of Biotechnology (Shanghai, China). ECL chemiluminescence kit was provided by New Cell and Molecular Biotech Co., Ltd. (Suzhou, China). AnnexinV-fluorescein isothiocyanate/propidium iodide (AV-FITC/PI) apoptosis detection kit was purchased from Keygene Biotech Co., Ltd. (Nanjing, China).

Animals

The animal experiments were performed in accordance with the guidelines for the welfare and use of laboratory animals and gained approval from the Animal Ethics Committee of the University of South China, according to the animal management regulations and specifications of the Laboratory Animal Center of University of South China. A total of 40 8-week-old male Sprague–Dawley (SD) rats were randomly assigned to four groups (n = 10). The type II diabetes rats model was established through the single intraperitoneal injection of STZ (35 mg/kg, Sigma-Aldrich; dissolved in citric acid-sodium citrate buffer, pH 4.5) combined with a diet high in fat and sugar for eight weeks before STZ intraperitoneal injection [28]. The Control group and the SO₂ group were given an equal amount of citric acid-sodium citrate buffer through intraperitoneal injection. One week after the injection, fasting blood glucose levels of the rats > 16.7 mM indicated that the type II diabetes rat model was successfully established, and diabetic rats were continuously given a diet high in sugar and fat. Subsequently, the diabetic cardiomyopathy (DC) + SO₂ and SO₂ groups were given exogenous SO₂ donor (Na₂SO₃/NaHSO₃, 85 mg/kg/d, Sigma-Aldrich) intraperitoneally for eight weeks [29], and the Control group and the DC groups were given equal amounts of saline intraperitoneally (Fig. 1). The heart tissues were collected after eight weeks of SO₂ intervention for follow-up assays, and the heart weight and the pre-execution weight were weighed to calculate the HW/BW value. 8-week-old male SD rats were purchased from HUNAN SJA Laboratory Animal Co. (Changsha, China).

Blood biochemistry analysis

After eight weeks of STZ injection, all rats were gavaged with 50% glucose solution for the glucose tolerance test, and tail vein blood specimens were collected at 30, 60, and 120 min to measure the blood glucose levels. Fasting blood glucose and fasting insulin levels were collected from the rats at the time of devotion and homeostasis model assessment of insulin resistance (HOMA-IR) values were obtained.

Echocardiography

After all rats were anesthetized with 4% chloral hydrate (400 mg/kg), the changes of left ventricular end-diastolic-dimension (LVEDD), left ventricular end-systolic dimension (LVESD), and left ventricular fractional shortening (LVFS) values were measured using M-mode ultrasound.

Histopathological staining

Myocardial tissues were fixed with 4% paraformaldehyde, paraffin-embedded after dehydration by gradient alcohol, made into 4 μm thin sections by microtome, and stained in accordance with the instructions of the Masson staining kit (Abiowell Biotech Co., Ltd., Changsha, Hunan), and sealed by treacle. The staining of the tissues was observed under a light microscope. The fixed and embedded myocardial tissues were made into 4 μm thin sections, and the corresponding operations were performed in accordance with the instructions of the TUNEL staining kit (Keygene Biotech Co., Ltd.). The staining was observed under a light microscope.

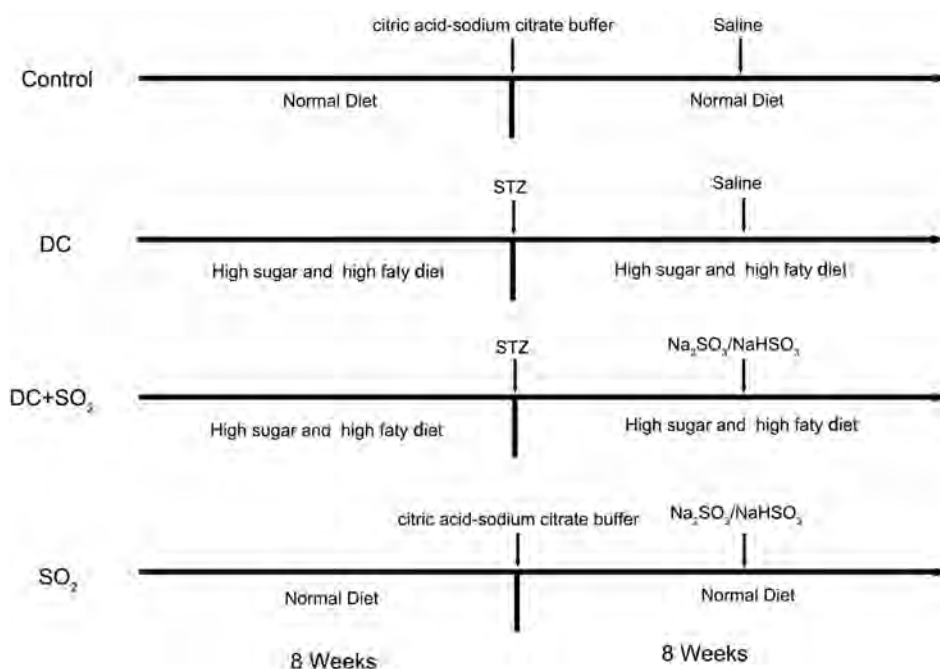


Fig. 1. Animal grouping and drug treatment. Sprague–Dawley (SD) rats were administered with 8 weeks of different dietary interventions followed by intraperitoneal injection of streptozotocin (STZ) or citric acid-sodium citrate buffer, and the dietary treatment was continued for 8 weeks after the successful establishment of the diabetic rats model, meanwhile intraperitoneal injection of exogenous sulfur dioxide (SO₂) donor (Na₂SO₃/NaHSO₃) or equivalent saline for 8 weeks. DC, diabetic cardiomyopathy.

Transmission electron microscopy

The myocardial tissues were fixed by 2.5% glutaraldehyde and cut into 50–100 nm thin sections, and phosphoric acid rinsing solution (Beyotime Institute of Biotechnology, Shanghai, China) was used. After being immersed in 1% osmium tetroxide (Absin Biosciences, Inc., Shanghai, China), the tissues were fixed and rinsed with phosphoric acid, followed by gradient acetone immersion. After being dehydrated and dried, the tissues were stained with 3% uranyl acetate and lead nitrate for 10–20 min and rinsed with distilled water. The ultrastructure was observed under transmission electron microscopy.

Cell culture

H9c2 cardiomyocyte cell line was purchased from ATCC (Manassas, VA, USA) and incubated in DMEM medium supplemented with 5.5 mM D-glucose with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO₂. The cells were inoculated in 6-well plates when the cell density was close to 1 × 10⁶ and incubated with a medium supplemented with high D-glucose (33 mM). The cells were assigned to the Control group, the HG group, the HG + SO₂ group, the HG + SO₂ + serine racemase inhibitor (HDX) group, the HG + SO₂ + 740 Y-P group, and the SO₂ group in accordance with different treatments. The Control group and SO₂ group were cultured with low D-glucose medium (5.5 mM), and the HG group and HG + SO₂ group, and HG + SO₂ + HDX group were treated with high D-glucose medium (33 mM) for 24 h at 37°C. The exogenous SO₂ donor Na₂SO₃/NaHSO₃ (50 μM, 3:1 mol rate) was introduced to the HG + SO₂ group and HG + SO₂ + HDX group for the 0.5-h pretreatment as described previously [30], and then co-incubated with high D-glucose medium. Besides, the HG + SO₂ + HDX group was co-incubated with the endogenous SO₂-produced enzyme inhibitor HDX (200 μM) [31]. In order to verify whether SO₂ activates autophagy by inhibiting the PI3K/AKT pathway, the HG + SO₂ + 740 Y-P group was incubated with PI3K/AKT activator 740 Y-P (30 μM) after the SO₂ pretreatment, and all groups were incubated with high D-glucose medium (33 mM), except for the Control group and the SO₂ control group.

Flow cytometry analysis

H9c2 cardiomyocyte apoptosis was detected using AV-FITC/PI Apoptosis Detection Kit (Keygene Biotech Co., Ltd.). In accordance with the manufacturer's instructions, the cells were digested with 0.25% EDTA-free trypsin (Gibco, Grand Island, NY, USA) and washed twice in cold phosphate buffer saline (PBS). Resuspension was performed with 500 μl binding buffer supplemented with 5 μl FITC and 5 μl PI for 10 min in a dark room. Flow cytometry (fluorescence-activated cell sorting [FACS] Calibur cytometer; BD Biosciences, San Diego, CA, USA) was

adopted to obtain the apoptosis rate. AV-FITC values were set as the horizontal axis, and PI values were set as the vertical axis. All experiments were performed in triplicate.

Immunofluorescence staining

Cells were fixed with 4% PFA for 40 min, permeabilized with 0.1% Triton X-100 for 5 min × 2, and blocked with 5% BSA for 1 h, incubated with rabbit anti-Beclin1 primary antibody (1:200) overnight at 4°C, then incubated with Cy3 (1:200)-conjugated secondary antibody for 1 h at room temperature and counterstained using DAPI. Fluorescence was analyzed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Real-time q-PCR

Total RNA was extracted from the rat myocardial tissues using TRIzol (Thermo Fisher Scientific, Rockford, IL, USA) and then transcribed into cDNA with the Prime-Script RT kit (Takara, Shiga, Japan). Real-time qPCR was performed with SYBR green (Takara) under the standard conditions of 95°C for 30 sec, 40 cycles of 95°C for 5 sec, and 60°C for 30 sec. Real-time qPCR was conducted under the standard conditions, GAPDH was used as an internal reference, and the relative expression levels of target genes were obtained using the 2^{-ΔΔCt} method. The primer sequences are presented as follows:

Col3a1:

F: AAGGGCAGGGAACAACACTGAT, R: GGTGAAGCAGGGTGAGAAGA;

P62:

F: GCTCGACCCGTCCTTCACTCA, R: CAGGGATCAGTACCCGCTCT;

Caspase3:

F: TTAGAACTTGAATCCACGAG, R: TTCCAATAATAAAACACGACT;

Bax:

F: GCGATGAACTGGACAACAAC, R: AAAGTAGAAAAGGGCAACCAC.

GAPDH:

F: ACAGCAACAGGGTGGTGGAC, R: TTTGAGGGTGCAGCGAACTT

Western blotting

The rat myocardial tissues and the cell precipitates were lysed through radioimmunoprecipitation assay (RIPA) lysis buffer containing cold protease inhibitor, the supernatant was collected after centrifugation, and the protein concentration of the supernatant was quantified and leveled using the BCA protein quantification kit based on the manufacturer's guidelines. The samples were denatured after heating at 95°C for 10 min, separated in

10%–12% polyacrylamide electrophoresis gels, and electrophoretically transferred to a PVDF membrane (polyvinylidene fluoride membrane; Millipore Co., Bedford, MA, USA). The membranes were blocked with 5% skim milk at RT for 1 h and treated with primary antibody at 4°C overnight. The membranes were washed three times (5 min × 3) with tris-buffered saline (TBST) containing 20 and incubated with the corresponding species of horseradish peroxidase (HRP)-conjugated secondary antibody at RT for 1 h. After TBST washing (5 min × 3), the exposure shots were taken with ECL chemiluminescent solution, and image signals were obtained using the Bio-Rad XRS+ imaging system (Bio-Rad, Hercules, CA, USA). The relative data were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

The data were expressed as mean ± standard error (SEM). The statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software Inc., San Diego, CA, USA). The differences between group means were evaluated through one-way ANOVA Test. $p < 0.05$ was considered statistically significant.

RESULTS

Test results of related biochemical markers of blood

Tables 1 and 2 list the results of the glucose tolerance test and the values of fasting blood glucose, insulin and HOMA-IR of the rats in the respective group, respectively. The fasting blood glucose, insulin levels and HOMA-IR values of the rats in the DC group and the DC + SO₂ group increased significantly compared

with those of the Control group ($p < 0.05$), which suggested that the rat model of type II diabetes was successfully established. There were significant abnormalities of blood glucose and insulin in both groups. The fasting blood glucose, insulin levels and related HOMA-IR values in the DC + SO₂ group tended to decrease compared with those of the DC group, whereas the difference between the two groups did not achieve statistical significance ($p > 0.05$). The fasting blood glucose, insulin level and HOMA-IR value of the rats in the SO₂ group did not change significantly compared with those of the Control group ($p > 0.05$).

Changes in the expression of endogenous SO₂-generating enzymes AAT_{1/2}

Western blot was used to detect the changes in protein expression of SO₂-generating enzyme AAT_{1/2} in the myocardial tissues of the rats in the respective group (Fig. 2A, C). As revealed by the results, compared with the Control group, the expression of AAT_{1/2} in the myocardial tissues of the rats in the DC group decreased significantly ($p < 0.05$), while the expression of AAT_{1/2} in the myocardial tissues of the rats in DC + SO₂ group increased after the intervention of exogenous SO₂ donor Na₂SO₃/NaHSO₃ ($p < 0.05$). There was no statistically significant difference in the expression of AAT_{1/2} between the SO₂ control group and the Control group.

Besides, similar changes were also identified in the cell experiments *in vitro* (Fig. 2B, D). AAT_{1/2} in H9c2 cardiomyocytes of the HG group had significantly lower expression than that in the Control group ($p < 0.05$), while the expression of AAT_{1/2} in the HG + SO₂ group was significantly up-regulated as compared with that in the HG group ($p < 0.05$). After the cardiomyocytes of the HG + SO₂ + HDX group were intervened with the endogenous

Table 1. Test results of glucose tolerance test of the rats in the respective group

Groups	Number	0.5 h (mM)	1 h (mM)	2 h (mM)
Control	9	5.57 ± 0.24	5.68 ± 0.37	4.27 ± 0.16
DC	7	31.36 ± 0.62*	31.09 ± 0.40*	27.59 ± 1.34*
DC + SO ₂	8	30.62 ± 1.53*	30.63 ± 0.44*	24.83 ± 1.12*
SO ₂	9	6.94 ± 0.49	5.82 ± 0.89	5.54 ± 0.10

Values are presented as mean ± SD. SO₂, sulfur dioxide; DC, diabetic cardiomyopathy. * $p < 0.05$ vs. Control.

Table 2. Comparison of values of fasting blood glucose, insulin and HOMA-IR of the rats in the respective group

Groups	Number	Fasting blood glucose (mM)	Fasting insulin level (μIU/ml, 10 ³)	HOMA-IR
Control	9	3.70 ± 0.32	25.00 ± 10.00	4.00 ± 0.80
DC	7	23.15 ± 1.49*	436.67 ± 79.46*	363.03 ± 68.61*
DC + SO ₂	8	22.32 ± 1.27*	316.67 ± 56.25*	312.48 ± 58.59*
SO ₂	9	3.60 ± 0.28	44.00 ± 5.48	7.00 ± 0.63

Values are presented as mean ± SD. HOMA-IR, homeostasis model assessment of insulin resistance; SO₂, sulfur dioxide; DC, diabetic cardiomyopathy. * $p < 0.05$ vs. Control.

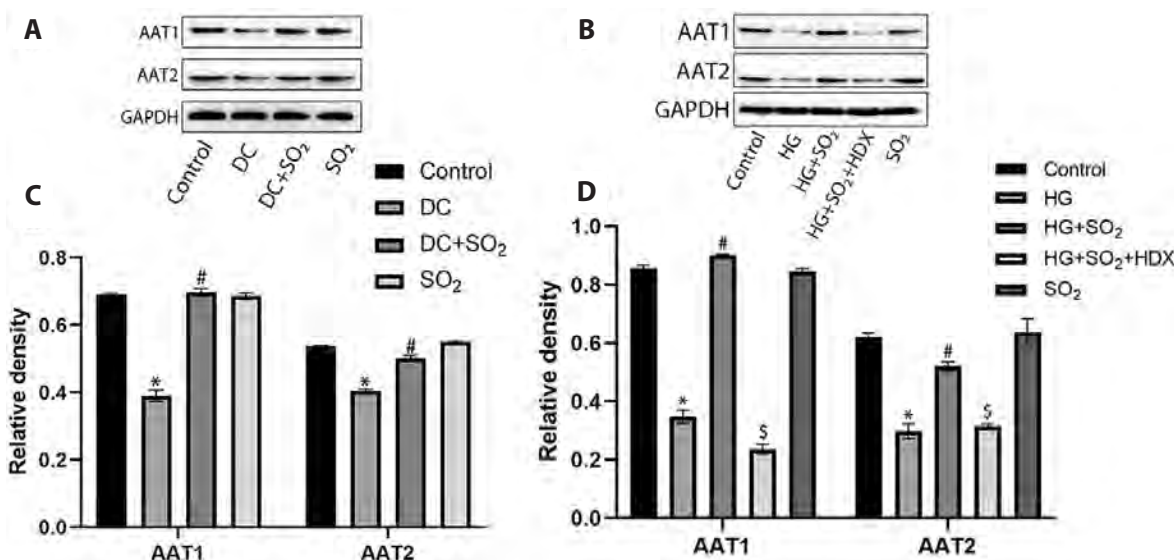


Fig. 2. Exogenous sulfur dioxide (SO₂) can up-regulate the expression level of aspartate aminotransferase (AAT)_{1/2}. (A, C) Western blot analysis of the expression changes of AAT_{1/2} in the myocardial tissue of type II diabetes rats. *n* = 3, **p* < 0.05 vs. Control; #*p* < 0.05 vs. DC. (B, D) Western blot analysis of the expression changes of AAT_{1/2} in H9c2 cardiomyocytes; *n* = 3, **p* < 0.05 vs. Control; #*p* < 0.05 vs. HG; §*p* < 0.05 vs. HG + SO₂. Values are presented as mean ± SD. DC, diabetic cardiomyopathy; HG, high glucose.

Table 3. Cardiac LVFS values of the rats in the respective group

Groups	Number	LVEDD (mm)	LVESD (mm)	LVFS
Control	9	5.53 ± 0.31	3.30 ± 0.15	39.97 ± 2.03
DC	7	4.94 ± 0.19	3.35 ± 0.10	31.96 ± 2.35
DC + SO ₂	8	5.02 ± 0.24	3.07 ± 0.10	38.26 ± 3.45
SO ₂	9	4.96 ± 0.20	2.97 ± 0.05	39.83 ± 1.91

Values are presented as mean ± SD. LVFS, left ventricular fractional shortening; LVEDD, left ventricular end-diastolic-dimension; LVESD, left ventricular end-systolic dimension; SO₂, sulfur dioxide; DC, diabetic cardiomyopathy.

Table 4. The HW, BW and HW/BW values of the rats in the respective group

Groups	Number	BW (g)	HW (mg)	HW/BW (10 ⁻³)
Control	9	390.00 ± 2.58	1090.00 ± 93.63	2.79 ± 0.17
DC	7	236.25 ± 19.70	810.50 ± 47.50	3.48 ± 0.09*
DC + SO ₂	8	285.83 ± 24.13	914.67 ± 85.39	3.20 ± 0.11
SO ₂	9	400.00 ± 25.49	1017.20 ± 45.26	2.55 ± 0.09

Values are presented as mean ± SD. LVFS, left ventricular fractional shortening; SO₂, sulfur dioxide; DC, diabetic cardiomyopathy; BW, body weight; HW, heart weight. **p* < 0.05 vs. Control.

SO₂ inhibitor HDX, the expression of AAT_{1/2} was significantly lower than that of the HG + SO₂ group (*p* < 0.05), thus revealing that the production of SO₂ in the myocardial tissue and cardiomyocytes of diabetes rats was reduced based on high-glucose stimulation. Furthermore, exogenous SO₂ donors could up-regulate the expression of SO₂-producing enzyme AAT_{1/2} in the myocardial tissue and high-glucose-stimulated myocardial cells of diabetic rats.

Effects of SO₂ on cardiac structure and cardiac function in diabetic rats

In this paper, we detected the cardiac function and structural changes of the respective rats by echocardiography (Table 3), and found that compared with the Control group, the LVFS value of the DC group decreased, while the LVFS value of the DC + SO₂ group increased after SO₂ intervention. However, the difference between the groups was not statistically significant. By comparing the changes of HW/BW of the rats in the respective group, as listed in Table 4, we found that the HW/BW of the DC group

was significantly higher than that of the Control group ($p < 0.05$). However, after SO₂ intervention, the HW/BW of the DC + SO₂ group decreased; the difference was not statistically significant.

The effect of SO₂ on myocardial fibrosis in diabetic rats

As revealed by the result of Masson staining (Fig. 3A, B), compared with the control group, the blue-stained collagen fibers in the myocardial tissue of the diabetic rats in the DC group were significantly increased by about 4 times, and the myocardial fibers were disordered. However, after the intervention of exogenous SO₂, the number of blue-stained collagen fibers in the myocardial tissue of rats decreased by about 1/2 compared with the DC group ($p < 0.05$), and the myocardial cells were arranged relatively neatly. Moreover, the expression changes of fibrosis-associated proteins (including Collagen III, MMP3, MMP8, and MMP13) were detected through Western blot (Fig. 3C, D). It was

found that the expression of Collagen III, MMP3, MMP8, and MMP13 in the DC group was significantly higher than those in the Control group ($p < 0.05$). As revealed by the RT-qPCR results (Fig. 3E), the mRNA expression of Col3a1 in the DC group was about 3.8 times higher than that in the Control group; after the SO₂ intervention, the changing trend of the above fibrosis-associated proteins was significantly reversed ($p < 0.05$). However, no statistically significant difference was found between the Control and SO₂ groups. The above results indicated that there was obvious myocardial fibrosis in type II diabetes rats, and SO₂ intervention could ameliorate myocardial fibrosis in diabetic rats.

SO₂ can inhibit cardiomyocyte apoptosis induced by high glucose

In this paper, we observed the effect of high glucose on the apoptosis of H9c2 cardiomyocytes and the protective effect of SO₂. Flow cytometry was used to detect the apoptosis rate of cells

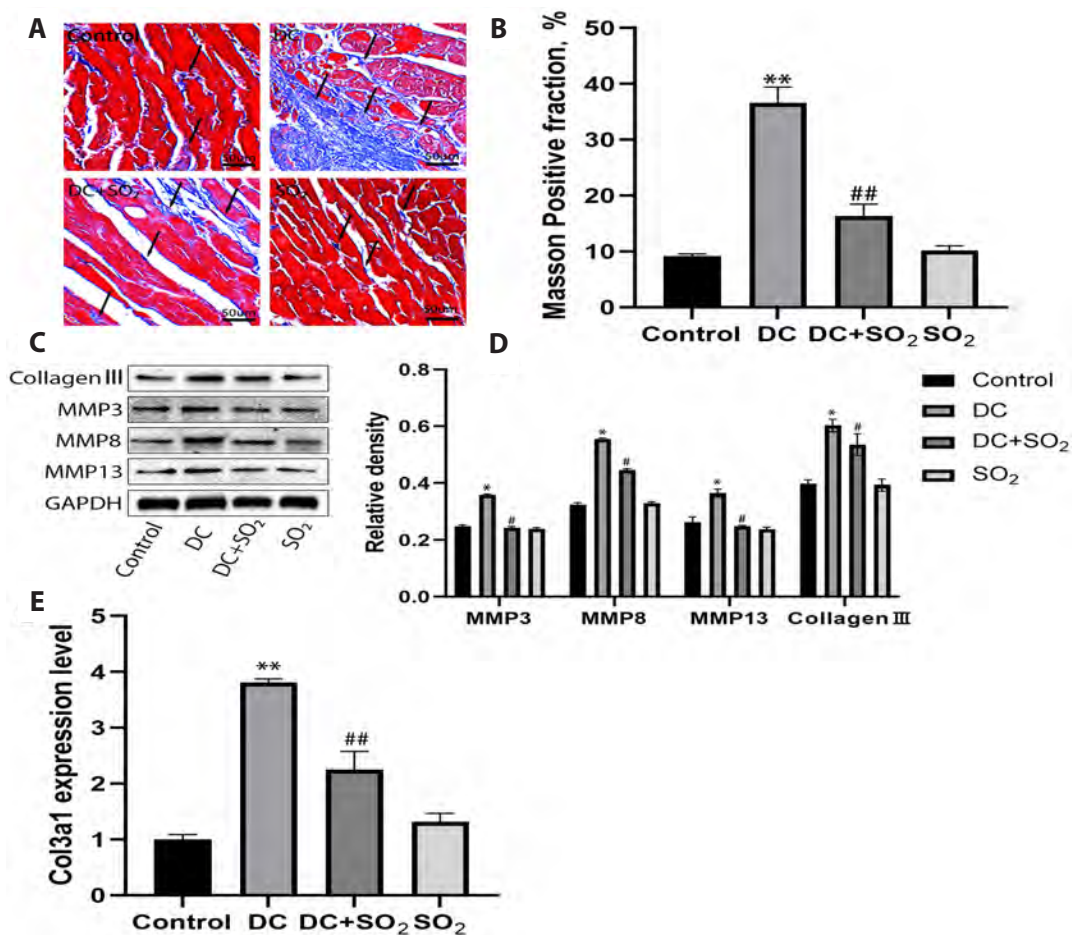


Fig. 3. Sulfur dioxide (SO₂) can reduce the formation of myocardial collagen fibers in type II diabetic rats. (A) Masson staining results of myocardial tissue of the rats in the Control, DC, DC + SO₂, and SO₂ groups. 10 × 40 magnification field of view (blue-stained part is collagen fibers, and black arrows are collagen fibers), $n = 3$; (B) Fraction of Masson-stained collagen volume, ** $p < 0.01$ vs. Control; ## $p < 0.01$ vs. DC. (C, D) Western blot analysis of the protein expression changes of MMP3, MMP8, MMP13, and Collagen III in the myocardial tissue of the rats in the respective group. $n = 3$, * $p < 0.05$ vs. Control; # $p < 0.05$ vs. DC. (E) RT-qPCR detection of the expression of Col3a1 in the myocardial tissue of the rats. $n = 3$, ** $p < 0.01$ vs. Control; ## $p < 0.01$ vs. DC. Take GAPDH as reference. Values are presented as mean \pm SD deviation. DC, diabetic cardiomyopathy.

in each group (Fig. 4A). It was found that the apoptosis of H9c2 cardiomyocytes in the HG group increased by about 4.5 times compared with the control group under high-glucose stimulation. The incidence of early and late apoptotic cells was significantly increased ($p < 0.05$). After the intervention of exogenous SO_2 , the apoptosis rate of cardiomyocytes in the HG + SO_2 group decreased by about 1/2, and the late apoptotic cells decreased significantly ($p < 0.05$). At the same time, the apoptosis-associated proteins were detected through Western blot (Fig. 4B–F): Bax/Bcl2, Cleaved Caspase3/Caspase3, Cleaved Caspase9/Caspase9 in the HG group were significantly higher than those in Control group ($p < 0.05$). After SO_2 intervention, the above apoptotic proteins were significantly down-regulated ($p < 0.05$), and HDX could significantly reverse the anti-apoptotic effect of SO_2 . The expressions of Bax/Bcl2, Cleaved Caspase3/Caspase3, and Cleaved Caspase9/Caspase9 in cardiomyocytes of the HG + SO_2 + HDX group were up-regulated ($p < 0.05$). The above results suggest that the high-glucose environment can induce the apoptosis of H9c2 cardiomyocytes, while SO_2 intervention can reduce the apoptosis of cardiomyocytes induced by high-glucose stimulation.

Similar changes were observed in *in vivo* experiments. By TU-

NEL staining on the myocardial tissue of diabetic rats (Fig. 5A, B), we found that the brown-stained apoptotic cardiomyocytes in the myocardial tissue of diabetic rats in the DC group increased by about 1.76 times compared with the control group ($p < 0.05$), after SO_2 intervention, TUNEL-positive apoptotic cardiomyocytes in the myocardial tissue were significantly reduced compared with DC group ($p < 0.05$). Compared with the Control group, the ratio of apoptosis-associated proteins Bax/Bcl2, Cleaved Caspase3/Caspase3, and Cleaved Caspase9/Caspase9 in the DC group increased significantly ($p < 0.05$), and the mRNA expression levels of Caspase3 and Bax were about 3 times higher than those in the Control group (Fig. 5H, I), while the changes of the above proteins in DC + SO_2 group were significantly reversed ($p < 0.05$).

SO_2 inhibits apoptosis and fibrosis in cardiomyocytes through upregulation of autophagy

The expression of autophagy-associated proteins in the myocardial tissue of the rats in the respective group was observed through Western blot. As revealed by the results (Fig. 6B, C), the expressions of Atg5, Atg16L1, LC3II/I, and Beclin1 in the myocar-

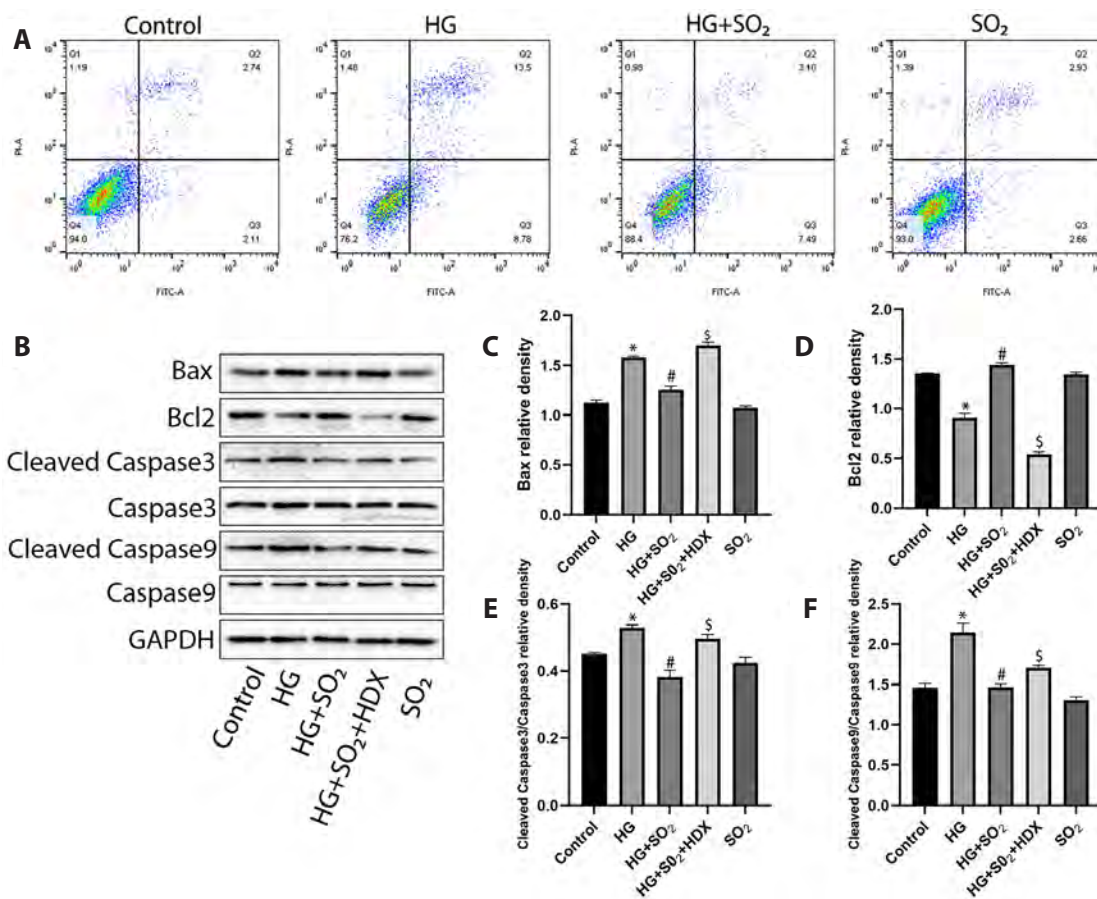


Fig. 4. SO_2 inhibits high-glucose-induced apoptosis in cardiac myocytes. (A) Flow cytometry to detect the apoptosis rate of H9c2 cardiomyocytes under high glucose environment (33 mM), Q2 + Q4 represents the incidence of apoptosis, $n = 3$; (B–F) The expression changes of apoptosis-associated proteins Bax, Bcl2, Caspase3, Cleaved-Caspase3, Caspase9, and Cleaved-Caspase9 in myocardial cells of each group were detected through Western blot. $n = 3$. Values are presented as mean \pm SD. SO_2 , sulfur dioxide; HG, high glucose. * $p < 0.05$ vs. Control; # $p < 0.05$ vs. HG; $^{\S}p < 0.01$ vs. HG + SO_2 .

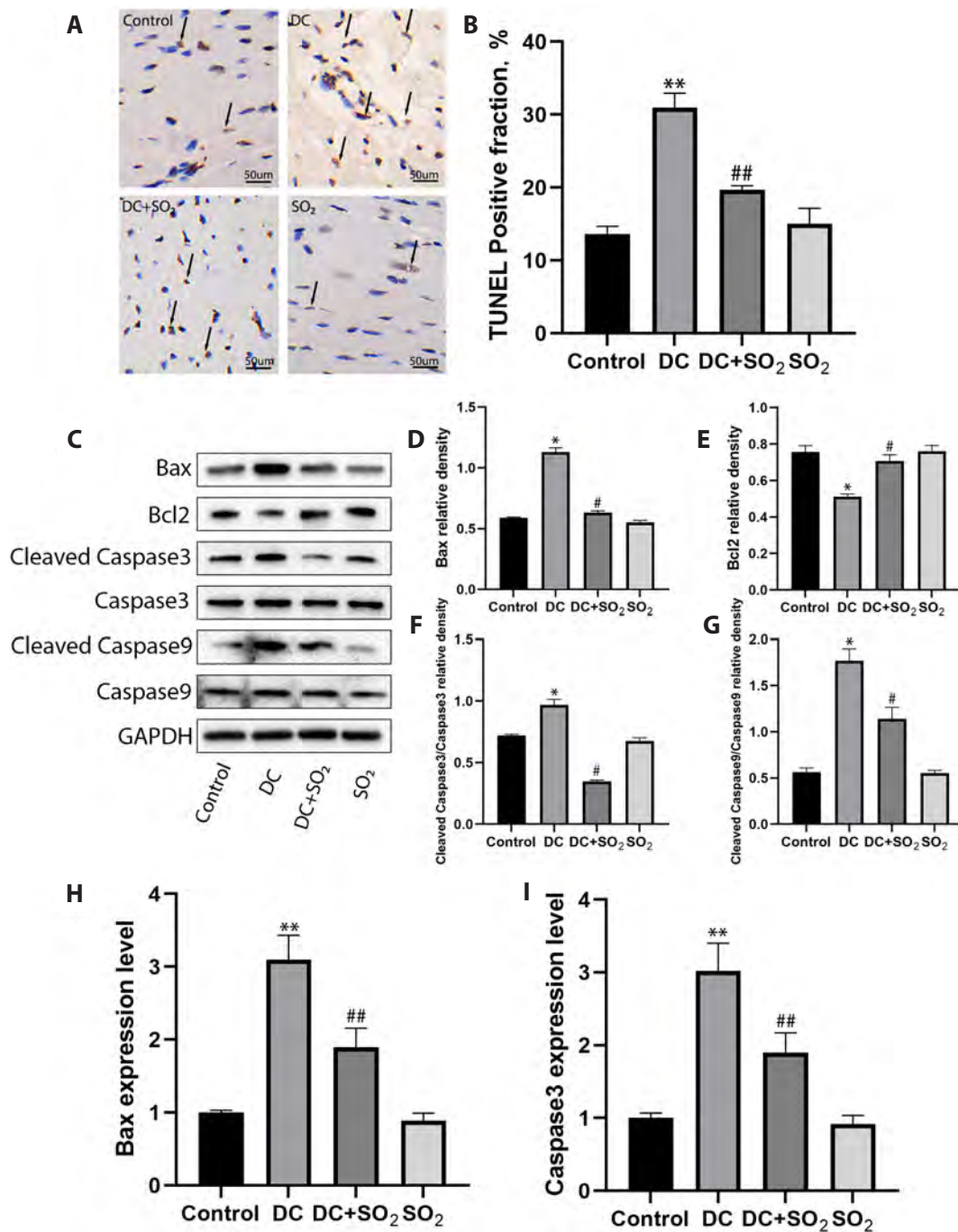


Fig. 5. SO₂ reduces cardiomyocyte apoptosis in diabetic rats. (A) TUNEL staining of myocardial tissue of the rats in the Control, DC, DC + SO₂ and SO₂ groups (apoptosis-positive cells in TUNEL staining are in brown, and they are indicated by black arrows), n = 3. Images were acquired at 10 × 40 magnification; (B) Quantitative results of TUNEL-stained positive cells in the myocardial tissue of the rats in the respective group, **p < 0.01 vs. Control. #p < 0.01 vs. DC; (C–G) Western blot detection of the expressions of Bax, Bcl2, Cleaved Caspase3, Caspase3, Cleaved Caspase9, Caspase9 in the myocardial tissue of the rats in the respective group. n = 3, *p < 0.05 vs. Control; #p < 0.05 vs. DC; (H, I) Detection of mRNA expression levels of Bax and Caspase3 in the myocardial tissue by RT-qPCR, **p < 0.01 vs. Control. #p < 0.01 vs. DC. GAPDH is taken as the internal parameter. Values are presented as mean ± SD. SO₂, sulfur dioxide; DC, diabetic cardiomyopathy.

dial tissue of the DC group were significantly lower than those of the Control group (p < 0.05). While the expression of P62 was significantly up-regulated (p < 0.05), and the above protein changes were reversed (p < 0.05) after SO₂ intervention. RT-qPCR results showed that the expression level of P62 in the DC group was

about 3.5 times higher than that in the Control group (Fig. 6G), and this change could be down-regulated after SO₂ intervention. Moreover, it was found through transmission electron microscopy (Fig. 6A) that compared with the control group, the myocardial tissues of the DC group were characterized by vacuolized

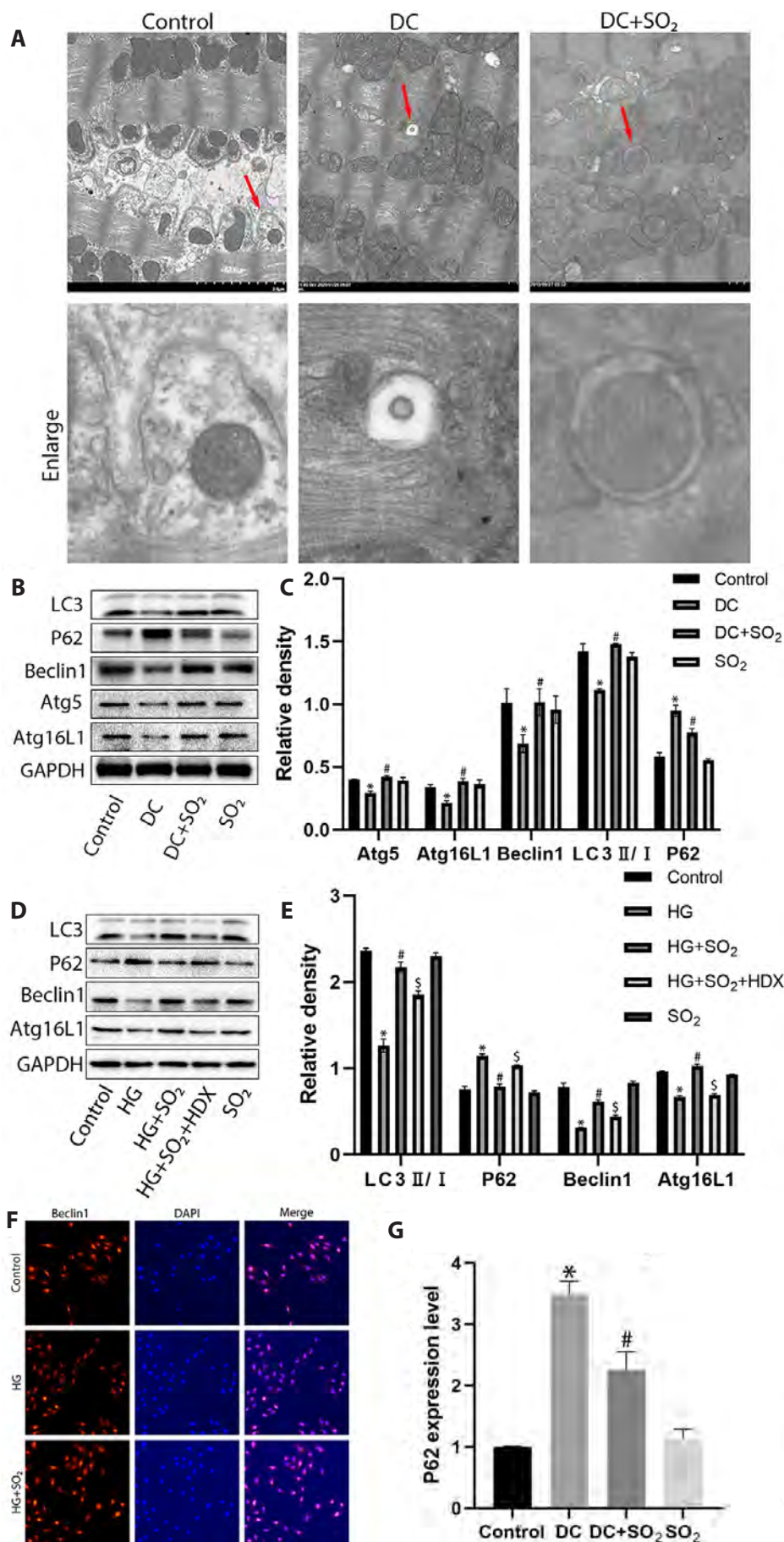


Fig. 6. Sulfur dioxide (SO₂) can up-regulate the level of autophagy in the myocardial tissue of type II diabetes rats. (A) The ultrastructure of cardiomyocytes and the changes of autophagosomes in type II diabetes rats were observed under transmission electron microscope. The arrows indicate autophagosomes, and the part indicated by the arrows in the upper figure is enlarged in the lower figure. The TEM figure was observed under $\times 5,000$ at $2 \mu\text{m}$ bar. (B, C) Western blot analysis of the expression changes of autophagy-associated proteins (including LC3II/I, Beclin1, Atg5, Atg16L1 and P62) in the myocardial tissue of the rats in the respective group. $n = 3$, * $p < 0.05$ vs. Control. # $p < 0.05$ vs. DC. (D, E) Western blot analysis of the expression changes of LC3II/I, Beclin1, Atg16L1 and P62 of H9c2 cardiomyocytes in the Control, HG, HG + SO₂, HG + SO₂ + HDX and SO₂ groups. $n = 3$; * $p < 0.05$ vs. Control. # $p < 0.05$ vs. HG. S $p < 0.05$ vs. HG + SO₂. (F) Immunofluorescence observation of Beclin1 expression in H9c2 cardiomyocytes from each group (magnification; $\times 10$). (G) RT-qPCR detection of P62 mRNA expression in rat myocardial tissue, * $p < 0.05$ vs. Control; # $p < 0.01$ vs. DC, take GAPDH as a reference. Values are presented as mean \pm SD. DC, diabetic cardiomyopathy; HG, high glucose.

autophagosomes, fewer autophagosomes, swollen mitochondria, and disordered arrangement of muscle fibers. Besides, the number of autophagosomes in the myocardial tissue of the rats after SO₂ intervention increased, vacuolization was rarely observed, and the arrangement of muscle fibers was relatively neat. The changes in autophagy-associated protein expression in the respective group of H9c2 cardiomyocytes were examined through Western Blot and immunofluorescence *in vitro* experiments (Fig. 6D–F) and found that autophagy-associated proteins (including LC3II/I, Beclin1, and Atg16L1) were significantly down-regulated ($p < 0.05$), the fluorescence intensity of Beclin1 was down-regulated in the HG group, and P62 was significantly up-regulated under high-glucose stimulation compared with the Control group ($p < 0.05$). However, after SO₂ intervention, the expression of LC3II/I, Beclin1, and Atg16L1 was significantly up-regulated ($p < 0.05$), and P62 was significantly down-regulated ($p < 0.05$) compared with the results of the HG group, while the fluorescence intensity of Beclin1 was significantly attenuated. HDX intervention could reverse the regulation effect of SO₂ on the above autophagy-associated proteins ($p < 0.05$). High glucose was found to lead to the decrease of the autophagy capacity of cardiomyocytes, while SO₂ intervention could partially up-regulate or restore autophagy.

SO₂ activates autophagy to antagonize cardiomyocyte apoptosis by downregulating PI3K/AKT

In this paper, the changes in PI3K/AKT protein expression in the myocardial tissue of the rats in the respective group were observed through Western blot (Fig. 7A, C). As revealed by the

results, compared with the Control group, the expressions of P-PI3K and P-AKT proteins in the DC group were significantly up-regulated ($p < 0.05$), and the ratios of P-PI3K/PI3K and P-AKT/AKT were also significantly increased. After SO₂ intervention, the expressions of P-PI3K and P-AKT proteins in the myocardium of the rats in the DC + SO₂ group were down-regulated ($p < 0.05$), and the ratios of P-PI3K/PI3K and P-AKT/AKT were decreased. There was no significant difference in the expression of P-PI3K and P-AKT between the SO₂ control group and the Control group ($p > 0.05$).

The results of the *in vitro* experiments also suggested that compared with the Control group (Fig. 7B, D), the P-PI3K and P-AKT proteins in the cardiomyocytes of the HG group were up-regulated ($p < 0.05$), while the ratios of P-PI3K/PI3K and P-AKT/AKT increased. After SO₂ intervention, the P-PI3K and P-AKT proteins in the HG + SO₂ group were down-regulated ($p < 0.05$), while the ratios of P-PI3K/PI3K and P-AKT/AKT were all decreased. At the same time, to further study whether SO₂ regulates autophagy and apoptosis via the PI3K/AKT pathway, we used 740 Y-P (30 μ M), an agonist of PI3K/AKT for intervention. It was found that the levels of LC3II/I, Beclin1, and Atg16L1 of cardiomyocytes in the HG + SO₂ + 740 Y-P group were significantly lower than those in the HG + SO₂ group ($p < 0.05$), and the level of P62 increased significantly ($p < 0.05$). The levels of Cleaved Caspase3/Caspase3, Cleaved Caspase9/Caspase9, and Bax/Bcl2 were significantly higher than those in the HG + SO₂ group ($p < 0.05$), as shown in Fig. 8C–G, which revealed that 740 Y-P, an agonist of PI3K/AKT, could reverse SO₂ and down-regulate PI3K/AKT to activate autophagy to antagonize hyperglycemia-induced apoptosis in cardiomyocytes, while SO₂ antagonized cardiomyo-

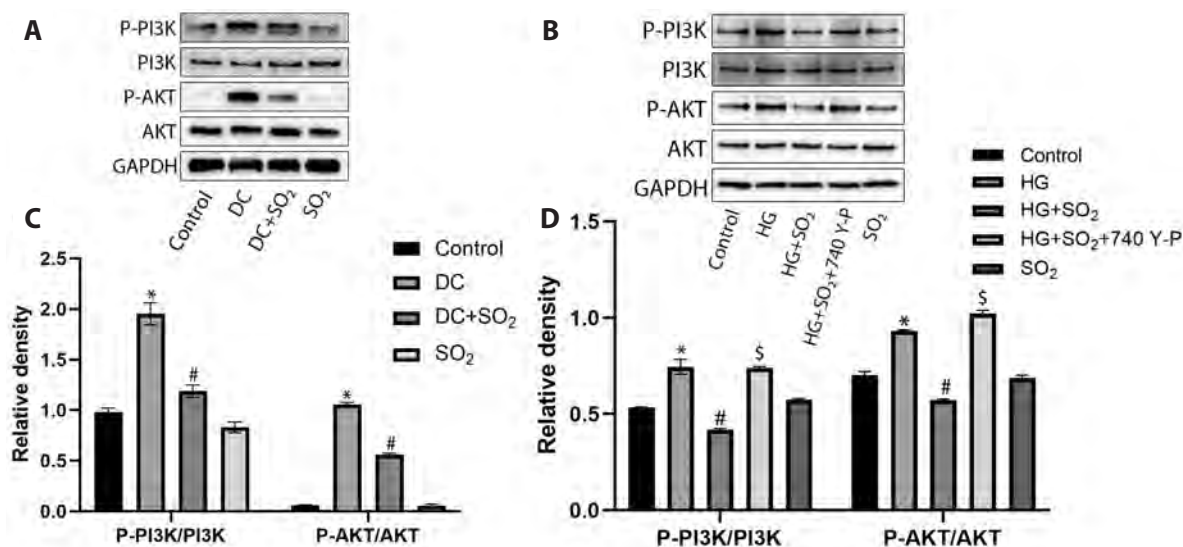


Fig. 7. Sulfur dioxide (SO₂) can reverse the up-regulation of phosphatidylinositol 3-kinase (PI3K)/AKT expression induced by high glucose. (A, C) Western blot detection to observe the expression changes of P-PI3K, PI3K, P-AKT, AKT in the myocardial tissues of the rats in the Control, DC, DC + SO₂, and SO₂ groups. $n=3$; * $p < 0.05$ vs. Control; # $p < 0.01$ vs. DC. (B, D) Western blot detection was used to observe the protein expression changes of P-PI3K, PI3K, P-AKT, and AKT of H9c2 cardiomyocytes in the Control, HG, HG + SO₂, HG + SO₂ + 740 Y-P and SO₂ group. $n = 3$; * $p < 0.05$ vs. Control. # $p < 0.05$ vs. HG. \$ $p < 0.05$ vs. HG + SO₂. Values are presented as mean \pm SD. DC, diabetic cardiomyopathy; HG, high glucose.

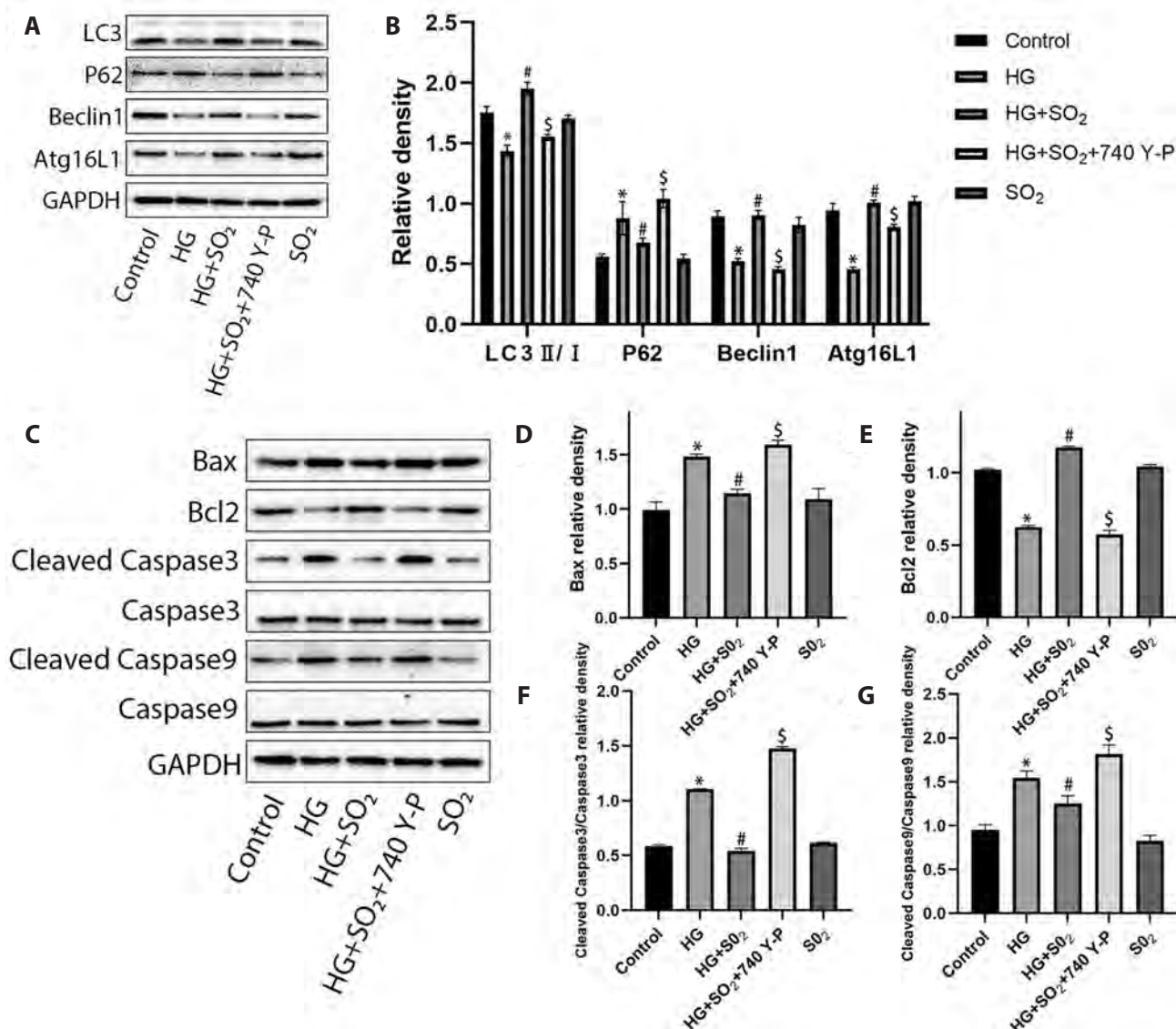


Fig. 8. Sulfur dioxide (SO₂) antagonizes apoptosis of H9c2 cardiomyocytes by down-regulating phosphatidylinositol 3-kinase (PI3K)/AKT and activating autophagy. (A, B) Western blot detection was used to observe the expression changes of autophagy-associated proteins (including LC3II/I, Beclin1, Atg16L1 and P62 of H9c2 cardiomyocytes) in the Control group, the HG group, the HG + SO₂ group, the HG + SO₂ + 740 Y-P group and the SO₂ group. (C–G) Western blot detection was used to observe the expression changes of apoptosis-associated proteins Bax, Bcl2, Caspase3, Cleaved-Caspase3, Caspase9, and Cleaved-Caspase9 of H9c2 cardiomyocytes in the Control, HG, HG + SO₂, HG + SO₂ + 740 Y-P, and SO₂ groups. n = 3. Values are presented as mean ± SD. DC, diabetic cardiomyopathy; HG, high glucose. *p < 0.05 vs. Control. #p < 0.05 vs. HG. §p < 0.05 vs. HG + SO₂.

cyte apoptosis by down-regulating PI3K/AKT and activating autophagy.

DISCUSSION

Type II diabetes is a metabolic disease characterized by hyperglycemia and insulin resistance. It has been recognized as a vital public health problem worldwide and has posed a serious hazard to people's health and quality of life [32–34]. Cardiomyopathy damage caused by diabetes has been found as one of the main causes of death in type II diabetes patients. Diabetic cardiomyop-

athy refers to a major cardiovascular disease that is characterized by myocardial abnormalities due to diabetes. Hyperglycemia and insulin resistance, two independent risk factors for DC, can cause abnormal myocardial metabolism and dysfunction and gradually develop into heart failure with the progression of the disease [35,36]. Existing studies have suggested that tissue inflammation, oxidative stress, and dysregulation of autophagy induced by high-glucose stimulation can promote dysfunction of cardiomyocytes and fibrosis of the myocardial interstitium, thus leading to diabetic cardiomyopathy [37,38]. Myocardial fibrosis is a vital pathological change leading to diabetic cardiomyopathy, which is primarily manifested as myocardial pathological changes charac-

terized by excessive deposition of myocardial extracellular matrix and myocardial structural disorder; it can develop into heart failure [39-41]. In this experiment, a model of rats with type II diabetes was established by a diet high in fat and sugar and through the intraperitoneal injection of STZ. The results of Masson staining showed that the DC group formed more collagen fibers than the normal group. Besides, the ultrasound results indicated that the cardiac function was lower than that of the normal group, thus revealing that myocardial fibrosis could cause obvious myocardial fibrosis and cardiac dysfunction in diabetic rats.

Apoptosis has been found as a key link between myocardial fibrosis and myocardial remodeling [42]. Since cardiomyocytes are permanent cells, it is difficult to repair and rebuild through proliferation and differentiation after myocardial injury, and effectively antagonizing cardiomyocyte apoptosis will help to improve myocardial fibrosis and inhibit myocardial remodeling [43]. The experimental result showed that the number of positive cells in the myocardium of diabetic rats after TUNEL staining increased significantly, and the pro-apoptotic protein in the myocardial tissue was significantly up-regulated compared with the Control group. The result of the *in vitro* experiments suggested that high-glucose stimulation could induce the apoptosis of H9c2 cardiomyocytes. Furthermore, the expression of pro-apoptotic proteins (Cleaved Caspase3, Cleaved Caspase9, and Bax) of H9c2 cardiomyocytes in the HG group was significantly up-regulated as compared with the Control group. As revealed by the above results, the high-glucose environment could promote cardiomyocyte apoptosis, and apoptosis might be a vital factor leading to myocardial fibrosis in diabetes rats, consistent with the study by Zou and Xie [44].

Autophagy means that cells engulf their own damaged organelles or proteins and fuse with lysosomes to meet the renewal and metabolic needs of the organelles of cells. Moderate autophagy is an important self-protection mechanism of damaged cells under stress [45], which helps maintain cellular physiological homeostasis. Insufficient autophagy may lead to the dysfunction of cardiomyocyte self-repair, which in turn leads to cardiomyocyte injury and even induces apoptosis [46]. Previous studies have suggested that autophagy is one of the important causes of diabetic cardiomyopathy. Autophagy-dependent cell death leads to the loss of cardiomyocytes and initiates the repair program of fibrosis, which is an important factor in the occurrence of diabetic cardiomyopathy [47]. However, recent studies have found that moderate autophagy can antagonize cellular inflammation, mitochondrial stress, oxidative stress, etc., thereby inhibiting cardiomyocyte apoptosis [48]. Dewanjee *et al.* [15] highlighted that autophagy is significantly correlated with diabetes-induced cardiomyopathy and may be a vital intervention target for diabetic cardiomyopathy. Previous studies have found that the disorder or deficiency of autophagy can induce cardiomyocyte apoptosis in diabetic rats/mice [17,49,50]. Our previous study also found that the level of autophagy in cardiomyocytes of diabetic rats was

down-regulated, and up-regulation of autophagy could antagonize high glucose-induced cardiomyocyte senescence [16]. In this study, we observed by transmission electron microscopy and immunoblotting that: autophagosomes, LC3II/I, Beclin1, and other autophagy-associated proteins in cardiomyocytes of diabetic rats were significantly down-regulated compared with those of the Control group. Through *in vitro* experiments, we also found that the level of autophagy in cardiomyocytes under high glucose stimulation was significantly down-regulated, which is consistent with other scholars and previous related studies. The down-regulation of autophagy can cause the dysfunction of cellular self-repair and induce cardiomyocyte apoptosis, which in turn leads to myocardial fibrosis.

The PI3K/Akt pathway has been found as a main regulatory pathway of autophagy. Existing studies have suggested that down-regulating the expression of the PI3K/AKT pathway can reduce diabetes-induced cardiac dysfunction and ameliorate myocardial fibrosis [24]. Its mechanism may be correlated with the inhibition of collagen synthesis in cardiac fibroblasts and cardiomyocyte hypertrophy [51]. Over-activation of the PI3K/AKT pathway can induce apoptosis, while down-regulation of PI3K/AKT can inhibit cardiomyocyte apoptosis and improve myocardial fibrosis [23], but the specific mechanism is still unclear. It has been found that inhibiting the excessive activation of PI3K/AKT can restore or upregulate the impaired autophagic flux and antagonize cardiomyocyte apoptosis [20]. *In vitro* experiments have also found that high-glucose stimulation can promote the phosphorylation of AKT and mTOR in H9c2 cardiomyocytes [13,52], inhibit autophagy, and promote oxidative stress and apoptosis. In the present study, we found that high-glucose stimulation induced apoptosis of H9c2 cardiomyocytes, accompanied by autophagy impairment. Western-blot detection also observed that the levels of P-PI3K and P-AKT in the HG group were significantly higher than those in the Control group, which was consistent with the previous research results of other scholars. It was also found in *in vivo* experiments that the expression levels of P-PI3K and P-AKT in the fibrotic myocardial tissue of diabetic rats were significantly increased, while the autophagy flux was decreased, and apoptosis-related proteins were significantly increased. It was suggested that the phosphorylation and excessive activation of PI3K/AKT might inhibit autophagy and induce apoptosis, thereby leading to diabetic myocardial fibrosis.

SO₂ was considered a harmful gas molecule in the past. With further research on endogenous SO₂, the physiological protective effect of SO₂ in the body has been gradually revealed. SO₂ has been considered the fourth endogenous gas signal molecule newly discovered after NO, CO, and H₂S. It exhibits the functions of vasodilator, anti-oxidation, anti-inflammatory, inhibition of apoptosis, and smooth muscle proliferation while having an important protective effect on the cardiovascular system at a proper concentration [30,53-55]. However, the correlation between SO₂ and myocardial fibrosis and remodeling remains unclear, and the

internal regulatory mechanism is unknown [56]. The endogenous enzyme AAT of SO₂ is widely expressed in tissues and organs, and it has the highest expression level in the myocardium. As an important endogenous regulator of cardiovascular homeostasis, exogenous SO₂ donors can upregulate the expression of endogenous SO₂-producing enzyme AAT₁, enhancing the synthesis of endogenous sulfur dioxide and inhibiting cardiomyocyte apoptosis, reducing inflammatory damage mediated by the NLRP3 inflammasome body and improve cardiac function [29]. We have previously found that exogenous SO₂ donors can upregulate the expression of GOT₁/AAT₁ increasing the synthesis of endogenous sulfur dioxide and improving myocardial fibrosis in rats with type 1 diabetes, and is related to the inhibition of cardiomyocyte apoptosis [26], but the internal regulatory mechanism is not very clear. In addition, some scholars have found that SO₂ can also inhibit endoplasmic reticulum stress and improve myocardial fibrosis [57], while it can inhibit the proliferation and migration of fibroblasts [27]. Therefore, some scholars regard endogenous SO₂ as a potential and promising anti-fibrotic treatment strategy, but the specific mechanism of action is unclear and needs to be further explored [25]. In this study, it was found that endogenous SO₂-producing enzyme AAT_{1/2} in myocardial tissue of diabetic rats and H9c2 cardiomyocytes stimulated by high glucose decreased significantly, suggesting that the production of endogenous SO₂ in cardiomyocytes of diabetic rats was insufficient. At the same time, it was found in the study that administration of exogenous SO₂ donor can improve the deposition of collagen fibers in the myocardial interstitium of diabetic rats, while the level of damaged autophagy is significantly up-regulated, and the apoptosis of cardiomyocytes is significantly antagonized. The study also found that the above changes may be related to the excessive activation of the PI3K/AKT pathway antagonized by SO₂. To investigate whether SO₂ regulates autophagy and inhibits cardiomyocyte apoptosis via the PI3K/AKT pathway, PI3K/AKT activator 740 Y-P was added, while cardiomyocytes were intervened in the high-glucose medium with SO₂. The results indicated that 740 Y-P significantly increased the phosphorylation level of PI3K/AKT in H9c2 cardiomyocytes, and the autophagy level of cardiomyocytes in the HG + SO₂ + 740 Y-P group decreased significantly, while the proportion of apoptotic cells and apoptosis-associated proteins increased significantly. The above results suggested that 740 Y-P, an activator of PI3K/AKT, could reverse the effect of SO₂ on inhibiting PI3K/Akt to activate autophagy and antagonize apoptosis, and SO₂ might inhibit cardiomyocyte apoptosis by antagonizing PI3K/AKT phosphorylation and increasing autophagic flux, which would ameliorate diabetic myocardial fibrosis.

In brief, this paper found that SO₂, a novel gas signaling molecule, could inhibit cardiomyocyte apoptosis by activating autophagy, thus improving myocardial fibrosis and myocardial remodeling in diabetic rats. Moreover, the mechanism might be correlated with the excessive activation of the PI3K/AKT path-

way that it antagonized. This study shows that endogenous SO₂ might play an important protective role in the occurrence and development of myocardial fibrosis in type 2 diabetes. Likewise, the down-regulation of endogenous SO₂ might play a certain role in the mechanism of myocardial remodeling, and the PI3K/AKT-mediated autophagy and its secondary myocardial apoptosis might be an essential link. This paper is expected to discover novel intervention targets for myocardial remodeling and heart failure, and more specific mechanisms (e.g., the mechanism by which SO₂ regulates PI3K/AKT phosphorylation) should be studied in depth.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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