Sulfur dioxide ameliorates rat myocardial fibrosis by inhibiting endoplasmic reticulum stress

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Summary. Myocardial remodeling occurs after myocardial infarction (MI), the leading cause of mortality worldwide. Although myocardial fibrosis plays an important role in the process of myocardial remodeling, there is not yet an effective method of reducing it. The aim of the present study was to determine the effects of sulfur dioxide (SO_2) on myocardial fibrosis and the possible mechanisms of these effects. SO_2 treatment reduced the extent of myocardial fibrosis and post-MI levels of collagens I and III in the left-ventricular myocardium. SO_2 also improved MI-induced thinning of the left ventricular wall while enlarging the left ventricular internal diameter. SO_2 was able to reduce matrix metalloproteinase (MMP)-9 activity and increase tissue matrix metalloproteinase inhibitor (TIMP)-1 content in myocardium after MI. However, the mechanism underlying these effects of SO_2 on myocardial fibrosis are unknown. Western blot analysis of endoplasmic reticulum (ER) stress-related proteins showed that glucose-regulated protein 78, C/EBP homologous protein, caspase-12, and phosphorylated eukaryotic initiation factor 2α expression levels were significantly increased in MI rats and decreased by SO_2 treatment. The ER stress promoter dithiothreitol reversed these effects of SO_2. In conclusion, SO_2 alleviated myocardial fibrosis in MI rats through a mechanism related to inhibition of excessive ER stress.

Key words: Sulfur dioxide, Myocardial fibrosis, Myocardial infarction, Endoplasmic reticulum stress, Glucose-regulated protein 78, Dithiothreitol

Introduction

Myocardial remodeling is a complex process involving myocardial necrosis and proliferation of the extracellular matrix (ECM), particularly myocardial fibers, ultimately leading to chronic heart failure. Myocardial fibrosis plays important roles during cardiac remodeling (Koca and Ari, 2008; Yavuz, 2008; Hausenloy et al., 2010; Mozaffarian et al., 2016). Myocardial fibrosis has also been seen in pediatric patients with Kawasaki disease (Potter et al., 2016). Excess degradation and disruption of the ECM play a key role in the process of myocardial fibrosis. Matrix metalloproteinases (MMPs) and tissue matrix metalloproteinase inhibitors (TIMPs) are the main regulators of ECM degradation and deposition. Additionally, recent studies have implicated endoplasmic reticulum stress (ERS) in myocardial fibrosis.

Endoplasmic reticulum (ER) is an important organelle that regulates cell-protein synthesis, posttranslational modifications, and calcium homeostasis. ERS is a subcellular pathological process that results from an imbalance in ER homeostasis. ERS can be induced by perturbation of calcium homeostasis

or redox status, glucose deprivation, or ischemia (Kaufman, 2002). C/EBP homologous protein (CHOP), caspase-12, and phosphorylated eukaryotic initiation factor 2α (p-eIF2α) are upregulated when ERS is severe or persistent (Vilatoba et al., 2005; Boyce and Yuan, 2006). Studies have shown that ERS plays an important role in the development of ischemic cardiomyopathy and fibrosis (Azfer et al., 2006; Lenna and Trojanowska, 2012).

Endogenous sulfur dioxide (SO$_2$) is generated by normal metabolism of sulfur-containing amino acids in mammals (Stipanuk et al., 2006). SO$_2$ converts to its derivatives bisulfite and sulfite (1:3 M/M) in neutral fluid and plasma (Meng et al., 2007; Meng and Zhang, 2007; Yargicoglu et al., 2007). Studies have demonstrated that SO$_2$ and its derivatives can regulate vascular tone (Meng et al., 2003; Li and Meng, 2009; Wang et al., 2009, 2017; Zhang and Meng, 2009), and that SO$_2$ can alleviate lung injury and improve pulmonary hypertension-induced structural remodeling of the pulmonary vascular system (Jin et al., 2008; Sun et al., 2010; Zhao et al., 2016). In addition, SO$_2$ can improve cardiac function and reduce myocardial injury induced by isoproterenol (Liang et al., 2011).

We previously showed that SO$_2$ preconditioning reduced myocardial ischemia-reperfusion injury via the ERS pathway *in vivo* and *in vitro* (Wang et al., 2011). However, the effects of SO$_2$ on myocardial fibrosis remain unknown. In the present study, we examined the effects of SO$_2$ on post-MI myocardial fibrosis in rats and the underlying mechanism of these effects.

Materials and methods

Animal preparation

Research was performed in accordance with the animal care guidelines of Capital Medical University and the Use of Laboratory Animals guidelines published by the US National Institutes of Health (NIH, No. 85-23, revised 1996). Healthy male Wistar rats (250±30 g) were obtained from Vital River (Beijing, China). Rats were anesthetized by intraperitoneal (i.p.) chloral hydrate (10%, 0.3 ml/100 g) and orally intubated with a polyethylene tube for artificial respiration (Kent Scientific, USA). Rats were subjected to ligation of the left anterior descending coronary artery to induce MI, as described previously. Thoracotomy was performed at the fourth intercostal space. A 5-0 silk suture was used to ligate the left anterior descending coronary artery approximately 2 mm from its origin. Sham-operated animals underwent the same procedure but without artery ligation.

After 1 week of acclimatization, 48 rats were randomly divided into six equally sized groups: 1) sham group, the sham-operated control group; 2) sham+DTT group, treatment of sham-operated group with 100 μmol/kg of the ERS inducer dithiothreitol (DTT); 3) MI group, induction of MI as described previously; 4) MI+S

group, induction of MI plus treatment with 5 μmol/kg SO$_2$ (NaHSO$_3$ and Na$_2$SO$_3$, 1:3 MM ratio); 5) MI+DTT group, induction of MI plus treatment with 100 μmol/kg DTT; and 6) MI+S+DTT group, induction of MI plus treatment with 5 μmol/kg SO$_2$ and 100 μmol/kg DTT. For animals receiving SO$_2$ or DTT, the treatment was administered by i.p. injection daily for 4 weeks. During the experiment, two rats in the MI group, three rats in the MI+DTT group, and one rat in MI+S+DTT group died. No rats died in the sham, sham+DTT, and MI+S groups.

Hematoxylin-eosin (HE) staining

The tissues of the heart were fixed with 10% formaldehyde and embedded in paraffin. Embedded tissue was cut into 5-μm-thick sections. Sections were stained with hematoxylin for 3-5 min and differentiated with 1% hydrochloric acid (HCl) in 70% alcohol after washing under running water. Sections were stained with eosin for 1-4 min, and then dehydrated and differentiated in alcohol. Finally, sections were observed under a microscope.

Masson trichrome staining

Sections were dewaxed, dehydrated in a graded alcohol series, stained with hematoxylin for 3 min, washed under running water, and differentiated in a 1% HCl alcohol solution. Sections were stained in warm Ponceau acid-Fuchsin solution for 3 min, washed with distilled water, and differentiated in a 1% phosphomolybdic acid solution for 1 min. Sections were stained with 0.1% brilliant green solution for 5 min, washed twice with 0.2% acetic acid, dehydrated in graded alcohol, dried, and mounted in neutral resin.

Changes in myocardial interstitial collagen content were observed and pictures taken with an stereomicroscope imaging system. Cardiac fibrosis was quantified by measuring the green fibrotic area. The ratio of cardiac fibrosis area to the whole left ventricular area was determined based on mesaurements taken in Image J software (USA).

Evaluation of cardiac remodeling

At the end of experiment, myocardial tissue sections were stained by Masson trichrome staining. Photomicrographs were captured for analysis of fibrosis of the left ventricular (LV) wall and determination of the LV internal diameter in Image J software (USA).

Enzyme-linked immunosorbent assay (ELISA) of collagen I, III, MMP-9 and TIMP-1

The apex tissues of heart were homogenized and centrifuged at 3000×g. Collagen I and III levels in the liquid supernatant were assayed by using a double-antibody sandwich ELISA. In brief, samples (100 μl)

and collagen I/III standards were added to the wells of a culture plate and incubated for 1 h at 37°C. Plates were washed with buffer (PBS, 10 mmol/L pH 7.4 and Tween-20, 0.1%), soaked for a few minutes, and washed with buffer five times. Anti-rat collagen I and III antibodies (50 µl) were added to each well and incubated for 60 min at 37°C. Plates were washed five times. Horseradish peroxidase (HRP, 100 µl) was added to the wells and incubated for 60 min at 37°C. Plates were washed five times, and tetramethylbenzidine (100 µl) substrate was added to each well. Plates were shaken for 10 s and incubated in the dark for 5-10 min. Stop solution (100 µl) was added to each well. A protocol similar to that used for collagen I and III detection was applied for assaying MMP-9 activity and TIMP-1 content in myocardial tissues.

Optical density was measured by an ELISA reader (Bio-Rad, Richmond, CA, USA) at 492 nm. Data were calculated by plotting the logarithms of the concentrations of collagen I and III vs. the logarithm of the optical density.

Western blot analysis

Rat myocardial tissue homogenate was prepared for western blot analysis (Liu et al., 2014). Myocardial tissue was homogenized in lysis buffer, boiled in Laemmli loading buffer, and centrifuged. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Primary antibodies against GRP78, caspase-12, p-eIF2α, eIF2α (Santa Cruz, CA, USA), and CHOP (Cell Signaling Technology, Boston, MA, USA) were added at a dilution of 1:1000. Second antibody (Santa Cruz) was used at a dilution of 1:10,000. Enhanced chemiluminescence was used to detect immunoreactions on X-ray film (Kodak Scientific).

Statistical analysis

All results are expressed as the mean ± standard deviation. Statistical analysis was performed by using one-way analysis of variance (ANOVA) comparisons test. ANOVA was used to compare between groups. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Histology

In the sham group, HE staining revealed neatly arranged, well-organized cardiomyocytes with few intercellular spaces and fibroblasts (Fig. 1A,D). In the
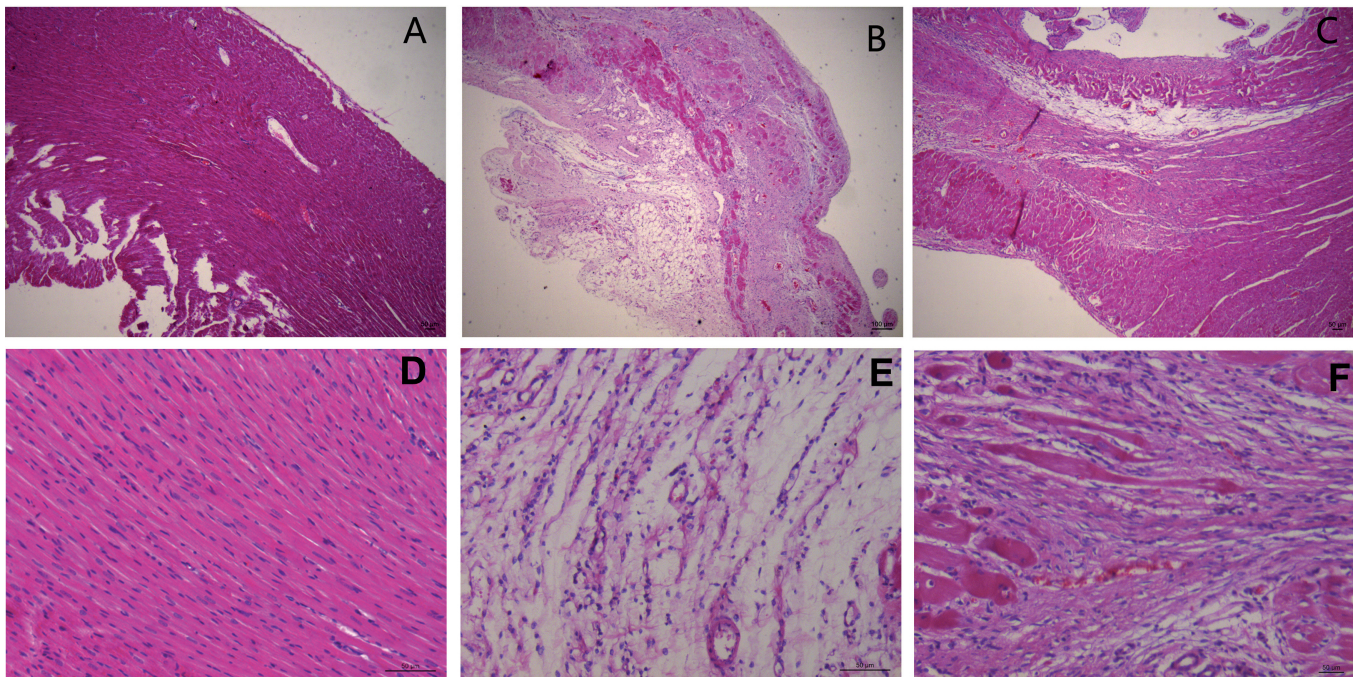

Fig. 1. Microstructural changes in cardiomyocytes by HE staining. **A, D.** Sham group showed normal microstructure of cardiomyocytes. **B, E.** MI group had damaged myocardial structure, with disordered arrangement, nucleolysis, and blurred boundaries. **C, F.** In MI+S group, the myocardial structure showed less disordered arrangement, nucleolysis, and less blurred boundaries than those in MI group. A-C, × 4; D-F, × 20.

MI group, the fibroblasts and cardiomyocytes showed a disordered arrangement, nucleolysis, and blurred boundaries (Fig. 1B,E). In the MI+S group, the fibroblasts and cardiomyocytes showed less disordered arrangement, nucleolysis, and fewer blurred boundaries than those in MI group (Fig. 1C,F).

SO₂ alleviates myocardial fibrosis in MI rats

Masson trichrome-staining revealed post-MI cardiac fibrosis (Fig. 2). In the sham and sham+DTT groups, there were no areas of cardiac fibrosis. In the MI group, a large area of interstitial collagen was observed in the
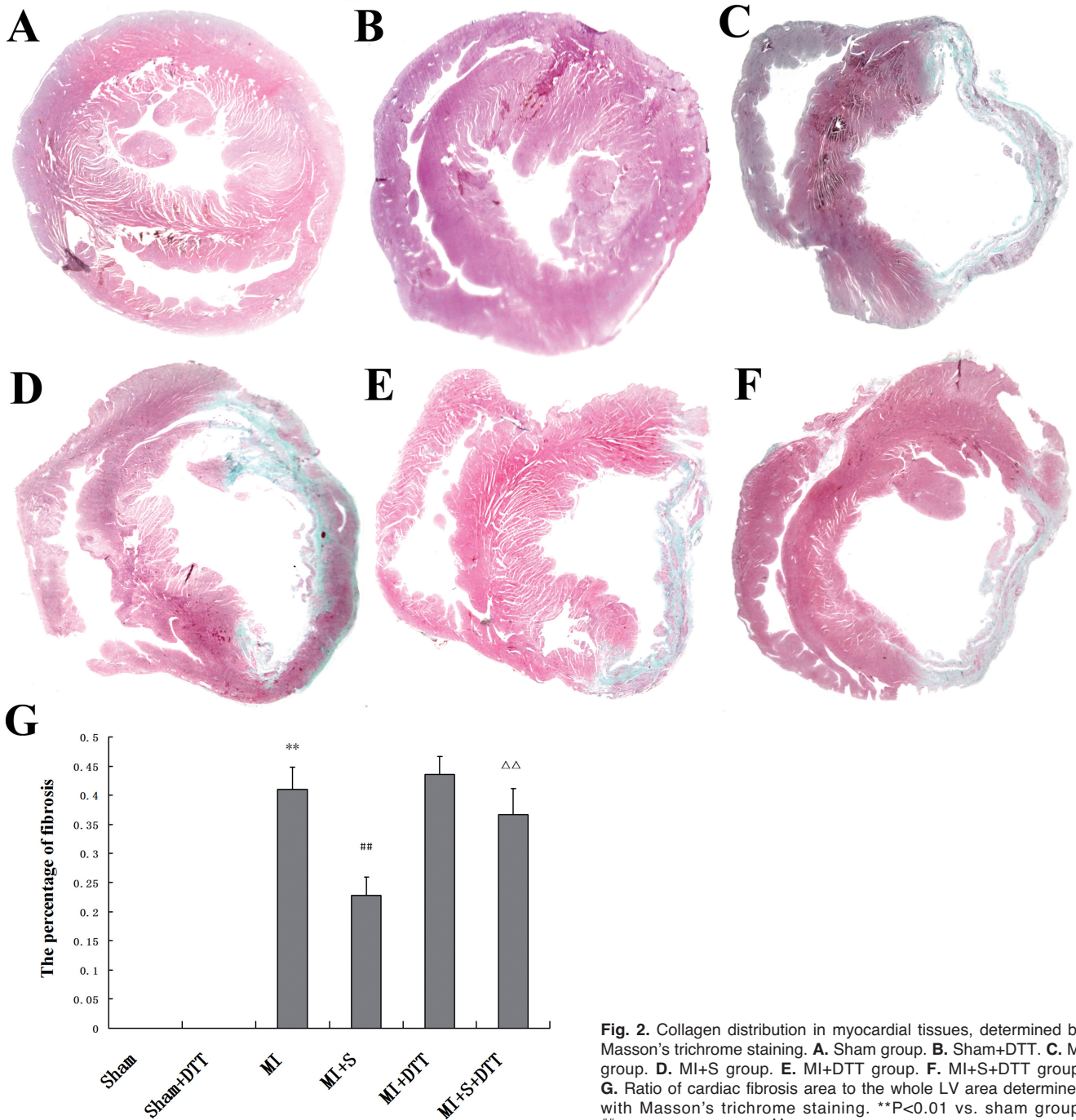

Fig. 2. Collagen distribution in myocardial tissues, determined by Masson's trichrome staining. **A.** Sham group. **B.** Sham+DTT. **C.** MI group. **D.** MI+S group. **E.** MI+DTT group. **F.** MI+S+DTT group. **G.** Ratio of cardiac fibrosis area to the whole LV area determined with Masson's trichrome staining. **P<0.01 vs. sham group; ##P<0.01 vs. MI group; △△P<0.01 vs. MI+S group.

myocardial tissues, less in the MI+S group than in the MI group, and greater in the MI+S+DTT group than in the MI+S group. There was no difference of cardiac fibrosis area between the MI+DTT and MI groups (Fig. 2G).

SO₂ improves myocardial remodeling in MI rats

The LV wall was thinner in the MI group than that in

sham group, but thicker in the MI+S group than in the MI group (Fig. 3A). The LV internal diameter was significantly greater in the MI group than that in sham group, but smaller in the MI+S group than in the MI group (Fig. 3B).

SO₂ reduces collagen I and III contents in MI rats
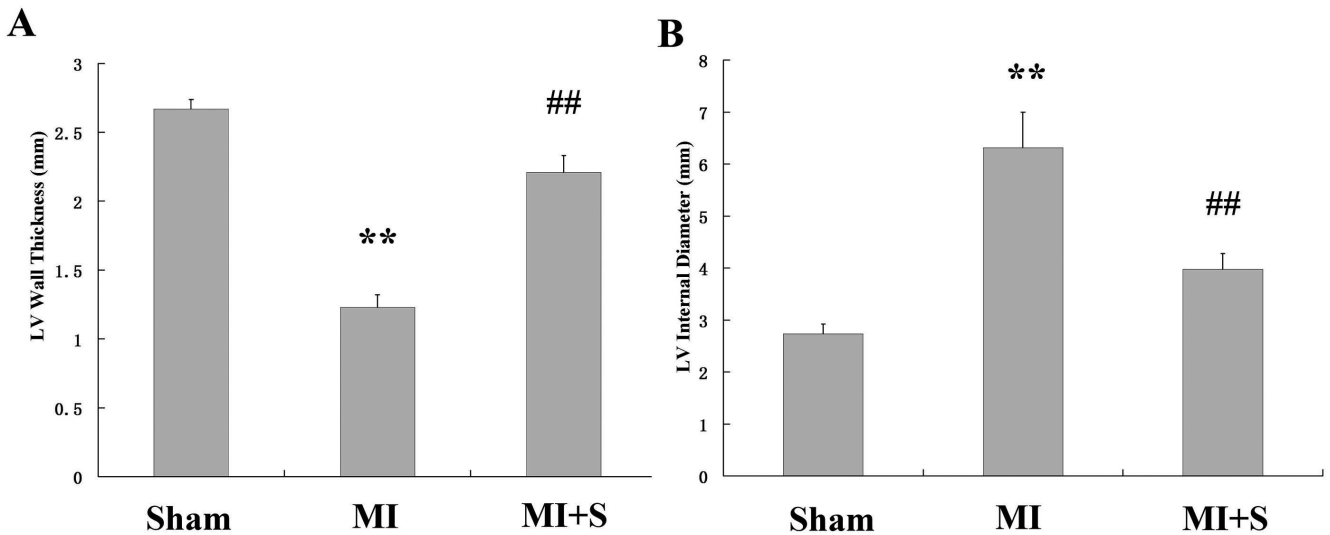
ELISA was used to quantify the collagen I and III

Fig. 3. A. Thickness of fibrotic LV wall tissue by Masson's trichrome staining. **B.** Changes in LV internal diameter. **P<0.01 vs. sham group; ##P<0.01 vs. MI group.
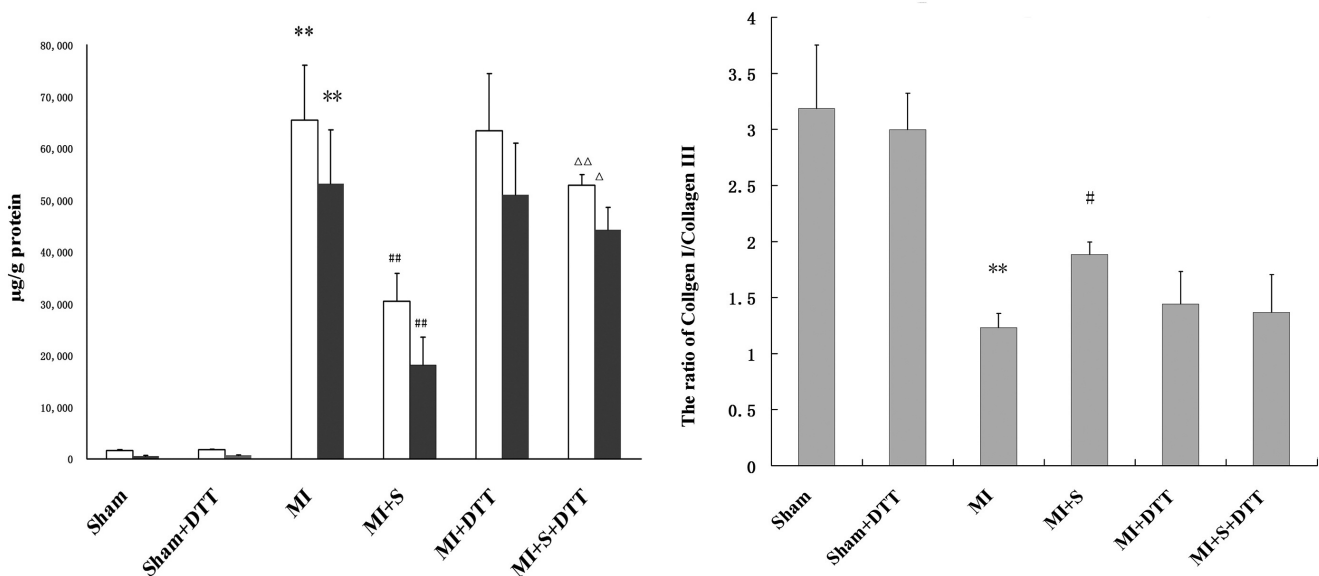

Fig. 4. A. SO₂ associated reduction of collagen I and III levels in myocardial tissues. **B.** Collagen I-to-collagen III ratios in myocardial tissues. **P<0.01 vs. sham group; #P<0.05, ##P<0.01 vs. MI group; ΔP<0.05, ΔΔP<0.01 vs. MI+S group.

contents in the LV myocardial tissue. However, in the MI+S group, the contents of collagens I and III in the LV myocardial tissues were significantly decreased compared to the MI group. Collagen I and III contents in the MI+S+DTT group were significantly increased compared to contents in the MI+S group. There was no difference in collagen I and III contents between the MI and the MI+DTT groups or between the sham and sham+DTT groups (Fig. 4A). In sham rats, the content of collagen I was more than three times the collagen III content level in myocardial tissues. Compared with sham rats, collagen I-to-collagen III ratio was significantly decreased in MI group rats, but increased in the MI+S group (Fig. 4B).

MMP-9 activity and TIMP-1 content in myocardial tissues

ELISAs of myocardial tissues indicated that MMP-9

activity was significantly increased in the MI group compared to the sham group, but decreased in the MI+S group compared to the MI group (Fig. 5A). Meanwhile, TIMP-1 content was significantly decreased in the MI group relative to that in sham group, but increased in the MI+S group relative to that in the MI group (Fig. 5B).

SO₂ suppresses myocardial ERS in MI rats

Expression levels of ERS-associated proteins were detected by Western blot analysis. Expression levels of GRP78, p-eIF2α, CHOP, and caspase-12 were significantly higher in the MI and MI + DTT groups and nonsignificantly higher in the sham+DTT group, than levels in the sham group. SO₂ treatment of MI rats (MI+S group) resulted in significant decreases in expression levels of these proteins compared to the MI group. DTT treatment reversed the above effects of SO₂.
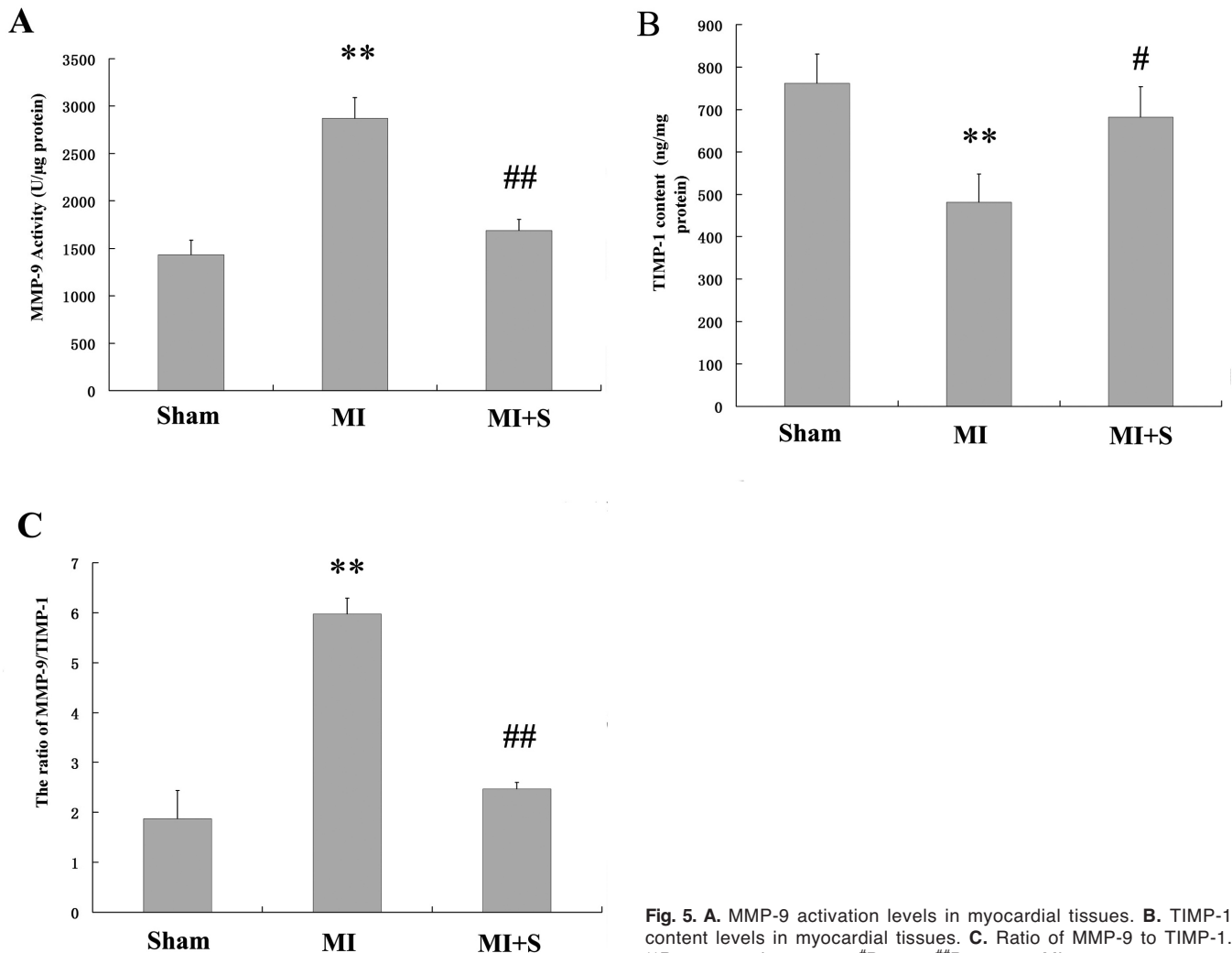
Fig. 5. A. MMP-9 activation levels in myocardial tissues. **B.** TIMP-1 content levels in myocardial tissues. **C.** Ratio of MMP-9 to TIMP-1. **P<0.01 vs. sham group; #P<0.05, ##P<0.01 vs. MI group.

Expression levels of GRP78, p-eIF2α, CHOP, and caspase-12 were significantly higher in the MI+S+DTT group than in the MI+S group, whereas no difference in expression levels was observed between the MI and MI+DTT groups (Fig. 6).

Discussion

Myocardial fibrosis is a pathological process of MI. More than two-thirds of myocardial tissues contain cardiomyocytes, particularly cardiac fibroblasts. Fibroblasts are essential "sensory cells" in the myocardium. Myocardial remodeling is a healing process in MI. This process is characterized by an increase in the amounts of fibroblasts and ECM deposits. In previous studies, SO₂ was shown to improve

pulmonary vascular remodeling induced by pulmonary hypertension, through a mechanism related to inhibition of the MAPK pathway. In addition, SO₂ can inhibit proliferation of vascular smooth muscle cells by downregulating the Erk/MAPK pathway mediated by the cAMP/PKA pathway (Liu et al., 2014). Furthermore, SO₂ can alleviate collagen remodeling by inhibiting the transforming growth factor-β1/Smad pathway in vascular smooth muscle cells and by downregulating the SO₂/AAT1 pathway in pulmonary fibroblasts (Huang et al., 2016; Liu et al., 2016).

In MI rats, the cardiac fibroblast phenotype changes to the dominant cell type. Under this condition, fibroblasts show a synthetic, myofibroblast-like phenotype with increased synthesis of ECM proteins. Compared to the sham group, HE staining showed that
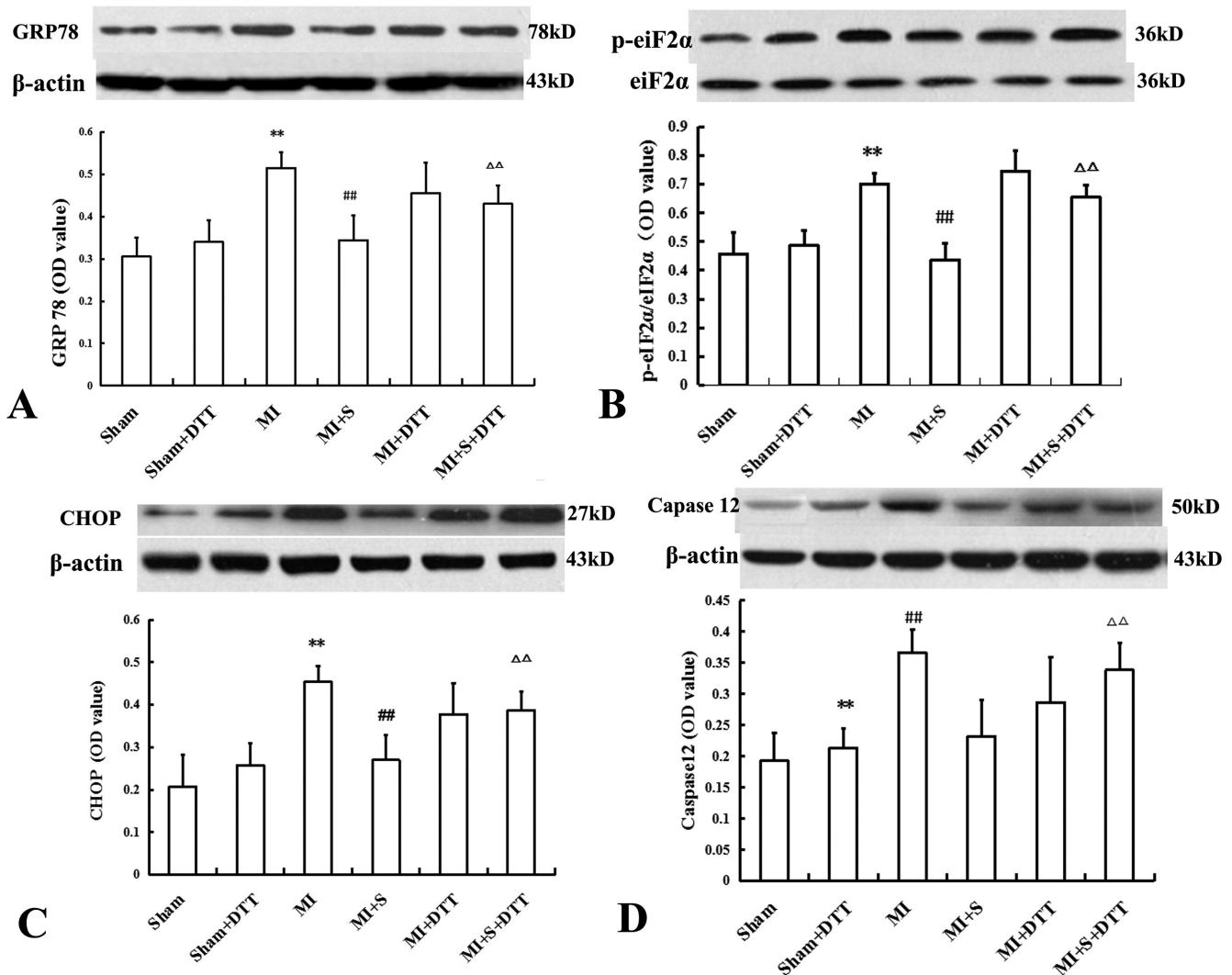

Fig. 6 SO₂ reduced expression levels of ERS-related proteins, which are induced by MI in myocardial tissues. **A.** GRP78. **B.** p-eIF2α. **C.** CHOP. **D.** caspase-12. *$P<0.05$, **$P<0.01$ vs. sham group; #$P<0.05$, ##$P<0.01$ vs. MI group; Δ$P<0.05$, ΔΔ$P<0.01$ vs. MI+S group.

the fibroblasts and cardiomyocytes had disordered arrangement, nucleolysis, and blurred boundaries in the MI group. The fibroblasts showed less disordered arrangement, nucleolysis, and fewer blurred boundaries in the MI+S group than those in MI group. Masson trichrome staining showed that the interstitial fibrotic content was significantly increased in the MI group. SO$_2$ treatment significantly decreased the size of cardiac fibrosis. Taken together, these data indicate that SO$_2$ reduced myocardial fibrosis in MI rats.

Our examination of the effects of SO$_2$ on myocardial remodeling showed that SO$_2$ attenuated MI-induced LV wall thinning, while also reducing the enlarged LV internal diameter induced by MI. SO$_2$ treatment reduced interstitial fibrosis, collagen I levels, and collagen III levels in LV myocardium. Furthermore, MI-associated reduction in the ratio of collagen I to collagen III was reversed in MI+S group.

Together, collagen I and III account for 90% of cardiac collagen in adults. They provide the structural integrity that enables cardiac tissue to withstand large dynamic pressure changes. Collagen I is a sturdy protein that can resist extension, whereas Collagen III is a fine, soft compliant fiber (Limon-Miranda et al., 2014). Additionally, SO$_2$ opposed the increasing and decreasing effects of MI on MMP-9 activity and TIMP-1 content, respectively. The MMP-9 to TIMP-1 ratio was also decreased after SO$_2$ treatment, compared to the non-treated MI group. These SO$_2$ effects on MMP-9 and TIMP-1 in rat MI myocardium resemble the effects of curcumin and losartan (Ma et al., 2017; Gay-Jordi et al., 2013). Together, these findings suggest that SO$_2$ improves myocardial remodeling in rats with MI.

To investigate the mechanisms responsible for the protective effects of SO$_2$ in MI, we determined the levels of the myocardial ERS markers GRP78, CHOP, p-eIF2α, and caspase-12 using Western blot analysis. In MI rats, myocardial expression levels of these four proteins in the LV myocardium were significantly higher than levels in the sham group. DTT can mimic augmentation of ERS and promote expression of myocardial GRP78 and p-eIF2α (Liang et al., 2006; Kim et al., 2007; Peyrou and Cribb, 2007). We found that DTT treatment reversed the inhibitory effects of SO$_2$ on myocardial fibrosis in MI rats, while simultaneously increasing the amounts of interstitial fibrosis in myocardium. Thus, the protective effects of SO$_2$ on myocardial fibrosis were related to inhibition of excess ERS.

In conclusion, we have demonstrated that SO$_2$ alleviates myocardial fibrosis through a mechanism that involves the inhibition of excess ERS induced by MI. Further studies are needed to investigate the potential mechanism of SO$_2$ on ERS.

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