

Inhibitory Effects of Sulfur Dioxide on Rat Myocardial Fibroblast Proliferation and Migration

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Abstract

Background: Myocardial fibrosis is an important pathological change in many heart diseases, but its pathogenesis is very complex and has not yet been fully elucidated. The study was designed to examine whether endogenous sulfur dioxide (SO₂) is a novel myocardial fibroblast proliferation and migration inhibitor.

Methods: Primary rat myocardial fibroblasts were isolated and transfected with aspartate aminotransferase (AAT1 and AAT2) knockdown lentivirus or empty lentivirus. SO₂ content in the supernatant was determined with high-performance liquid chromatography, and the expressions of AAT1, AAT2, proliferating cell nuclear antigen (PCNA), phosphorylated extracellular signal-regulated protein kinase (p-ERK), and total ERK (T-ERK) in the cells were detected. Cell migration was detected by wound healing test. Independent sample *t*-test (for two groups) and one-way analysis of variance (three or more groups) were used to analyze the results.

Results: Both AAT1 and AAT2 knockdown significantly reduced SO₂ levels ($F = 31.46$, $P < 0.01$) and AAT1/2 protein expression (AAT1, $t = 12.67$, $P < 0.01$; AAT2, $t = 9.61$, $P < 0.01$), but increased PCNA expression and Cell Counting Kit-8 (CCK-8) activity as well as the migration in rat primary myocardial fibroblasts ($P < 0.01$). Supplementation of SO₂ rather than pyruvate significantly inhibited the increase in proliferation and migration caused by AAT knockdown ($P < 0.01$). Mechanistically, the ratio of p-ERK to T-ERK was significantly increased in the AAT1/2 knockdown groups compared with that in the empty lentivirus group (AAT1, $t = -7.36$, $P < 0.01$; AAT2, $t = -10.97$, $P < 0.01$). Whereas PD98059, an inhibitor of ERK activation, successfully blocked AAT knockdown-induced PCNA upregulation ($F = 74.01$, $P > 0.05$), CCK-8 activation ($F = 50.14$, $P > 0.05$), and migration augmentation in myocardial fibroblasts (24 h, $F = 37.08$, $P > 0.05$; 48 h, $F = 58.60$, $P > 0.05$).

Conclusion: Endogenous SO₂ might be a novel myocardial fibroblast proliferation and migration inhibitor via inhibiting the ERK signaling pathway.

Key words: Endogenous; Fibroblast; Migration; Proliferation; Sulfur Dioxide

INTRODUCTION

Myocardial fibrosis is an important pathological change in myocardial remodeling and involved in the development of many cardiovascular diseases such as myocardial hypertrophy, hypertension, myocardial infarction, myocarditis, and heart failure.^[1-4] Clarifying the mechanism for cardiac fibrosis is a very important issue in the cardiovascular field. Myocardial fibrosis is caused by abnormal deposition of extracellular matrix in the myocardial interstitium. Cardiac fibroblasts are major sources of extracellular matrix synthesis and secretion. Under physiological conditions, cardiac fibroblasts are at rest status. However, in the process of many cardiac diseases, the quiescent fibroblasts are stimulated to transform into

activated cells and then produce more extracellular matrix. The excessive proliferation and migration of myocardial fibroblasts were regarded as the typical changes of activated myocardial fibroblasts.^[5,6] A plenty of previous studies showed that transforming growth factor- β (TGF- β)/small

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mothers against decapentaplegic, FAK/TAK/JNK, and PI3K/Akt/Rac signaling pathway are involved in the regulation of myocardial fibroblasts.^[7-9] However, the molecular mechanism responsible for cardiac fibroblast proliferation and migration has not been fully elucidated.

Recently, sulfur dioxide (SO₂), formerly known as an exhaust gas, can be endogenously produced from the metabolic pathway of sulfur-containing amino acids.^[10] The previous studies showed that SO₂ was considered as the fourth gasotransmitter following nitric oxide, carbon monoxide, and hydrogen sulfide.^[11,12] Du *et al.*^[13] found that its endogenous-generating enzyme was aspartate aminotransferase (AAT) in the cardiovascular system. AAT has two subtype isoenzymes including AAT1 and AAT2. AAT1 is mainly localized in the cytoplasm and AAT2 localized in the mitochondria.^[14] Particularly, endogenous SO₂ was reported to be generated from the cardiomyocyte and involved in the regulation of cardiac function under physiological and pathophysiological conditions. For example, endogenous SO₂ was found to prevent the isopropanol-induced myocardial injury, acting as an antioxidant and a protector against the mitochondrial dysfunction.^[15,16] Chen *et al.*^[17] found that SO₂ prevented angiotensin II-induced myocardial hypertrophy accompanied by downregulating cardiomyocyte autophagy. Liu *et al.*^[18] found that there was endogenous SO₂ in the pulmonary artery fibroblast and deficiency of endogenous SO₂/AAT1 pathway mediated mechanical stretch-stimulated abnormal collagen accumulation. However, whether endogenous SO₂/AAT presents in the myocardial fibroblasts has not been clear. What is more, whether endogenous SO₂ has a regulatory impact on the proliferation and migration of cardiac fibroblasts has not been understood so far.

Extracellular signal-regulated protein kinases (ERK)/mitogen-activated protein kinase (MAPK) is widely involved in cell proliferation and differentiation, migration, senescence, apoptosis, and so on.^[19] ERKs are rapidly phosphorylated and activated in response to a variety of extracellular stimuli in many different cell types. The kinases that activate ERK/MAPK, the MAPK/ERK kinases, are also activated by phosphorylation. On activation, the phosphorylated ERK enzyme migrates into the nucleus, where they activate various transcription factors, control the expression of target genes, and then regulate cell proliferation, apoptosis, and migration.^[20] Barathi *et al.*^[21] found that the activated ERK/MAPK pathway was involved in the promotive effect of carbachol on the proliferation of mouse scleral fibroblast cells. Feng *et al.*^[22] found that the collagen production and proliferation of human embryonic lung fibroblasts enhanced by silicon dioxide were partially mediated by ERK/MAPK signal pathway. Our previous studies^[23,24] found that SO₂ inhibited the proliferation of vascular smooth muscle cells via suppressing ERK signaling. Therefore, we supposed that SO₂ derived from cardiac fibroblasts might inhibit the proliferation and migration of cardiac fibroblasts through the ERK pathway.

In the present study, we attempted to demonstrate the existence of endogenous SO₂/AAT pathway in the myocardial fibroblasts. Then, we downregulated AAT in the myocardial fibroblasts by the transfection with AAT1/2 shRNA lentivirus to investigate the effect of endogenous SO₂ on the migration and proliferation of myocardial fibroblasts. Furthermore, we tried to explore the possible mechanisms targeting on the ERK/MAPK pathway. PD98059, an inhibitor of the MAPK signaling pathway, was used to verify the significance of ERK/MAPK pathway in the regulation of the cardiac fibroblast proliferation and migration by SO₂.

METHODS

Ethical approval

This study was approved by the Animal Ethics Committee of Peking University First Hospital (No. J 201713) and was conducted in strict accordance with applicable regulations of the Animal Ethics Committee of Peking University. The license number for the use of laboratory rats was 11400700271350 SCXK (Beijing) 2016-0006.

Cell model and groups

Healthy specific pathogen-free Sprague-Dawley rats, weighing 120–150 g, were sacrificed. Heart tissue was then harvested under sterile conditions and placed in ice-cold phosphate-buffered saline (PBS) to remove residual blood. Next, the heart tissue was cut into 1.0 mm × 1.0 mm × 1.0 mm tissue blocks, which were placed in a culture flask 0.5 cm apart. F12/Dulbecco's Modified Eagle Medium (DMEM) low glucose medium containing 20% fetal bovine serum was slowly added to the culture flask without causing the tissue blocks to float. The culture flask was then placed in an incubator at 37°C and 5% CO₂. Three days later, rat primary myocardial fibroblasts had migrated out from the tissue blocks, the medium was replaced, and the myocardial fibroblasts were passaged at a 1:3 ratio. Primary myocardial fibroblasts at 100% confluence were used in this study.

The primary fibroblasts were transfected with empty virus or an AAT knockdown virus, and the cells were divided into the following groups: empty virus group, AAT1 knockdown group, AAT2 knockdown group, AAT1 knockdown + SO₂ group, AAT2 knockdown + SO₂ group, AAT1 knockdown + Pyruvate group, AAT2 knockdown + Pyruvate group, empty virus + PD98059 group, AAT1 knockdown + PD98059 group, and AAT2 knockdown + PD98059 group. The transfected cells were seeded into 6-well plates, cultured in F12/DMEM medium containing 10% fetal bovine serum, and then synchronized in serum-free F12/DMEM medium once the cells had reached 60–70% confluence. Twenty-four hours later, 20 μmol/L PD98059 was added, and cells were cultured for additional 24 h. SO₂-treated cells were with culture medium containing 100 μmol/L SO₂ for 24 h, and pyruvate -treated cells were with culture medium containing 100 μmol/L pyruvate for 24 h. Cell proteins and cell supernatants were then collected. In this study, the viruses were

purchased from Shandong Weizhen Co., (Jinan, Shandong, China), including vector pLV (shRNA)-EGFP-T2, empty virus (ID: VB 151214-10025), AAT1 knockdown virus (ID: VB 151210-10015), and AAT2 knockdown virus (ID: VB 151210-10016). PD98059 was purchased from Promega Corporation (V119A Lot # 307219; Madison, Wisconsin, USA).

Measurement of sulfur dioxide in cell supernatant with high-performance liquid chromatography

Seventy microliters of 0.212 mol/L sodium borohydride was added to 100 μ l of cell supernatant. After incubating for 30 min at room temperature, 5 μ l of 70 mmol/L mBrB (Sigma, St. Louis, MO, USA) was added and mixed, and the mixture was incubated at 42°C for 10 min. Next, 40 μ l of 1.5 mol/L perchloric acid was added, and the mixture was centrifuged at 12,400 $\times g$ for 10 min at room temperature. Next, 10 μ l of Tris-HCl (2.0 mol/L, pH 3.0) was added to 100 μ l of cell supernatant and mixed, and the mixture was centrifuged at 12,400 $\times g$ for 10 min at room temperature. Then, 100 μ l of the cell supernatant was added to a special brown glass flask to determine the SO₂ content in a high-performance liquid chromatography (HPLC; Agilent, California, USA) analyzer. During analysis, a 10 μ l sample was added and analyzed using the following conditions: mobile phase A with methanol: acetic acid: water (5.00:0.25:94.75 [v/v/v], pH: 3.4); mobile phase B with methanol and methanol gradient elution, at a flow rate of 1.0 ml/min, an excitation wavelength of 392 nm, and a detection wavelength of 479 nm. The SO₂ content in the sample was calculated and analyzed using a standard curve.

Expression of aspartate aminotransferase, proliferating cell nuclear antigen, phosphorylated extracellular receptor kinase, and total extracellular receptor kinase in rat primary fibroblasts by Western blot

After the medium was discarded, the 6-well plates were gently washed with 0.01 mol/L PBS at 4°C three times to remove any residual medium and dead cells. After all PBS was removed, 60 μ l of lysis buffer was added to each well, and the cells were incubated at 4°C for 20 min. Next, the cells were collected into the Eppendorf tube, and an equal volume of 2 \times sodium dodecyl sulfate loading buffer (containing 5% β -mercaptoethanol) was added and mixed. The mixture was then boiled at 100°C for 10 min. After cooling down to room temperature, protein samples (equivalent volumes) were loaded into polyacrylamide gels and separated by electrophoresis to identify proteins of different molecular weights. The proteins were transferred to a nitrocellulose membrane, blocked with 50 g/L skim milk for 1 h, and incubated with a primary antibody at 4°C overnight. After washed with TPBS for 3 times, the secondary antibody was added, and the membranes were incubated for 1 h. After washed with TPBS for 4 times, enhanced chemiluminescence reagents were added, and the protein bands were observed. AlphaEaseFC was used to analyze

the gray value of the protein bands, which was corrected according to the gray value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primary antibodies used in this study included anti-AAT1 and anti-AAT2 (1: 1000; Sigma, St. Louis, MO, USA), anti-proliferating cell nuclear antigen (PCNA) (1: 1000; Anbo, California, USA), anti-p-ERK and anti-ERK (1: 500; Beyotime Biotech Co., Shanghai, China), and anti-GAPDH (1: 4000; Kangcheng, Shanghai, China).

Cell Counting Kit-8 activity of rat myocardial fibroblasts

Cells were seeded in 96-well plates with 100 μ l of cell suspension (2000 cells) in each well. After the cells adhered to the well, the medium was replaced with serum-free medium to synchronize the cells for 24 h. Then, 10 μ l of enhanced Cell Counting Kit-8 (CCK-8) solution was added to each well, and the cells were incubated in cell incubator for additional 2 h. Then, the optical absorbance was measured at 450 nm using a microplate reader. The more the cells proliferated, the darker the color after incubation and the greater the optical absorbance value. In this study, the enhanced CCK-8 kit was purchased from Beyotime Biotech Co., Ltd. (Shanghai, China).

Migration of rat myocardial fibroblasts detected by wound healing test

Rat myocardial fibroblasts transfected with AAT1 knockdown virus, AAT2 knockdown virus, or empty virus were seeded into the cell migration chamber. After the cells adhered to the chamber, the medium was replaced with serum-free medium to synchronize the cells for 24 h. The migration chamber was then removed, the medium was replaced with complete medium, and the length of cell scratch was photographed under a microscope immediately and again at 24 and 48 h to determine cell migration. In this study, the migration chamber (25 Culture-inserts; 2 wells for self-insertion) was purchased from Ibidi USA, Inc. (Madison, USA).

Statistical analysis

SPSS 20.0 (SPSS Institute Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were expressed as mean \pm standard deviation (SD) and were analyzed with Levene's homogeneity-of-variance test (for two groups), independent samples *t*-test (for two groups), one-way analysis of variance (ANOVA; three or more groups), and least significant difference method (for two groups). A *P* < 0.05 was considered statistically significant.

RESULTS

Endogenous sulfur dioxide/aspartate aminotransferase pathway existed in the primary rat myocardial fibroblasts

We detected protein expression of AAT1 and AAT2 in cardiac fibroblasts and also detected the generation of SO₂ in the cell supernatant, suggesting that there was an endogenous SO₂/AAT pathway in cardiac fibroblasts. For further investigation, primary rat myocardial fibroblasts were

transfected with empty lentivirus or an AAT knockdown lentivirus. We found that the expression of AAT was decreased by 79% in the AAT1 knockdown group ($t = 12.67$, $P < 0.01$) and 77% in the AAT2 knockdown group ($t = 9.61$, $P < 0.01$) compared with the empty virus group. Consistently, the level of SO_2 in the cell supernatant was decreased by 84.18% in the AAT1 knockdown group and 82.49% in the AAT2 knockdown group compared with the empty lentivirus group [$F = 31.46$, $P < 0.01$; Figure 1].

Endogenous sulfur dioxide inhibited the proliferation of rat primary myocardial fibroblasts

We observed the proliferation of primary rat myocardial fibroblasts by detecting the protein expression of PCNA and the activity of CCK8. In rat primary myocardial fibroblasts, the PCNA expression was significantly increased in the AAT1 knockdown group ($F = 31.70$, $P < 0.01$) and in the AAT2 knockdown group compared with the empty virus group ($F = 18.93$, $P < 0.01$). Similarly, the activity of CCK-8 was much higher in the AAT1 knockdown group and the AAT2 knockdown group than in the empty virus group [$F = 56.99$, $P < 0.01$; Figure 2a-2c].

Endogenous sulfur dioxide inhibited the migration of rat primary myocardial fibroblasts

Two wells for self-insertion migration chambers were used to detect the migration of rat primary myocardial fibroblasts. The images and data both showed that the width of wound in the cultured cells of AAT1 knockdown group at 24 h ($F = 53.01$, $P < 0.01$) and 48 h ($F = 43.35$, $P < 0.01$) was narrower than that of empty group at the same time point. In the cells of AAT2 knockdown groups, the similar results were observed ($P < 0.01$), suggesting that the migration of primary rat myocardial fibroblasts was promoted by the downregulation of endogenous SO_2 /AAT pathway [Figure 2d].

Supplementation of sulfur dioxide rather than pyruvate significantly inhibited the increase in proliferation and migration caused by aspartate aminotransferase knockdown

Compared with the AAT1 knockdown lentivirus group, the expression of PCNA and the activity of CCK8 were significantly decreased in the AAT1 knockdown + SO_2 group. The images and data both showed that the migration distance in the cultured cells of AAT1 knockdown group at 24 h ($F = 53.01$, $P < 0.01$) and 48 h ($F = 43.35$, $P < 0.01$) was smaller than that of SO_2 group at the same time point. However, compared with the AAT1 knockdown lentivirus group, the expression of PCNA, the activity of CCK8, and the migration distance had no significant difference with AAT1 knockdown + pyruvate group ($P > 0.05$). In the cells of AAT2 knockdown groups, the similar results were observed [both $P < 0.01$; Figure 2].

Endogenous sulfur dioxide inhibited extracellular receptor kinase phosphorylation in rat primary myocardial fibroblasts

Compared with the empty lentivirus group, the phosphorylated ERK in the rat primary myocardial fibroblasts of AAT1 knockdown lentivirus group and AAT2 knockdown lentivirus group was significantly increased, respectively. However, there were no significant differences in the expression of T-ERK protein in the rat primary myocardial fibroblasts ($P > 0.05$). Therefore, the ratio of p-ERK and ERK was significantly increased by 119% in the fibroblasts of AAT1 knockdown group ($t = -7.36$, $P < 0.01$) and 259% in the fibroblasts of AAT2 knockdown group ($t = -10.97$, $P < 0.01$) compared with the empty lentivirus group [Figure 3].

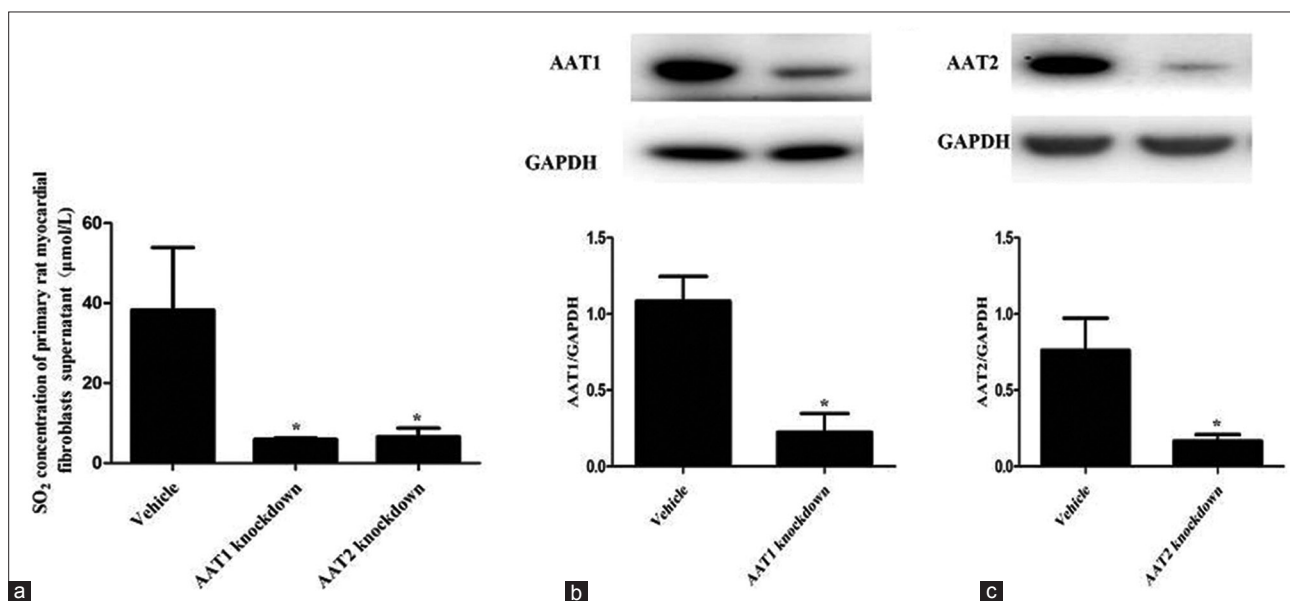


Figure 1: Endogenous SO_2 /AAT pathway existed in the primary rat primary fibroblasts. (a) The level of SO_2 in supernatant of rat primary myocardial fibroblast; (b) AAT1 expression in the rat primary myocardial fibroblast; (c) AAT2 expression in the rat primary myocardial fibroblast. * $P < 0.01$, compared with vehicle. SO_2 : Sulfur dioxide; AAT: Aspartate aminotransferase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

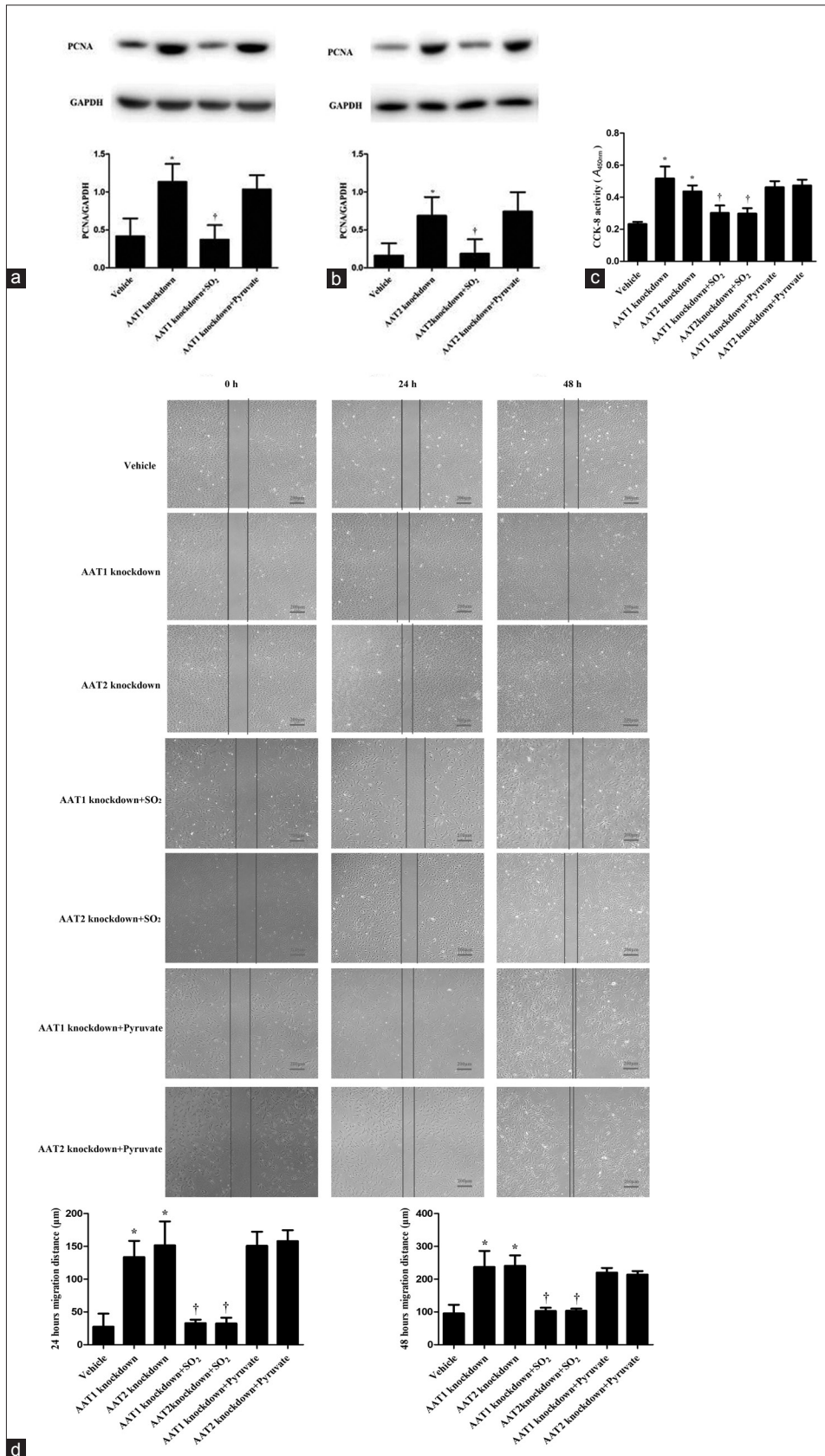


Figure 2: Endogenous SO₂ inhibited PCNA expression, CCK-8 activity, and the migration of primary myocardial fibroblasts. (a) PCNA expression in the rat primary myocardial fibroblast transfected with empty virus or AAT1 knockdown virus; (b) PCNA expression in the rat primary myocardial fibroblast transfected with empty virus or AAT2 knockdown virus; (c) CCK-8 activity in each group; (d) migration distance at 24 and 48 h (original magnification ×40). **P* < 0.01, compared with vehicle. †*P* < 0.01, compared with AAT knockdown group. SO₂: Sulfur dioxide; AAT: Aspartate aminotransferase; PCNA: Proliferating cell nuclear antigen; CCK-8: Cell Counting Kit-8; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

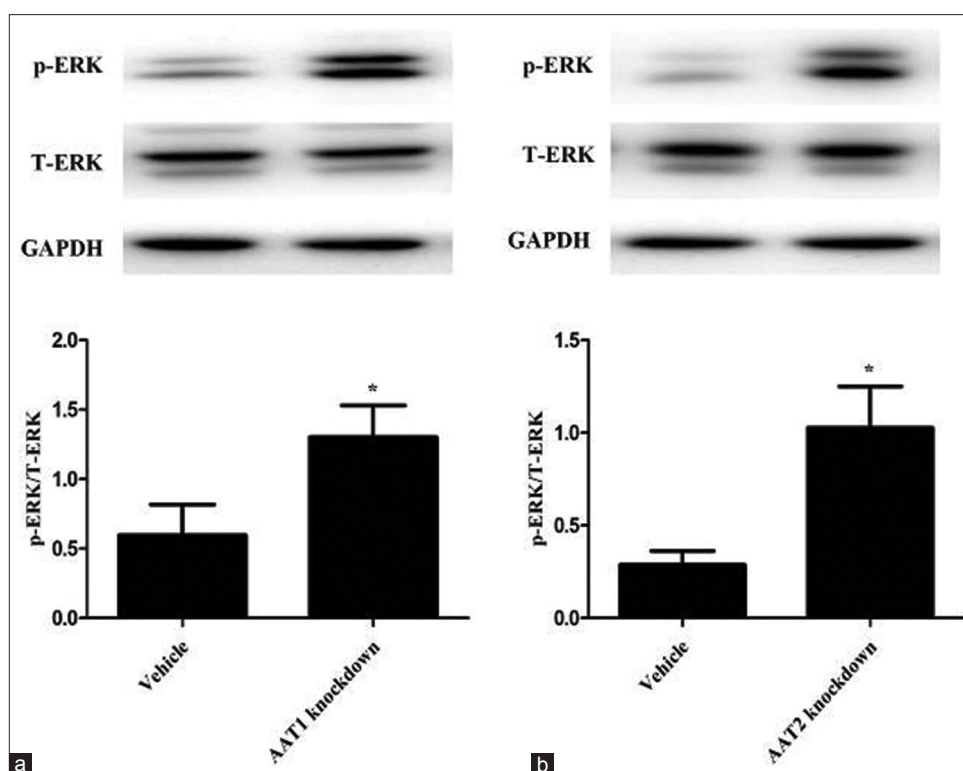


Figure 3: Endogenous SO_2 inhibited ERK phosphorylation in rat primary fibroblasts. (a) p-ERK and T-ERK levels in the empty virus group and the AAT1 knockdown group; (b) p-ERK and T-ERK levels in the empty virus group and the AAT2 knockdown group. * $P < 0.01$, compared with vehicle. SO_2 : Sulfur dioxide; AAT: Aspartate aminotransferase; p-ERK: Phosphorylated extracellular signal-regulated protein kinase; T-ERK: Total extracellular signal-regulated kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

PD98059 inhibited the enhanced proliferation and migration of rat primary myocardial fibroblasts induced by aspartate aminotransferase knockdown

PD98059, an inhibitor of the MAPK signaling pathway, was used to block the activation of ERK in the primary rat myocardial fibroblasts induced by AAT knockdown. The data showed that there were no significant differences in the expression of PCNA ($F = 74.01$, $P > 0.05$), ERK phosphorylation ($F = 27.83$, $P > 0.05$), CCK-8 activity ($F = 50.14$, $P > 0.05$), and the migration distance in the primary rat myocardial fibroblasts at 24 h ($F = 37.08$, $P > 0.05$) and 48 h ($F = 58.60$, $P > 0.05$) between the PD98059 group and PD98059+AAT1 knockdown group, and between the PD98059 group and PD98059+AAT2 knockdown group [Figure 4].

DISCUSSION

In recent years, it has been found that SO_2 is generated endogenously, catalyzed by AAT in the sulfur-containing amino acid metabolic pathway in mammals, and plays an importantly regulatory role in cardiovascular system.^[10,25-27] In particular, endogenous SO_2 was reported to be involved in the important cardiac regulation. For example, SO_2 preconditioning could reduce I/R-induced myocardial injury *in vivo* and *in vitro*.^[28] Endogenous SO_2 restored calcium homeostasis disturbance in rat with isoproterenol-induced myocardial injury.^[29] In diabetic rats, SO_2 was found to alleviate myocardial fibrosis.^[30] Our previous study

also proved that endogenous SO_2 prevented angiotensin II-induced myocardial hypertrophy. All the above-mentioned studies suggested that cardiomyocyte-derived SO_2 had an important protective effect on the heart. However, there was no study about the relationship between endogenous SO_2 and other important heart cells, cardiac fibroblasts, which played an important role in heart damage and repair.^[31,32] Therefore, in the present study, we attempted to investigate whether endogenous SO_2 also existed in cardiac fibroblasts and its possible role. Fortunately, in the cardiac fibroblasts, we confirmed the existence of endogenous SO_2 /AAT pathway, demonstrated by the results that the expression of AAT1 and AAT2 in the cardiac fibroblasts was detected by Western blot, and about 30 $\mu\text{mol/L}$ SO_2 content in the cell supernatant was also measured by HPLC method. Moreover, either AAT1 knockdown or AAT2 knockdown significantly reduced the generation of endogenous SO_2 , which further supported that cardiac fibroblast-derived SO_2 was generated from the enzymatic reaction catalyzed by AAT.

As well known, the proliferation and migration of cardiac fibroblast were the iconic characteristics of the activation of cardiac fibroblast during the development of cardiac diseases. When the heart injury occurs, more quiescent fibroblasts are transformed to the activated cells and migrate to the injured myocardial site for the active proliferation, extensive production, and deposition of collagen and other matrix proteins. Cardiac fibrosis and adverse remodeling are mostly due to the excessive proliferative and profibrotic response.^[33]

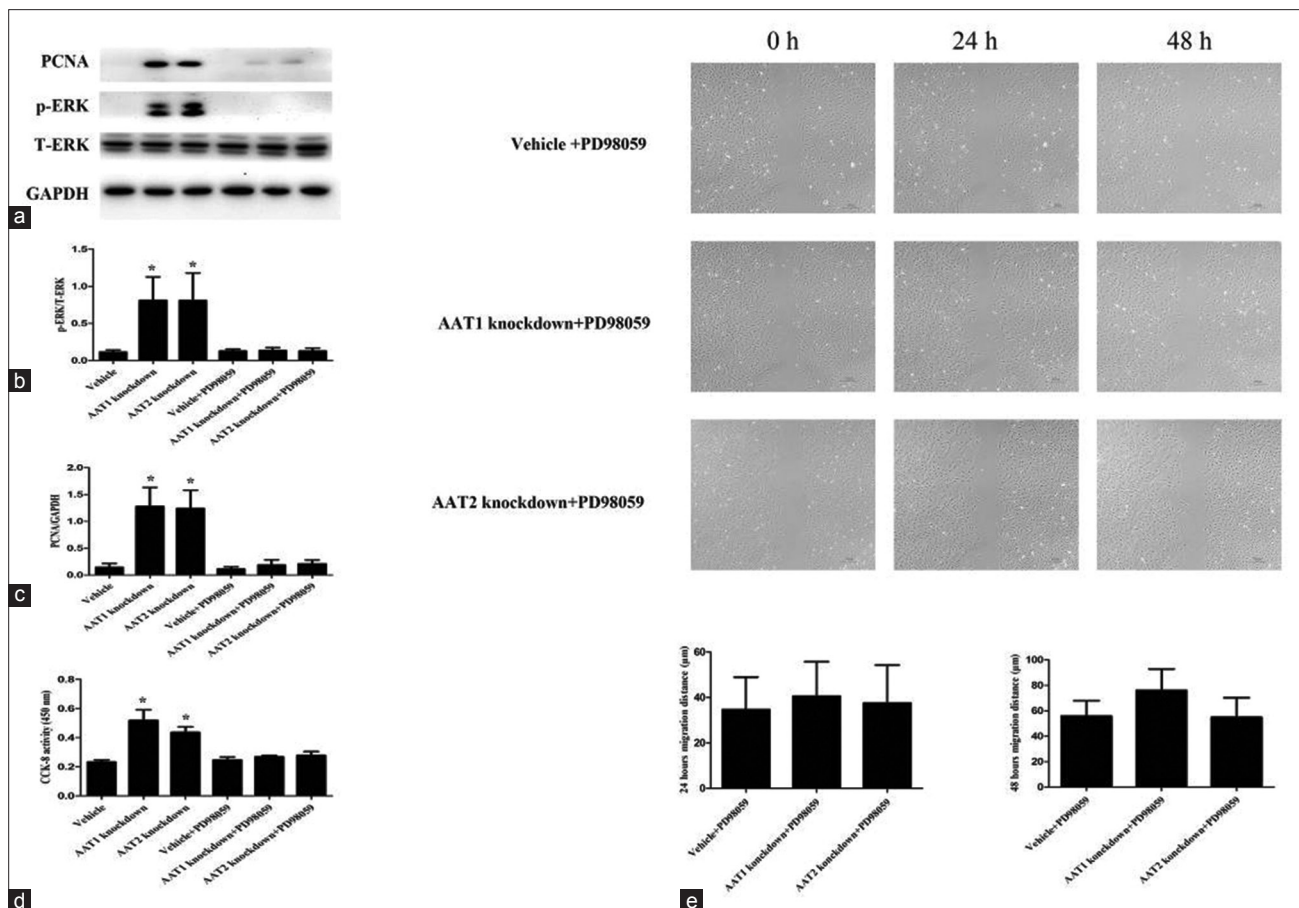


Figure 4: PD98059 blocked AAT knockdown-induced enhanced PCNA expression, activated ERK phosphorylation, enhanced CCK-8 activity, and migration in rat primary myocardial fibroblasts. (a) The expression of PCNA, p-ERK, and T-ERK in each group; (b) statistics of p-ERK/T-ERK; (c) statistics of PCNA/GAPDH; (d) CCK-8 activity in each group; (e) migration distance at 24 and 48 h after PD98059 treatment (original magnification $\times 40$). $*P < 0.01$. SO₂: Sulfur dioxide; AAT: Aspartate aminotransferase; PCNA: Proliferating cell nuclear antigen; p-ERK: Phosphorylated extracellular signal-regulated protein kinase; T-ERK: Total extracellular signal-regulated kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; CCK-8: Cell Counting Kit-8.

Therefore, we observed the effect of myocardial fibroblast derived-endogenous SO₂ on the proliferation and migration of cardiomyocytes. In the present study, we detected the PCNA expression and CCK-8 activity to represent the change of cell proliferation. We found that PCNA expression was significantly upregulated after the downregulation of the endogenous SO₂/AAT pathway. Moreover, the CCK-8 assay showed an enhanced cell proliferation after downregulation of the endogenous SO₂/AAT pathway. Since the migration of myocardial fibroblasts is an important factor for excessive accumulation of myocardial extracellular matrix, we performed cell wound-healing assay and found that cell migration was markedly promoted by the downregulation of endogenous SO₂/AAT pathway. However, AAT catalyzes the production of endogenous SO₂ accompanied by the formation of pyruvate. In order to observe whether the increase in the proliferation and migration caused by AAT knockdown cardiac fibroblasts is due to a decrease in SO₂ or a decrease in pyruvate, we supplemented AAT knockdown cardiac fibroblasts with SO₂ and pyruvate, respectively. The results showed that SO₂ supplementation could significantly inhibit the proliferation and migration of cardiac fibroblasts induced by AAT knockdown. However,

pyruvate supplementation did not significantly inhibit the proliferation and migration of cardiac fibroblasts induced by AAT knockdown. Those results indicated that endogenous SO₂ inhibited the proliferation and migration of myocardial fibroblasts.

Furthermore, we designed an experiment attempt to explore the mechanism by which endogenous SO₂ inhibited the migration and proliferation of myocardial fibroblasts. As well known, cell growth, development, and differentiation are tightly regulated, during which the MAPK signaling pathway plays an important role. ERK is a member of the MAPK family, and the ERK signaling pathway plays a crucial role in regulating cell growth, development, and differentiation. ERK is normally located in the cytoplasm, and once activated, it is translocated into the nucleus to regulate the activity of transcription factors and exert cellular effects.^[15] Previous studies have showed that endogenous SO₂ inhibits vascular smooth muscle cell proliferation via the ERK signaling pathway.^[19,20] Now, here, the problem is that whether ERK/MAPK pathway mediated the inhibitory effect of endogenous SO₂ on the cardiac fibroblast proliferation and migration. To resolve the above problem, we first detected

the activation of ERK pathway representing by ERK phosphorylation and discovered that phosphorylated ERK was significantly increased in the AAT1/2 knockdown rat primary myocardial fibroblasts. Furthermore, PD98059, an ERK inhibitor, was used. The data showed that PD98059 successfully blocked AAT knockdown-induced enhanced proliferation and migration of myocardial fibroblasts, indicating that SO₂ inhibited the proliferation and migration of myocardial fibroblasts by inhibiting the ERK signaling pathway.

In summary, our study reported that endogenous SO₂ was present in cardiac fibroblasts, and endogenous SO₂ pathway inhibited the proliferation and migration of cardiac fibroblasts. Mechanistically, ERK pathway might be involved in the mechanisms by which endogenous SO₂/AAT pathway protected against excessive proliferation and migration of cardiac fibroblasts. This study suggested that endogenous SO₂ might be a novel proliferation and migration inhibitor in myocardial fibroblasts, which would be of value in the understanding of the pathogenesis of myocardial fibrosis.

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Conflicts of interest

There are no conflicts of interest.

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二氧化硫对大鼠心肌成纤维细胞增殖和迁移的抑制作用

摘要

背景: 心肌纤维化是许多心脏疾病的重要病理变化,但其发病机制非常复杂,目前尚未完全阐明。该研究旨在研究内源性二氧化硫(SO₂)是否是一种新型心肌成纤维细胞增殖和迁移抑制剂。

方法: 分离原代大鼠心肌成纤维细胞采用天冬氨酸氨基转移酶(AAT1和AAT2)敲低的慢病毒和空慢病毒分别转染原代大鼠心肌成纤维细胞。用高效液相色谱法测定上清液中SO₂含量,蛋白印迹法检测细胞中AAT1、AAT2、增殖细胞核抗原(PCNA)、磷酸化胞外受体激酶(p-ERK)和总ERK(T-ERK)蛋白含量。通过细胞划痕实验检测细胞迁移。使用独立样本*t*检验(对于两组)和单因素方差分析(三个或更多组)分析结果。

结果: AAT1和AAT2敲低均显著降低SO₂水平($F=31.46, P<0.01$)和AAT1/2蛋白表达(AAT1, $t=12.67, P<0.01$; AAT2, $t=9.61, P<0.01$),但显著增加PCNA的蛋白表达(AAT1, $F=31.70, P<0.01$; AAT2, $F=18.93, P<0.01$)和CCK-8活性($F=56.99, P<0.01$)以及大鼠原代心肌成纤维细胞的迁移(24小时, $F=53.01, P<0.01$ 和48小时, $F=43.35, P<0.01$)。SO₂而非丙酮酸的补充可显著抑制由AAT敲低引起的增殖和迁移的增加(P 均 <0.01)。从机制上看, AAT1/2敲除组p-ERK与T-ERK的比值显著高于空病毒组(AAT1, $t=-7.36, P<0.01$; AAT2, $t=-10.97, P<0.01$),而ERK激活的抑制剂PD98059阻断了AAT敲低诱导的PCNA上调($F=74.01, P>0.05$)、CCK-8激活($F=50.14, P>0.05$)以及心肌成纤维细胞迁移(24小时, $F=37.08, P>0.05$ 和48小时, $F=58.60, P>0.05$)。

结论: 内源性SO₂可能是一种新的心肌成纤维细胞增殖和迁移抑制剂,其抑制心肌成纤维细胞增殖和迁移的机制可能是通过抑制ERK信号通路。