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Hydrogen sulfide suppresses transforming growth factor-β1-induced differentiation of human cardiac fibroblasts into myofibroblasts

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In heart disease, transforming growth factor-β1 (TGF-β1) converts fibroblasts into myofibroblasts, which synthesize and secrete fibrillar type I and III collagens. The purpose of the present study was to investigate how hydrogen sulfide (H_2S) suppresses TGF-β1-induced differentiation of human cardiac fibroblasts to myofibroblasts. Human cardiac fibroblasts were serum-starved in fibroblast medium for 16 h before exposure to TGF-β1 (10 ng mL^{-1}) for 24 h with or without sodium hydrosulfide (NaHS, 100 μmol L^{-1}, 30 min pretreatment) treatment. NaHS, an exogenous H_2S donor, potently inhibited the proliferation and migration of TGF-β1-induced human cardiac fibroblasts and regulated their cell cycle progression. Furthermore, NaHS treatment led to suppression of fibroblast differentiation into myofibroblasts, and reduced the levels of collagen, TGF-β1, and activated Smad3 in TGF-β1-induced human cardiac fibroblasts *in vitro*. We therefore conclude that H_2S suppresses TGF-β1-stimulated conversion of fibroblasts to myofibroblasts by inhibiting the TGF-β1/Smad3 signaling pathway, as well as by inhibiting the proliferation, migration, and cell cycle progression of human cardiac myofibroblasts. These effects of H_2S may play significant roles in cardiac remodeling associated with heart failure.

human cardiac fibroblasts, hydrogen sulfide, transforming growth factor β1, myofibroblasts

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Cardiac fibroblasts (CFs) are the most common cell type in the adult myocardium, accounting for about two-thirds of the cell population. They are embedded within the extracellular matrix (ECM) of the connective tissue and are, to a large extent, responsible for its synthesis and degradation [1]. However, CFs have received relatively little attention compared to their more famous neighbors, the cardiomyocytes. Emerging studies reveal that fibroblasts are fundamentally involved in cardiac remodeling in normal ageing heart and in damaged myocardium [2,3]. Other recent studies have suggested a "sentinel" role for CFs that is intimately associated with myocardial response to a broad range of stimuli during cardiac development and disease, including hypoxia, and changes in chemical, electrical, and mechanical signals. Due to their potential role in the regulation of global myocardial function, fibroblasts have become a promising therapeutic target in heart disease.

Hydrogen sulfide (H_2S) was previously regarded as a toxic gas. However, recent evidence suggests that H_2S is an emerging endogenous gasotransmitter in mammals that

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promotes vasorelaxation, cardioprotection, neurotransmission, and anti-inflammatory action in the gastrointestinal tract [4,5]. These findings, which indicate that H_2S is an endogenous signaling molecule (second messenger) capable of modulating various physiological processes including vasodilation, and that it parallels the action of nitric oxide (NO), prompted us to investigate the potential of H_2S as a cardioprotective agent [6,7]. We recently reported that transplantation of exogenous H_2S-pretreated mesenchymal stem cells (MSCs) could reduce infarct size and increase left ventricular function in a rat myocardial infarction model [8]. However, little is known about the mechanism(s) by which H_2S affects cell growth and function. Therefore, in the present study, we tested the hypothesis that direct administration of the H_2S donor-sodium hydrosulfide (NaHS) affects the proliferation, migration, and differentiation of transforming growth factor-β1 (TGF-β1)-induced CFs into myofibroblasts *in vitro*.

1 Materials and methods

1.1 Cell Culture

Human Cardiac Fibroblasts-adult ventrical cells (HCF-av, Catalog# 6310) were obtained from ScienCell Research Laboratories (San Diego, USA) and cultured in fibroblast media (FM) supplemented with 2% fetal bovine serum, 1% fibroblast growth supplement (FGS), and 1% penicillin/streptomycin solution (P/S) according to the manufacturer's protocol (ScienCell Research Laboratories). Cells were subcultured when they became more than 90% confluent. Cells at passage 3 to 6 were used for experiments [9].

1.2 Experimental design

HCF-av cells were serum-starved in FM for 16 h prior to experiments. Cells were then stimulated with TGF-β1 (10 ng mL^{-1}, Sigma, USA) for 24 h to promote the cardiac myofibroblast phenotype [9,10] in the absence or presence of NaHS (100 μmol^{-1}, 30 min pretreatment; Sigma).

1.3 Cell proliferation assay

Cell proliferation was determined by Bromodeoxyuridine (BrdU) incorporation using a colorimetric kit from Roche Applied Science, according to the manufacturer's instructions. Briefly, 5×10^3 cells per well were seeded on flat-bottom 96-well plates. Cells were then starved in serum-free media for 16 h. Proliferation was induced by culturing cells in TGF-β1 in the absence or presence of NaHS. Cells were then exposed to the BrdU labeling solution (10 μmol^{-1}), fixed, and incubated with an anti-BrdU-POD (peroxidase) working solution, followed by a detection substrate. Emission at 450 nm was immediately measured on a standard enzyme-linked immunosorbent assay (ELISA) plate reader (Infinite 200, TECAN).

1.4 Cell migration assay

The migration of HCF-av cells was determined using a wound-healing assay, as previously described [11]. Briefly, confluent cultured HCF-av cells were grown in 6-well plates. After the cells were serum-starved in FM media for 16 h, they were scratched with a sterile 1 mL pipette tip and then washed twice with phosphate buffered saline (PBS) to remove unattached cells. The cells were then cultured in FM supplemented with TGF-β1 (10 ng mL^{-1}) in the absence or presence of NaHS (100 μmol L^{-1}). To standardize the position of the wound for imaging, small indentations were made at the bottom of the plate. Cell migration was determined after 24 h by counting cells that had moved out of the initial area.

1.5 Measurement of cell cycle distribution

Exponentially growing HCF-av cells cultured in T-25 flasks were either treated with TGF-β1 (10 ng mL^{-1}) or left untreated, in the absence or presence of NaHS (100 μmol L^{-1}). 24 h after treatment, the cells were harvested by trypsinization, washed twice in PBS, and fixed in ice-cold ethanol (70%, v/v) overnight at 4°C. Cells were stained with propidium iodide (PI, 50 μg L^{-1}; Sigma) for 30 min in the presence of RNase A (100 μg mL^{-1}, Sigma) and Triton X-100 (0.1%, v/v). The cell cycle distribution of HCF-av cells was determined by flow cytometry (FACS Calibur, BD Biosciences, USA). Flow cytometry data were acquired using CellQuest software (10,000 cells were recorded for each sample), and the cell profile was assessed using ModFit software.

1.6 Apoptosis assay

An Annexin V apoptosis detection kit (BD Biosciences) was used to measure apoptosis of HCF-av cells by following the manufacturer's instructions. Briefly, after treatment, cells were washed twice with cold PBS and then resuspend in 1×binding buffer at a concentration of 1×10^6 cells mL^{-1}. A 100 μL aliquot of this cell solution (1×10^5 cells) was transferred to a 5 mL culture tube and incubated with fluorescein isothiocyanate (FITC)-Annexin V (5 μL) and PI (5 μL) for 15 min at RT in the dark. The cells were then analyzed by flow cytometry within 1 h of staining.

1.7 Immunocytochemistry

Immunocytochemistry was performed as previously described [12]. Cells plated on chamber slides were fixed in

4% paraformaldehyde for 15 min at room temperature, permeabilized with Triton X-100, and blocked with albumin from bovine serum (BSA). The cells were then incubated with a primary antibody against α-smooth muscle actin (α-SMA, Sigma) overnight at 4°C and subsequently washed with PBS three times, followed by incubation with a FITC-conjugated secondary antibody (Jackson Immunoresearch) for 1 h at room temperature. After washing with PBS, cells were incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed by fluorescent microscopy. Cells were counted and scored from five randomly selected areas of each slide, and quantitative analysis was performed using Image-Pro Plus software.

1.8 Western blot analysis

Western blot analysis was performed as previously described [13]. Cells were washed twice with PBS and harvested in radio immune precipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Thermo Scientific) and phenylmethanesulfonyl fluoride (PMSF; 1 mmol L^{-1}). Equal amounts (30 μg) of samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF, Millipore, USA) using a Trans-blot apparatus (Bio-Rad, USA). Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at RT and were subsequently incubated with anti-α-SMA (Sigma; 1∶1000 dilution), anti-Collagen I (ABcam; 1∶5000 dilution), anti-Collagen III (ABcam; 1∶5000 dilution), anti-TGF-β1 (ABcam; 1∶800 dilution), and anti-phospho-Smad3 (CST; 1∶1000 dilution) primary antibodies overnight at 4°C. Blots were washed with TBST, followed by incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch). Protein signals were detected using enhanced chemiluminescence reagents and were quantified by densitometry.

1.9 Statistics analysis

Data are expressed as $\bar{x}$ ±SE. Different groups were compared by one-way ANOVA followed by the Student-Newman-Keuls post-hoc test. Comparison between two groups was assessed by a Student's *t* test, and a $P<0.05$ indicated statisitcal significance.

2 Results

2.1 H_2S inhibits TGF-β1-induced proliferation of HCF-av cells

To determine the effect of NaHS on the proliferation of HCF, cells were stimulated with TGF-β1 followed by BrdU labeling. Cell proliferation of TGF-β1 stimulated cells was substantially increased as indicated by the increase in BrdU-positive cells, compared to the control. However, NaHS treatment dramatically reduced cell proliferation (Figure 1).

2.2 H_2S antagonizes TGF-β1-induced migration of HCF-av cells

To investigate whether H_2S inhibits migration of HCF-av cells, we performed a wound-healing assay. Cells in culture were scraped with a 1,000 μL pipette tip and a wide acellular area was produced. Figure 2A illustrates that the wound was completely closed in HCF-av cells treated with 10 ng mL^{-1} TGF-β1 for 24 h. The number of CFs migrating into

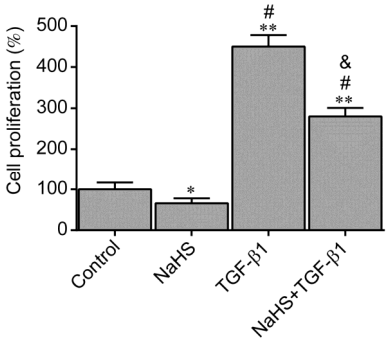

Figure 1 H_2S inhibits TGF-β1-induced proliferation of HCF-av cells. Proliferation of cells with or without TGF-β1 (10 ng mL^{-1}) treatment, and in the absence or presence of NaHS (100 μmol L^{-1}). *, $P<0.05$; **, $P<0.01$ vs. Control; #, $P<0.01$ vs. NaHS; &, $P<0.01$ vs. TGF-β1 ($n=6$).

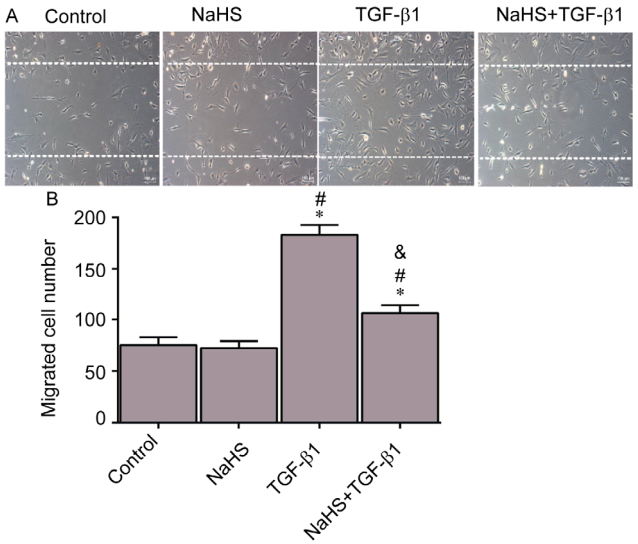

Figure 2 H_2S antagonizes TGF-β1-induced migration of HCF-av cells. A, Wound-healing migration assay on HCF-av cells. Broken white lines indicate the initial acellular wound regions. B, Mean values for the number of migrated HCF-av cells counted in the areas defined in A. *, $P<0.01$ vs. Control; #, $P<0.01$ vs. NaHS; &, $P<0.01$ vs. TGF-β1 ($n=6$).

the acellular area was calculated and expressed as the number of migrated cells (Figure 2B). Similarly, a substantial number of cells populated the scratched area. However, H_2S markedly antagonized TGF-β1-induced migration.

2.3 Role of H_2S in cell cycle distribution

Subconfluent cycling HCF-av cells were stimulated with TGF-β1 in the absence or presence of NaHS and collected 24 h after treatment. The percentage of cells in each cell cycle phase (G_0/G_1, S, and G_2/M) was determined by flow cytometry (Figure 3A). Figure 3B demonstrates that there was no significant difference in the number of cells in the G_0/G_1 phase in all treatment groups compared to the control. However, exposure to TGF-β1 increased the percentage of S phase cells, but NaHS blocked this increase (Figure 3C). NaHS and/or TGF-β1 treatment resulted in a higher number cells in the G_2/M phase compared to the control (Figure 3D).

2.4 Apoptosis

We evaluated whether NaHS or TGF-β1 could induce apoptosis in HCF-av cells. However, there was no significant difference in the number of apoptotic cells (Annexin V^+PI^+) between all experimental groups, indicating that neither TGF-β1 nor NaHS could induce apoptosis in HCF-av cells (Figure 4A, B).

2.5 H_2S suppresses TGF-β1-induced differentiation of HCF-av cells to myofibroblasts

TGF-β1 is the most potent stimulor of fibroblast differentiation to myofibroblasts that express α-SMA microfilaments, which confer contractile behavior to myofibroblasts. As shown in Figure 5A and 5B, TGF-β1 upregulated α-SMA expression 3.9±0.8 fold, compared to the control (P<0.01). Furthermore, the addition of NaHS potently abrogated α-SMA expression induced by TGF-β1. This effect was confirmed by immunocytochemical analysis of α-SMA protein expression, which showed that 24 h TGF-β1 exposure potently increased the expression and organization of α-SMA microfilaments and led to larger cells, which is consistent with the myofibroblast phenotype (Figure 5C). However, TGF-β1-induced myofibroblast differentiation was abrogated by NaHS (Figure 5D).

2.6 H_2S blocks TGF-β1-induced Collagen I and Collagen III expression in HCF-av cells

Collagen protein expression in response to TGF-β1 or H_2S stimulation was assessed by western blotting (Figure 6A).

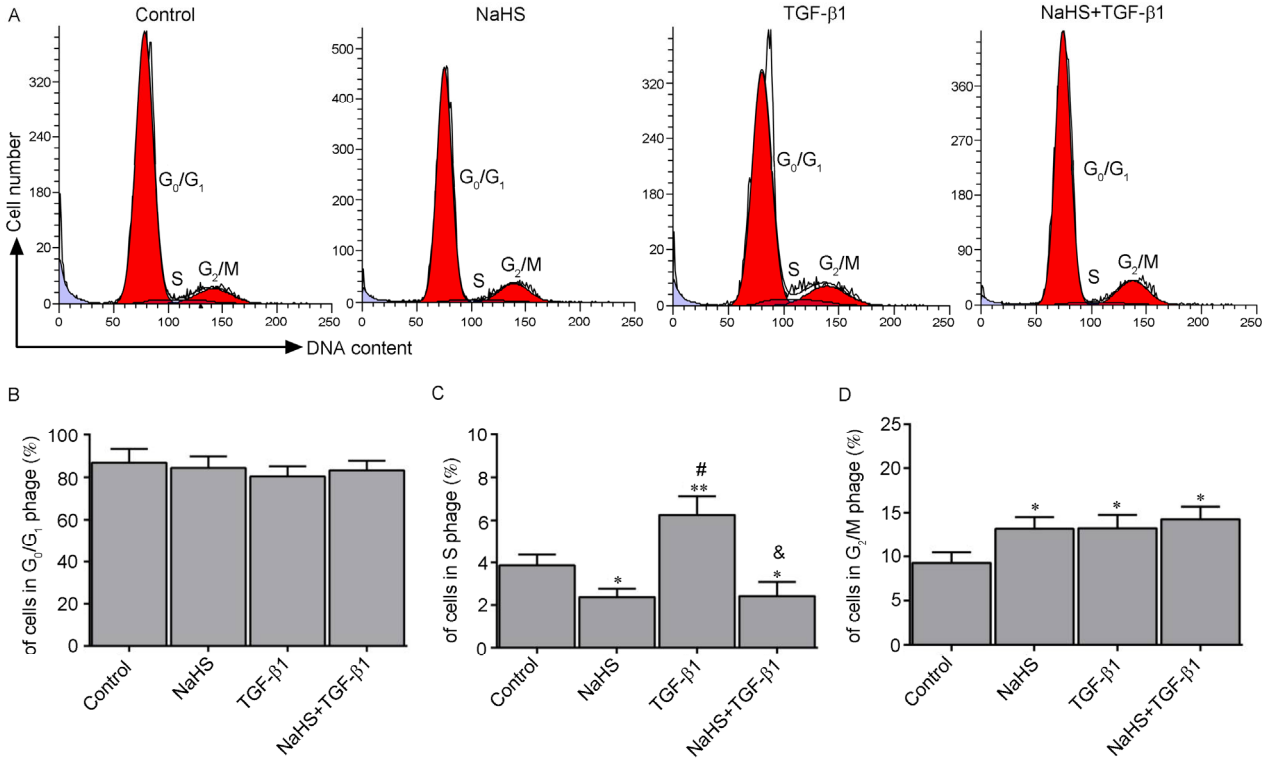

Figure 3 Effect of H_2S on cell cycle progression in TGF-β1-induced HCF-av cells. A, Representative image of PI stained cells showing DNA distribution as analyzed by flow cytometry. B, Quantitative comparison of the fraction of HCF-av cells in G_0/G_1, S, and G_2/M cell cycle stages. *, P<0.05; **, P<0.01 vs. Control; #, P<0.01 vs. NaHS; &, P<0.01 vs. TGF-β1 (n=6).

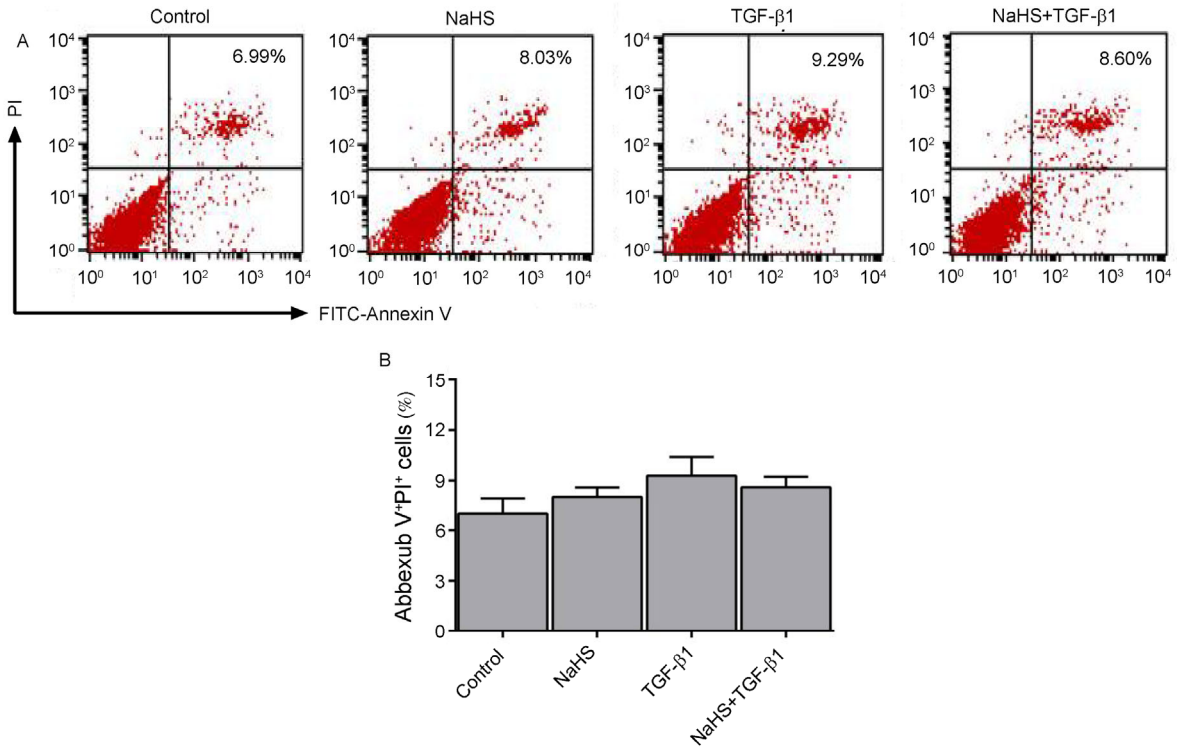

Figure 4 Exposure to TGF-β1 with or without H_2S treatment did not induce apoptosis of HCF-av cells. A, Representative flow cytometric analysis of HCF-av apoptotic cells stained for Annexin V/PI. B, Summaries of the rate of apoptosis, represented by histograms (n=6).

We observed that TGF-β1 treatment for 24 h produced more Collagen I and Collagen III than control cells, which confirmed the differentiation patterns shown in Figure 5 (α-SMA positive cells). H_2S significantly abrogated the TGF-β1 induced increase in Collagen I and Collagen III.

2.7 H_2S inhibits TGF-β1-induced Smad3 activation of HCF-av cells

To investigate the role of H_2S during TGF-β1-induced myofibroblast differentiation, the TGF-β1 expression was assessed by western blot. As shown in Figure 7A and 7B, cells exposed to TGF-β1 exhibited significant increase in TGF-β1 expression compared to the control, while NaHS treatment dramatically reduced TGF-β1 expression.

Next, to further examine the mechanistic role of TGF-β1, we investigated its effect on Smad3 phosphorylation. As shown in Figure 7C and 7D, TGF-β1 (10 ng mL^{-1}) induced a robust increase in phospho-Smad3, which was dramatically reduced after NaHS treatment.

3 Discussion

Fibroblasts are embryologically distinct from cardiomyocytes in their origin. Following myocardial infarction they become activated, migrate to the injury site, and proliferate [14]. Interest in CFs has grown with the recognition that cardiac fibrosis is a prominent contributor to diverse forms of myocardial disease. Excessive interstitial fibrosis of failing hearts is caused by activated myofibroblasts in response to mechanical stretch, autocrine, and paracrine factors generated within the myocardium (for example, angiotensin II [Ang II] [15] and TGF-β1), and circulating hormones such as aldosterone [16]. It has therefore been hypothesized that the phenotypic conversion of CFs into specialized myofibroblasts is a critical process mediating cardiac remodeling. Previous studies show that TGF-β1 is the most potent stimulator of the activation and differentiation of fibroblasts into myofibroblasts [17]. After fibroblast maturation to myofibroblasts, an increase in the synthesis and secretion of fibronectin is observed. Therefore, in the current study, we utilized TGF-β1 to induce myofibroblast differentiation, as this cytokine is a critical mediator of the excessive fibrogenic reaction in CF culture, animals, and humans with heart failure [9]. In cardiac remodeling, CFs exhibit various functions, including proliferation, migration, and secretion of proinflammatory cytokines and growth factors [18]. Furthermore, intense proliferative activity has been documented in fibroblasts infiltrating the infarcted heart. In the present study, TGF-β1 induced the proliferation and migration of cultured HCF-av cells and exogenous

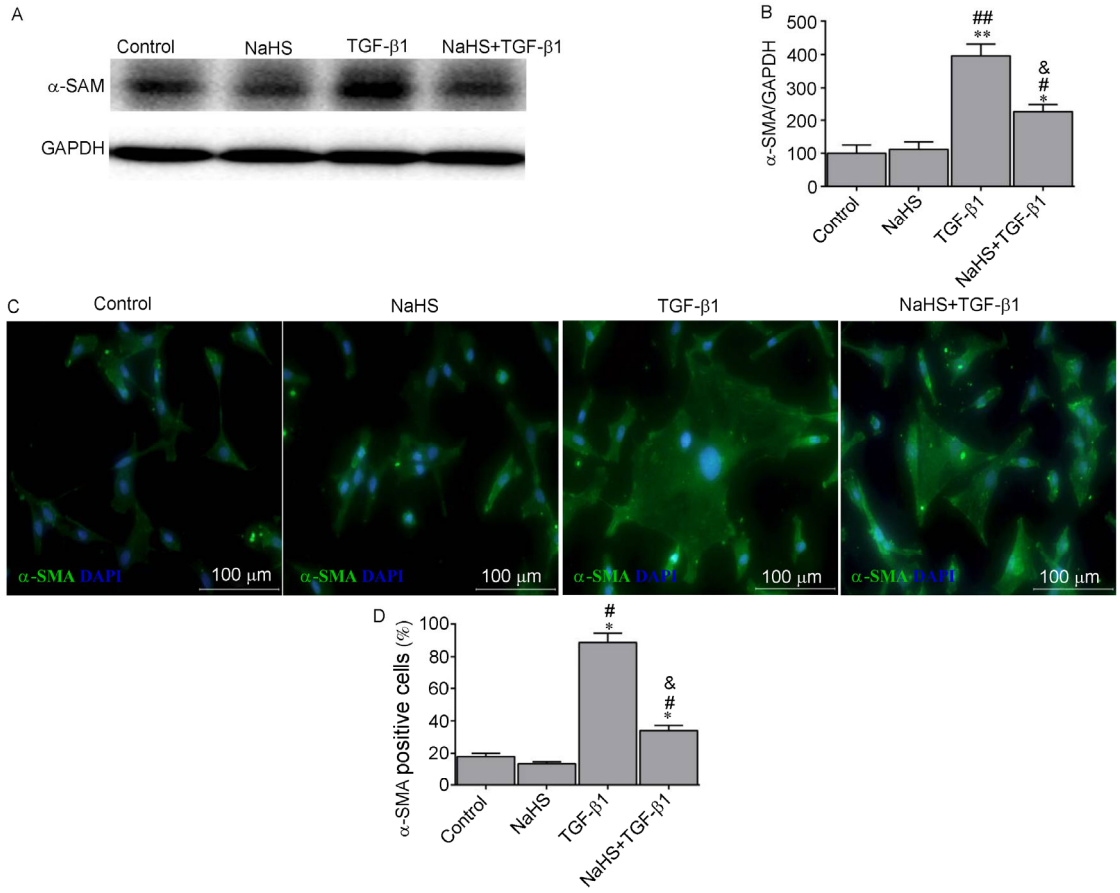

Figure 5 H_2S suppresses TGF-β1-induced differentiation of HCF-av cells into myofibroblasts. A, HCF-av cells were treated with NaHS (100 μmol L^{-1}), exposed to TGF-β1 (10 ng mL^{-1}) for 24 h, harvested, and then proteins were extracted. Western blot analysis was performed for α-SMA relative to GAPDH. B, The results are representative of four independent experiments after normalization to GAPDH. C, Immunofluorescence staining of α-SMA (green) in cells (400×). Nuclei are stained in blue with DAPI. D, The data represent the results of 6 independent experiments. *, P<0.05, **, P<0.01 vs. Control; #, P<0.05, ##, P<0.01 vs. NaHS; &, P<0.01 vs. TGF-β1.

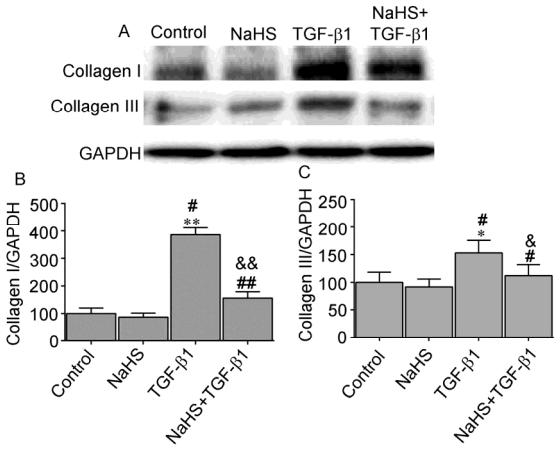

Figure 6 H_2S prevents TGF-β1-induced Collagen I and Collagen III expression in HCF-av cells. A, Representative immunoblots of Collagen I and Collagen III expression in HCF-av cells in response to stimulation with TGF-β1 (10 ng mL^{-1}) with or without NaHS (100 μmol L^{-1}) for the indicated times. B and C, Densitometric analysis of cellular Collagen I (B) and Collagen III (C) normalized to GAPDH. *, P<0.05; **, P<0.01 vs. Control; #, P<0.05; ##, P<0.01 vs. NaHS; &, P<0.05; &&, P<0.01 vs. TGF-β1 (n=4).

H_2S strongly inhibited these effects.

Cell cycle checkpoints are major control mechanisms that maintain proper execution of cell cycle events. Following DNA damage, cells arrest at the transition from the G_1 to S phase or from the G_2 to M phase [19]. The proportion of cells that arrest at G_1 to S or G_2 to M phases depends on the cell type, growth conditions, and checkpoint controls operating in the cell [20], and cell arrest often leads to cell death. Deplancke and Gaskins [21] demonstrated that exposure to H_2S significantly increased the fraction of cells in the S and G_2/M cell cycle phases in the rat intestinal crypt IEC-18 cell line. In addition, they found that exposure to 0.05–1 mmol L^{-1} NaHS did not significantly alter the percentage of apoptotic cells. However, treatment with 5 mmol L^{-1} NaHS increased the apoptotic cell population. Yang et al. [22] demonstrated that H_2S-induced apoptosis in human aorta smooth muscle cells (HASMCs) was concentration dependent, with significant apoptosis detected at concentrations of 200 μmol L^{-1} and higher. In contrast, Baskar et al. [23] demonstrated that NaHS decreased the percentage

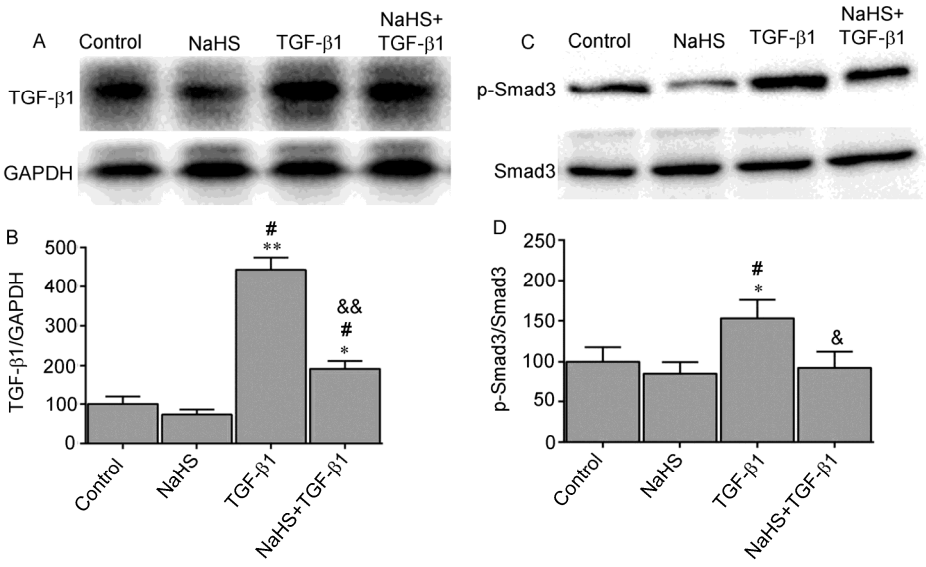

Figure 7 H_2S inhibits Smad3 activation evoked by TGF-β1 in HCF-av cells. Western blot analysis was used to assess the expression of actived TGF-β1 (A) and phospho-Smad3 (C) levels in cultured HCF-av cells exposed to TGF-β1 (10 ng mL^{-1}) with or without NaHS treatment (100 μmol L^{-1}). Densitometric analysis of cellular TGF-β1 normalized to GAPDH (B) and p-Smad3 normalized to Smad3 (D). *, P<0.05; **, P<0.01 vs. Control; #, P<0.01 vs. NaHS; &, P<0.05; &&, P<0.01 vs. TGF-β1 (n=4).

of normal human lung primary fibroblast (MRC-5) cells in G_1 and S phases and induced a slightly higher incidence of cells in the G_2/M phase. Furthermore, exposure to 50–75 μmol L^{-1} NaHS significantly increased the number of apoptotic cells. In the present study, we demonstrate that TGF-β1 stimulation for 24 h significantly increased the number of HCF-av cells in the S phase, while NaHS abrogated this effect. Additionally, we did not observe a significant difference in the number of apoptotic cells (Annexin V^+PI^+) with TGF-β1 and/or NaHS treatment. H_2S plays a different role in cell cycle distribution, which may vary depending on the cell type.

Myofibroblasts (mainly differentiated from fibroblasts) that express α-SMA microfilaments and exhibit proliferative, migratory, and secretory properties are abundant in cardiac fibrosis. Therefore, preventing the differentiation of proliferating fibroblasts to myofibroblasts has been an attractive strategy for limiting cardiac fibrosis. Previous studies have shown that α-SMA expression is regulated by TGF-β1, a locally generated cytokine in heart failure that is downstream of many of the pro-fibrotic actions of other fibroblast growth factors, including Ang II, aldosterone, and norepinephrine [24]. TGF-β1 is upregulated in failing human hearts and various experimental models of cardiac hypertrophy [24,25]. Functional blockade of TGF-β1 prevents cardiac interstitial fibrosis induced by pressure overload in rats [26]. Previous studies have demonstrated that inhibition of TGF-β1 function by anti-TGF-β1 antibodies reduced myofibroblasts and reduced fibrosis. In addition, H_2S was found to inhibit TGF-β1-induced transformation of MRC-5 lung fibroblasts to myofibroblasts [27], and suppress endothelin-induced proliferation of rat aortic smooth muscle cells [28]. Moreover, a recent study showed that H_2S attenuated human atrial fibroblast proliferation via suppression of K^+ channel activity and moderated their differentiation to myofibroblasts [29]. In agreement with these recent findings, the present study showed that NaHS effectively inhibited TGF-β1 expression and inhibited proliferation of HCF-av cells in response to TGF-β1 stimulation. Furthermore, NaHS ameliorated fibroblast differentiation to myofibroblasts and significantly suppressed α-SMA expression and their associated stress fibers.

Collagen proteins found in the normal myocardium include types I, III, IV, V, and VI. Fibrillar type I and III collagens are major components of the cardiac ECM. SDS-PAGE revealed that type I collagen, the most predominant (>70%) in the normal myocardium of nonhuman primates and rodents, has the tensile strength of steel. Types III and V collagens account for up to 10%–15% and less than 5%, respectively. In the heart, collagen is produced primarily by CFs. Previous studies have shown that type I and III collagen mRNAs in the infarcted myocardium are simultaneously increased with myofibroblast expression, suggesting that myofibroblasts are responsible for collagen synthesis in the repairing myocardium. In a previous study [30], we reported that recombinant human bone morphogenetic protein-2 (rhBMP-2) could attenuate pressure overload (transverse aortic constriction) induced collagen deposition, improve cardiac function, and reduce TGF-β1-dependent activation of PKC-δ and Smad3. In the current study, con-

sistent with changes in the number of myofibroblasts (α-SMA positive), we found that collagen I, collagen III, and p-Smad3 activation were increased upon stimulation with TGF-β1, however, administration of NaHS, attenuated collagen increase. In addition, TGF-β1 can amplify its own expression in fibroblasts and myofibroblasts. Therefore, our results suggest that the TGF-β1/Smad3 signaling pathway plays an important role in myofibroblast differentiation during cardiac fibrosis.

In conclusion, the present study shows that NaHS inhibited TGF-β1 secretion and TGF-β1 induced Smad3 activation as well as prevented TGF-β1-induced proliferation, migration, cell growth regulation, transformation to myofibroblasts, and collagen synthesis in an *in vitro* human cardiac fibroblasts-to-myofibroblasts assay. These effects provide insight into novel mechanisms with potential therapeutic implications for cardiac fibrosis of injured hearts.

The authors declare that they have no conflict of interest.

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