

# Hydrogen Sulfide Inhibits Transforming Growth Factor Beta-1 Induced Bronchial Epithelial-mesenchymal Transition

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**Key words:** Chronic Obstructive Pulmonary Disease; Epithelia-mesenchymal Transition; Hydrogen Sulfide; Small Airway Fibrosis

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death in the developed world and associated with a high individual and socioeconomic burden.<sup>[1]</sup> It is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Peri-bronchiolar fibrosis was occurred in small airways in the early state of COPD, and then followed by structure changes, and finally became persistent airflow limitation.<sup>[2]</sup> Recent researches have shown that epithelial-mesenchymal transition (EMT) is one of the leading causes of fibrosis in various diseases.

EMT is a process when epithelial cells gradually transform into mesenchymal-like cells losing their epithelial functionality and characteristics. EMT is thought to be involved in the pathogenesis of several chronic lung conditions such as asthma, COPD, bronchiolitis obliterans syndrome, and lung fibrosis.<sup>[3,4]</sup> It has been confirmed that COPD is accompanied by inflammation and tissue remodeling which is characterized by emphysema, and small airway remodeling with per-bronchiolar fibrosis.

Hydrogen sulfide (H<sub>2</sub>S), the third gas transmitter, together with nitric oxide (NO) and carbon monoxide (CO), is involved in many pathophysiological processes.<sup>[5]</sup> Our previous studies have demonstrated that endogenous H<sub>2</sub>S is involved in the pathogenesis of airway obstruction in COPD, and its alteration in level may be associated with disease activity and severity.<sup>[6]</sup> However, whether H<sub>2</sub>S could attenuate the fibrosis of small airway is still unknown. Therefore, this study aimed to investigate

the effect of H<sub>2</sub>S on inhibiting small airway fibrosis in bronchial epithelium.

## METHODS

### Materials

GY4137 (Cayman, USA) is a H<sub>2</sub>S slowly released donor. Its formal name is (p-methoxyphenyl) morpholinophosphinodithioic acid and molecular formula is C<sub>11</sub>H<sub>16</sub>NO<sub>2</sub>·PS<sub>2</sub>C<sub>4</sub>H<sub>10</sub>NO. Transforming growth factor beta-1 (TGF-β1) was from R&D (USA). Antibodies against E-cadherin, alpha-smooth muscle actin (α-SMA) were from Abcam (USA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Zhongshan Biotechnology (Beijing, China). IRDye 800CW-conjugated goat anti-rabbit IgG or goat anti-mouse IgG were from LI-COR Biosciences (USA). RPMI 1640 (Gibico, USA), fetal bovine serum (FBS) (Hyclone, USA), antibiotics (North China pharmaceutical Group Corporation, China) cell-lysis buffer (Applygen Technologies Inc., China), bicinchoninic acid assay (BCA) protein assay reagent (Pierce, USA) were applied in the research.

### Cell culture and treatment

16-HBE, a cell line of human bronchial epithelia, was purchased from Shanghai Bogoo Biotechnology. Co., Ltd. (Shanghai,

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China). Cells were maintained in RPMI 1640 containing 10% FBS and antibiotics at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After each passage, the cells grew to confluence within 1–2 days. Cells were maintained in FBS-free RPMI 1640 for 24 h before stimulation with TGF-β1. After overnight culture, cells were treated with TGF-β1 in serum-free medium as indicated. In all experiments, cells at 80–90% confluence were treated with GYY4137.

### Western blotting analysis

Cells were lysated with cell-lysis buffer. The protein content was assayed by BCA protein assay reagent (Pierce Biotechnology, IL, USA). Total 30 μg protein was loaded to 10% (wt./vol.) sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to membrane. The expression of E-cadherin, α-SMA and GAPDH were detected. The primary antibodies E-cadherin (1:2000) and α-SMA (1:500) and GAPDH (1:1000) were used, followed by a 1:10,000 dilution of IRDye 800CW-conjugated goat anti-rabbit IgG or goat anti-mouse IgG for 1 h. Protein bands were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) as previously reported.

### Wound healing assay

Cells (1 × 10<sup>5</sup>) were seeded onto 60-cm<sup>2</sup> culture plates. When the cells reached 90–100% confluence, wounds were mechanically generated by scraping with a sterile pipette tip. Photomicrographs were taken at 72 h after wound generation.

### Statistical analysis

The results were expressed as mean ± standard deviation (SD). Comparisons were analyzed using one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. A *P* <

0.05 was considered statistically significant. SPSS 20.0 was applied for statistical analysis (SPSS, Inc., Chicago, IL, USA).

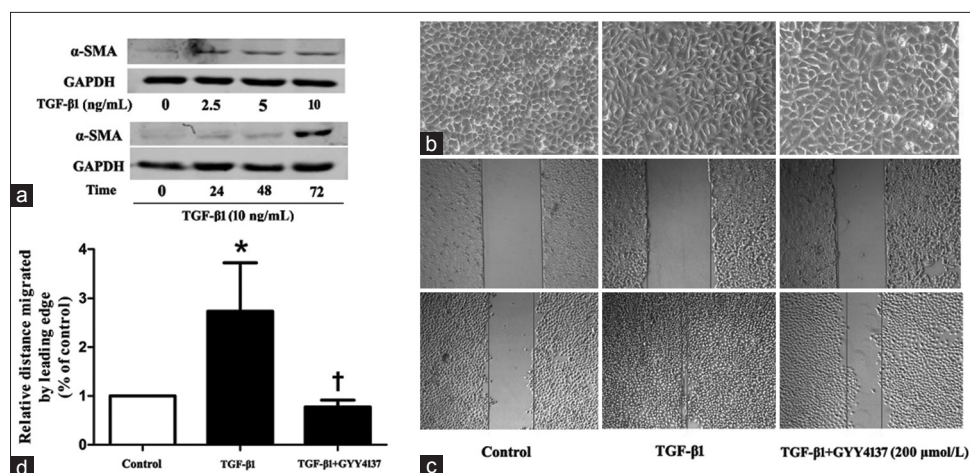
## RESULTS

### Exogenous H<sub>2</sub>S inhibits 16-HBE cells morphologic changes and motility induced by transforming growth factor beta-1

We first examined the effects of TGF-β1 on mesenchymal marker α-SMA in 16-HBE cells. Cells were pretreated with TGF-β1 (2.5, 5.0, 10.0 ng/ml) for 72 h or incubated with TGF-β1 (10 ng/ml) for various time (24, 48, 72 h). TGF-β1 resulted in dose-related and time-related increased in α-SMA expression [Figure 1a]. TGF-β1 (10 ng/ml) treatment for 72 h were used in the following experiments. We then assessed whether exogenous H<sub>2</sub>S could inhibit the morphologic changes and motility induced by TGF-β1. Untreated 16-HBE cells showed a cobblestone epithelial morphology and were tightly attached to each other. TGF-β1 treated cells showed an elongated shape, and many cells lost contact with each other and displayed spindle-shape, fibroblast-like morphologic features. While with GYY4137 treatment, cells maintained a classic cobblestone epithelial morphology [Figure 1b]. Wound healing experiments showed that TGF-β1 obviously enhanced motility of 16-HBE cells, whereas GYY4137 treatment eliminated the effects of TGF-β1 [Figure 1c and 1d].

### Exogenous H<sub>2</sub>S inhibits 16-HBE cells epithelial-mesenchymal transition induced by transforming growth factor beta-1

As GYY4137 treatment inhibits 16-HBE cells morphologic changes and motility induced by TGF-β1. We then examined whether GYY4137 could affect the expression of EMT



**Figure 1:** (a) Western blotting analysis of α-SMA in 16-HBE cells treated with different concentration TGF-β1 for 72 h or incubated with different time. (b) Morphologic changes observed under a light microscope in 16-HBE cells with control, TGF-β1 (10 ng/ml) or TGF-β1 (10 ng/ml) and GYY4137 (200 μmol/L) treatment for 72 h (original magnification ×100). (c) Wound healing assay observed on light microscopy in 16-HBE cells with control, TGF-β1 (10 ng/ml) or TGF-β1 (10 ng/ml) and GYY4137 (200 μmol/L) treatment for 72 h (original magnification ×50). The area between lines indicated the wound area. (d) Quantitative assay of wound healing experiment. Data are shown as mean ± standard deviation. *n* = 3. TGF-β1 (10 ng/ml) or TGF-β1 (10 ng/ml) and GYY4137 (200 μmol/L) treatment were for 72 h. \**P* < 0.05 (vs. control), †*P* < 0.05 (treatment vs. TGF-β1). α-SMA: Alpha-smooth muscle actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TGF-β1: Transforming growth factor beta-1.

markers in 16-HBE cells. Cells were pretreated with GYY4137 (50, 100, 200  $\mu\text{mol/L}$ ) for 1 h and then incubated with TGF- $\beta$ 1 (10 ng/ml) for 72 h. TGF- $\beta$ 1 treatment significantly increased the expression of mesenchymal marker  $\alpha$ -SMA and decreased the expression of epithelial marker E-cadherin. Compared with TGF- $\beta$ 1 group, GYY4137 could reverse these markers by reducing the expression of  $\alpha$ -SMA by 45.1% ( $P < 0.05$ ) and increasing the expression of E-cadherin by 57.6% ( $P < 0.05$ ) [Figure 2].

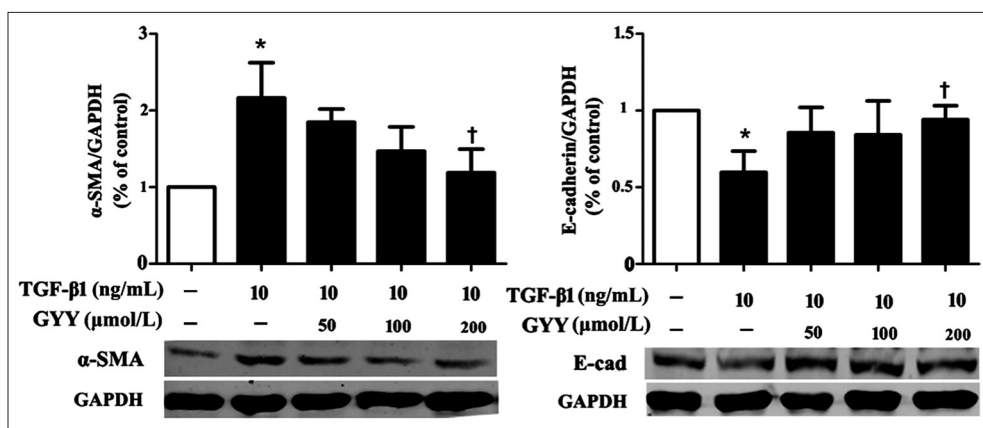
## DISCUSSION

COPD is defined as an irreversible expiratory airflow limitation, which is caused by various degrees of the following two main features: First, small airway disease, which includes airway inflammation and remodeling; and second, emphysema, which is characterized by airspace enlargement.<sup>[7,8]</sup> Arguably, airway remodeling is one of the most intractable and the most pressing problems in the disease, which leads to irreversible loss of lung function.<sup>[9]</sup> Current therapeutics could ameliorate inflammation, but there is no available therapy proven to prevent or reverse airway remodeling.

The airway epithelium is the primary target for the inhaled environmental factors and pathogens. An important function of epithelial cells is to respond to micro-environmental cues by undergoing epithelial to mesenchymal transition. It has been accepted that TGF- $\beta$  signaling has played critical functional roles in lung development, injury, and repair.<sup>[10-12]</sup> Several studies have reported an increased expression of TGF- $\beta$ 1 in the airway epithelium of smokers, as well as in patients with chronic bronchitis or COPD.<sup>[13]</sup> Our results found that TGF- $\beta$ 1 stimulated 16-HBE transformed into mesenchymal-like cells. In addition, markers of epithelial cells such as E-cadherin are lost and markers of mesenchymal cells such as  $\alpha$ -SMA are present. These results proved that EMT occurred in epithelial cells with TGF- $\beta$ 1 treatment.

$\text{H}_2\text{S}$  was believed to be a toxic environmental pollutant with no physiological significance. However, in the past few years, it has been identified as a physiologically or pathophysiologically relevant endogenous gaseous transmitter, third in line to NO and CO.<sup>[6,14]</sup> In a chronic cigarette smoke-induced COPD rat model, we found that endogenous  $\text{H}_2\text{S}$  has played a protective role in anti-inflammation and bronchodilation.<sup>[6]</sup> In this study, we have further demonstrated that exogenous  $\text{H}_2\text{S}$  attenuates the EMT in bronchial epithelial cells. Although  $\text{H}_2\text{S}$  performs many critical functions in the lung, the role of  $\text{H}_2\text{S}$  in the airway epithelium has not been well characterized. Several studies have proved that both endogenous and exogenous  $\text{H}_2\text{S}$  attenuate the EMT in alveolar epithelial cells.<sup>[15]</sup> The mechanism by which  $\text{H}_2\text{S}$  affects airway fate and protects against small airway fibrosis is not clear now. In rats with streptozotocin-induced diabetic kidney injury, sodium hydrosulfide inhibited albuminuria, TGF- $\beta$  expression and matrix accumulation; moreover,  $\text{H}_2\text{S}$  donor blocked the differentiation of quiescent renal fibroblasts to myofibroblasts by inhibiting the TGF- $\beta$ 1-Smad and mitogen-activated protein kinase signaling pathways.<sup>[16,17]</sup> In addition, NaHS treatment following TGF- $\beta$ 1 administration also resulted in decreasing human breast cancer cell invasion and decreasing EMT, which was indicated by decreasing Snail protein expression.<sup>[18]</sup> Preincubation with  $\text{H}_2\text{S}$  decreased Smad2/3 phosphorylation in A549 cells stimulated by TGF- $\beta$ 1, and  $\text{H}_2\text{S}$ -inhibited alveolar EMT was mimicked by treatment with SB505124, a Smad2/3 inhibitor, but not pinacidil, a KATP opener.<sup>[15]</sup> Until now, we still need more exploration into the role of  $\text{H}_2\text{S}$  in COPD.

Our research found that  $\text{H}_2\text{S}$  inhibits TGF- $\beta$ 1-induced cell morphological changes and EMT in 16-HBE cells. However, the underlying mechanism needs further study. A greater understanding of the  $\text{H}_2\text{S}$  pathway and airway EMT will help us better harness the potent actions of  $\text{H}_2\text{S}$ -antagonized airway fibrosis as a therapeutic target of COPD.



**Figure 2:** Dose-dependent changes in E-cadherin and  $\alpha$ -SMA in 16-HBE cells treated with TGF- $\beta$ 1 (10 ng/ml) and GYY4137 (50, 100, 200  $\mu\text{mol/L}$ ). Cells were pretreated with GYY4137 for 1 h following treated with TGF- $\beta$ 1 for 72 h. Relative protein expression levels were normalized to GAPDH. Data are shown as mean  $\pm$  standard deviation.  $n = 3$ . \* $P < 0.05$  (vs. control), † $P < 0.05$  (treatment vs. TGF- $\beta$ 1).  $\alpha$ -SMA: Alpha-smooth muscle actin; E-cad: E-cadherin; GYY: GYY4137; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TGF- $\beta$ 1: Transforming growth factor beta-1.

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## Conflicts of interest

There are no conflicts of interest.

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