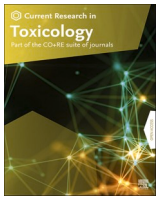
Current Research in Toxicology 7 (2024) 100199

Contents lists available at ScienceDirect

Current Research in Toxicology

journal homepage: www.journals.elsevier.com/current-research-in-toxicology



Hydrogen sulfide donor NaHS inhibits formaldehyde-induced epithelial-mesenchymal transition in human lung epithelial cells via activating TGF-β1/Smad2/3 and MAPKs signaling pathways

Haopei Wang [1], Miaomiao Jia [1], Yuxin Chang, Xingwei Ling, Wenyan Qi, Hongtao Chen, Feipeng Chen, Haiyang Bai, Yuhan Jiang, Chengfan Zhou [*]

Department of Occupational Health and Environmental Health, School of Public Health, Anhui Medical University, Hefei 230032, Anhui, PR China

A R T I C L E I N F O

Keywords:
Formaldehyde
Hydrogen sulfide
Epithelial-mesenchymal transformation
Lung epithelial cells
Transforming growth factor-β1**1. Introduction**

A B S T R A C T

Formaldehyde (FA) long term exposure leads to abnormal pulmonary function and small airway obstruction of the patients. Hydrogen sulfide (H_2S) is one of the recognized gaseous transmitters involved in a wide range of cellular functions. It is unknown the involvement of H_2S in FA-induced lung injury. The purpose of this study is to investigate the therapeutic potential and mechanism of H_2S on FA-induced epithelial-mesenchymal transition (EMT) of human lung epithelial cells. The cell viability of Beas2B and A549 cells after FA treatment were assessed using MTT assay. The endogenous H_2S was visualized by fluorescence microscopy using of the 7-azido-4-methylcoumarin (AzMC). Cell morphology was observed under phase contrast microscope. The mRNAs and proteins level were evaluated by reverse transcription-polymerase chain reaction and western blotting assays. FA treatment downregulated the endogenous H_2S levels and the mRNAs and proteins level of H_2S synthesizing enzymes, such as cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) in Beas2B and A549 cells. FA treatment changed the cell morphology of Beas2B cells from cuboid to a spindle-shape, while declined the protein level of E-cadherin and increased the protein level of Vimentin. Moreover, FA treatment increased the proteins level of transforming growth factor-β1 (TGF-β1), phosphorylated-Smad2 (p-Smad2), phosphorylated-Smad3 (p-Smad3), phosphorylated-extracellular signal-regulated kinase (p-ERK), phosphorylated-c-Jun N-terminal kinase (p-JNK), and phosphorylated-P38 (p-P38). Furthermore, the inhibitors of TGF-β receptor type 1 (TGFβRI) and mitogen-activated protein kinases (MAPKs) signaling pathways reversed FA-induced decrease in E-cadherin expression and increase in Vimentin expression in Beas2B cells. Sodium hydrogen sulfide (NaHS) increased the level of H_2S, while reversed FA-induced the low expression of E-cadherin and the high expression of Vimentin, TGF-β1, p-Smad2, p-Smad3, p-ERK, p-JNK, and p-P38. These findings indicates FA treatment downregulating the endogenous H_2S in human lung epithelial cells. NaHS may inhibit FA-induced EMT in human lung epithelial cells via modulating TGF-β1/Smad2/3 and MAPKs signaling pathways. Therefore, we demonstrated that supplementation of exogenous H_2S may inhibit FA-induced lung injury.

1. Introduction

Formaldehyde (FA) is one of major chemicals in the global economy, based on its wide applications in construction, furniture, wood processing, carpeting, textiles and chemical industry (Tang et al., 2009). Today, public concern regarding the health effects of FA exposure in the environmental, residential, occupational, and food continues to grow in the worldwide (Huang et al., 2021; Soltanpour et al., 2021; Gelbke et al., 2019). FA is recognized to be acutely toxic, causing irritation and other immunotoxic effects (Paustenbach et al., 1997). FA is also a group 1 human carcinogen, classified by the International Agency for Research on Cancer (IARC), based on adequate evidence that chronic exposure is closely related to nasopharyngeal cancer in 2004 (Formaldehyde, 2006) and leukemia in 2012 (Humans IWGotEoCRt, 2012). Moreover, long-

* Corresponding author at: Department of Occupational Health and Environmental Health, School of Public Health, Anhui Medical University, 81 Meishan Road, Hefei 230032, Anhui, PR China.
E-mail address: zhouchengfan@sohu.com (C. Zhou).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.crtox.2024.100199
Received 12 June 2024; Received in revised form 30 September 2024; Accepted 10 October 2024
Available online 24 October 2024

term exposure to FA can cause symptoms such as neurasthenia, low blood counts, abnormal lung function, and small airway obstruction (Mathur and Rastogi, 2007). Recently, accumulating evidence shows that FA exposure is associated with the progression and occurrence of asthma in children and adults (Yu et al., 2020; Lam et al., 2021; Neamtiu et al., 2019). Therefore, FA causes detrimental effects on respiratory system and potential treatment of the respiratory disease induced by FA should be explored.

Hydrogen sulfide (H_2S), one of the recognized gaseous transmitters, is involved in a wide range of cellular functions, as well as the physiological and pathological processes in various diseases. Recently, several studies have indicated that H_2S inhibits FA-induced neurotoxicity (Zhu et al., 2019; Chen et al., 2017; Jiang et al., 2015; Li et al., 2014; Tang et al., 2013; Tang et al., 2012; Tang et al., 2023) and cognitive dysfunction (Li et al., 2020; Tang et al., 2013). In addition, H_2S also has significant therapeutic effect in respiratory diseases such as obstructive respiratory disease, lung injury, lung inflammation, emphysema, pulmonary fibrosis, bronchial asthma and bronchiectasis (Khattak et al., 2021). H_2S in the respiratory tract induces anti-apoptosis and anti-inflammatory effects and regulates vascular permeability (Chen et al., 2009). FA exposure may cause the activation of inflammatory pathways in respiratory tissues, which in turn causes the production of pro-inflammatory mediators and the recruitment of immune cells (Bhat et al., 2024). However, the therapeutic potential and mechanism of H_2S on FA-induced respiratory diseases have not been fully investigated.

The epithelial-mesenchymal transition (EMT) is a phenomenon in which polarized epithelium lost cell-to-cell contact from the basal membrane differentiates into fibroblast-type mesenchymal cells. This process promotes extracellular matrix (ECM) deposition, leading to fixed bronchial obstruction (Pain et al., 2014). Evidence has shown that repeated inflammation of airway epithelial cells, leading to EMT, is considered to play a crucial role in airway remodeling in asthma following environmental challenge (Hackett, 2012). FA-induced EMT mediated the growth and migration of human choriocarcinoma cells (Lee et al., 2017). H_2S has anti-inflammatory and cyto-protective effects due to its ability to act as an antioxidant and a reducing agent, and its scavenging features (Khattak et al., 2021). However, the potential mechanism of EMT mediated the therapeutic potential of H_2S on FA causes respiratory diseases in lung epithelial cells remains unclear. In the present study, we researched the effects and underlying mechanisms of sodium hydrogen sulfide (NaHS), an exogenous H_2S donor, on FA-induced EMT in human lung epithelial cells.

2. Materials and methods

2.1. Materials

Formaldehyde (FA; 37.0 % in water, FW 30.03) was obtained from Xilong Scientific Co., Ltd. (Guangdong, China). Sodium hydrogen sulfide (NaHS, an exogenous hydrogen sulfide donor) and 3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide (MTT) (Purity 98.0 %) were supplied by Sigma-Aldrich, Inc. (St. Louis, MO, USA). 7-azido-4-methylcoumarin (AzMC), a fluorescent probe for H_2S, was provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The chemical inhibitors, including LY364947 (CAS No. 396129–53-6), U0126 (CAS No. 109511–58-2), SP600125 (CAS No. 129–56-6) and SB203580 (CAS No. 152121–47-6), were all obtained from MedChemExpress (MCE). The primary antibodies against E-cadherin (#3195), Vimentin (#5741), cystathionine-β-synthase (CBS) (#14782), cystathionine-γ-lyase (CSE) (#19689), transforming growth factor-β1 (TGF-β1) (#3711), phosphorylated-Smad2 (p-Smad2) (#3108 s), phosphorylated-Smad3 (p-Smad3) (#9520 s), Smad2/3 (#8685), phosphorylated-extracellular signal-regulated kinase (p-ERK) (#4370), ERK (#4695), phosphorylated-c-Jun N-terminal kinase (p-JNK) (#9251 s), JNK (#9252), phosphorylated-P38 (p-P38) (#4511), P38 (#8690), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#5174) were all supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA). The 3-mercaptopyruvate sulfurtransferase (3-MST) (sc-374326) and goat anti-rabbit IgG/HRP antibody were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) (1 ×) were obtained from Gibco, Inc. (Rockville, MD, USA).

2.2. Cell culture and treatment

Beas2B cell, an immortalized lung bronchial epithelial cell line, was supplied by American Type Cell Culture Collection (Manassas, VA, USA). A549 cell, a human lung adenocarcinoma epithelial cell line, was obtained from the Type Culture Collection Cell Bank, Chinese Academy of Sciences Committee (Shanghai, China). Beas2B cells and A549 cells were cultured in medium (prepared from 90 % DMEM, 10 %FBS, 100 μg/mL penicillin and streptomycin mixture) in an incubator maintained at 37 ℃ and 5 % CO_2. When the cells were grown and confluent to 80–90 % density, each group of Beas2B cells was dosed with 100 μM FA for different time (0, 6, 12, 24 and 36 h) or at gradient concentrations (0 μM, 25 μM, 50 μM, 75 μM and 100 μM) for 24 h in 6-well plates. When the cells were grown and confluent to 80–90 % density, each group of A549 cells was dosed with 200 μM FA for different time (0, 6, 12, 24 and 36 h) or at gradient concentrations (0 μM, 50 μM, 100 μM, 200 μM and 400 μM) for 24 h in 6-well plates. We choose to expose A549 cells to a higher dose than BEAS-2B cells duo to their differing sensitivities and biological characteristics. A549 cells, derived from lung carcinoma, often exhibit greater tolerance to certain toxic agents compared to BEAS-2B cells, which are normal bronchial epithelial cells. To assess the effect of NaHS for FA causes EMT, the Beas2B cells were treated with NaHS or/and FA. For the control group, the Beas2B cells were cultured with medium only. For the NaHS group, the Beas2B cells were treated with 100 μM NaHS for 1 h prior to further addition of normal medium. For the FA group, the Beas2B cells were exposed to 100 μM of FA for 24 h. For the FA + NaHS group, the Beas2B cells were treated with 100 μM NaHS for 1 h prior to further exposure to 100 μM of FA for 24 h.

2.3. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is one of the most frequently used methods for measuring cell viability (Liu et al., 1997). The cell viability of Beas2B and A549 cells after FA treatment was evaluated by MTT assay. Briefly, Beas2B and A549 cells were incubated overnight at 37 ℃ with 5 % CO_2 in a 96-well flat-bottomed plate containing 1.0×10^4 cells/well. After FA treatment, wash the cells with PBS and then add 20 μL of MTT (0.5 mg/mL) into each well and maintained at 37℃ for 4 h. After discarding the medium, add 150 μL dimethyl sulfoxide (DMSO) and then shake for 10 min to dissolve the intracellular formazan product, followed by analysis on a microplate reader at 570 nm (Bio-Tek Instruments Inc., VT, USA). Cell viability of Beas2B or A549 cells in each concentration FA group was evaluated by comparing with the control group which was cultured with medium only.

2.4. Measurement of H_2S production

The endogenous H_2S was visualized using of the fluorescent H_2S probe AzMC and fluorescence microscopy in Beas2B cells. The Beas2B cells were dosed with 100 μM FA for 0, 12, 24, and 36 h. Then, the Beas2B cells were removed FA and incubated with 50 mM AzMC for 30 min at 37℃ after rinsing with PBS and incubation in phenol red free medium for 30 min. After three times rinsed with PBS, the fluorescence of Beas2B cells was observed and photographed by excitation wavelength of 405 nm under inverted fluorescence microscope. All images were captured using the same exposure time.

2.5. Western blotting

Western blotting was used to evaluate the proteins expression level. Briefly, Beas2B or A549 cells after FA treatment were isolated in the ice-cold lysis buffer for total protein extraction and detected by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). Then, the total proteins were subject to SDS-PAGE separation and next all transferred to PVDF membranes, afterwards, probed with specific primary antibodies and proper HRP-conjugated secondary antibodies. At last, the protein expression level was discovered by the enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific Inc.).

2.6. Reverse transcription-polymerase chain reaction

The Beas2B cells were dosed with 100 μM FA for 0, 12, 24, and 36 h. Then, the total RNA was isolated from the cultured Beas2B cells using TRIzol reagent. The total RNA concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific Inc.). DNase (Promega, Madison, WI, USA) was used to remove contaminating genomic DNA. DNase-treated total RNA (1.0 μg) was reversely transcribed into cDNA with AMV (Promega) in the presence of oligo(dT). The cDNA was used for semiquantitative polymerase chain reaction (PCR) to detect the *cbs*, *cse* and *3-mst* gene expressions using 2 × Power Taq PCR MasterMix (BioTeke, Beijing, China). The primer sequences have been described in our previous research (Bai et al., 2019). The thermocycling conditions for PCR were as follows: initial denaturation at 95℃ for 5 min, followed by 40 amplification cycles denaturation at 95℃ for 10 s, anneal at 60℃ for 30 s and extension at 72℃ for 30 s. Each assay was performed in triplicate for each sample, and the *gapdh* expression served as an internal control.

2.7. Statistical analysis

All data were presented as mean ± SEM of three replicates. Differences between the multiple groups were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's test compared to control group. Differences were regarded as significant when $p < 0.05$.

3. Results

3.1. FA exposure on human lung epithelial cells viability

MTT assay was used to evaluate the effect of FA exposure on human lung epithelial cells viability. As shown in Fig. 1A, when FA concentration increased to 100 μM, Beas2B cells viability were significantly decreased compared to the PBS control group until at 36 h, but the cell viability was still over 80 %. However, when FA concentration was increased to 200 μM, cell viability was significantly decreased at 24 and 36 h compared with the PBS control group. As for A549 cells, when FA concentration increased to 200 μM, the cell viability was still over 80 %. However, when FA concentration increased to 400 μM, A549 cells viability was significantly decreased compared to the PBS control group at 12, 24 and 36 h (Fig. 1B). Therefore, 100 μM FA was used in Beas2B cells, while 200 μM FA was used in A549 cells for our next study.

3.2. Effect of FA exposure on endogenous H_2S and H_2S-generating enzymes in human lung epithelial cells

To evaluate the effect of FA exposure on endogenous H_2S production, Beas2B cells were treated with 100 μM FA for 0, 12, 24 or 36 h and visualized using of the fluorescent H_2S probe AzMC. As shown in Fig. 2A&B, FA has significantly decreased the endogenous H_2S level in the Beas2B cells, presenting a time-dependent manner. Furthermore, CBS, CSE and 3-MST (the synthase enzymes of H_2S) were evaluated by Western blotting. As shown in Fig. 2C-F, FA has decreased the proteins level of, CBS, CSE and 3-MST in Beas2B cells in an obviously time- and dose-dependent manner. The mRNAs expression of CBS, CSE and 3-MST were also significantly inhibited by FA in a time-dependent manner in Beas2B cells (Fig. 2G&H). Moreover, A549 cells were dosed with 200 μM FA for different time (0, 6, 12, 24 and 36 h) or at gradient concentrations (0 μM, 50 μM, 100 μM, 200 μM and 400 μM) for 24 h. As shown in Fig. 2I-L, FA also has decreased the proteins level of, CBS, CSE and 3-MST in A549 cells in an obviously time- and dose-dependent manner.

3.3. Effect of FA exposure on the EMT process, TGF-β1/Smad2/3 and MAPKs signaling pathways activation in human lung epithelial cells

To evaluate the effect of FA exposure induces the EMT process of lung epithelial cells, cell morphology of Beas2B was inspected using phase contrast microscope. As shown in Fig. 3A, after treatment with 100 μM FA, the shape of Beas2B cells changed from cuboid to spindle. Moreover, after treated with 100 μM FA for different times or different concentrations FA for 24 h, the proteins level of E-cadherin (the epithelial marker) and Vimentin (the mesenchymal marker) in Beas2B cells were assessed by Western blotting. As shown in Fig. 3B-E, FA expoure significantly downregulated the expression of E-cadherin, while upregulated the expression of Vimentin, presenting a time- and dose-dependent manner.

To evaluate the effect of FA exposure on TGF-β1/Smad2/3 and MAPKs signaling pathways activation, the proteins level of TGF-β1, p-Smad2, p-Smad3, Smad2/3, p-ERK, ERK, p-JNK, JNK, p-p38 and p38 were evaluated after Beas2B cells were exposed to 100 μM FA for different times or at different concentrations FA for 24 h. As shown in Fig. 3F-K, FA exposure upregulated the proteins level of TGF-β1, p-

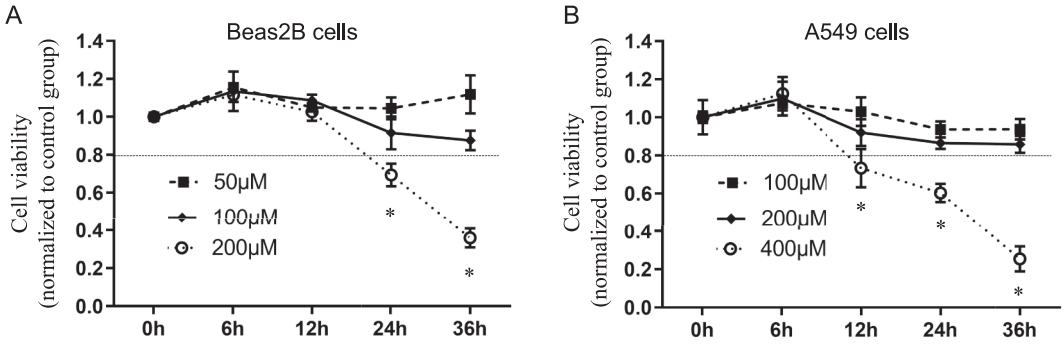


Fig. 1. The time- and dose-dependent effects of FA exposure on human lung epithelial cells viability. Beas2B cells were treated with PBS, 50 μM, 100 μM, and 200 μM of FA, while A549 cells were treated with PBS, 100 μM, 200 μM, and 400 μM of FA, the cells viability were assessed using MTT assay at 0, 6, 12, 24 and 36 h. *, Significant difference from PBS control group.

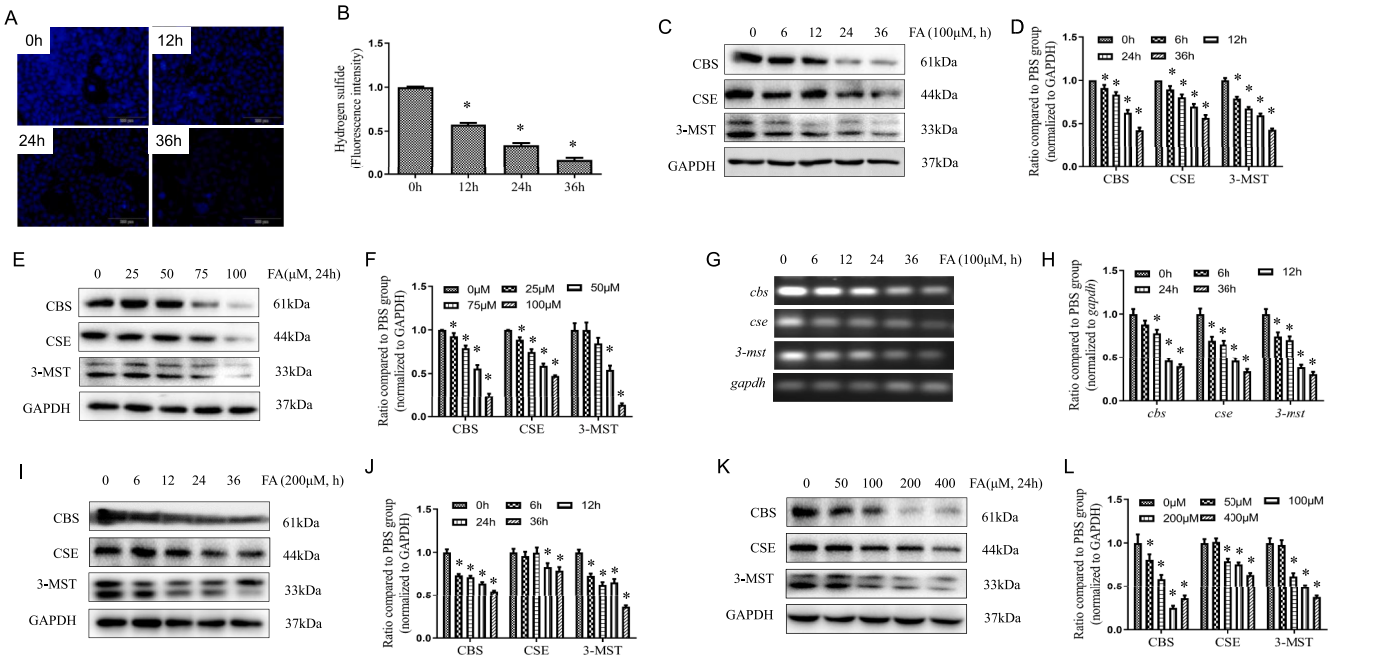


Fig. 2. The effects of FA exposure on endogenous H_2S and H_2S-generating enzymes in human lung epithelial cells. (A) Beas2B cells were dosed with 100 μM FA for 0, 12, 24 or 36 h and then incubated with the fluorescent H_2S probe AzMC for 30 min. Then, the cells were observed and (B) analyzed by fluorescence microscopy (magnification, ×100). (C) Western blotting and (D) densitometry analysis of CBS, CSE and 3-MST, compared to the GAPDH control in Beas2B cells were dosed with 100 μM FA for 0, 6, 12, 24 and 36 h. (E) Western blotting and (F) densitometry analysis of CBS, CSE and 3-MST, compared to the GAPDH control in Beas2B cells were dosed with FA at gradient concentrations (0 μM, 25 μM, 50 μM, 75 μM, and 100 μM) for 24 h. (G) Semiquantitative polymerase chain reaction and (H) densitometry analysis of CBS, CSE and 3-MST mRNA expression levels after Beas2B cells were dosed with 100 μM FA for 0, 6, 12, 24 and 36 h. (I) Western blotting and (J) densitometry analysis of CBS, CSE and 3-MST, compared to the GAPDH control in A549 cells were dosed with 200 μM FA for 0, 6, 12, 24 and 36 h. (K) Western blotting and (L) densitometry analysis of CBS, CSE and 3-MST, compared to the GAPDH control in A549 cells were dosed with FA at gradient concentrations (0 μM, 50 μM, 100 μM, 200 μM, and 400 μM) for 24 h. *, Significant difference from PBS control group.

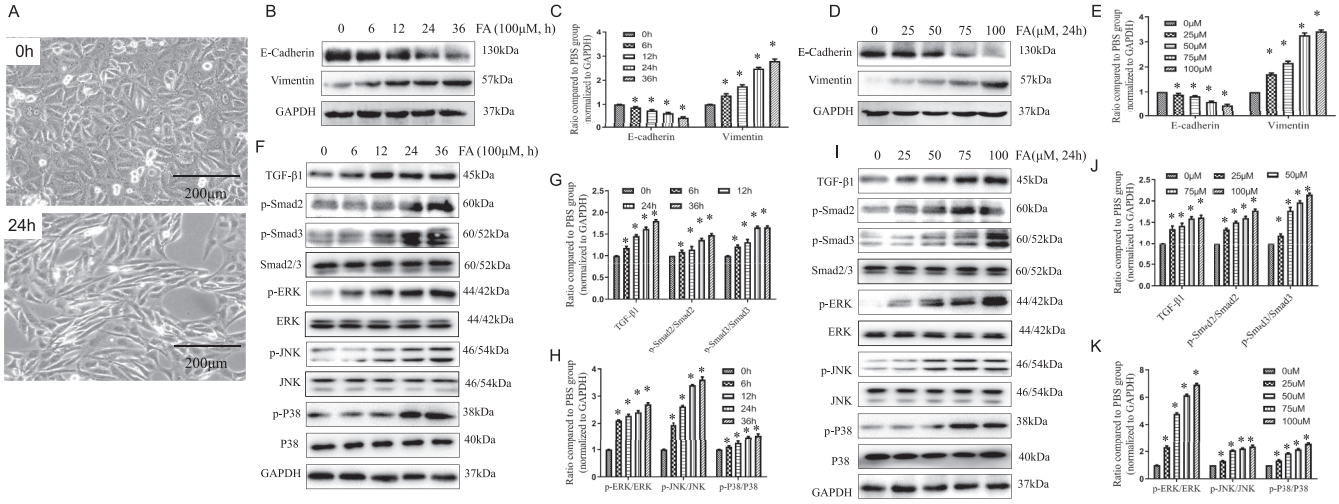


Fig. 3. The effect of FA exposure on the EMT process, TGF-β1/Smad2/3 and MAPKs signaling pathways activation in human lung epithelial cells. (A) The change of Beas2B cells morphology have been observed after being treated with 100 μM of FA for 24 h, and compared with the control group. (B) Western blotting and (C) densitometry analysis of E-cadherin and Vimentin, compared to the GAPDH control in Beas2B cells were dosed with 100 μM FA for 0, 6, 12, 24 and 36 h. (D) Western blotting and (E) densitometry analysis of E-cadherin and Vimentin, compared with the GAPDH control in Beas2B cells were dosed with FA at gradient concentrations (0 μM, 25 μM, 50 μM, 75 μM, and 100Mm) for 24 h. (F) Western blotting and (G&H) densitometry analysis of TGF-β1, p-Smad2, p-Smad3, Smad2/3, p-ERK, ERK, p-JNK, JNK, p-P38, and P38, compared to the GAPDH control in Beas2B cells were dosed with 100 μM FA for 0, 6, 12, 24 and 36 h. (I) Western blotting and (J&K) densitometry analysis of TGF-β1, p-Smad2, p-Smad3, Smad2/3, p-ERK, ERK, p-JNK, JNK, p-P38, and P38, compared to the GAPDH control in Beas2B cells were dosed with FA at gradient concentrations (0 μM, 25 μM, 50 μM, 75 μM and 100 μM) for 24 h. *, Significant difference from PBS control group.

Smad2, p-Smad3, p-ERK, p-JNK, and p-p38 presenting an obviously time- and dose-dependent manner, while the total protein levels of Smad2/3, ERK, JNK, and P38 did not change.

3.4. Effect of inhibitors of TGF-β1/Smad2/3 and MAPKs signaling pathways on FA-induced EMT in human lung epithelial cells

To evaluate the effect of LY364947, an inhibitor of TGF-β receptor

type 1 (TGFβRI), on FA exposure induced the EMT of lung epithelial cells, the proteins level of TGF-β1, E-cadherin and Vimentin in Beas2B cells were evaluated after treatment with 10 μM LY364947 or/and 100 μM FA. As shown in Fig. 4A&B, LY364947 treatment obviously inhibited the proteins level of TGF-β1 and Vimentin, while reversed the level of E-cadherin protein.

To evaluate the effect of MAPKs signaling pathway inhibitors on FA exposure induce the EMT of lung epithelial cells, the proteins level of p-ERK, ERK, p-JNK, JNK, p-P38, P38, E-cadherin and Vimentin were evaluated after the Beas2B cells were respectively exposed to 10 μM U0126 (an inhibitor of MEK-1/2 activation), 10 μM SP600125 (an inhibitor of JNK activation), 10 μM SB203580 (an inhibitor of P38 activation), or/and 100 μM FA. As shown in Fig. 4C-H, U0126, SP600125, or SB203580 treatment respectively inhibited the proteins level of p-ERK, p-JNK, p-P38 and Vimentin, while reversed the level of E-cadherin protein.

3.5. Effect of NaHS pretreatment on FA-induced EMT process, TGF-β1/Smad2/3 and MAPKs signaling pathways activation in human lung epithelial cells

To evaluate the effect of NaHS, one of the exogenous hydrogen sulfide donors, on H_2S level in cell, Beas2B cells visualized using of the fluorescent H_2S probe AzMC. As shown in Fig. 5A&B, 100 μM NaHS, dose referenced from our previous studies (Bai et al., 2019; Ye et al., 2020), has significantly increased the H_2S level, which downregulating by the FA treatment. Moreover, 100 μM NaHS treatment significantly reversed FA downregulating the protein expression of E-cadherin and upregulating the protein expression of Vimentin in Beas2B cells (Fig. 5C&D).

To evaluate the effect of NaHS treatment on TGF-β1/Smad2/3 and MAPKs signaling pathways affected by FA exposure, the proteins level of TGF-β1, p-Smad2, p-Smad3, Smad2/3, p-ERK, ERK, p-JNK, JNK, p-P38 and P38 were evaluated after the cells were pretreated with 100 μM NaHS or/and 100 μM FA for 24 h. As shown in Fig. 5E-H, NaHS treatment obviously inhibited FA upregulating the proteins level of TGF-β1, p-Smad2, p-Smad3, p-ERK, p-JNK, and p-P38 in Beas2B cells, while the total protein levels of Smad2/3, ERK, JNK, and P38 did not change. Taken together, these findings suggested that NaHS attenuates FA causes EMT process of lung epithelial cells (Beas2B) by regulating the TGF-β1/Smad2/3 and MAPKs signaling pathway, which has been illustrated in Fig. 6.

4. Discussion

FA is a ubiquitous environmental and occupational pollutant, many people in the world might be continuously exposed to FA above 0.1 mg/m^3-the indoor limit recommended by the World Health Organization (Tang et al., 2009). The impact of FA exposure on lung tissues has been reported in many studies (Liu et al., 2021; Sholapuri et al., 2020; Leal et al., 2018; Duan et al., 2020). Moreover, there was "sufficient evidence of toxicity" for associations between exposure to FA and asthma in both children and adults (Lam et al., 2021). Lately, airway remodeling of asthma has been shown to be associated with dysregulation of epithelial-mesenchymal transition (EMT) (He et al., 2021). H_2S, one of the recognized gaseous transmitters, is involved in a broad range of cellular functions, and physiological and pathological processes in various respiratory diseases (Khattak et al., 2021). Therefore, H_2S may mediate FA exposure affect the EMT of lung epithelial cells. To best our knowledge, our current study is the first scientific publication that illustrated FA exposure reduces endogenous H_2S production and induces the EMT of lung epithelial cells. In addition, we discovered that NaHS attenuates FA causes EMT process of lung epithelial cells by regulating the TGF-β1/Smad2/3 and MAPKs signaling pathway. Since we exposed Beas2B cells to 100 μM FA(about 3.1 mg/m^3), this concentration is significantly higher than the WHO recommended indoor limit of 0.1 mg/m^3. While such high concentrations may help shed light on the cellular mechanisms and toxicological effects of FA, it also raises questions about the relevance of the findings to real-world exposure scenarios. Therefore, additional experiments may be necessary to validate this discovery.

H_2S was considered for decades only as a toxic gas, and more recently as a gasotransmitter that exerts numerous physiologic and pathophysiologic effects, *e.g.* vasodilation, antioxidant and anti-inflammation (Wallace and Wang, 2015). To explore the roles of H_2S in the aforementioned physiological events; spurring significant efforts has make in the development of fluorescent probes for H_2S detection in biological settings (Feng and Dymock, 2015). AzMB, an elegant aliphatic azide, undergoes reduction subsequent intramolecular attack on the

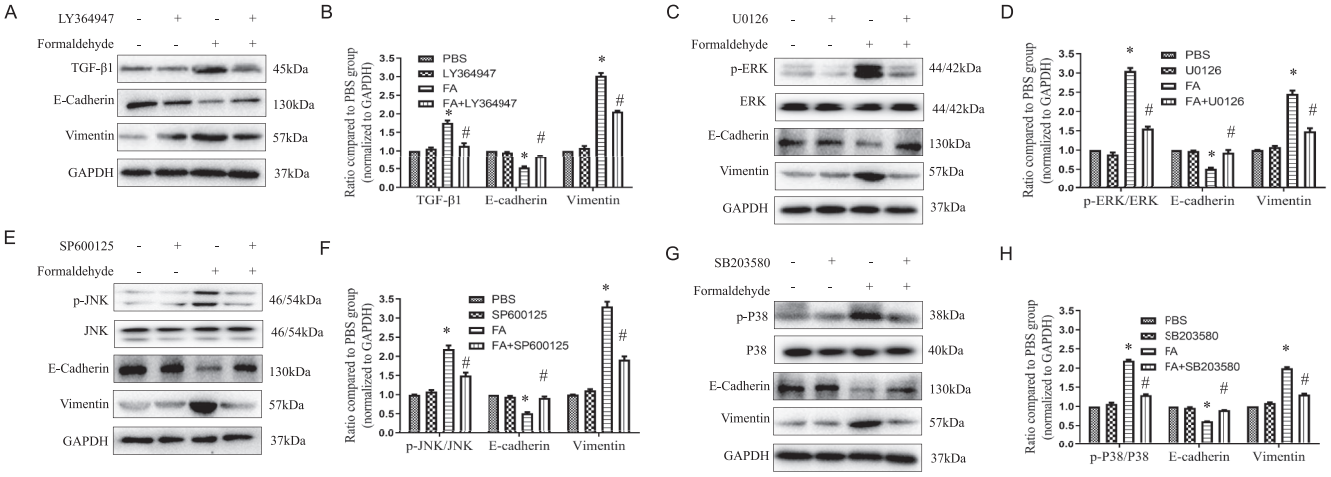


Fig. 4. The effect of inhibitors of TGF-β1/Smad2/3 and MAPKs signaling pathways on FA-induced EMT in human lung epithelial cells. (A) Western blotting and (B) densitometry analysis of TGF-β1, E-cadherin and Vimentin, compared to the GAPDH control in Beas2B cells were dosed with 10 μM LY364947, an inhibitor of TGFβR-I, or/and 100 μM FA for 24 h. (C) Western blotting and (D) densitometry analysis of p-ERK, ERK, E-cadherin and Vimentin, compared to the GAPDH control in Beas2B cells were dosed with 10 μM U0126, an inhibitor of MEK-1/2 activation, or/and 100 μM FA for 24 h. (E) Western blotting and (F) densitometry analysis of p-JNK, JNK, E-cadherin and Vimentin, compared to the GAPDH control in Beas2B cells were dosed with 10 μM SP600125, an inhibitor of JNK activation, or/and 100 μM FA for 24 h. (G) Western blotting and (H) densitometry analysis of p-P38, P38, E-cadherin and Vimentin, compared to the GAPDH control in Beas2B cells were dosed with 10 μM SB203580, an inhibitor of P38 activation, or/and 100 μM FA for 24 h. *, Significant difference from PBS control group. #, Significant difference from FA treatment group.

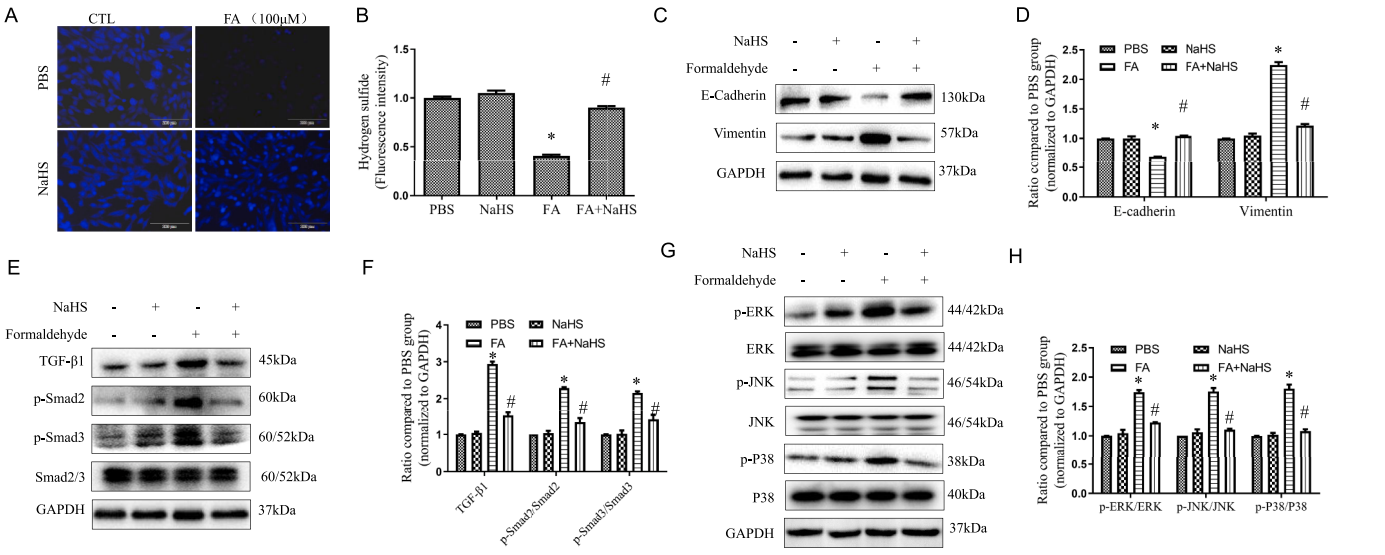


Fig. 5. The effect of NaHS on FA-induced EMT and FA-activated TGF-β1/Smad2/3 and MAPKs signaling pathways in human lung epithelial cells. (A) Beas2B cells were treated with 100 μM NaHS or/and 100 μM FA for 24 h and then incubated with the fluorescent H_2S probe AzMC for 30 min following by rinsed. The cells were finally observed and (B) analyzed by fluorescence microscopy (magnification, ×100). (C) Western blotting and (D) densitometry analysis of E-cadherin and Vimentin, compared to the GAPDH control in Beas2B cells were dosed with 100 μM NaHS or/and 100 μM FA for 24 h. (E) Western blotting and (F) densitometry analysis of TGF-β, p-Smad2, p-Smad3 and Smad2/3, compared to the GAPDH control in Beas2B cells were dosed with 100 μM NaHS or/and 100 μM FA for 24 h. (G) Western blotting and (H) densitometry analysis of p-ERK, ERK, p-JNK, JNK, p-P38, and P38, compared to the GAPDH control in Beas2B cells were dosed with 100 μM NaHS or/and 100 μM FA for 24 h. *, Significant difference from PBS control group. #, Significant difference from FA treatment group.

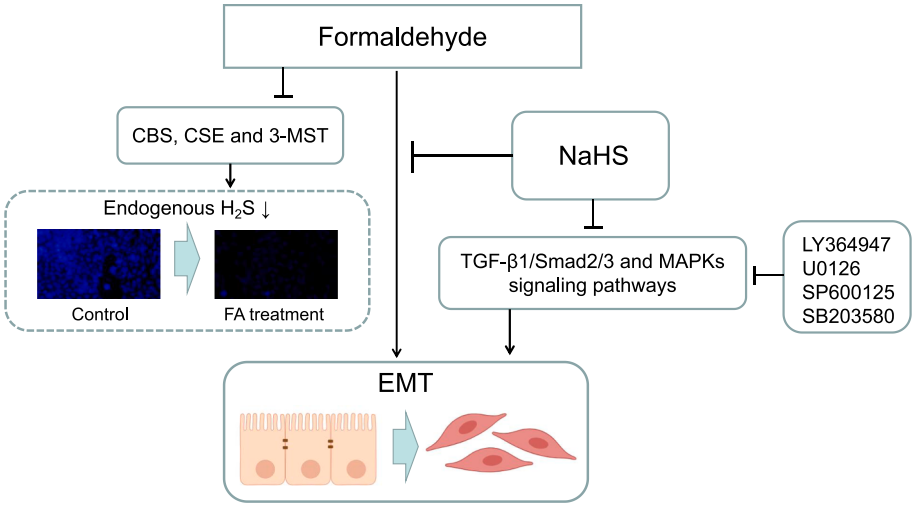


Fig. 6. Schematic illustration of NaHS attenuates FA causes EMT process of lung epithelial cells. Exogenous hydrogen sulfide donor NaHS increased H_2S level in cell, which downregulating by the FA treatment. Moreover, NaHS treatment inhibited FA causes EMT process of lung epithelial cells by regulating the TGF-β1/Smad2/3 and MAPKs signaling pathway. LY364947, a potent ATP-competitive inhibitor of TGFβR-I; U0126, a selective non-competitive inhibitor of MAPK that potently inhibits MEK-1 and MEK-2; SP600125, a selective ATP-competitive JNK-MAPK inhibitor; SB203580, a pyridinyl imidazole inhibitor normally applied to clarify the effects of P38-MAPK, treatment respectively inhibited the protein expression level of TGF-β1, p-ERK, p-JNK, p-P38, while reversed the protein level of Vimentin and E-cadherin. The figure was modified with BioRender scientific illustration software (https://biorender.com/).

nearby ester following by jetting fluorescent 7-hydroxy-4-methylcoumarin as well as the harmless by-product isoindolin-1-one (Wu et al., 2012). In this study, exposure to 100 μM FA resulted in over 80 % inhibition of fluorescence of 7-hydroxy-4-methylcoumarin compared to the control group, which was clearly observed in Beas2B cells. From cysteine, H_2S is generated through the direct action of two pyridoxal 5′-phosphate (PLP)-dependent enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), or the indirect action of 3-mercaptopyruvate sulfurtransferase (3-MST), in mammalian cells (Wallace and Wang, 2015). Moreover, FA exposure also has obviously decreased the mRNAs and proteins level of CBS, CSE and 3-MST in Beas2B cells presenting a time-and-dose-dependent manner. As for A549 cells, when FA concentration increased to 200 μM, the cell viability was still over 80 %. FA exposure also has obviously decreased the proteins level of CBS, CSE and 3-MST in A549 cells presenting a time-and-dose-dependent manner. Therefore, FA declined the proteins level of CBS, CSE and 3-MST, which might contribute to reduce the H_2S content in human lung epithelial cells. Since Beas2B cells are derived from bronchial epithelium, while A549 cells are derived from alveolar epithelium. Bronchial epithelial cells are often the first line of defense against inhaled irritants and may exhibit a stronger inflammatory response to FA compared to alveolar cells. The MTT assay results also indicated

Beas2B cells were more sensitive to FA than A549 cells, so we chose Beas2B cells to investigate the molecular mechanism in the present study.

EMT is a dynamic process whereby epithelial cells gradually acquire mesenchymal characteristics after losing their epithelial phenotype (Nieto et al., 2016). Emerging evidences show that airway epithelial cells can promote airway remodeling after environmental challenge through the EMT process (Zhao et al., 2021; Zou et al., 2013; Zou et al., 2014; Jiang et al., 2021). The low expression of E-cadherin, which maintains cell adhesion and epithelial structural integrity, is thought to be the typical event that characterizes EMT (Wheelock and Johnson, 2003). Upregulation of Vimentin-an intermediate filament protein, is characteristic in cells undergoing EMT and might be a precondition for EMT induction (Ivaska, 2011). Therefore, EMT is marked by the high expression of E-cadherin-the epithelial marker and the low expression of Vimentin-the mesenchymal marker. In our present study, FA exposure changed the shape of Beas2B cells from cuboid to spindle, and significantly reduced the expression level of E-cadherin, while enhanced the expression level of Vimentin presenting a time- and dose-dependent effect. These above results shown that the EMT process may involve in the pathophysiology of FA-induced airway remodeling, which agrees with the effect of FA on EMT in JEG-3 human choriocarcinoma cells (Lee et al., 2017).

Transforming growth factor-β1 (TGF-β1), with anti-inflammatory and profibrotic effect, is an important cytokine in asthma participated in airway inflammation as well as airway remodeling (Al-Alawi et al., 2014). TGF-β1 normally acts through a complex receptor, consisting of TβRII (type II; TGFR2) and TβRI (type I; TGFR1) receptors, that activated Smad2 and/or Smad3, and then bind to Smad4 to form trimeric Smad complexes, which activate the expression while enhancing the activity of EMT transcription factors (Lamouille et al., 2014). Moreover, TGF-β1 also activates complementary non-Smad signaling such as mitogen-activated protein kinase (MAPK) pathways that could conduce to the induction and development of EMT (Heldin and Moustakas, 2016). In the present study, FA exposure upregulated the protein level of TGF-β1, p-Smad2, p-Smad3, p-ERK, p-P38, and p-JNK in an obviously time- and dose-dependent effect. Furthermore, LY364947, a potent ATP-competitive inhibitor of TGFβR-I (Sawyer et al., 2003); U0126, a selective non-competitive inhibitor of MAPK that potently inhibits MEK-1 and MEK-2 (Favata et al., 1998); SP600125, a selective ATP-competitive JNK-MAPK inhibitor (Bennett et al., 2001; Cuenda et al., 1995), or SB203580, a pyridinyl imidazole inhibitor normally applied to clarify the effects of P38-MAPK51, treatment respectively inhibited the protein expression level of TGF-β1, p-ERK, p-JNK, p-P38, while reversed the protein level of Vimentin and E-cadherin. These results suggested that TGF-β1/Smad2/3 and MAPKs signaling pathways mediated FA-induced EMT in Beas2B cells.

Endogenous H_2S plays a crucial role in regulating EMT through its influence on various signaling pathways. Moreover, several studies have indicated that, the therapeutic and protective effects of the exogenous H_2S donor on fibroproliferative diseases and syndromes of organs, mainly as a result of its antioxidant, anti-inflammatory and antifibrotic property, such as sodium hydrosulfide (NaHS) (Zhang et al., 2015; Powell et al., 2018). NaHS serves as a valuable tool for studying these effects, as it allows simulating the presence of endogenous H_2S and observing its impact on cellular processes. Our former research indicated that 100 μM NaHS, a physiological concentration of H_2S in plasma (Wallace, 2007); attenuated paraquat (PQ), a kind of herbicide, induced EMT of A549 cells through inhibiting the TGF-β1/Smad2/3 signaling pathway (Bai et al., 2019). Furthermore, our recent study also suggested that 100 μM NaHS pretreatment prevented the EMT progress and migration of A549 cells induced by Nickel chloride through modulating TGF-β1/Smad2/3, but not the MAPKs, signaling pathway (Ye et al., 2020). In our present study, NaHS has significantly increased the H_2S level, which downregulating by the FA treatment. Moreover, 100 μM NaHS treatment significantly inhibited FA downregulating the expression level of E-cadherin and upregulating the expression levels of Vimentin, TGF-β1, p-Smad2, p-Smad3, p-ERK, p-JNK, and p-P38 in Beas2B cells. These above results suggested that NaHS reduced FA-induced EMT through regulating TGF-β1/Smad2/3 and MAPKs signaling pathways in Beas2B cells. However, it still warrants further investigation to elucidate the role of H_2S synthesizing enzymes in endogenous H_2S protect against FA-induced EMT in lung epithelial cells *in vivo*. In addition, it would be interesting to clarify whether NaHS protects against FA-induced lung injury model *in vivo*, which needs to be further investigated. Meanwhile, H_2S is known to be toxic at higher concentrations (e.g., above 100 μM), so concentrations should remain well below this level. It is of great significance to select more suitable concentrations of H_2S, and to facilitate the use of H_2S in the clinical setting for potential therapeutic use. Conducting human studies is a crucial step in the translational research process for H_2S, allowing for a thorough evaluation of its safety and therapeutic potential. Of course, proper research design and compliance with regulations will be the keys to success.

5. Conclusions

Our findings demonstrated that FA exposure decreases endogenous H_2S production and induces the EMT process of lung epithelial cells. FA down-regulated the proteins level of CBS, CSE and 3-MST, which might be contributed to decrease the H_2S content in the Beas2B and A549 cells. Moreover, NaHS could significantly increase the H_2S level, which attenuated FA-induced EMT through regulating TGF-β1/Smad2/3 and MAPKs signaling pathways activation in Beas2B cells. The above results may shed new light on therapies for FA-induced lung toxicity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was partly supported by grants from the National Natural Science Foundation of China (81773464), the Natural Science Foundation of Anhui province (1708085MH195), the Key Projects of Anhui Province University Outstanding Youth Talent Support Program (gxyqZD2021100). We also want to thanks the platform of environmental exposure and life health research in Anhui Medical University.

Data availability

Data will be made available on request.

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