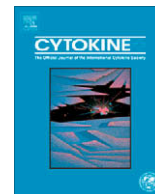




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Endogenous hydrogen sulfide reduces airway inflammation and remodeling in a rat model of asthma

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ABSTRACT

Endogenous hydrogen sulfide (H₂S) is hypothesized to have an important role in systemic inflammation. We investigated if endogenous H₂S may be a crucial mediator in airway inflammation and airway remodeling in a rat model of asthma and if endogenous H₂S may exert its anti-inflammatory effect by inhibiting inducible nitric oxide synthase (iNOS)/NO pathway. Cystathionine-γ-lyase (CSE; a H₂S-synthesizing enzyme) was mainly expressed in airway and vascular smooth muscle cells in rat lung tissue. Levels of endogenous H₂S was decreased in pulmonary tissue in ovalbumin (OVA)-treated rats. Exogenous administration of NaHS alleviated airway inflammation and airway remodeling: peak expiratory flow (PEF) increased, goblet cell hyperplasia and collagen deposition score decreased, with decreased total cells recovered from bronchoalveolar fluid (BALF) and influx of eosinophils and neutrophils. The H₂S levels of serum and lung tissue were positively correlated with PEF and negatively correlated with the level of eosinophils and neutrophils in BALF, score of lung pathology. NaHS treatment significantly attenuated pulmonary iNOS activation in OVA-treated rats. These results suggest that the CSE/H₂S pathway plays an anti-inflammatory and anti-remodeling part in asthma pathogenesis and could be a novel target in prevention and treatment of asthma.

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1. Introduction

Asthma is an inflammatory disorder of the airways. Chronic inflammation causes characteristic structural changes in the airways ("airway remodeling"). Airway remodeling includes subepithelial fibrosis, and hyperplasia and hypertrophy of airway smooth muscle, which results in relatively irreversible narrowing of the airways [1]. The mechanism of airway remodeling is incompletely understood, but nitric oxide (NO) has been found to have an important role in airway inflammation and remodeling in asthma. Inflammatory and oxidant stimuli induce the cellular expression of inducible nitric oxide synthase (iNOS) and iNOS activation. The NO generated by iNOS shifts the cellular redox potential to a more oxidized state. Inhibition of iNOS attenuates bronchoconstriction and inflammatory and remodeling processes during asthma [2].

Hydrogen sulfide (H₂S) has long been recognized as a water and industrial air pollutant, its major effects were on the central nervous system and the respiratory system. Recent studies have shown that endogenous H₂S, along with NO and carbon monoxide, may be an endogenous "signaling gasotransmitter", and act as a vasodilator and neurotransmitter [3]. Our previous study showed

that endogenous H₂S concentration was increased in patients with stable chronic obstructive pulmonary disease (COPD) and decreased in patients with acute exacerbation of COPD (AECOPD); this indicates that endogenous H₂S may be involved in the pathogenesis of airway inflammation and airflow obstruction in COPD [4]. H₂S in airway inflammation and airway remodeling in asthma has not been evaluated. We investigated if endogenous H₂S may be a crucial mediator in airway inflammation and airway remodeling in a rat model of asthma.

2. Materials and methods

2.1. Rat model of asthma

Animal care and experimental protocols were in compliance with animal management guidelines set by the Government of People's Republic of China. Male Sprague–Dawley (SD) rats (180–200 g) were supplied by the Animal Center, Health Science Center, Peking University.

Rats were randomly divided into three groups of eight. Ovalbumin (OVA, Sigma; St. Louis, MO, USA)-treated and sodium hydrosulfide-ovalbumin (NaHS/OVA)-treated groups underwent immunization by intraperitoneal injection with 1 mL 10% OVA saline (OVA, 100 mg; Al(OH)₃, 100 mg; deactivated pertussis bacillus vaccine (5 × 10⁹ colony-forming units (CFU)) on days 1 and 8. On

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day 15, animals were challenged with 1% OVA for 30 min/day for 2 weeks. For this, animals were placed in a plastic chamber (30 × 30 × 15 cm) and given aerosolized 1% OVA solution via an ultrasonic nebuliser (MDSIN Medical Technology; Shanghai). Freshly prepared NaHS (14 μmol/kg; Sigma) was administered intraperitoneally 30 min before OVA challenge in the NaHS/OVA-treated group. The saline-treated control group underwent an identical schedule for sham sensitization and challenge, but received saline rather than OVA. Animals were killed 1 h after the last challenge on day 29 [5].

2.2. Lung function test

Lung function was measured as previously described [6] but with modification. Rats were anesthetized by intraperitoneal injection of 20% (w/v) urethane (5 mL/kg) and fixation. A “Y”-type trachea tube connected to flow sensors and pressure sensors was inserted into the trachea. A ventilator was connected to a flow sensor; the tidal volume was 10 mL/kg and the respiratory rate was 60 times/min. The Powerlab multi-lead physical instrument was connected to flow and pressure sensors. Peak expiratory flow (PEF), peak inspiratory flow (PIF), intra-pressure (IP) and maximum rising slope of IP (IP slope) were analyzed using Chart 4.1 software (AD Instruments, Australia).

2.3. Bronchoalveolar lavage (BAL)

BAL was performed as previously described. Briefly, 5 mL of saline was injected into the right lung lobe using a sterile lavage catheter after clamping the left main bronchi. BAL cells were recovered by gentle suction. A small aliquot of the pooled, non-centrifuged lavage fluid (BALF) was used to determine the total number of nucleated cells. Cytocentrifuged samples underwent Wright–Giemsa staining. A minimum of 200 cells per slide were counted for the differential cell count.

2.4. Histology

Immediately after BAL, the thoracic cavity was opened and 10% (w/v) formalin was infused into the trachea. The left lung was inflated and maintained at 25–30 cm H₂O for 24 h. Tissues were embedded in paraffin and cut into 5 μm sections. The grading system described by Henderson was used [7]. Briefly, the lung sections were stained with hematoxylin and eosin (H&E) to assess the inflammatory cell infiltrate (0–4+ scale), Masson's trichrome to determine the collagen deposition in the lungs, and alcian blue, pH 2.5, with nuclear fast-red counterstaining to identify airway goblet cells and the degree of mucus plugging of the airways (0.5–0.8 mm in diameter) with the percent occlusion of airway diameter by mucus classified on a 0–4+ scale. Airway smooth muscle thickness was determined in H&E-stained sections by measuring the thickness of the smooth muscle cell layer beneath the airway epithelial cell base membrane at three sites tangential to each airway cross section examined. Scores were done by two experienced pathologist blinded to testing. Two scores were taken and the average value noted.

2.5. Immunohistochemistry

Formaldehyde-fixed and paraffin-embedded sections of tissue from left lower lung were stained with monoclonal antibody against the H₂S-generating enzyme cystathionine-γ-lyase (CSE, Abnova, Taiwan). Briefly, deparaffined sections were treated with 0.3% H₂O₂ for 30 min at room temperature to inactivate endogenous peroxidase and dried. Each section was blocked with 10% of normal goat serum for 10 min and incubated with anti-CSE anti-

body overnight at 4 °C (1:400). The control slide was incubated with a matched control. The sections were washed with PBS to remove unbound antibody and incubated with peroxidase-conjugated secondary antibody (rabbit anti-murine IgG). The bound peroxidase was detected colorimetrically using diaminobenzidine as the substrate [8].

2.6. Measurement of H₂S content in plasma and lung tissue

H₂S content in plasma and tissue from the right lung was measured by a sulfide-sensitive electrode (Model 9616, Orion Research; Beverly, MA, USA). H₂S concentration was calculated against the calibration curve of standard H₂S solutions [9].

2.7. Measurement of CSE activity in lung tissue

Tissue CSE activity was measured as described previously [10], with minor modifications. Right lung tissue was homogenized in 50 mmol/L ice-cold potassium phosphate buffer (pH 6.8). The reaction mixture contained (in mmol/L) 100 potassium phosphate buffer (pH 7.4), 10 L-cysteine, 2 pyridoxal 5'-phosphate, and 10% (w/v) tissue homogenate. Cryovial test tubes (2 mL) were used as the center wells; each contained 0.5 mL of 1% zinc acetate as trapping solution and a filter paper of 2.0 × 2.5 cm² to increase the air to liquid contact surface. The reaction was performed in a 25-mL Erlenmeyer Pyrex flask. The flasks containing reaction mixture and center wells were flushed with N₂ before being sealed with a double layer of Parafilm. The reaction was initiated by transferring the flasks from ice to a shaking water bath at 37 °C. After incubation at 37 °C for 90 min, 0.5 mL of 50% (w/v) trichloroacetic acid was added into the reaction mixture to stop the reaction. The flasks were sealed again and incubated at 37 °C for another 60 min to ensure complete trapping of the H₂S released from the mixture. The contents of the center wells were then transferred to test tubes. Each test tube contained 3.5 mL water. Subsequently, 0.5 mL of 20 mmol/L N,N-dimethyl-p-phenylenediamine sulfate in 7.2 mol/L HCl was added, immediately followed by 0.4 mL of 30 mmol/L FeCl₃ in 1.2 mol/L HCl. The absorbance of the resulting solution at 670 nm was measured on spectrophotometry (Beckman DU 640; USA). The H₂S concentration was calculated against the calibration curve of the standard H₂S solutions.

2.8. Measurement of iNOS activity in lung tissue

iNOS activity in tissue from the right lung was measured by ELISA kit according to manufacturer instructions (Jianchen Biotechnology Institute; Nanjing, China). Measurement was based on conversion of L-arginine to L-citrulline using spectrophotometric detection of nitrate and nitrite. Data were represented as units per milligram of protein.

2.9. Protein expression of CSE and iNOS

Tissue from the right middle lung was homogenized in 50 mmol/L Tris/HCl (pH 7.4) containing 0.25 mmol/L EDTA, 0.5 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL benzamidin. Protein was determined by BIORAD protein assay. The proteins were solubilized by boiling in SDS–PAGE sample buffer [0.0625 mmol/L Tris/HCl (pH 6.8) containing 10% (v/v) glycerol, 1% (w/v) SDS, 1% (w/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol blue], then proteins (10 μg per lane) were resolved by electrophoresis in 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The nitrocellulose was blocked overnight at 4 °C in 10% (w/v) dried milk protein in PBS containing 0.05% (v/v) Tween 20. The blots were washed in PBS containing 0.05% (v/v) Tween 20 and incubated for 1 h in the pres-

ence of monoclonal anti-CSE (1:4000) (Abnova; Taiwan). The blots were washed extensively, then incubated for 1 h with anti-mouse IgG conjugated to horseradish peroxidase (1:10,000) (Sigma). The bands were visualized using an enhanced chemiluminescence (ECL) kit following the manufacturer's instructions (Applygen Technologies; Beijing). The expression of the housekeeping protein β -actin was measured using an anti- β -actin antibody (Santa Cruz Biotechnology), and results were expressed as a ratio of the expression of the measured protein to that of β -actin. For iNOS, primary antibody was rabbit-anti-rat iNOS (1:1000) (Santa Cruz Biotechnology) [11].

2.10. Measurement of CSE mRNA in lung tissue by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total lung tissue RNA was prepared by *in situ* lysis with Trizol reagent. One microgram of total tissue RNA was reverse-transcribed into a single-stranded cDNA with M-MuLV reverse transcriptase and oligo (dT) 15 primers. PCR was performed in a tube containing 2 μ L tissue cDNA, 5 μ mol/L of each CSE-S and CSE-A primer mixture 1 μ L (CSE-S, 5'-TCC GGA TGG AGA AAC ACT TC-3', and CSE-A, 5'-GCT GCC TTT AAA GCT TGA CC-3'; Sai Bai Sheng, Beijing), 2.5 mmol/L of each dNTP mixture 1 μ L, 1.5 mmol/L $MgCl_2$ 1.5 μ L, 10 $\times$ PCR buffer 2.5 μ L, and 1.25 unit Taq DNA polymerase, in a total volume of 25 μ L. After being denatured at 95 $^{\circ}$ C for 5 min, the solution underwent PCR at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s for 30 cycles, then 72 $^{\circ}$ C for 5 min. Seven microliters of PCR product was separated in a 1.5% agarose gel and stained with ethidium bromide. The optical density of the 400-bp band was measured by the Gel Documentation System (Bio-Rad, Hercules, CA). Amplification of CSE cDNA was confirmed by use of CSE primer measuring ribonucleotide sequence analysis. For calibrating sample loading, 2 μ L of PCR product was amplified again with the 2 rat β -actin primers (β -actin-S, 5'-ATC TGG CAC CAC ACC TTC-3', and β -actin-A, 5'-AGC CAG GTC CAG ACG CA-3'; Sai Bai Sheng) at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s for 23 cycles, and then 72 $^{\circ}$ C for 5 min, the optical density of the β -actin band (291 bp) was measured.

2.11. Statistical analysis

Data were expressed as means $\pm$ SD. Comparisons among groups involved one-way ANOVA followed by Student–Newman–Keuls test. Comparisons between two groups involved unpaired Student's *t*-test. Pearson's correlation analysis was used to assess correlation. SPSS 10.0 (SPSS, Chicago, IL, USA) was used for analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Endogenous CSE was expressed in lung tissues

Immunohistochemical staining showed that CSE expression was mainly in the airway and vascular smooth muscle cells in rat peripheral lung tissues (Fig. 1).

3.2. Endogenous generation of H_2S was downregulated in an OVA-induced asthma model

Pulmonary functions decreased in OVA-treated rats. PEF was 55.4% lower in OVA-treated rats than in control, whereas IP slope was 33.3% higher (all $P < 0.01$) (Table 1).

Light microscopy revealed extensive infiltration of eosinophils and mononuclear cells around pulmonary blood vessels and airways in the lung interstitium in OVA-treated rats (Fig. 2A and B). Goblet cell hyperplasia and airway mucus secretion was higher (Fig. 2D and E) and the basement membrane thicker (Fig. 2G and H). Using morphometry, scores of the infiltration of inflammatory cells, collagen deposition, and goblet cell hyperplasia were higher in OVA-treated rats than in control (2.50 ± 0.54 vs. 0.63 ± 0.52 , 2.50 ± 0.54 vs. 0.50 ± 0.54 , and 2.00 ± 1.07 vs. 0.0 ± 0.0 , respectively, all $P < 0.01$).

Total cells recovered from BALF were 2.3-fold higher in OVA-treated rats than in control ($P < 0.01$). Of the BALF cells in OVA-treated animals, $6.13\% \pm 1.15\%$ were eosinophils, compared with 0% in control ($P < 0.01$). The proportion of neutrophils and lymphocytes was 66-fold and 14-fold higher, and that of macrophages 59.6% lower, than in control (all $P < 0.01$) (Table 2).

H_2S level was 81.1% lower in serum and 79.8% lower in lung tissue in OVA-treated rats than in control (all $P < 0.01$) (Table 3). CSE activity was 42.6% lower in lung tissue in OVA-treated animals than in control (1.01 ± 0.08 vs. 1.76 ± 0.10 μ mol/min/mg protein, $P < 0.01$). Western blot analysis revealed expression of CSE was 78.3% lower in lung tissue in OVA-treated rats than in control

Table 1

Effect of NaHS on lung function in an OVA-induced rat asthma model ($\bar{X} \pm S$, $n = 8$).

Group	PIF (L/S)	PEF (L/S)	IP (L)	IP slope (L/S)
Control	1.38 ± 0.30	6.48 ± 0.11	14.97 ± 1.36	1.44 ± 0.13
OVA-treated	$1.62 \pm 0.16^*$	$2.89 \pm 0.71^{**}$	$10.06 \pm 2.47^{**}$	$1.92 \pm 0.13^{**}$
NaHS/OVA	$1.77 \pm 0.20^{**}$	$5.67 \pm 0.49^{**\#}$	$14.59 \pm 4.68^{\#}$	$2.06 \pm 0.14^{**}$

Compared with control, $^*P < 0.05$, $^{**}P < 0.01$; compared with OVA-treated group, $^{\#}P < 0.01$.

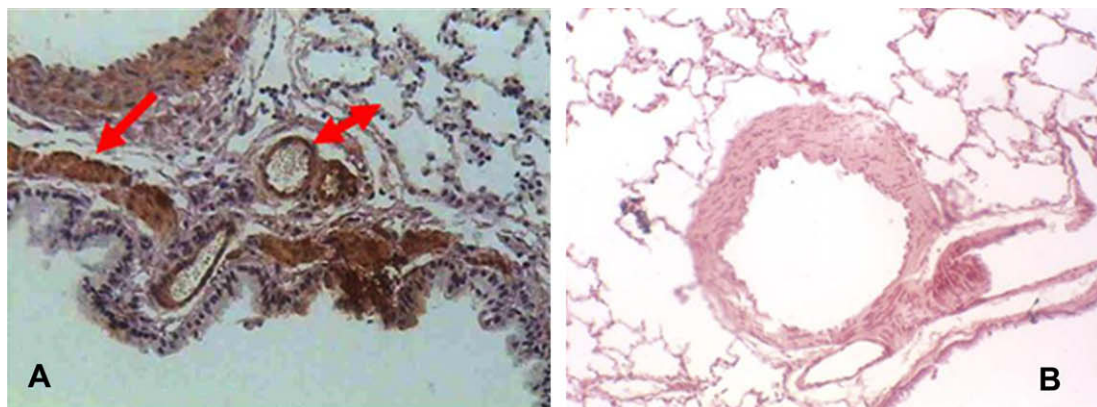


Fig. 1. CSE distribution in the lung tissue of normal rats. Immunohistochemical staining showed that expression of CSE was mainly located in airway (→) and vascular (↔) smooth muscle cells in peripheral lung tissues (A). No positive staining was found in control slide (B). Magnification $\times 400$.

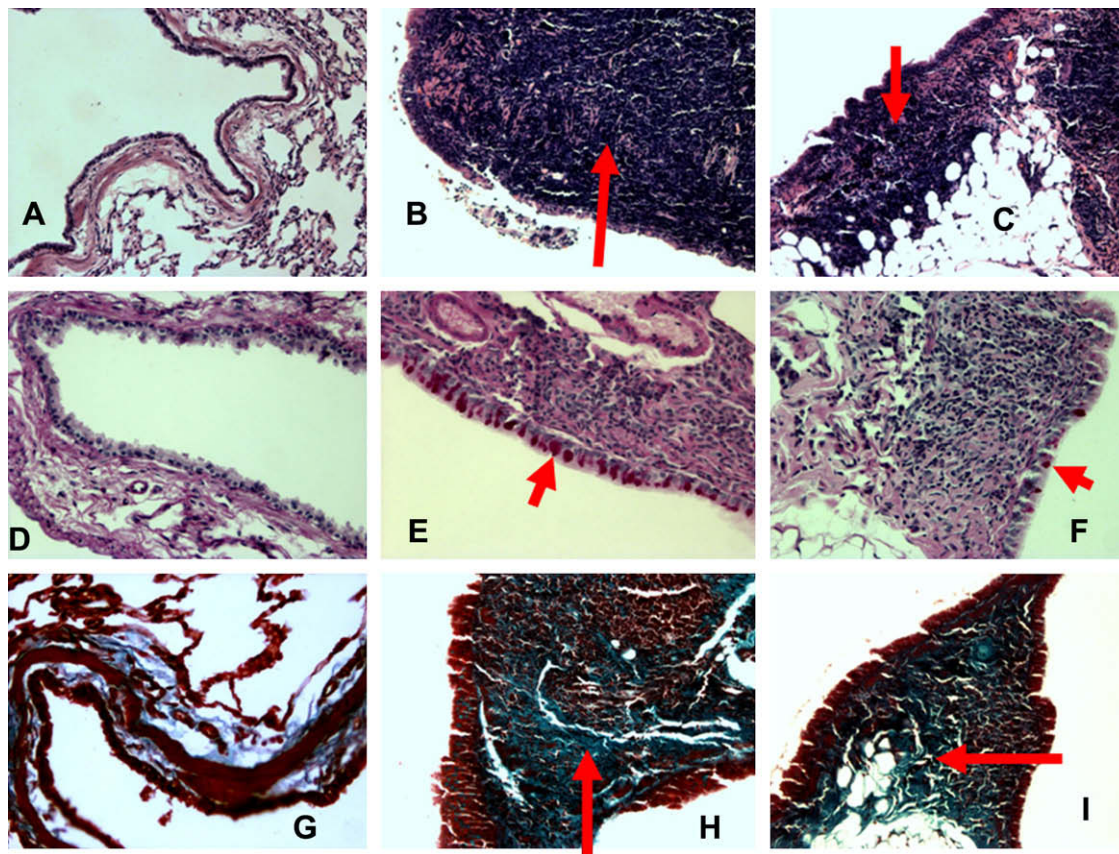


Fig. 2. Lung pathology in OVA-treated rats in the absence or presence of NaHS. Lung tissue was obtained from saline-treated rats (control, A, D, and G) and OVA-treated rats in the absence (OVA, B, E, and H) or presence of NaHS (NaHS/OVA, C, F, and I) stained with hematoxylin and eosin (A–C), alcian blue with nuclear fast-red counterstaining (D–F), Masson's trichrome (G–I), and examined by light microscopy. Magnification $\times 200$.

Table 2

Effect of NaHS on cell differential counts in BALF ($\bar{X} \pm S$, $n = 8$).

Group	Total cell counts (10^4 cells/ml)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)
Control	30.84 ± 8.20	98.43 ± 0.49	0.28 ± 0.34	1.23 ± 0.30	0 ± 0
OVA-treated	$72.16 \pm 48.91^*$	$58.71 \pm 5.64^{**}$	$18.35 \pm 3.34^{**}$	$16.81 \pm 2.82^{**}$	$6.13 \pm 1.15^{**}$
NaHS/OVA	39.59 ± 20.31	$65.42 \pm 4.38^{**}$	$12.69 \pm 2.45^{**\#}$	$19.55 \pm 2.19^{**}$	$2.34 \pm 0.68^{**\#}$

Compared with control, $^*P < 0.05$, $^{**}P < 0.01$; compared with OVA-treated group, $^\#P < 0.01$.

Table 3

Serum and lung tissue H_2S levels in an OVA-induced asthma model ($\bar{X} \pm S$, $n = 8$).

Group	Serum H_2S level ($\mu\text{mol/L}$)	Lung tissue H_2S level ($\mu\text{mol/L}$)
Control	53.90 ± 9.90	24.05 ± 7.96
OVA-treated	$10.20 \pm 3.22^*$	$4.85 \pm 1.28^*$
NaHS/OVA	$16.50 \pm 3.64^\#$	$15.34 \pm 4.02^{**\#}$

Compared with control, $^*P < 0.01$; compared with OVA-treated group, $^\#P < 0.05$, $^{**}P < 0.01$.

($n = 3$, $P < 0.01$) (Fig. 3) and RT-PCR revealed CSE mRNA expression was 63.4% lower ($P < 0.01$) (Fig. 4).

3.3. Administration of NaHS alleviated airway inflammation and airway remodeling in the rat asthma model

Our previous study showed that NaHS alone had no effect on pulmonary function, cell counts in BALF, and lung pathology in normal rats (Supplementary data). PEF was 96.2% greater with NaHS/OVA treatment than with OVA treatment alone ($P < 0.01$)

(Table 1). NaHS/OVA treatment attenuated infiltration of inflammatory cells (Fig. 2C), goblet cell hyperplasia, airway mucus secretion (Fig. 2F) and subepithelial fibrosis (Fig. 2I) compared with OVA treatment alone. Morphometry revealed 37.5% lower goblet cell hyperplasia score (1.25 ± 0.46 vs. 2.00 ± 1.07 , $P < 0.05$) and 54.8% lower collagen deposition (1.13 ± 0.35 vs. 2.50 ± 0.54 , $P < 0.01$) with NaHS/OVA treatment than with OVA treatment alone. Inflammatory cell infiltration score did not differ (2.00 ± 0.54 vs. 2.50 ± 0.54 , $P > 0.05$). NaHS/OVA-treated rats showed fewer total cells recovered from BALF, and a lower influx of eosinophils and neutrophils by 45.1% ($P < 0.05$), 61.8% ($P < 0.01$) and 30.8% ($P < 0.01$), respectively, than OVA-treated rats (Table 2).

Compared with OVA-treatment alone, with NaHS/OVA treatment, levels of H_2S in serum and lung tissue were 61.8% and 2.2-fold more, respectively ($P < 0.05$ and 0.01 , respectively) (Table 3), and pulmonary CSE activity was 60.4% more (1.62 ± 0.18 vs. 1.01 ± 0.08 $\mu\text{mol/min/mg}$ protein, $P < 0.01$). Western blot analysis revealed that CSE protein in lung tissue was 5.6-fold higher in NaHS/OVA-treated rats ($n = 3$) than with OVA treatment alone ($P < 0.01$) (Fig. 3). RT-PCR revealed that the mRNA level of CSE in lung was 82.5% higher in NaHS/OVA-treated rats ($P < 0.05$) (Fig. 4).

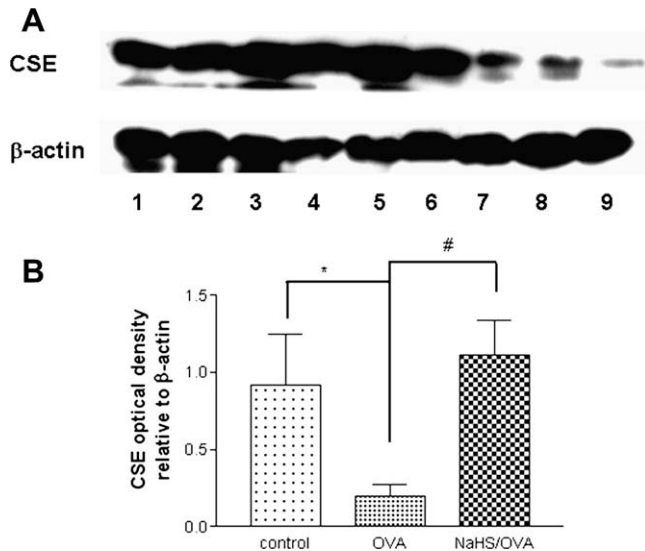


Fig. 3. Effect of NaHS on expression of CSE protein in lung tissue. (A) Total protein was isolated from the lungs of saline-treated rats (lane 1–3) and OVA-treated rats in the absence (lane 7–9) or presence (lane 4–6) of NaHS. Expression of β -actin was the reference. (B) Densitometric analysis of CSE protein expression relative to that of β -actin ($n = 3$, respectively, in each group). Compared with control, * $P < 0.01$; compared with OVA-treated group, # $P < 0.01$.

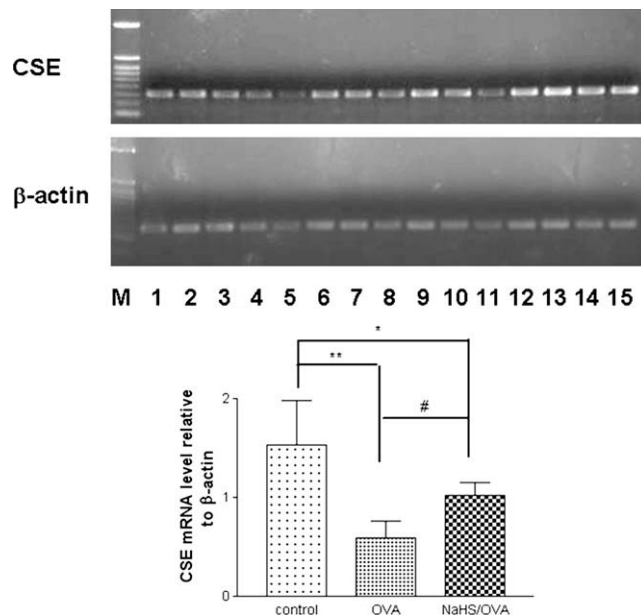


Fig. 4. Effect of NaHS on level of CSE mRNA in lung tissue. Total RNA was isolated from the lungs of saline-treated rats (lane 11–15) and OVA-treated rats in the absence (lane 1–5) or presence (lane 6–10) of NaHS. The top panel represents CSE; the middle panel shows β -actin. The densitometric analysis of CSE mRNA levels relative to that of β -actin ($n = 5$, respectively, in each group) is shown in the lower panel. Compared with control, * $P < 0.05$, ** $P < 0.01$; compared with OVA-treated group, # $P < 0.05$.

3.4. Correlation between endogenous generation of H_2S and airway inflammation

The serum level of H_2S was positively correlated with H_2S level in lung tissues ($r = 0.752$, $P < 0.01$). H_2S levels in serum and lung tissue were positively correlated with PEF ($r = 0.709$ and 0.758 , respectively, all $P < 0.01$), negatively correlated with IP slope ($r = -0.761$ and -0.507 , respectively, $P < 0.01$ and 0.05), proportion

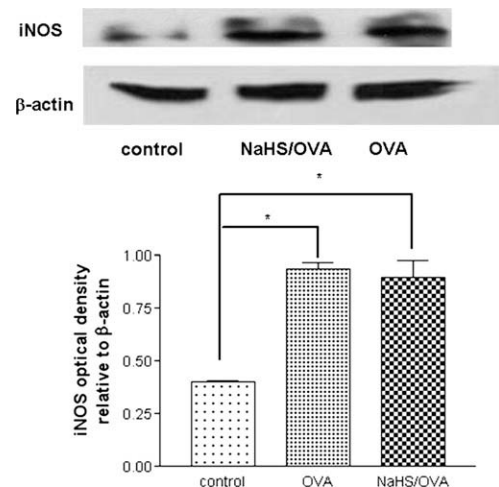


Fig. 5. Effect of NaHS on expression of iNOS protein in lung tissue. Total protein was isolated from the lungs of saline-treated rats (lane 1) and OVA-treated rats in the presence (lane 2) or absence (lane 3) of NaHS. Expression of iNOS protein (top) was determined by Western blot analysis, β -actin expression was the reference. (lower) Densitometric analysis of iNOS protein expression relative to that of β -actin ($n = 3$ in each group) was undertaken. The level of iNOS protein in the NaHS/OVA-treated group was similar to that of the OVA-treated group. Compared with control, * $P < 0.01$.

of eosinophils ($r = -0.811$ and -0.801 , respectively, all $P < 0.01$), neutrophils in BALF ($r = -0.917$ and -0.764 , respectively, all $P < 0.01$), score of inflammatory cells infiltration ($r = -0.837$ and -0.743 , respectively, all $P < 0.01$), collagen deposition ($r = -0.860$ and -0.779 , respectively, all $P < 0.01$), goblet cell hyperplasia ($r = -0.785$ and -0.710 , respectively, all $P < 0.01$), and airway smooth muscle hyperplasia ($r = -0.481$, $P < 0.05$).

3.5. Endogenous generation of H_2S did not affect iNOS expression, but decreased iNOS activity

Western blot analysis showed that expression of iNOS protein was 135% more in the OVA-treated group than in control ($P < 0.01$). Activity of iNOS was higher in OVA-treated rats than in control (0.21 ± 0.05 vs. 0.12 ± 0.01 U/mg protein, $P < 0.01$). The NaHS/OVA-treated group had levels of iNOS protein similar to the OVA-treated group ($P > 0.05$) (Fig. 5). However, iNOS activity was lower in NaHS/OVA-treated rats than in OVA-treated rats (0.13 ± 0.02 vs. 0.21 ± 0.05 U/mg protein, $P < 0.01$).

iNOS activity was positively correlated with goblet cell hyperplasia ($r = 0.701$, $P < 0.01$), infiltration of inflammatory cells ($r = 0.600$, $P < 0.01$), collagen deposition ($r = 0.760$, $P < 0.01$), total cell counts ($r = 0.801$, $P < 0.01$), percentage of neutrophils ($r = 0.581$, $P = 0.011$) and eosinophils in BALF ($r = 0.666$, $P = 0.003$). It was negatively correlated with PEF ($r = -0.817$, $P = 0.000$), H_2S levels in serum and lung tissue ($r = -0.65$, $P < 0.01$ and $r = -0.503$, $P < 0.05$), and pulmonary CSE activity ($r = -0.793$, $P < 0.01$).

4. Discussion

The present study showed that CSE was expressed in normal lung tissue of rats. Endogenous pulmonary H_2S generation was down-regulated in OVA-treated rats. Exogenous administration of the donor NaHS could alleviate airway inflammation and airway remodeling. Endogenous H_2S may exert its anti-inflammatory effect, at least in part by decreasing iNOS activity.

H_2S was recently found to be endogenously generated by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine

β -synthase (CBS) and CSE with L-cysteine used as a major substrate. H₂S is now considered to be a novel gasotransmitter with many functions. The endogenous CSE/H₂S pathway was altered during hypoxic pulmonary hypertension and high pulmonary blood flow-induced pulmonary hypertension in rats, and H₂S relaxed pulmonary artery smooth muscle cells and inhibited their proliferation [10,12,13]. Our recent published study showed that endogenous H₂S may be involved in the pathogenesis of airway obstruction in COPD, and that its alteration may be connected with the activity and severity of this disease. This effect may not be fully explained by the hemodynamic effect of H₂S. H₂S could be an important mediator or modulator of airway inflammation [4]. The changes in the endogenous CSE/H₂S pathway and its role in the pathogenesis of airway inflammation and airway remodeling in asthma remain to be clarified.

In the present study, we found that the H₂S-generating enzyme CSE was mainly distributed in airway and vascular smooth muscle cells in rat peripheral lung tissues. This suggested that endogenously generated H₂S could have a direct effect on airway smooth muscle. Pulmonary function was reduced in the OVA-treated rats, with lower PEF and higher IP slope; the proportion of eosinophils in BALF was increased. Histological analysis demonstrated airway inflammation and airway remodeling, characteristic features of asthma. Our study showed a decreased level of H₂S in serum and lung tissue, CSE activity, CSE protein, and mRNA expression in lung tissues, which showed that endogenous generation of H₂S was downregulated in the OVA-induced asthma rat model. H₂S levels in serum and lung tissue were positively correlated with PEF, and negatively correlated with IP slope, the proportion eosinophils and neutrophils in BALF, and scores for inflammatory cell infiltration, collagen deposition and goblet cell hyperplasia. Treatment with the H₂S donor NaHS increased PEF, indicating alleviation of airway obstruction. BALF cell counts and histological analysis showed that NaHS administration had an inhibitory effect on airway inflammation and airway remodeling. Taken together, these results suggest that endogenous H₂S was involved in the pathogenesis of airway inflammation and airway remodeling, and may be an endogenous protective mediator or modulator in the rat model of asthma.

The role of endogenous H₂S in systematic inflammation is controversial: it may have a pro- or anti-inflammatory effect under different conditions [14,15]. Recent studies show that H₂S can be an antagonist of oxidative damage in the brain and heart [16–18]. H₂S donors induced neutrophil apoptosis [19]. H₂S produced an anti-inflammatory effect in lipopolysaccharide (LPS)-stimulated microglia and astrocytes [20]. These findings have important implications for the development of anti-inflammatory drugs. H₂S has a pro-inflammatory role in regulating sepsis severity [21,22]. H₂S stimulates the activation of human monocytes with the generation of pro-inflammatory cytokines, and this response is (at least in part) through the extracellular-regulated kinase mediated NF- κ B signaling pathway [23,24]. The significance of endogenous H₂S in the development of airway inflammation and airway remodeling in asthma deserves further investigation.

Recently, NO has been found to be very useful in asthma assessment [25]. The relationship between H₂S and NO must be clarified. In the present study, we found that administration of NaHS had no effect on the expression of iNOS protein, but there was decreased iNOS activity in the OVA-induced asthma rat model. iNOS activity was positively correlated with scores of lung pathology, total cell counts, neutrophils and eosinophils proportion in BALF, and negatively correlated with PEF, serum and lung tissue H₂S levels, and pulmonary CSE activity. We speculate that interaction between H₂S and NO in our model may be at a post-translation modification level instead of a translation level. Endogenous H₂S may exert its anti-inflammatory effect by inhibiting iNOS activity. The relation-

ship between H₂S and NO in asthmatic airway inflammation could be tested in the presence of inhibitors (e.g., L-NAME) or enhancers (e.g., increased dietary arginine) of NOS activity. Recent study investigated the relation among H₂S, NO and CO in hypoxia pulmonary hypertension. H₂S could play a regulatory role in the pathogenesis of hypoxia pulmonary hypertension by up-regulating the CO/HO pathway and down-regulating the NO/NOS system. In addition, the NO donor nitroflurbiprofen down-regulates H₂S biosynthesis, which suggests the existence of cross-talk between NO and H₂S in the model of endotoxemic shock. H₂S can inhibit NO production and NF- κ B activation in LPS-stimulated macrophages through a mechanism that involves the action of HO-1/CO [26–28].

In conclusion, our study showed that generation of pulmonary endogenous H₂S was downregulated in an OVA-induced asthmatic rat model. Exogenous administration of NaHS could alleviate airway inflammation and airway remodeling. Endogenous H₂S may exert its anti-inflammatory effect by inhibiting iNOS activity. Further study will be done whereby H₂S synthesis is inhibited *in vivo* and *in vitro* to investigate the mechanism of endogenous H₂S in airway inflammation and airway remodeling in asthma.

5. Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyto.2008.11.009.

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Supplemental materials

Effect of NaHS alone on the airways of normal rats

Twelve rats were randomly divided into saline-treated control group (control) and NaHS-treated group. Control rats underwent the same regimen for sham sensitization and challenge, but received saline rather than OVA. NaHS-treated rats underwent the same regimen as NaHS/OVA-treated rats, but received saline rather than OVA for sham sensitization and challenge. Rats were killed 1 h after the last challenge on day 29. NaHS alone had no effect on pulmonary function, cell counts in BALF, and lung pathology in normal rats (Table 1, Figure 1).

Table 1. Effect of NaHS alone on airway inflammation parameters ($\bar{X} \pm S$, n=6)

	Saline-treated (control)	NaHS-treated
Pulmonary function		
PIF (L/S)	1.14±0.03	1.16±0.03
PEF (L/S)	6.54±0.28	6.08±0.48
IP (L)	15.90±2.20	16.97±1.14
IP slope (L/S)	1.54±0.20	1.44±0.23
Cell counts in BALF		
Total cells counts (10 ⁴ cells/ml)	47.90±6.20	53.97±6.63
Macrophages (%)	94.92±2.82	96.81±1.56
Neutrophils (%)	0	0
Lymphocytes (%)	4.91±2.99	3.27±1.78
Eosinophils (%)	0	0

Compared with saline-treated group, * $P < 0.01$.

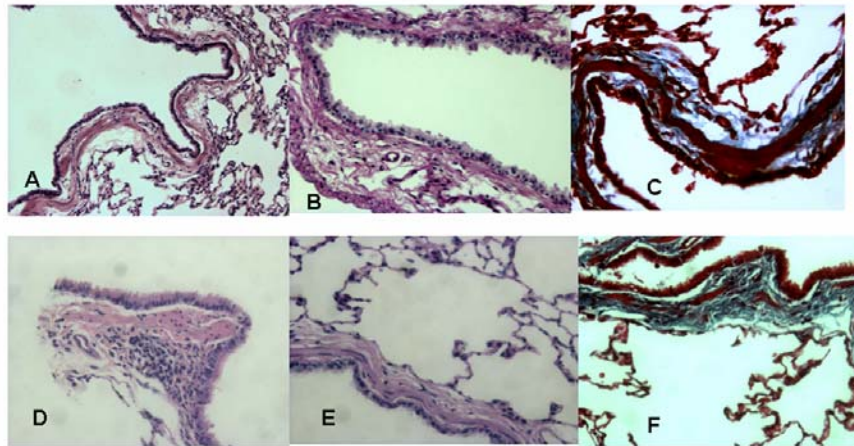


Figure 1

Figure 1 Effect of NaHS on lung pathology in normal rats. Lung tissue was obtained from saline-treated rats in the absence (control, A, B, C) or presence of NaHS (NaHS, D, E, F) stained with hematoxylin and eosin (A, D), alcian blue with nuclear fast-red counterstaining (B, E), Masson's trichrome (C, F), and examined by light microscopy. Magnification $\times 200$.