



CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

The Inhibitory Role of Hydrogen Sulfide in Airway Hyperresponsiveness and Inflammation in a Mouse Model of Asthma

Gensheng Zhang,^{*} Peipei Wang,^{*} Guangdong Yang,[†] Qihui Cao,^{*} and Rui Wang^{*}

From the Department of Biology^{*} and the School of Kinesiology,[†] Lakehead University, Thunder Bay, Ontario, Canada

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Address correspondence to Rui Wang, M.D., Office of Vice President (Research, Economic Development and Innovation), Lakehead University, 955 Oliver Rd., Thunder Bay, Ontario, P7B 5E1 Canada.
E-mail: rwang@lakeheadu.ca.

Cystathionine γ -lyase (CSE) is one of the major enzymes producing hydrogen sulfide (H_2S) in lungs, participating in the regulation of respiratory functions. The role of CSE-derived H_2S in eosinophil-dominant inflammation in allergic diseases has been unclear. The objective of this study was to explore the protective role of H_2S against allergen-induced airway hyperresponsiveness (AHR) and inflammation. CSE expression and H_2S production rate were assessed in mouse lung tissues with ovalbumin (OVA)-induced acute asthma. AHR, airway inflammation, and Th2 response in wild-type (WT) mice were compared with those in CSE gene knockout (KO) mice. The effect of NaHS, an exogenous H_2S donor, was also evaluated on these parameters. CSE expression was absent and H_2S production rate was significantly lower in the lungs of CSE KO mice when compared with WT littermates. OVA challenge decreased lung CSE expression and H_2S production in WT mice. CSE deficiency resulted in aggravated AHR, increased airway inflammation, and elevated levels of Th2 cytokines such as IL-5, IL-13, and eotaxin-1 in bronchoalveolar lavage fluid after OVA challenge. The aforementioned alterations were reversed by exogenous H_2S treatment. More importantly, NaHS supplement rescued CSE KO mice from the aggravated pathological process of asthma. The CSE/ H_2S system plays a critical protective role in the development of asthma. A new therapeutic potential for asthma via targeting CSE/ H_2S metabolism is indicated. (*Am J Pathol* 2013, 182: 1188–1195; <http://dx.doi.org/10.1016/j.ajpath.2012.12.008>)

Hydrogen sulfide (H_2S), traditionally known for centuries as a noxious and toxic gas is now recognized as a third gas-transmitter after nitric oxide and carbon monoxide for its important regulatory roles in chronic respiratory diseases, cardiovascular, and metabolic disorders.^{1–5} The role of H_2S in inflammation, however, is still controversial.^{2,6} Some studies have showed that exogenous H_2S has a pro-inflammatory effect in various inflammatory models including lipopolysaccharide-induced endotoxemia, acute pancreatitis, and cecal ligation and puncture-induced sepsis.^{7–9} Others have reported its anti-inflammatory effects.^{10–12} The concentration and the release rate of exogenous H_2S donors are the main factors in determining whether H_2S is anti-inflammatory or pro-inflammatory. The high concentration and fast-releasing rate of H_2S results in a pro-inflammatory effect, and at low concentration and slow-releasing rate H_2S is anti-inflammatory.^{12,13}

Asthma is one of the most common chronic inflammatory diseases, and its prevalence has markedly increased

throughout the past 2 decades.¹⁴ Eosinophilia and Th2 cytokine production are two hallmarks of allergic asthma.¹⁵ Increased Th2 cytokines, such as IL-4, IL-5, and IL-13, cause immunoglobulin isotype switching of B cells to produce IgE, promote the growth, maturation, and activation of Th2 cells and eosinophils, and result in airway hyperresponsiveness (AHR), mucus production, and airway remodeling.¹⁵ Previous studies have shown the existence of cystathionine γ -lyase (CSE) and/or cystathionine β -synthase (CBS) in peripheral lung tissues of mice and rats.^{16,17} Decreased CSE expression and serum H_2S level are observed in the asthma model of rats and in asthmatic

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G.Z. and P.W. contributed equally to this study.

Current address of G.Z., Department of Critical Care Medicine, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China.

patients.^{17–19} Serum H₂S level is positively correlated with the lung function parameters such as the forced expiratory volume in 1 second, both in adult and pediatric asthma,^{18,19} and negatively correlated with sputum count of total cells and the percentage of sputum neutrophils.¹⁸ These results suggest a possible role of endogenous H₂S in the development of asthma. All of these previous studies, however, were based on neutrophil-dominant airway inflammation,^{17,18} which is different from eosinophil-dominant airway inflammation in classically allergic asthma.¹⁵ Whether and how endogenous or exogenous H₂S plays a role in the development of eosinophil-dominant inflammation such as allergic asthma and atopic dermatitis remains unknown.

Our previous studies have found that CSE is the main H₂S-producing enzyme in peripheral tissues such as liver, pancreases, and aorta tissues.^{3,4} In CSE knockout (KO) mice, endogenous H₂S levels were decreased by 50% in serum, and ~80% in vascular tissues and pancreases.^{3,4} Lower level of endogenous H₂S causes hypertension,⁴ delays the onset of streptozotocin-induced diabetes,³ promotes proliferation of smooth muscle cells,²⁰ and inhibits vascular endothelial growth factor-induced microvessel formation and wound healing²¹ in CSE KO mice. To date, the exact role of endogenous H₂S in systemic or local inflammation has not been studied by knocking out the *CSE* gene. Given the critical regulatory role of the CSE/H₂S system in various physiological and pathological conditions, we hypothesized that the endogenous CSE/H₂S pathway may play an important role in the pathogenesis of allergic asthma.

In the present study, we examined the alterations of CSE lung expression and H₂S production, and changes in AHR, airway inflammation, and Th2 response, in CSE KO mice in comparison with wild-type (WT) littermates in a mouse model of asthma. In addition, the effect of exogenous H₂S treatment on the development of asthma was also observed in both WT and CSE KO mice.

Materials and Methods

Mice

CSE KO mice on C57BL/6J × 129SvEv background were generated as we previously described.⁴ CSE KO offspring and age-matched male WT littermates were used. All animal experiments were in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care Committee of Lakehead University, Thunder Bay, Ontario, Canada.

OVA-Induced Asthma Model and Experimental Design

WT and CSE KO mice at 8 to 10 weeks were sensitized i.p. with 20 µg chicken ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) emulsified in 100 µL Imject Alum (Pierce, Rockford, IL) on days 0 and 7 and then challenged by 100 µg OVA in 40 µL saline administered intranasally after a short

anesthesia with isoflurane inhalation on days 28, 29, and 30 (termed as WT-OVA and KO-OVA mice). Sensitized mice were challenged with saline as the control (termed as WT-CON and KO-CON mice). In another group of experiments, mice were given 14 µmol/kg NaHS i.p. (Sigma-Aldrich), 14 µmol/kg NaHS i.p. plus 40 µmol/kg glibenclamide i.p., 20 minutes before NaHS, or vehicle (dimethyl sulfoxide) 30 minutes before and 8 hours after each OVA challenge.

Assessment of AHR

Twenty-four hours after the last OVA challenge, AHR to inhaled methacholine (MCh) (Sigma-Aldrich) was invasively measured. Mice were anesthetized with a combination of 100 mg/kg ketamine hydrochloride and 8.5 mg/kg xylazine, and the tracheas were cannulated and connected to an inline aerosol administrator and ventilator, which were attached to a preamplifier and computer (Buxco, Wilmington, NC). Mice were nebulized with PBS, followed by increasing doses of MCh (3.125, 6.25, 12.5, and 25 mg/mL, respectively). At each dose, the values of lung resistance, reflecting the degree of AHR, were calculated.

Analysis of BALF

A collection of bronchoalveolar lavage fluid (BALF) was performed after AHR measurement. The trachea was cannulated, and inflammatory cells were obtained by lavage of the airway lumen with 0.8 mL saline three times. Cytospin slides were prepared by Wright-Giemsa staining. The number of total and differential cells was counted in a blind fashion.

Lung Histology

Paraffin-embedded lung tissue sections (5 µm) were stained with H&E (Sigma-Aldrich) to evaluate pulmonary inflammatory cell infiltration.

Western Blot

Isolated lung tissues were homogenized and lysed. Equal amounts of proteins were boiled and separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The dilutions of primary antibodies were 1:1000 for CSE (12217-1-AP; Proteintech Group Inc., Chicago, IL), 1:1000 for CBS (14787-1-AP; Proteintech Group Inc.), and 1:10,000 for β-actin (Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich) were used at 1:10,000. The immunoreactions were visualized by enhanced chemiluminescence and exposed to X-ray film (Kodak Scientific Imaging film; VWR International, Mississauga, Canada).

H₂S Production Rate

The measurement of H₂S production rate was previously described.²² Briefly, isolated lung tissues were homogenized

in 50 mmol/L ice-cold potassium phosphate buffer (pH 6.8). The homogenate was first incubated with L-cysteine (10 mmol/L) for 90 minutes at 37°C, and then trichloroacetic acid was added to stop the reaction. The level of methylene blue generated by the interaction of H₂S with N,N-dimethyl-p-phenylenediamine sulfate was determined at 670 nm with a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH, Offenburg, Germany).

Lymphocytes Isolation and Cell Culture

Lung draining lymph nodes were isolated from OVA-challenged WT and CSE KO mice. Single-cell suspensions were obtained by gently mincing these isolated lymph nodes using the plunger of a 5-mL syringe followed by passing through a 40- μ m cell strainer. Lymphocytes were separated with Lympholyte-M cell separation media (Cedarlane Laboratories, Hornby, ON, Canada). These lymphocytes at 2×10^5 cells per well in 96-well plates were cultured in 1640 medium at 37°C in 5% CO₂ and stimulated with 80 μ g/mL OVA in the presence or absence of NaHS (50 to 500 μ mol/L). Seventy-two hours later, cell culture supernatant was harvested and stored at -80°C for cytokines measurement. In another independent experiment, these lymphocytes were cultured for cell viability assay. Seventy-two hours later, 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well and the cells were incubated for another 4 hours. The cell culture medium was removed, and 200 μ L per well dimethyl sulfoxide was added. The absorbance of formazan products at 570 nm was measured in a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTECH).

Analysis of Cytokines

The concentrations of IL-4, IL-5, IL-13, and eotaxin-1 in BALF and culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using the DuoSet ELISA development kit (R&D Systems, Minneapolis, MN), according to the manufacturer's protocols.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Data were detected for their normality by the Kolmogorov-Smirnov test and were analyzed by one-way analysis of variance or by an unpaired Student's *t*-test using the SPSS software version 13.0. A value of *P* < 0.05 was considered statistically significant.

Results

OVA Challenge Reduces CSE Expression and H₂S Production Rate in the Lung

In KO mice, CSE proteins were not detectable in the lung (Figure 1A). Sensitized WT mice displayed a slight, but significant decrease in CSE expression in the lung after

OVA challenge (0.70 ± 0.05 versus 0.52 ± 0.03 ; *P* < 0.05) (Figure 1A). The level of CBS expression, another H₂S-producing enzyme, was similar in the lungs of WT- and KO-CON mice. Even after OVA challenge, CBS expression was not changed in either WT or KO mice (Figure 1A). Endogenous H₂S production rate in lungs of KO mice was 20% of that from WT mice (0.27 ± 0.04 versus 1.38 ± 0.06 nmol/g per minute; *P* < 0.05) (Figure 1B). H₂S production rate was reduced by 40% in lungs of WT-OVA mice in comparison with that of WT-CON mice (0.83 ± 0.10 versus

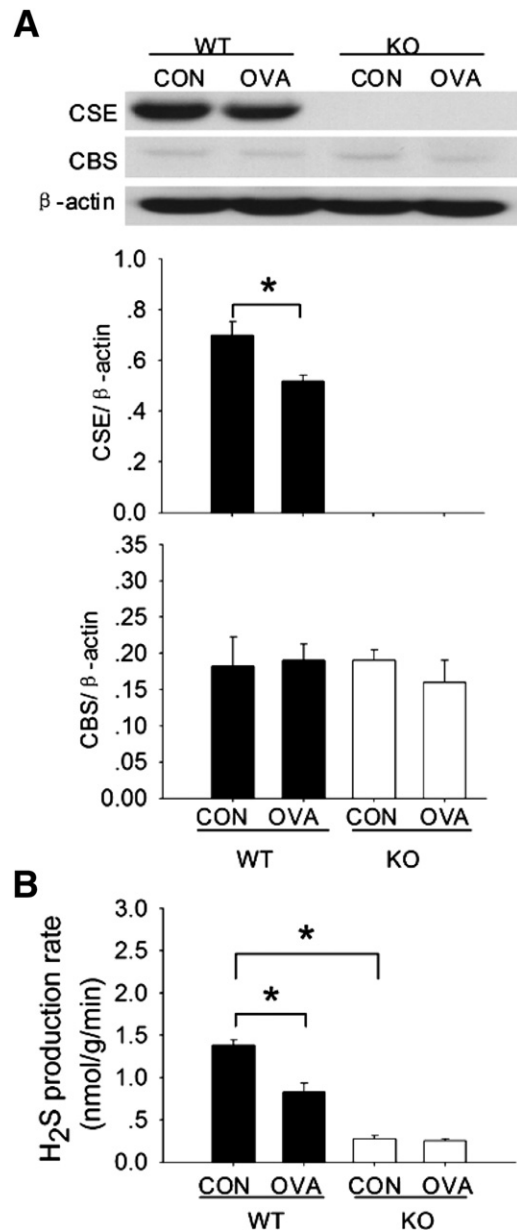


Figure 1 The OVA challenge reduced CSE expression and H₂S production in mouse lungs. OVA-sensitized WT and CSE KO mice were challenged with OVA (WT-OVA and KO-OVA). Sensitized mice challenged with saline were used as a control (WT-CON and KO-CON). Twenty-four hours after the last OVA challenge, lung tissues were dissected for Western blot analysis (A) and H₂S production detection (B). Six to ten mice were used for each group. **P* < 0.05.

1.38 ± 0.06 nmol/g per minute; $P < 0.05$) (Figure 1B). In CSE KO mice, OVA challenge has no effect on lung H_2S production (Figure 1B).

CSE Deficiency Promotes the OVA-Induced AHR, Aggravates Airway Inflammation, and Elevates Cytokine Levels in BALF

No difference was detected in AHR in response to increased doses of MCh between WT and CSE KO control mice (Figure 2A). OVA challenge induced a significant increase in AHR in both WT and KO mice with the latter being more severe (Figure 2A). The RL in response to MCh at 12.5 mg/mL and 25 mg/mL was 2- and 1.62-fold higher, respectively in CSE KO-OVA mice in comparison to that in WT-OVA mice (13.13 ± 1.58 versus 6.52 ± 0.56 and 26.25 ± 3.47 versus 16.18 ± 0.98 cmH₂O/mL per second, respectively, both $P < 0.05$) (Figure 2A).

The number of leukocytes in BALF was not different between WT and CSE KO control mice. However, OVA challenge resulted in significantly greater counts of total cells and eosinophils in BALF in CSE KO mice than in WT mice (Figure 2B). The number of eosinophils in KO-OVA mice

was almost threefold higher than that in WT-OVA mice (148.70 ± 16.63 versus $50.66 \pm 11.35 \times 10^4$ /mL; $P < 0.05$) (Figure 2B). Histological examination revealed widespread patchy inflammatory infiltrates around peribronchial and perivascular areas in lungs of WT-OVA and KO-OVA mice with more serious infiltrates in the latter (Figure 2C).

IL-4, IL-5, IL-13, and eotaxin-1 in BALF were hard to detect or at very low levels in WT-CON or KO-CON mice and were significantly increased after OVA challenge (Figure 2, D–G). Compared to WT-OVA mice, IL-5 and IL-13 were 3.49-fold and 1.75-fold higher, respectively in KO-OVA mice (Figure 2, D and E). However, there was no significant difference in IL-4 level between WT-OVA and KO-OVA groups (Figure 2F). Among OVA-challenged mice, eotaxin-1 level in BALF was higher in KO mice than in WT mice (90.25 ± 4.23 versus 41.13 ± 3.45 pg/mL; $P < 0.05$) (Figure 2G).

NaHS Treatment Alleviates OVA-Induced AHR and Airway Inflammation and Lowers Cytokine Levels in WT Mice

NaHS treatment of WT mice attenuated OVA-induced AHR in response to MCh at 12.5 and 25 mg/mL compared with vehicle treatment (both $P < 0.05$) (Figure 3A). The numbers of total and differential cell counts including eosinophils, macrophages, and lymphocytes were significantly decreased in BALF by NaHS treatment (Figure 3B). Of note, the number of eosinophils was reduced almost by 50% after NaHS treatment compared to vehicle treatment (70.23 ± 7.41 versus $36.32 \pm 8.71 \times 10^4$ /mL; $P < 0.05$) (Figure 3B). Histological sections of the lung also showed lesser inflammatory cell infiltration in OVA-challenged WT mice after NaHS treatment (Figure 3C). In view of cytokines in BALF, NaHS treatment significantly decreased the OVA-induced increase in IL-5, IL-13, and eotaxin-1 levels compared with vehicle treatment, whereas it had no significant effect on IL-4 (Figure 3, D–G). As shown in Figure 3, pretreatment with glybenclamide did not counteract or reverse the inhibitory effect of NaHS on the OVA-induced AHR, airway inflammation, and cytokine levels in BALF.

NaHS Supplement Reduces Asthmatic Severity in KO-OVA Mice

AHR in response to MCh at 12.5 and 25 mg/mL in KO-OVA mice was decreased by 41.68% and 32.40%, respectively after NaHS treatment (10.94 ± 1.42 versus 6.38 ± 0.91 and 22.93 ± 2.57 versus 15.16 ± 1.77 cmH₂O/mL per second, respectively; both $P < 0.05$) (Figure 4A). NaHS application significantly reduced the total and differential cell counts in BALF in comparison with those with vehicle treatment (Figure 4B). Particularly, eosinophils count in KO-OVA mice was significantly reduced by 51% after NaHS treatment (105.2 ± 9.7 to $51.5 \pm 6.7 \times 10^4$ /mL; $P < 0.05$). Decreased inflammatory cell infiltrates in lung tissues were also observed after NaHS treatment in KO-OVA mice

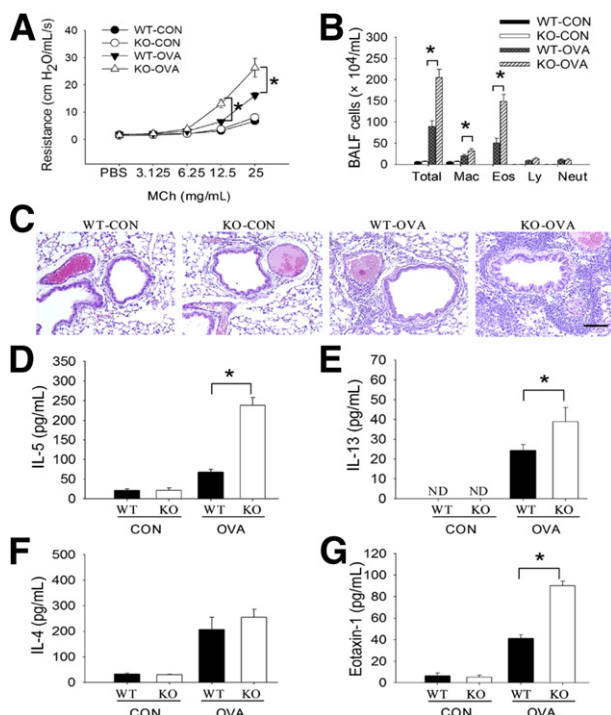


Figure 2 CSE gene deficiency and asthmatic damage. **A:** Increased AHR in KO-OVA mice. Twenty-four hours after last OVA challenge, AHR in response to inhaled methacholine (MCh) was measured by a resistance and compliance system. **B:** Total and differential cell counts in BALF. BALF was recovered and total cell counts were determined after AHR measurement. Cytospins of leukocytes were stained with Wright-Giemsa and differential cell counts were performed for 200 cells. Eos, eosinophils; Ly, lymphocytes; Mac, macrophages; Neut, neutrophils. **C:** Representative H&E staining of lung tissues. Scale bar = 100 μ m. **D–G:** Levels of IL-5, IL-13, IL-4, and eotaxin-1 in BALF were assessed by ELISA. For each group, 8 to 10 mice were used. * $P < 0.05$. ND, not detected.

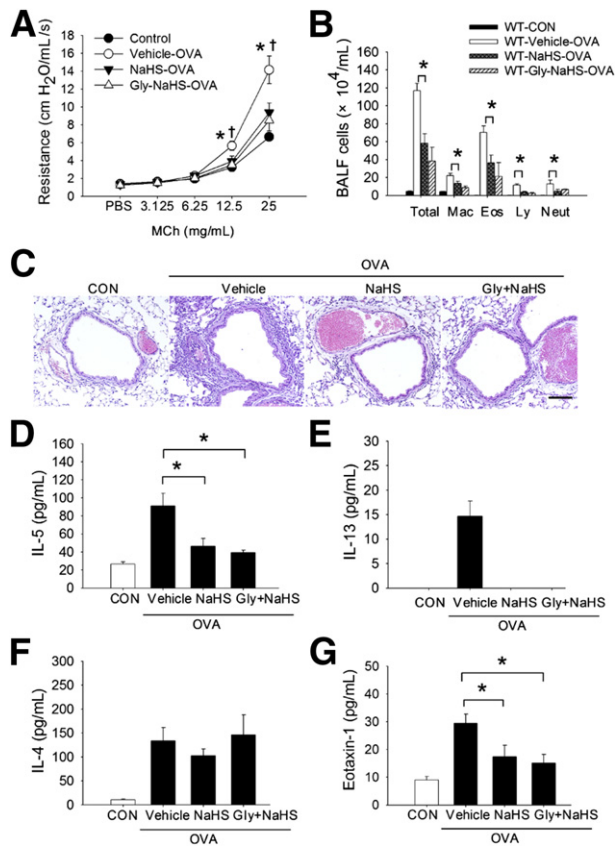


Figure 3 NaHS treatment reversed OVA-induced AHR, airway inflammation, and cytokines levels in BALF in WT mice. OVA-sensitized WT mice were injected with 14 $\mu\text{mol/kg}$ NaHS i.p., 40 $\mu\text{mol/kg}$ glibenclamide (Gly) i.p., plus 14 $\mu\text{mol/kg}$ NaHS, or vehicle (dimethyl sulfoxide) 30 minutes before and 8 hours after each OVA challenge. Sensitized mice were challenged with saline as the control. **A:** Decrease in OVA-induced AHR after NaHS treatment was not reversed by pretreatment of Gly. Twenty-four hours after the last OVA challenge, AHR in response to inhaled methacholine (MCh) was measured by a resistance and compliance system. $^*P < 0.05$ compared to WT-NaHS-OVA group; $^\dagger P < 0.05$ compared to WT-Gly-NaHS-OVA group. **B:** Total and differential cell counts in BALF. After AHR measurement, BALF was recovered and total cell counts were determined. Cytospins of leukocytes were stained with Wright-Giemsa and differential cell counts were performed for 200 cells. Eos, eosinophils; Ly, lymphocytes; Mac, macrophages; Neut, neutrophils. **C:** Representative H&E staining of lung tissues from the control group, and OVA-challenged groups treated with vehicle, NaHS alone and Gly plus NaHS. Scale bar = 100 μm . **D–G:** Levels of IL-5, IL-13, IL-4, and eotaxin-1 in BALF were assessed by ELISA. For each group, 8 to 10 mice were used. $^*P < 0.05$.

(Figure 4C). NaHS treatment significantly decreased the levels of IL-5, IL-13, and eotaxin-1 in BALF compared to vehicle treatment, whereas it had no effect on IL-4 (Figure 4D). Moreover, NaHS treatment decreased IL-13 in CSE KO-OVA mice to the basal level (Figure 4D).

CSE Deficiency Promotes But NaHS Suppresses Cell Viability and Secretion of OVA-Specific Th2 Cytokines of Lymphocytes *in Vitro*

As seen in Figure 5A, OVA stimulation increased lymphocyte viability from both WT and CSE KO mice,

which was more obvious in the later. NaHS treatment at higher concentrations ranging from 100 to 500 $\mu\text{mol/L}$ markedly reduced the viability of lymphocytes from both WT-OVA and KO-OVA mice, whereas lower concentration of NaHS at 50 $\mu\text{mol/L}$ only significantly inhibited the viability of KO lymphocytes (Figure 5A).

To further determine the effects of endogenous and exogenous H₂S on the Th2 response, we observed the secretion of OVA-specific Th2 cytokines of lymphocytes from lung draining LN in both WT-OVA and KO-OVA mice *in vitro*. Without OVA stimulation, the levels of IL-5 and IL-13 were low or not detectable in the supernatant of cultured lymphocytes (Figure 5, B and C). After OVA stimulation, a significant increase in IL-5 and IL-13 levels was observed from WT and CSE KO lymphocytes (Figure 5, B and C). Compared with WT lymphocytes, the levels of IL-5 and IL-13 from CSE KO lymphocytes were 1.14-fold and 1.60-fold higher, respectively (both $P < 0.05$). NaHS treatment at higher concentrations (100 to 500 $\mu\text{mol/L}$) markedly reduced IL-5 and IL-13 in lymphocytes from both WT-OVA and KO-OVA mice (Figure 5). NaHS at a lower concentration of 50 $\mu\text{mol/L}$ only significantly inhibited the levels of IL-5 and IL-13 from KO lymphocytes, but not from WT lymphocytes.

Discussion

CSE is a major H₂S-production enzyme in the mouse lung, although at least one other H₂S-producing enzyme (CBS)

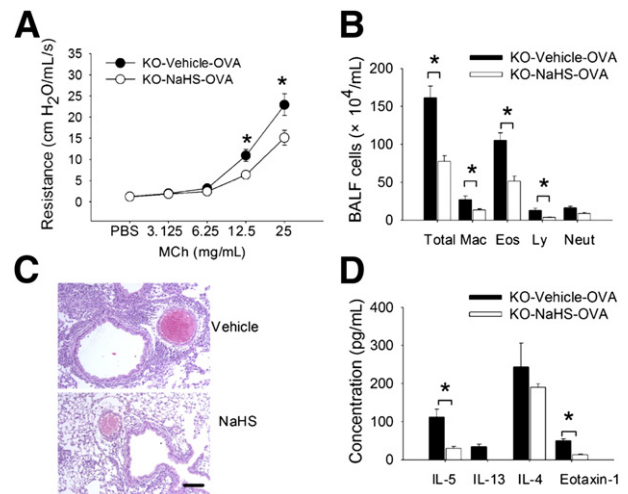


Figure 4 Reverse of asthmatic severity in CSE KO mice by NaHS supplement. OVA-sensitized CSE KO mice were given 14 $\mu\text{mol/kg}$ NaHS i.p. or vehicle 30 minutes before and 8 hours after each OVA challenge. **A:** Decrease in OVA-induced AHR in CSE KO mice after NaHS treatment. Twenty-four hours after last OVA challenge, AHR in response to inhaled MCh was measured by a resistance and compliance system. **B:** Total and differential cell counts in BALF. After AHR measurement, BALF was recovered and total cell counts were determined. Cytospins of leukocytes were stained with Wright-Giemsa and differential cell counts were performed for 200 cells. Eos, eosinophils; Ly, lymphocytes; Mac, macrophages; Neut, neutrophils. **C:** Representative H&E staining of lung tissues. Scale bar = 100 μm . **D:** Levels of IL-5, IL-13, IL-4, and eotaxin-1 in BALF. For each group, 8 to 10 mice were used. $^*P < 0.05$.

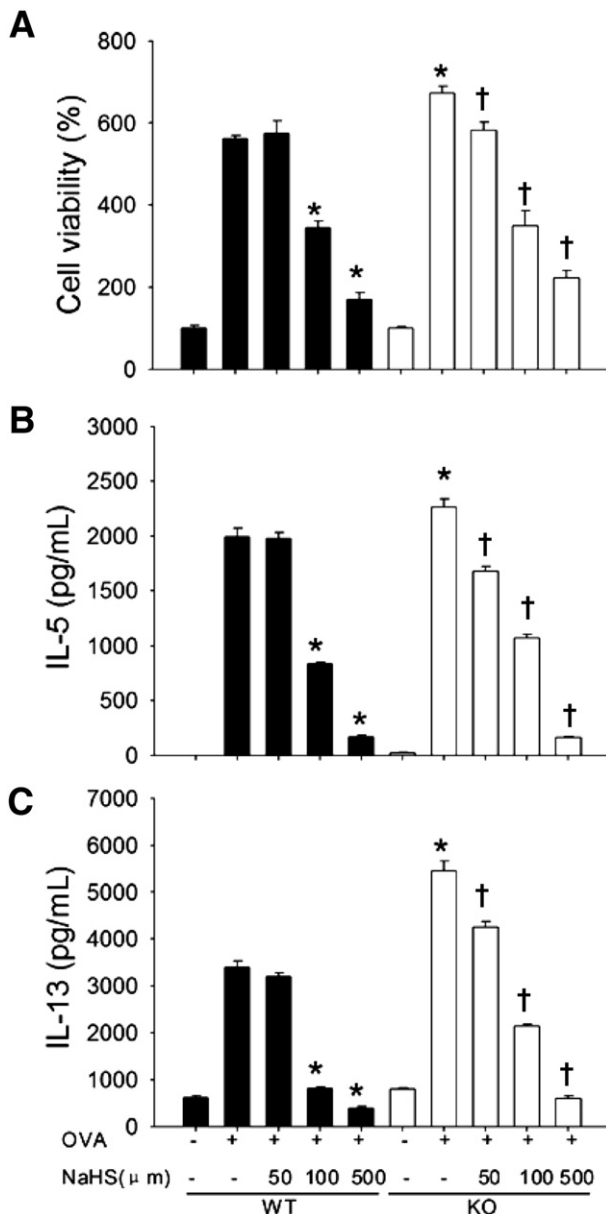


Figure 5 The effect of H₂S on the viability and Th2 cytokine production of lymphocytes. Lymphocytes from lung draining lymph node were isolated from OVA-challenged WT and CSE KO mice, and cultured in RPMI 1640 medium at 2×10^5 cells per well in 96-well plates with OVA (80 μ g/mL) or without it. NaHS at indicated concentrations was given immediately to the cell cultures. **A:** Then, 72 hours later, the cell viability was measured by using 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for assay. The lymphocytes from OVA-challenged WT mice incubated without OVA and NaHS were considered 100% viable. **B and C:** In another experiment, the supernatant was collected 72 hours after culture, and levels of IL-5 (**B**) and IL-13 (**C**) were evaluated by ELISA. The results are from three independent experiments and $n = 5$ for each independent experiment. * $P < 0.05$ compared with WT plus OVA; † $P < 0.05$ compared with KO plus OVA.

also exists in the lung.^{5,16} The lung CSE expression in WT mice was significantly reduced after the OVA challenge, accompanied with a significant decrease in H₂S production (Figure 1B). The OVA challenge, however, had no effect on

lung CBS expression, even in the condition of CSE deficiency (Figure 1A). Based on these results, it is suggested that the decrease in endogenous H₂S production in WT mice is due to reduced CSE expression after OVA challenge.

Accumulating evidence has shown altered endogenous H₂S metabolism and a potentially protective role of exogenous H₂S in many conditions of neutrophil-dominant inflammation.^{10–12} Herein, we investigated the role of the CSE/H₂S system in eosinophil-dominant airway inflammation. In CSE KO mice, CSE deficiency and lower H₂S production in the lung cause aggravated AHR, profound airway inflammation, and elevated levels of Th2 cytokines after OVA challenge (Figure 2), whereas exogenous H₂S supplement suppresses these alterations (Figure 4). Although CSE gene is present in WT mouse lungs, the expression and activity of CSE proteins (reflected by H₂S production) were inhibited by OVA challenge (Figure 1). Administration of exogenous H₂S also alleviates these OVA-mediated pathological changes in WT mice (Figure 2). These results demonstrated that CSE-generated endogenous H₂S is a protective factor against the development of OVA-induced allergic airway inflammation. Decreased lung CSE expression and H₂S production were seen in an asthma model of rats.¹⁷ Increased CSE protein expression and H₂S production, however, are often seen in other inflammatory conditions such as sepsis, endotoxic shock, acute pancreatitis, and colitis.^{7–9,11} As endogenous CSE expression and H₂S production are inducible *in vivo* and *in vitro* in different inflammatory conditions,^{7–9,23} there are different inflammatory events that may yield different patterns of CSE expression and H₂S production. How, and whether, OVA-induced inflammatory events decrease CSE expression and H₂S production merits further investigation.

Development and maintenance of AHR in asthma depend on large eosinophil infiltration and Th2 cytokine production, particularly IL-13 and to a lesser extent IL-4.^{24–27} In the present study, CSE deficiency significantly increases OVA-induced AHR to inhaled MCh. This is associated with profound eosinophilic airway inflammation (Figure 2B) and elevated IL-13 (Figure 2E). In addition, exogenous H₂S supplement decreased or reversed OVA-induced AHR in WT-OVA mice or KO-OVA mice, respectively, which was also corrected with reduced IL-13 production and decreased eosinophils. Although it is also involved in the development of AHR in allergic asthma, IL-4 does not play an important role in the protective role of H₂S against OVA-induced AHR in the present experiment. Our study and others have shown that H₂S relaxes vascular smooth muscle by increasing K_{ATP} channel currents and hyperpolarizing membrane.^{22,28} H₂S, however, can also relax nonvascular smooth muscle in a K_{ATP} channel-independent manner.^{29,30} Chen et al³⁰ have shown that exogenous NaHS directly relax airway smooth muscle pre-contracted with acetylcholine and potassium chloride, which cannot be reversed by glybenclamide. In our study, glybenclamide did not affect the inhibitory effect of exogenous H₂S on

OVA-induced AHR. Taken together, these data imply that endogenous or exogenous H₂S reduces AHR via relaxing airway smooth muscle in a K_{ATP} channel-independent manner and inhibiting Th2 cytokine production and eosinophil infiltration.

Eotaxins and Th2 cytokines are two important factors for eosinophil accumulation into asthmatic lungs.^{15,31,32} In CSE KO mice, the OVA challenge resulted in a significant increase in eosinophilic airway inflammation, accompanied with a large production of IL-5, IL-13, and eotaxin-1. In contrast, exogenous H₂S supplementation reduced, even rescued, OVA-induced eosinophilic airway inflammation in WT-OVA mice or in KO-OVA mice, respectively. Accordingly, cytokine levels of IL-5, IL-13, and eotaxin-1 were significantly decreased after NaHS treatment. It is suggested by these results that the loss (or insufficiency) of CSE expression in KO mice or WT-OVA mice results in deficiency of endogenous H₂S production, which in turn promotes Th2-associated cytokine expression and recruits eosinophils and other inflammatory cells into the allergic airway. The IL-4 level in BALF maintains a similar high level regardless of CSE deficiency or exogenously supplied H₂S after OVA challenge. This suggests a less important role of IL-4 in inhibiting the effect of H₂S against the pathogenesis of asthma. The inhibition of OVA-induced cytokine secretion of IL-5 and IL-13 by endogenous or exogenous H₂S from *in vivo* results was further confirmed by *in vitro* experiments. When lymphocytes from WT-OVA and KO-OVA mice were stimulated with OVA, increased cytokine levels of IL-5 and IL-13 were seen in the supernatant in the CSE deficiency condition, whereas the NaHS treatment reduced these cytokine levels (Figure 5, B and C). The pattern of lymphocyte viability in the condition of CSE deficiency or NaHS treatment was consistent with that of Th2 cytokine levels (Figure 5). CSE deficiency increased the lymphocytes viability, whereas NaHS treatment inhibited it (Figure 5A). It is suggested by these results that inhibition of lymphocyte viability or proliferation by NaHS treatment may contribute to the lower levels of Th2 cytokines observed in the present study. In addition, NaHS at 50 μ mol/L significantly inhibited the cell viability and secretion of IL-5 and IL-13 of CSE-KO lymphocytes, but not of WT lymphocytes. This implies that NaHS-induced inhibition in these parameters is more sensitive in the condition of endogenous H₂S deficiency. Similarly, an increased sensitivity for proliferation inhibition in CSE-KO smooth muscle cells by NaHS treatment has been reported in our previous study.²⁰ It has been reported that H₂S inhibits leukocyte adherence to and migration across the vascular endothelium, playing an important anti-inflammatory role.³³ In the present study, endogenously-generated or exogenously-supplied H₂S inhibits the recruitment of inflammatory cells into the OVA-induced lung (Figure 2, B and C, Figure 3, B and C, and Figure 4, B and C). These results suggest that inhibition of inflammatory cell migration across the vascular endothelium may be another important mechanism for the observed

effects of H₂S in asthma. The exact molecular mechanism underlying the inhibitory role of H₂S in inflammation is still not well understood. Studies have shown that H₂S is involved in regulation of anti-inflammation through inhibition of NF- κ B activation.^{12,34} Recently, a direct effect of endogenous H₂S on NF- κ B activation has been reported.³⁵ CSE-generated H₂S directly sulphydrates the p65 subunit of NF- κ B at cysteine-38, and then antagonizes tumor necrosis factor- α -induced cell death.³⁵ NF- κ B also plays an important role in allergic inflammation.^{36,37} Thus, it would be interesting to investigate whether H₂S plays its anti-asthmatic role via sulphydration of NF- κ B.

Our animal asthma model in the current study is suitable for the study of acute inflammatory events of human asthma,³⁸ although it cannot mimic the chronic features of human asthma as with epithelial detachment, increased smooth muscle mass, or peribronchial collagen deposit.³⁹ Next we will investigate the effect of endogenous H₂S on the development of airway remodeling during asthma with CSE KO mice using an OVA-mouse model recently reported.⁴⁰

In summary, CSE is a main H₂S-production enzyme in the mouse lung. CSE-derived endogenous H₂S is a protective factor against the development of asthma via inhibition of Th2 cytokines and eotaxin-1 secretion. Exogenous H₂S supplement suppresses asthmatic pathogenesis. These results establish, for the first time, a critical role of endogenous CSE/H₂S system in the development of allergic asthma, and shed insight into a new therapeutic potential for allergic disorders such as asthma through targeting the endogenous CSE/H₂S pathway.

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