Involvement of endogenous hydrogen sulfide in cigarette smoke-induced changes in airway responsiveness and inflammation of rat lung

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ABSTRACT

Hydrogen sulfide (H₂S), recently considered the third endogenous gaseous transmitter, may have an important role in systemic inflammation. We investigated whether endogenous H₂S may be a crucial modulator in airway responsiveness and inflammation in a rat model of chronic exposure to cigarette smoke (CS). Rats randomly divided into control and CS-exposed groups were treated with or without sodium hydrosulfide (NaHS, donor of H₂S) or propargylglycine (PPG, inhibitor of cystathionine-γ-lyase [CSE], an H₂S-synthesizing enzyme) for 4-month exposure. Serum H₂S level and CSE protein expression in lung tissue were higher, by 2.04- and 2.33-fold, respectively, in CS-exposed rats than in controls (P < 0.05). Exogenous administration of NaHS to CS-exposed rats alleviated airway reactivity induced by acetylcholine (Ach) or potassium chloride (KCl) by 17.4% and 13.8%, respectively, decreased lung pathology score by 32.7%, inhibited IL-8 and TNF-α concentrations in lung tissue by 34.2% and 31.4%, respectively, as compared with CS-exposed rats (all P < 0.05). However, blocking endogenous CSE with PPG in CS-exposed rats increased airway reactivity induced by Ach or KCl, by 24.1% and 24.5%, respectively, and aggravated lung pathology score, by 44.8%, as compared with CS-exposed rats (all P < 0.01). Incubation in vitro with NaHS, 1–3 mmol/L, relaxed rat tracheal smooth muscle precontracted by Ach or KCl. However, the NaHS-induced relaxation was not blocked by glibenclamide (10⁻⁴ mol/L), L-NAME (10⁻⁴ mol/L) or denudation of epithelium. Endogenous H₂S may have a protective role against inflammation and bronchodilation in chronic CS-induced pulmonary injury.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation due to chronic bronchitis or emphysema. Cigarette smoking is the major risk factor of COPD [1]. It induces an inflammatory response in the airways that might play a key role in the pathogenesis of COPD. Furthermore, multi-center clinical trials showed that current smokers with functional evidence of early COPD have airway hyper-responsiveness [2,3]. Similarly, a dose-dependent effect of cigarette smoking on airway responsiveness was reported [4]. Airway hyper-responsiveness is an independent predictor of mortality in COPD patients [4]. However, the mechanisms of such hyper-responsiveness in COPD are less understood than are those in bronchial asthma.

The major effects of hydrogen sulfide (H₂S), long recognized as a toxic gas with a strong odor of rotten eggs in water pollution and industrial air pollution, are intoxication of the central nervous system and inhibition of the respiratory system [5]. Recently, H₂S was found endogenously generated in various mammalian tissues and may be a functional regulator in nervous and cardiovascular systems. H₂S is synthesized endogenously in various mammalian tissues by two pyridoxal-5'-phosphate-dependent enzymes responsible for metabolizing L-cysteine: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). The substrate of CBS and CSE, L-cysteine, can be derived from dietary sources or can be liberated from endogenous proteins. It can also be synthesized endogenously from L-methionine through the trans-sulphuration pathway, with homocysteine being an intermediate in the process. Because of its endogenous metabolism and physiological functions, H₂S is well positioned in the novel family of endogenous gaseous transmitters and, in addition to nitric oxide (NO) and carbon monoxide (CO),
might be the third endogenous signaling gasotransmitter [5]. Pulmonary tissue is rich in the H2S synthase, cystathionine γ-lyase (CSE), and generates endogenous H2S. Our previous study showed endogenous H2S concentration increased in patients with stable COPD and decreased in patients with acute exacerbation of COPD; therefore, endogenous H2S may be involved in the pathogenesis of airway inflammation and airflow obstruction in COPD [6,7].

In this study, we investigated whether endogenous H2S may be a crucial mediator in airway hyper-responsiveness and airway inflammation in a chronic cigarette smoke (CS)-exposed rat model.

2. Methods

2.1. Chronic cigarette smoke-exposed rat model

All animal care and experimental protocols were in compliance with the PR China Animal Management Rule (documentation 55, 2001, Ministry of Health of PR China) and the Third Hospital, Peking University Guide for the Care and Use of Laboratory Animals.

Male Sprague–Dawley rats weighing 200–250 g were supplied by the Animal Center, Health Science Center, Peking University, and randomly divided into six groups (each n = 6) for treatment: control, CS, sodium hydrosulfide (NaHS; an H2S donor), NaHS + CS, propargylglycine (PPG; CSE inhibitor), and PPG + CS. The rats in the CS groups were exposed to whole-body mainstream CS generated from 20 commercial unfiltered cigarettes, which yielded 1.2 mg nicotine, 17 mg tar each, in a dynamic smoke exposure box (diameter 700 mm, Tianjin Hope Corp., Tianjin, China) for 4 h/day, 7 days/week. The PM 2.5 levels were 25 mg/m³ after the exposure. Freshly prepared NaHS (14 μmol/kg; Sigma) and PPG (37.5 μmol/kg, Sigma) were intraperitoneally administered 30 min before CS exposure in the NaHS + CS and PPG + CS groups, respectively. The control group underwent an identical schedule for sham exposure, but received air rather than smoke. The NaHS and PPG groups underwent the same schedule as NaHS + CS and PPG + CS groups, respectively, but received air rather than smoke for sham exposure. Rats were anesthetized by intraperitoneal injection of 20% (w/v) urethane (5 mL/kg) 24 h after 4-month exposure.

2.2. Measurement of airway responsiveness [8]

The trachea of rats was removed and put into ice-cold Krebs–Henseliet (K-H) solution of the following composition (mmol/L): 110 NaCl, 25 NaHCO3, 4.8 KCl, 1.2 KH2PO4, 1.2 MgSO4, 11 D(+)-glucose, 2.5 CaCl2, pH 7.4. After removing adhering fat and connective tissue, the trachea was cut into several 3-cartridge segments.

Tissue was then suspended in a 10-mL organ bath containing K-H solution. The K-H solution was maintained at 37 °C and gassed with 95% O2 and 5% CO2. Tissue was suspended under isotonic tension of 0.5 g and allowed to equilibrate for at least 1 h, during which the K-H solution was changed every 15 min.

Concentration–effect curves were obtained by exposing the tissue to cumulative increasing concentrations of acetylcholine (Ach) or potassium chloride (KC1). Airway responsiveness was measured by use of a Powerlab polygraph (AD Instruments, Australia).

2.3. Histology analysis

The left lung tissues were fixed with being injected 2 mL paraformaldehyde into the bronchi, and then put into paraformaldehyde solution and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E), periodic acid-Schiff, and Masson trichrome. The following nine parameters were evaluated as described [9], with modification: constriction or occlusion of the small airway lumen, abscission or ulceration, squamous cell metaplasia and goblet cell proliferation of epithelium, inflammatory cell infiltration, pigment deposits, connective tissue proliferation and smooth muscle hypertrophy in the airway wall, and emphysema of lung tissue. Three membranous bronchioles of each rat were selected at random for evaluation. Scores from 0 (normal) to 3 (most abnormal) were assigned to each pathologic feature. The total pathologic score is the sum of the score for all nine individual parameters. Scoring was performed by two experienced pathologists blinded to testing. Two scores were assigned, and the mean value was recorded.

2.4. Measurement of IL-8 and TNF-α concentrations in lung tissue

Lung tissues were homogenized on ice in 1:10 (w/v) 0.01 mol/L of ice-cold potassium phosphate buffer (pH 7.2) with use of a glass homogenizer. The homogenates were centrifuged at 4000 rpm for 10 min. The liquid supernatants were obtained for testing IL-8 and TNF-α concentrations. Cytokine level was assayed using double-antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA), following the manufacturer’s instructions. ELISA kits for detection of IL-8 and TNF-α were purchased from R&D systems (Minneapolis, MN, USA). They were expressed by pg/g lung tissue.

2.5. Measurement of H2S content in plasma and lung tissue

H2S content in plasma was measured with use of a sulfide-sensitive electrode (PX5-270; Shanghai). Briefly, sulfide antioxidant buffer (SAOB II) was added into the standard or sample at a ratio of 1:1, then stirred thoroughly. Electrodes were rinsed in distilled water, blotted dry and placed into the standard and sample. When a stable reading was displayed, the mV value was recorded. The H2S concentration was calculated against the calibration curve of the standard H2S solution [6]. The H2S content in lung tissue was measured as described previously, with modification [10]. Briefly, lung tissues were homogenized in a 10-fold volume (w/v) of ice-cold potassium phosphate buffer (pH 6.8). The reaction was performed in a 25 mL Erlenmeyer flask. Cryovial test tubes (2 mL) were used as the center wells, each containing 0.5 mL of 1 mol/L NaOH. The reaction mixture contained lung tissue homogenate and 1 mol/L HCl at a ratio of 1:5. The flasks containing reaction mixture and central wells were flushed with N2 for 30 s before being sealed with a double layer of parafilm. The reaction was initiated by transferring the flask from ice to a 37 °C shaking water bath. After incubation at 37 °C for 4 h, the contents of the central wells were transferred to 10-mL beakers, each containing 0.5 mL antioxidant solution. Then the solution was analyzed by use of sulfide-sensitive electrodes (PX5-270; Shanghai), and the lung tissue H2S content was calculated against the calibration curve of the standard H2S solution [6].

2.6. Protein expression of CSE

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 benzamidine. 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 membranes were blocked overnight at 4 °C in 10% (w/v) dried milk protein (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 presence of monoclonal anti-CSE (1:2000) (Abnova; Taiwan), washed extensively, then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (1:4000) (Santa Cruz, USA). The bands were visualized by use of an enhanced chemiluminescence kit following the manufacturer’s instructions (Applygen Technologies; Beijing). The expression of the housekeeping protein β-actin was measured with use of an anti-β-actin antibody (Santa Cruz Bio-technology), and results were expressed as a ratio of the expression of the measured protein to that of β-actin [10].

2.7. Effect of NaHS on airway tension in vitro

The tracheae in normal rats (n = 6) were prepared as described previously. To denude epithelium, the epithelium of the trachea was removed by inserting a moistened cotton wire into the lumen of trachea and gently rubbing with a corkscrew motion 5–6 times. The removal of epithelium was confirmed by H&E staining. The effects of cumulatively applied NaHS were observed in resting preparations and in preparations precontracted with Ach, 10–4 mol/L, or KCl, 80 mmol/L (ATP-sensitive K+ channel [KATP] inhibitor); L-NAME, 10–4 mol/L (inhibitor of nitric oxide synthase [NOS]), and ODQ, 1 μmol/L (inhibitor of soluble guanylyl cyclase [sGC]) were applied to the bath solution 15 min before the addition of Ach (11,12). To examine whether the epithelium was involved in the relaxant effect of NaHS, only animals in the epithelium-denuded group were denuded of epithelium. To quantify the essential NaHS-induced inhibition of muscle contraction, concentration–effect curves for Ach and KCl were calculated after preparations were pre-incubated with NaHS for 30 min. The relaxant activity of NaHS in the preparations precontracted with Ach or KCl was represented as a percentage of the initial tension before the addition of NaHS.

2.8. Statistical analysis

Data are expressed as mean ± SD. Comparisons among groups involved one-way ANOVA followed by Student–Newman-Keuls test. Comparisons between two groups involved unpaired Student’s t test. SPSS v10.0 (SPSS, Chicago, IL, USA) was used for analysis. P < 0.05 was considered statistically significant.

3. Results

3.1. Chronic exposure to CS induced H₂S/CSE upregulation

As compared with controls, the NaHS-alone and NaHS + CS groups showed increased plasma levels of H₂S, by 2.70-fold and 3.29-fold, respectively, but the PPG-alone group showed decreased level, by 77.1% (all P < 0.01) (Fig. 1A). Exogenous administration of NaHS in NaHS + CS group increased the plasma level of H₂S, by 61.4%, whereas blockade of endogenous CSE with PPG in the PPG + CS group decreased the level, by 50.9%, as compared with the CS-alone group (all P < 0.01). Although the H₂S level of lung tissue in each group did not significantly differ (P > 0.05) (Fig. 1B), plasma H₂S level and CSE protein expression in lung tissue were higher, by 2.04- and 2.33-fold, respectively, for the CS than control group (all P < 0.05) (Fig. 1C and D).

3.2. Administration of NaHS alleviated and PPG aggravated lung injury and airway hyper-responsiveness in rats exposed to CS

In our study, CS induced necrosis and erosion of bronchial epithelium, metaplasia of airway goblet cells, inflammatory cell infiltration, proliferation of airway smooth muscles and fibrous connective tissues of airway wall, and emphysema to different degrees. The lung pathology score for the CS group was 2.63-fold higher than for the control group (Fig. 2 and 3). Controls and the NaHS-alone group did not differ in lung pathology score (P > 0.05), whereas the score was 3.29-, 5.26-, and 2.44-fold higher, respectively, in the PPG-alone, PPG + CS, and NaHS + CS groups.
than control group (all $P < 0.01$) (Fig. 2). The lung pathology score was lower by 32.7% with exogenous administration of NaHS in the NaHS + CS group, whereas blockade of endogenous CSE by PPG in the PPG + CS group increased the score by 44.8% than in the CS group (all $P < 0.01$).

IL-8 concentration in lung tissue in CS group was higher by 60.2% than control group ($P < 0.05$) (Table 1). IL-8 and TNF-$\alpha$ concentrations in lung tissue were lower by 34.2% and 31.4%, respectively, in NaHS + CS group than in CS group (all $P < 0.05$). Both pro-inflammatory cytokines were lower by 39.5% and 34.3%, respectively, in NaHS + CS group than in PPG + CS group (all $P < 0.05$) (Table 1).

Trachea from rats in the CS group showed significantly higher Ach or KCl-induced contraction than for controls ($P < 0.05$). The contraction induced by 3 $\times$ $10^{-4}$ mol/L Ach was 1.49 ± 0.13 g and 2.24 ± 0.27 g for the control and CS groups, respectively ($P < 0.05$). Similarly, the contraction induced by 75 mmol/L KCl was 1.833 ± 0.053 g and 1.498 ± 0.049 g, respectively ($P < 0.05$) (Fig. 4). Airway hyper-responsiveness induced by Ach or KCl did not differ between the NaHS-alone and control groups (all $P > 0.05$) (Fig. 4). Exogenous administration of NaHS in the NaHS + CS group alleviated airway hyper-responsiveness induced by Ach or KCl by 17.4% and 13.8%, respectively, as compared with control group (all $P < 0.05$), and blockade of endogenous CSE with PPG in the PPG + CS group increased airway responsiveness induced by Ach or KCl by 24.1% and 24.5%, respectively (all $P < 0.01$).

3.3. Relaxing effect of NaHS on airway smooth muscle in vitro

In rat tracheal rings without precontraction, incubation with the H$_2$S donor NaHS, applied cumulatively in a range of $10^{-6}$–$10^{-2}$ mol/L, did not affect muscle tension. The medium pH values after incubation for the NaHS and control groups were 7.368 ± 0.010 and 7.398 ± 0.011, respectively ($P > 0.05$). However, when the preparations were precontracted with Ach, 10$^{-4}$ mol/L, or KCl, 80 mmol/L (the level of force generated by Ach and KCl was 1.833 ± 0.053 g and 1.498 ± 0.049 g, respectively), NaHS, (1–3)$\times$ $10^{-3}$ mol/L, produced marked relaxant activity in a concentration-dependent manner (Fig. 5A). Pre-exposure of the tracheal ring to NaHS, 1$\times$ $10^{-3}$ or 3$\times$ $10^{-3}$ mol/L, did not alter the contractions induced by Ach or KCl (Fig. 5B). NaHS, 3$\times$ $10^{-3}$ mol/L, incubation had no effect on histological structure (Fig. 5C).

The concentration–response curve for the relaxant effect of NaHS in the rat trachea was not affected by pre-incubation with glybenclamide, 10$^{-4}$ mol/L; L-NAME, 10$^{-4}$ mol/L; or ODQ, 1 $\mu$mol/L (Fig. 6). To test whether the relaxation effect of H$_2$S was mediated by epithelium, epithelium was removed from isolated trachea. However, the relaxant effect was not affected in such trachea (Fig. 6).

4. Discussion

The present study suggested that chronic exposure to CS can induce rat pulmonary injury and airway hyper-responsiveness.

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Endogenous H₂S generation was upregulated in the CS group. Administration of NaHS alleviated and PPG, inhibitor of CSE, aggravated lung injury and airway hyper-responsiveness in the CS group. The NaHS-induced relaxation in the rat tracheal was not blocked by inhibitors of KATP, NOS, sGC, or denudation of epithelium.

H₂S has been generally considered a toxic gas found in the contaminated environmental atmosphere. In recent years, however, increasing studies have suggested that endogenous H₂S in physiological concentration is a gasotransmitter. H₂S was 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. The endogenous H₂S/CSE 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 [11–15]. Our previous study showed increased endogenous H₂S concentration in patients with stable COPD and decreased concentration in those with acute exacerbation of COPD; therefore, endogenous H₂S may be involved in the pathogenesis of airway inflammation and airflow obstruction in COPD [6]. CSE was mainly expressed in airway and vascular...
smooth muscle cells in rat lung tissue [10]. Levels of endogenous H$_2$S were decreased in pulmonary tissue in ovalbumin (OVA)-treated rats [10]. Exogenous administration of NaHS alleviated airway inflammation and airway remodeling: peak expiratory flow increased, goblet cell hyperplasia and collagen deposition score decreased, and decreased number of total cells recovered from bronchoalveolar lavage fluid treatment with influx of eosinophils and neutrophils. The H$_2$S levels of serum and lung tissue were positively correlated with peak expiratory flow and negatively correlated with the level of eosinophils and neutrophils in bronchoalveolar lavage fluid, score of lung pathology. NaHS treatment significantly attenuated pulmonary inducible nitric oxide synthase (iNOS) activation in OVA-treated rats. These results suggest that the H$_2$S/CSE pathway plays an anti-inflammatory and anti-remodeling part in asthma pathogenesis and could be a novel target in prevention and treatment of asthma [10]. However, whether endogenous H$_2$S is involved in CS-induced changes in the structure and function of rat lung was unknown.

Cigarette smoking is the major risk factor of COPD. Here we found that chronic CS exposure can induce necrosis and erosion of bronchial epithelium, metaplasia of airway goblet cells, airway lumen occlusion by mucus and cells, inflammatory cell infiltration, proliferation of airway smooth muscle and fibrous connective tissues of the airway wall, and different degrees of emphysema, all characteristics of COPD. IL-8 concentration in lung tissue was increased in CS group. The airway responsiveness induced by Ach or KCl was increased in CS group. These results were similar to those in the literature [16]. CSE protein expression in lung tissues and the H$_2$S levels of plasma were increased in the CS group. These results suggest that the H$_2$S/CSE pathway in lung tissues was upregulated after chronic CS exposure in rats. It is known that the H$_2$S level of lung tissue may be influenced by many factors, including CSE activity, CSE protein expression, and mRNA expression. Further study need to be done to illustrate the factors influencing the generation of CSE/H$_2$S pathway in lung tissues.

In the present study, the lung pathology score was not affected with NaHS-alone treatment but was increased with the CSE inhibitor PPG alone as compared with controls. Exogenous administration of NaHS reduced the lung pathology score, and blockade of endogenous CSE with PPG increased the score, as compared with CS group. IL-8 and TNF-α levels in lung tissue were lower in NaHS + CS group than in CS group. These results indicated that H$_2$S may have provided pulmonary protection in terms of lung morphology and pro-inflammatory cytokines after chronic CS exposure.

One of the main functions of H$_2$S in mammalian peripheral tissues is to modulate smooth muscle tone. For example, H$_2$S can relax rat aortic tissues and mesenteric artery beds [17–19]. However, the role of H$_2$S in airway responsiveness in a rat model of chronic CS exposure was unknown. We investigated the responsiveness of airway smooth muscles to Ach and KCl and found that...
The mechanism of bronchodilation triggered by H$_2$S is unclear. Previous study showed that H$_2$S at relatively high concentrations relaxes vascular smooth muscle, seems to involve a small, variable component of NO-related mechanisms, and have a K$_{ATP}$-channel factor-like effect. H$_2$S also induces intracellular acidification via activation of Cl$^-$/HCO$_3$$^-$ exchanger, which is responsible, at least in part, for H$_2$S-mediated vasorelaxation. H$_2$S-mediated vasorelaxation also depends in part on calcium entry. H$_2$S facilitates erectile function in part through the cyclic adenosine monophosphate pathway [15,18,19,28,29]. In this study, the concentration–response curve for the relaxant effect of NaHS in the rat trachea was not affected by the K$_{ATP}$ inhibitor glibenclamide, the NOS inhibitor L-NAME, or the sGC inhibitor ODQ. To test whether the relaxation effect of H$_2$S was mediated by epithelium, epithelium was removed from isolated trachea, but the relaxant effect was not affected in such trachea. Obviously, the mechanism for trachea response to H$_2$S is not clear. Differences in distributions of H$_2$S targets, K$_{ATP}$ channel densities, sensitivities of voltage-dependent Ca$^{2+}$ channels to intracellular calcium levels may be mechanisms for further exploration. More in-depth analyses to elucidate the mechanisms in bronchodilation triggered by H$_2$S are in progress.

In conclusion, our study showed that exogenous administration of NaHS could alleviate airway hyper-responsiveness, lung pathology changes and pro-inflammatory cytokines expression induced by chronic exposure to CS in rats, and those changes were aggravated when endogenous H$_2$S was inhibited. In vitro, NaHS relaxed the rat tracheal smooth muscle precontracted with Ach or KCl. We suggest a possible protective role of anti-inflammation and bronchodilation for H$_2$S in the pathogenesis of COPD due to chronic CS exposure.

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