



Hydrogen sulfide inhalation ameliorates allergen induced airway hypereactivity by modulating mast cell activation



Fiorentina Roviezzo^{a,1}, Antonio Bertolino^{a,1}, Rosalinda Sorrentino^b, Michela Terlizzi^b, Maria Matteis^d, Vincenzo Calderone^c, Valentina Mattera^a, Alma Martelli^c, Giuseppe Spaziano^d, Aldo Pinto^b, Bruno D'Agostino^d, Giuseppe Cirino^{a,*}

^a Università di Napoli Federico II, Italy

^b Dipartimento di Farmacia, Università di Salerno, Italy

^c Dipartimento di Farmacia Università di Pisa, Italy

^d Dipartimento di Medicina Sperimentale, Sezione di Farmacologia L. Donatelli, Seconda Università degli Studi di Napoli, Italy

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ABSTRACT

Compelling evidence suggests that hydrogen sulfide represents an important gaseous transmitter in the mammalian respiratory system. In the present study, we have evaluated the role of mast cells in hydrogen sulfide-induced effects on airways in a mouse model of asthma. Mice were sensitized to ovalbumin and received aerosol of a hydrogen sulfide donor (NaHS; 100 ppm) starting at day 7 after ovalbumin challenge. Exposure to hydrogen sulfide abrogated ovalbumin-induced bronchial hypereactivity as well as the increase in lung resistance. Concomitantly, hydrogen sulfide prevented mast cell activity as well as FGF-2 and IL-13 upregulation. Conversely, pulmonary inflammation and the increase in plasmatic IgE levels were not affected by hydrogen sulfide. A lack of hydrogen sulfide effects in mast cell deficient mice occurred. Primary fibroblasts harvested from ovalbumin-sensitized mice showed an increased proliferation rate that was inhibited by hydrogen sulfide aerosol. Furthermore, ovalbumin-induced trans-differentiation of pulmonary fibroblasts into myofibroblasts was reversed. Finally, hydrogen sulfide did abrogate in vitro the degranulation of the mast cell-like RBL-2H3 cell line. Similarly to the in vivo experiments the inhibitory effect was present only when the cells were activated by antigen exposure. In conclusion, inhaled hydrogen sulfide improves lung function and inhibits bronchial hyper-reactivity by modulating mast cells and in turn fibroblast activation.

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1. Introduction

Compelling evidence suggests that hydrogen sulfide represents an important gaseous transmitter in the mammalian respiratory system [1]. Hydrogen sulfide is an endogenous gaseous transmitter that is produced in many tissues primarily by cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE), two PLP dependent enzymes. A third enzyme involved in hydrogen sulfide production is 3-mercaptopyruvate sulfur transferase (MST) that also produces hydrogen sulfide but does not require PLP [2,3]. These enzymes are widely expressed in pulmonary and airway tissues, the expression patterns and the extent of these enzymes are variable depending

on the species and cell types [4]. It has been demonstrated that administration of the hydrogen sulfide-donor, sodium hydrosulfide (NaHS) directly relaxes airway smooth muscle [5]. In addition, intraperitoneal administration of an hydrogen sulfide donor or an inhibitor of hydrogen sulfide biosynthesis alleviates or aggravates allergen-induced airway hypereactivity, respectively [6,7]. Studies performed using CSE KO mice have further confirmed that lower hydrogen sulfide levels worsen airway hyperresponsiveness [7]. Recently, Huang et al. [5] showed that treating mice with an hydrogen sulfide donor by aerosol, airway hyperresponsiveness (AHR) was significantly relieved. This effect on airway hypereactivity has been associated to a direct relaxant effect of hydrogen sulfide on airway smooth muscle. Moreover, Zhang et al. [7] demonstrate that systemic administration of hydrogen sulfide donor, in addition to the direct relaxing effect, modulates eosinophil infiltration as well as Th2 cytokine production. Hydrogen sulfide can relax also human airway smooth muscle [8] and a positive correlation among

* Corresponding author.

E-mail address: cirino@unina.it (G. Cirino).

¹ These authors have equally contributed.

the decline in lung function, the decreases in CSE expression and the endogenous plasma hydrogen sulfide concentrations has been found in asthmatic patients [9].

AHR is the main asthma feature. Several causative factors have been proposed such as chronic inflammation, airway remodeling, smooth muscle growth and epithelial damage, but the mechanisms are not as yet clear [10]. This is probably because AHR is multifactorial and therefore different in distinct asthma phenotypes [11]. An essential role of mast cells in the development of mouse airway hyperresponsiveness has been widely proposed. It is well established that cross-linking of IgE Abs on mast cells by antigen triggers the release of chemical mediators, which in turn cause the early allergic reactions. Activation of mast cells also leads to the synthesis of cytokines, which in turn contribute to chronic inflammation. Several studies have demonstrated elevated number of mast cells in different sites in the lung of the asthmatic patients and their correlation with inflammation and impairment of pulmonary function [12–14]. Recently it has been also demonstrated that hydrogen sulfide prevents heart failure development via inhibition of renin release from mast cells in isoproterenol-treated rats [15].

In light of these findings, we investigated if the hydrogen sulfide effect on airways involves mast cells. The study has been performed by using a well known murine model of asthma induced by ovalbumin. The contribution of mast cells in the development of AHR has been also assessed by using mast cell-deficient mice. Hydrogen sulfide has been administered by aerosol and both airway reactivity and pulmonary inflammation have been evaluated.

2. Material and methods

2.1. RBL-2H3 cell culture and β -hexosaminidase measurement

RBL-2H3 (Rat Basophilic Leukemia Mast Cell Line; Japan Health Sciences Foundation) [16] were sensitized with the monoclonal anti-dinitrophenyl antibody anti-DNP IgE (0.50 μ g/ml). The H₂S-donor NaHS (10, 100 μ M and 1 mM; 5 min) or the vehicle were added after 24 h. RBL-2H3 cell degranulation was induced by (i) the antigen dinitrophenyl-seralbumin 10 ng/ml (DNP); (ii) ionomycin 1 μ M and (iii) thapsigargin 1 μ M. Triton-X-100 was added to cause exhaustive release of β -hexosaminidase. All reagents were purchased from Sigma-Aldrich, Milan Italy.

2.2. Mice

BALB/c, mast cell-deficient Kit^{W-sh/W-sh} mice [17,18] and C57Bl6/J mice (Charles River) were housed in a controlled environment and provided with standard rodent chow and water. All mouse strains (20–25 g) were housed with a 12 h light dark cycle and were allowed food and water ad libitum. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986). All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and were approved by the local committee.

2.2.1. Antigen exposure and drug treatment of mice

Mice received subcutaneous administration of ovalbumin (OVA, 100 μ g) adsorbed onto Al(OH)₃ at day 0 and 7. In a preliminary screening, we determined that the optimal dose to be given by aerosol of hydrogen sulfide was 100 ppm of NaHS. Rodents were exposed to aerosolized NaHS or vehicle for up to 5 min, daily for 2 weeks. NaHS was administered to mice using an aerosol exposure device (In Tox Products, N. Mex.). The system consisted of a central chamber having separate aerosol supply and exhaust paths. The central chamber had 24 ports that were directly connected to the

aerosol supply system. Mice were placed into individual aerosol exposure tubes and restrained with an adjustable push plate and end cap assembly so they could not turn around or back away from the end of the tube. The restraint tubes containing the mice were loaded onto ports on the central chamber and the air flow adjusted to 1 l/min. The air (breathable quality air) flow to the PARI LC Star nebulizer was held constant at 6.9 l/min. NaHS nebulized at 0.2 ml/min and delivered to the mice via the aerosol supply path. Additional air (hereafter referred to as dilution air) was delivered to nebulizer (where) to balance the over pressure of the air used to deliver aerosolized drugs to the mice. Twenty four hours after the last administration mice were anesthetized and subjected to euthanasia (Fig. 2A).

2.3. Airway reactivity

2.3.1. Bronchial reactivity

Mice were sacrificed and bronchi were rapidly dissected and cleaned from fat and connective tissue. Rings of 1–2 mm length were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O₂–5% CO₂) Krebs solution at 37 °C and mounted to isometric force transducers (type 7006, Ugo Basile, Comerio, Italy) and connected to a Powerlab 800 (AD Instruments). Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to a 0.5 g and bathing solution was periodically changed. In a preliminary study, a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. In each experiment, bronchial rings were previously challenged with acetylcholine (10^{−6} mol/l) until the responses were reproducible. Subsequently, bronchi were challenged with carbachol.

2.3.2. Isolated perfused mouse lung preparation

The mouse lungs were perfused in a non-recirculating fashion through the pulmonary artery at a constant flow of 1 ml/min resulting in a pulmonary artery pressure of 2–3 cm H₂O. The perfusion medium used was RPMI 1640 lacking phenol red (37 °C). The lungs were ventilated by negative pressure (−3 and −9 cm H₂O) with 90 breath min^{−1} and a tidal volume of about 200 μ l. Every 5 min a hyperinflation (−20 cm H₂O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45-24) and airflow velocity with pneumotachograph tube connected to a differential pressure transducer (Validyne DP 45-15). The lungs respiration humidified air. The arterial pressure was continuously monitored by means of a pressure transducer (Isotec Healthdyne) which was connected with the cannula ending in the pulmonary artery. All data were transmitted to a computer and analysed with the Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany). The data were analyzed through the following formula: $P = V \times C^{-1} + R_L \times dV \times dt^{-1}$, where P is chamber pressure, C pulmonary compliance, V tidal volume, R_L airway resistance. The airway resistance value registered was corrected for the resistance of the pneumotacometer and the tracheal cannula of 0.6 cm H₂O s ml^{−1}. Lungs were perfused and ventilated for 45 min without any treatment in order to obtain a baseline state. Subsequently, lungs were challenged with carbachol. Repetitive dose response curve of carbachol was administered as 50 μ l bolus, followed by intervals of 15 min, in which lungs were perfused with buffer only.

2.4. Flow Cytometry Analysis

Lungs were digested with collagenase (Sigma-Aldrich, Italy). Pulmonary inflammatory cells were determined by flow cytometry

(BD FacsCalibur, Italy) using CD11c-APC, CD11b-PeCy5.5, cKit-PeCy5.5 or- PE, IgE-FITC (Bioscience, San Diego, CA, USA).

2.5. Immunohistochemistry

Left lung lobes were paraffin-embedded and 7 µm sections were obtained. The degree of inflammation was scored by blinded observers by using hematoxylin and eosin (H&E) and Periodic acid/Alcian blue/Schiff staining (PAS). PAS Staining (Sigma-Aldrich, Milan Italy) was performed according to the manufacturer's instructions to detect glycoprotein. PAS+ cryosections were graded with scores 0–4 to describe low to severe lung inflammation as follows: 0: <5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: >75% positive staining/total lung area. FGF2 detection was performed by using anti-FGF-2 or IgG isotype control (Santa Cruz, USA). Mast cell recruitment was evaluated by means of toluidine blue staining (1% solution).

2.6. Measurement of serum IgE levels

Plasmatic serum IgE levels were measured by ELISA according to the manufacturer's instructions (BD Pharmingen, USA).

2.7. Fibroblast activity

2.7.1. Fibroblast culture

Mice were sacrificed and the thorax was opened, then lungs were removed. Lungs were minced and incubated with DMEM containing 15% FCS. Cultures were replaced three times weekly. All cultures were evaluated by immunohistochemistry to assess vimentin and all stained positively with vimentin. Selected cultures were evaluated. Fibroblasts were passaged with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA; GIBCO). Fibroblast between the third and the sixth passages were used for assays.

2.7.2. Fibroblast proliferation and differentiation

Pulmonary fibroblasts were harvested from mice treated with vehicle, OVA or OVA and exposed to hydrogen sulfide. Fibroblast proliferation was assessed by the MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl) tetrazolium bromide, colorimetric assay. Fibroblast differentiation was tested by incubating cells with mouse monoclonal antibody against α-SMA (Sigma-Aldrich, Italy) or rabbit polyclonal antibody against vimentin (Santa Cruz, CA). For detection fluorescein labeled anti-mouse IgG (ABNOVA, Italy) or Texas-Red labeled anti-rabbit IgG (ABNOVA, Italy) were used. The level of myofibroblastic differentiation was measured by counting at least 200 cells.

2.8. Statistical analysis

Data are means ± SEM from at least 6 mice in each group. The level of statistical significance was determined by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons by using the GraphPad Prism software (USA).

3. Results

3.1. Hydrogen sulfide inhibits antigen-induced degranulation in RBL-2H3 cells

A direct effect of hydrogen sulfide on mast cell degranulation was investigated in vitro by using mast cell-like RBL-2H3 cell line. The addition of the antigen DNP-HSA to pre-sensitized RBL-2H3 cells caused a significant degranulation evaluated as β-hexosaminidase release (Fig. 1A). An almost equivalent level of

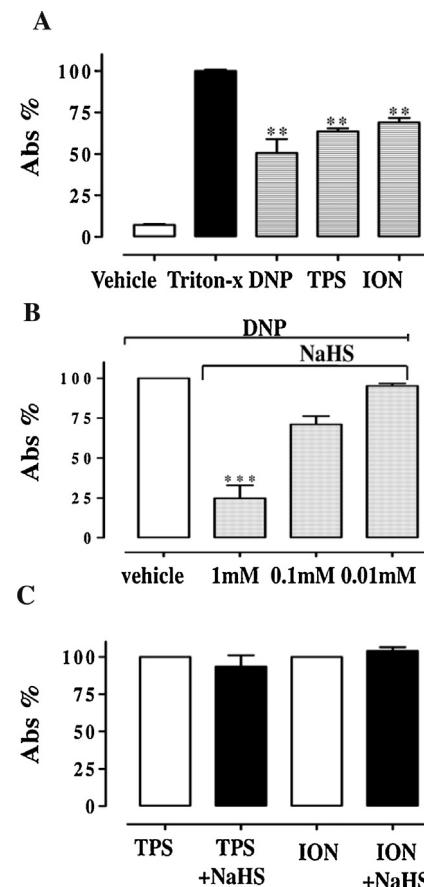


Fig. 1. Hydrogen sulfide reduces IgE-mediated RBL-H3 cells degranulation. (A) RBL-2H3 cell degranulation was pharmacologically induced by (i) the antigen dinitrophenyl-seralbumin 10 ng/ml (DNP), (ii) ionomycin (ION) 1 µM or (iii) thapsigargin (TPS) 1 µM. In matched wells, triton-X-100 was added, to elicit cell lysis and exhaustive release of β-hexosaminidase. The concentration of p-nitrophenate release of β-hexosaminidase was measured at 405 nm. (B) Cells were sensitised with DNP and after 24 h incubated for 5 min with either the H₂S-donor NaHS (10, 100 µM and 1 mM) or the vehicle. Cell degranulation was induced by DNP. (C) Cells were incubated either with NaHS (1 mM) or vehicle for 5 min and cell degranulation induced by ION or TPS. (**p < 0.01 vs vehicle; p < 0.001 vs vehicle).

degranulation was induced by the non-antigenic stimuli thapsigargin and ionomycin (Fig. 1A). Hydrogen sulfide caused a remarkable and concentration-dependent inhibition of the RBL-2H3 degranulation induced by DNP-HAS (Fig. 1B). In contrast, hydrogen sulfide did not exhibit any significant inhibitory activity on the degranulation induced either by thapsigargin or ionomycin (Fig. 1C).

3.2. Hydrogen sulfide inhibits OVA-induced airway hypereactivity

Sensitized mice were exposed to NaHS inhalation (100 ppm; 5 min) daily from day 7 to 21 (Fig. 2A). This dose was shown not to cause any effect "per se". Bronchial reactivity (Fig. 2B) and lung resistance (Fig. 2C) after carbachol challenge were measured. Bronchi harvested from OVA-sensitized mice showed an increased reactivity to carbachol in comparison to vehicle treated mice (Fig. 2B). Similarly, increased lung resistances was present as determined by using the isolated and perfused lung preparation (Fig. 2C). NaHS inhalation reversed both bronchial hypereactivity (Fig. 2B) and the increase of lung resistance (Fig. 2C) in OVA-treated mice. Conversely, hydrogen sulfide inhalation did not alter OVA-induced cell infiltration and inflammation (Fig. 2D). PAS positive staining was still visible in pulmonary histology of lungs harvested from mice sensitized and exposed to NaHS inhalation (Fig. 2E), as also observed by the H&E staining (Fig. 2D) showing a still altered

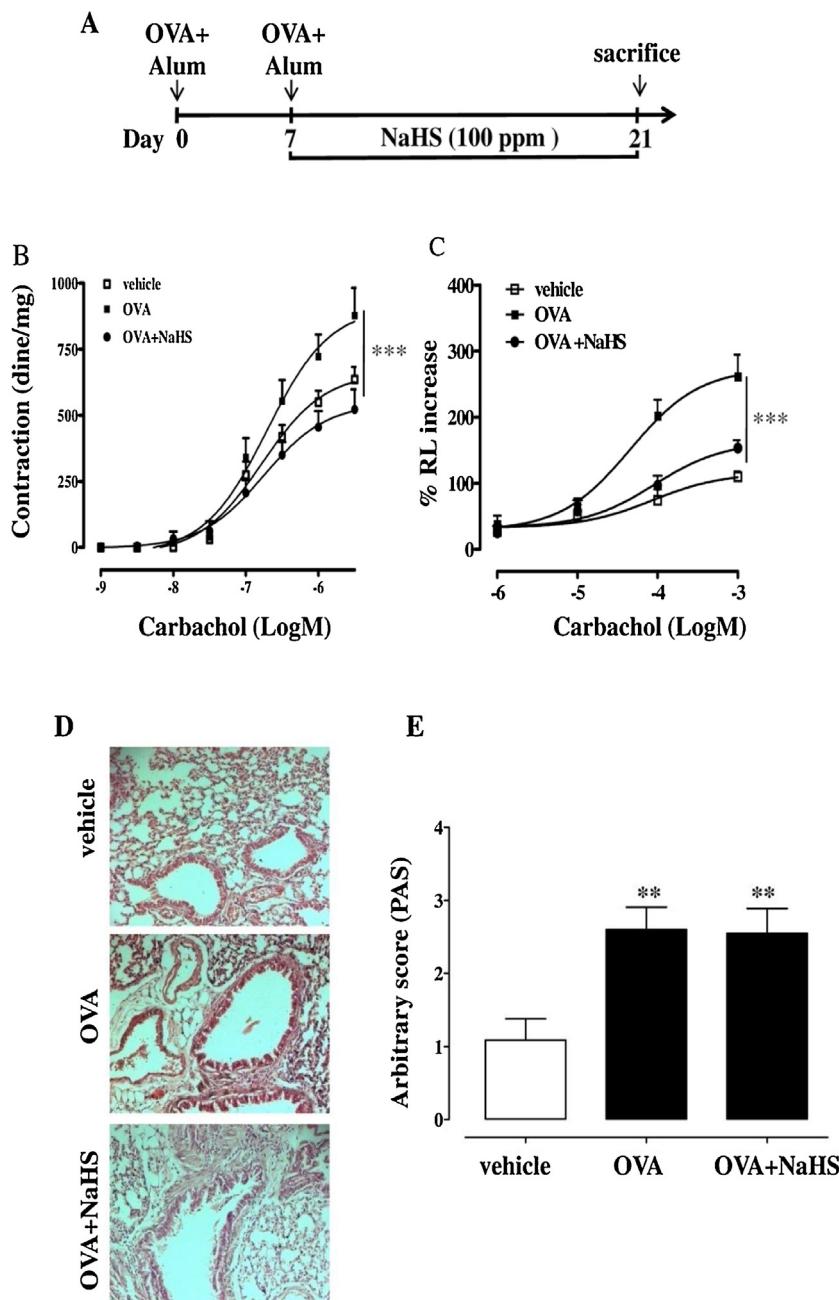


Fig. 2. Hydrogen sulfide inhalation abrogates OVA-induced airway hyper-reactivity. (A) Schematic representation of the protocol used. Mice received subcutaneous administration of OVA adsorbed onto alum at day 0 and 7. Aerosol administration of vehicle or hydrogen sulfide (NaHS, 100 ppm) was performed daily from day 7 to day 21. Twenty four hours after the last administration mice were sacrificed. (B) Measurement of bronchial reactivity to carbachol. (C) Measurement of lung resistance by using isolated perfused lung preparation. *** $p < 0.001$ vs vehicle. (D) Histological analysis of pulmonary sections (Hematoxylin & Eosin). (E) Goblet cell hyperplasia evaluation by periodic acid/Alcian blue/Schiff staining (** $p < 0.01$ vs vehicle). Lung sections have been photographed under light microscopy at 10 \times magnification, PAS positive cryosections were graded with scores 0–4 to describe low to severe lung inflammation (0: <5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: <75% positive staining/total lung area). Data are means \pm SEM from 6 mice in each group.

bronchial epithelium in OVA+NaHS treated mice compared to vehicle mice.

3.3. Hydrogen sulfide affects mast cell degranulation and inhibits fibrogenic cytokines production

Flow cytometry analysis of lungs harvested from OVA-sensitised mice demonstrated a significant increase of mast cell recruitment. The mast cells were identified as CD11c $+$ c – Kit $+$ IgE $+$ positive cells (Fig. 3A). Treatment of mice with NaHS neither affected mast cell count (Fig. 3B) nor plasmatic IgE levels (Fig. 1B). Conversely NaHS inhalation significantly affected mast cell degranulation as

evident by toluidine positive staining (Fig. 3C). Hydrogen sulfide reversed also OVA-induced up-regulation of two important fibrogenic cytokines IL-13 (Fig. 3D) and FGF-2 (Fig. 3E).

3.4. Mast cell-deficient $Kit^{W-sh/W-sh}$ mice fail to develop airway hyperresponsiveness

Mast cell-deficient $Kit^{W-sh/W-sh}$ mice exposed to OVA sensitization did not develop bronchial hypereactivity to carbachol (Fig. 4A). Exposure of mice to NaHS during sensitization did not further affect bronchial tone (Fig. 4A). On the other hand, a significant airway hyperreactivity was observed in the wild type mice

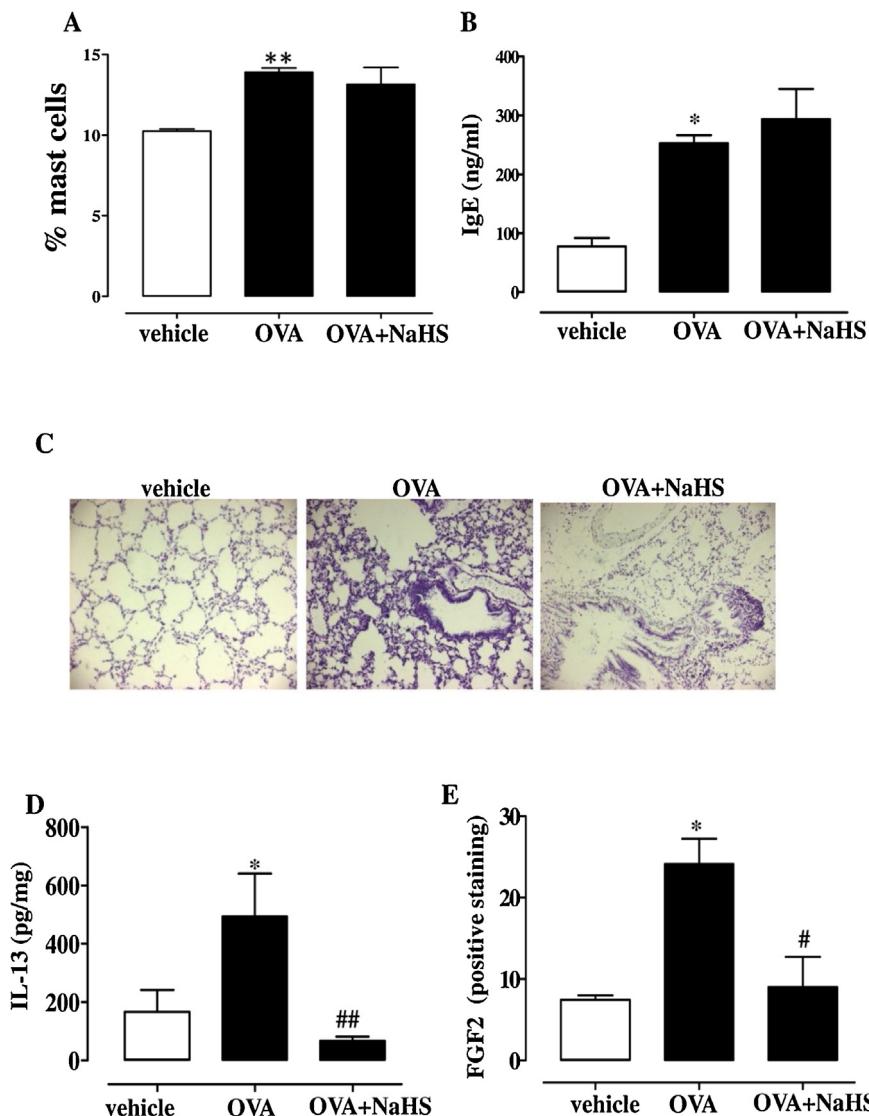


Fig. 3. Hydrogen sulfide inhibits mast cell degranulation and cytokines expression in the lung. (A) Quantification of lung mast cells by flow cytometry identified as CD11c⁺c – Kit + Igε + positive cells (***p* < 0.01 vs vehicle). (B) IgE sera levels were determined by using specific ELISA (**p* < 0.05 vs vehicle). (C) Toluidine blue staining of paraffin-embedded lung sections. (D) Pulmonary IL-13 expression has been determined by ELISA. (E) Immunohistochemistry of FGF-2 expression in lungs, (**p* < 0.05 vs vehicle, #*p* < 0.05 vs OVA, ##*p* < 0.01 vs OVA). Data are means ± SEM from 6 mice in each group.

(C57Bl6/J, data not shown). Similarly to what observed in BALB/c mice, NaHS treatment of sensitized C57Bl6/J mice reversed OVA-induced airway hypereactivity (data not shown). Sensitization of mast cell-deficient Kit^{W-sh/W-sh} mice failed to induce a significant increase of both IL-13 (Fig. 4B) and FGF-2 (Fig. 4B) expression in lung. Treatment of mast cell-deficient Kit^{W-sh/W-sh} mice with NaHS did not affect all parameters evaluated (Fig. 4A–D). These data imply that mast cell is the primary target of hydrogen sulfide.

In order to further address the role of mast cells we treated mice with OVA without alum using the protocol reported in supplemental Fig. 1A. This is a chronic allergic asthma model mainly mast cell dependent [19]. Similarly, to what we found in the other model the increase in mast cell recruitment driven by the sensitization procedure was not affected by NaHS (Supplemental Fig. 1B), while mast cell degranulation was blunted (Supplemental Fig. 1C). Also in this experimental setting bronchial hypereactivity to carbachol was abrogated by hydrogen sulfide aerosol (Supplemental Fig. 1D). PAS staining evidenced a weak increase of goblet cells suggesting a marginal role of mast cells in the regulation of mucus production (Supplemental Fig. 1E). Finally hydrogen sulfide treatment did not alter pulmonary morphology.

3.5. Fibroblasts harvested from mice exposed to NaHS displayed a reduced activation

Primary pulmonary fibroblasts were harvested from mice exposed to OVA and their proliferation rate and differentiation evaluated in vitro. Our data show that primary fibroblasts harvested from sensitized mice displayed an increased proliferation rate (Fig. 5A) as well as increased expression of α-SMA (Fig. 5B and C) as compared to vehicle treated mice. When fibroblasts were harvested from mice treated with hydrogen sulphide aerosol in vivo both the increased proliferation rate and differentiation were reverted (Fig. 5). Thus, mice exposed to hydrogen sulfide inhalation displayed a significant reduction of fibroblast activation beyond reduction of airway hyper-reactivity. In order to further demonstrate that the fibroblasts contribute to mast-cell-dependent airway hyper-reactivity, we evaluated fibroblast activation also in the model of mast cell-dependent hyper-reactivity using the protocol described in Supplemental Fig. 1A. Our data show that primary fibroblasts harvested from these mice displayed an increased rate of proliferation (Supplemental Fig. 2A) as well as an increased expression of α-SMA (Supplemental Fig. 2B and C). When mice

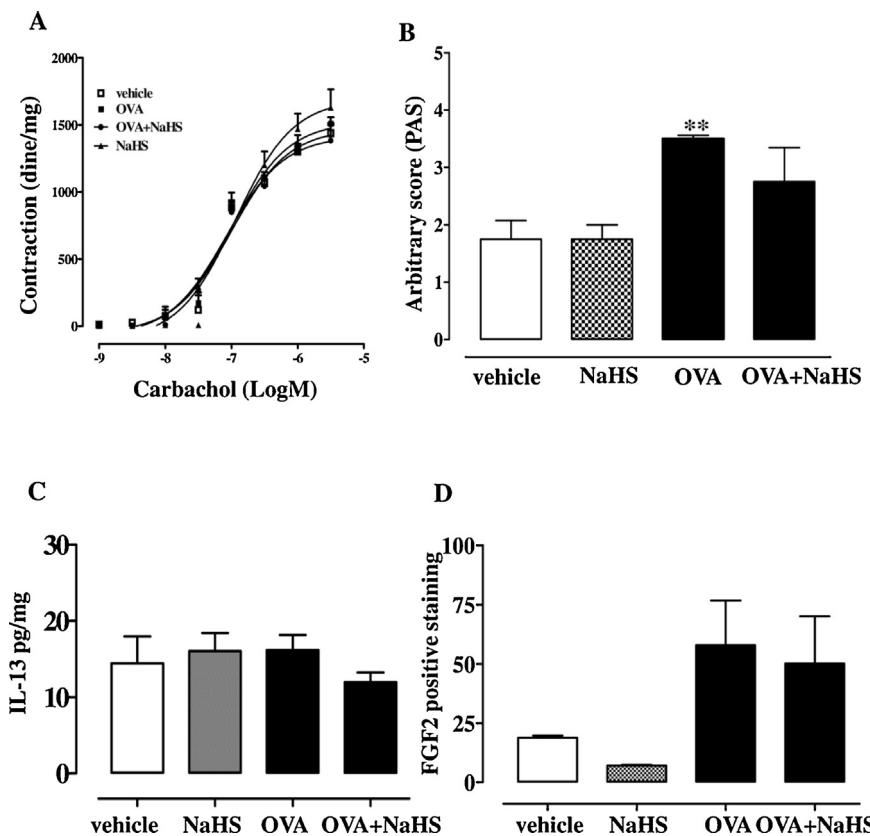


Fig. 4. Lack of effect of hydrogen sulfide in mast cell-deficient $Kit^{W-sh}/W-sh$ mice. Mast cell-deficient $Kit^{W-sh}/W-sh$ mice received subcutaneous administration of OVA adsorbed onto alum at day 0 and 7. Mice were treated with either vehicle or hydrogen sulfide aerosol daily from day 7 to 21. Twenty four hours after the last administration mice were sacrificed. (A) Bronchial reactivity to carbachol was assessed. (B) Goblet cell hyperplasia evaluation by periodic acid/Schiff staining. Lung sections have been photographed under light microscopy at 10 \times magnification, PAS positive cryosections were graded with scores 0–4 to describe low to severe lung inflammation (0: <5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: <75% positive staining/total lung area, ** p < 0.01 vs vehicle). (C) Pulmonary IL-13 expression has been determined by ELISA. (D) Immunohistochemistry of FGF-2 pulmonary expression. Data are means \pm SEM from 6 mice in each group.

were treated with aerosol of hydrogen sulfide, both the increased fibroblast proliferation and differentiation measured ex vivo were prevented (Supplemental Fig. 2) as well as the airway hyperreactivity.

4. Discussion

There is a growing body of evidence suggesting that hydrogen sulfide might be of clinical benefit in pulmonary diseases. In particular, a role for hydrogen sulfide in modulating airway hyperreactivity and remodeling has been suggested. Airway hyperreactivity is an important pathophysiological characteristic of asthma and it is linked to both airway inflammation and remodeling. Although the pathogenic mechanisms are not completely understood, an essential role is attributed to mast cells. Here we have assessed whether the beneficial effects of hydrogen sulfide on lung function are related to mast cell activation.

Preliminarily, we have determined the effect of NaHS on mast cells by using the mast cell-like RBL-2H3 cell line. Incubation of RBL-2H3 with NaHS did not have any effect by itself up to a dose of 1 mM. Conversely, hydrogen sulfide caused a remarkable and concentration dependent inhibition of the RBL-2H3 degranulation when cells were sensitized and challenged with antigen. Hydrogen sulfide did not affect either thapsigargin or ionomycin-induced RBL-2H3 degranulation. These results clearly suggest that hydrogen sulfide can explicate its action only on sensitized cells.

In order to better understand this phenomenon we tested the effect of hydrogen sulfide in vivo by using OVA-sensitized mice. Exposure of allergen-sensitized mice to hydrogen sulfide aerosol

reversed bronchial hyperactivity as already demonstrated by others [6]. In addition, we also show that the aerosol treatment abrogated the increase in lung resistance. Allergen-induced AHR was coupled to an increased presence of mast cells within the pulmonary tissue, as determined by FACS analysis. Aerosol administration of hydrogen sulfide neither influenced the recruitment of lung mast cells or plasma levels of IgE induced by OVA sensitization. However, in analogy with RBL-2H3 cells, hydrogen sulfide abrogated in vivo mast cell degranulation. This effect was coupled to inhibition of the pulmonary expression of FGF-2 as well as IL-13. On the other hand, hydrogen sulfide did not affect allergen-induced goblet cell metaplasia, mucus hypersecretion and pulmonary cell infiltration, all parameters indicative of lung inflammation [20,21]. Therefore, the hydrogen sulfide beneficial effects on airway hyperreactivity well correlates with the reversion of mast cell activation. Indeed, the data obtained with the experiments in vitro with RBL cells, taken together with the in vivo functional data and the modulation of IL-13 and FGF2 pulmonary expression were all indicative for a central role for mast cell in hydrogen sulfide-mediated actions. To further understand the link among hydrogen sulfide, mast cells and bronchial hyperreactivity, we tested hydrogen sulfide effect in mast cell-deficient $Kit^{W-sh}/W-sh$ mice. Bronchi, harvested from OVA-sensitized mast cell-deficient $Kit^{W-sh}/W-sh$ mice, did not develop bronchial hyperreactivity. The upregulation of IL-13 and FGF2 was absent, too. Conversely, the inflammatory features i.e. allergen-induced cell infiltration, goblet cell metaplasia and mucus hypersecretion were all still evident. Exposure of OVA-sensitized mast cell-deficient $Kit^{W-sh}/W-sh$ mice to hydrogen sulfide aerosol did not affect the inflammatory

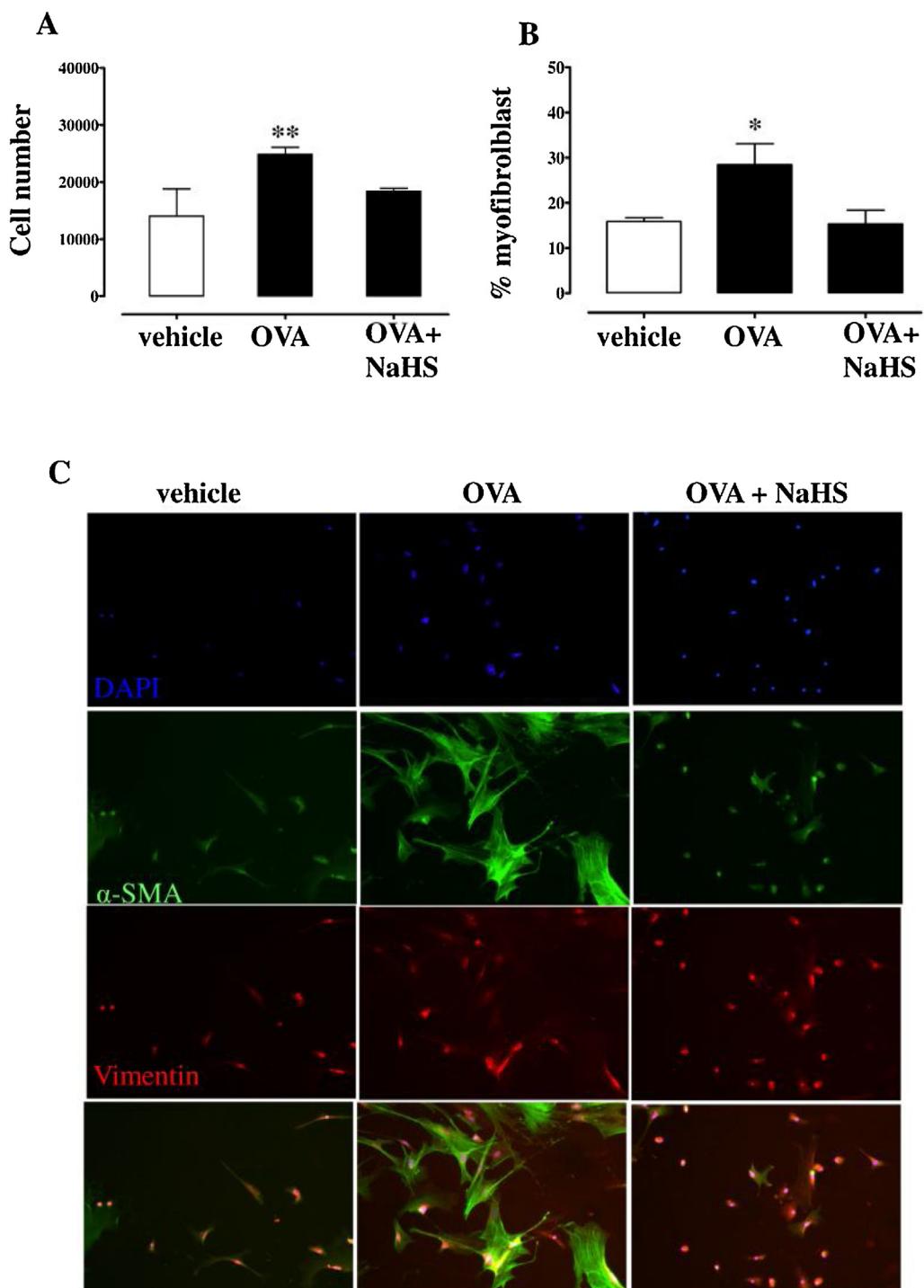


Fig. 5. Hydrogen sulfide inhibits fibroblast activation in the lung. (A) Lung primary fibroblasts were harvested from OVA-sensitized mice exposed to vehicle or NaHS aerosol. For the in vivo protocol see Fig. 2A. The MTT colorimetric assay was used to assess fibroblast proliferation in vitro (** $p < 0.01$ vs vehicle). (B) Percentage of myofibroblasts (* $p < 0.05$ vs vehicle). (C) Fibroblasts were labelled with anti-vimentin antibody (red). Nuclei were stained with DAPI (blue). Myofibroblast differentiation was determined by detection of expression of alpha SMA (green). Lowest panels represent the merge of upper and central panels. The images were acquired with confocal microscope (Leica). The percentage of myofibroblasts are easily distinguishable since they display well-developed, thick and alpha SMA-positive stress fibers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reaction. Therefore the lack of mast cells in $Kit^W-sh/W-sh$ mice mimics the hydrogen sulfide effects in OVA-sensitized mice. In addition these data imply that the upregulation of OVA-induced IL-13 and FGF-2 expression in the lung is mast cell dependent and therefore hydrogen sulfide effect is strictly dependent from antigen-activated mast cells. However, we still had to define how this hydrogen sulfide effect on mast cells translates into a beneficial effect on airway hypereactivity. Mast cells synthesize and secrete a large

number of pro-inflammatory cytokines which regulate both IgE synthesis and the development of inflammation and several profibrogenic cytokines. Some of these mast cell products e.g., IL-13 are known to induce bronchial hypereactivity in the mouse independent of the inflammatory response and enhanced the responses in cultured human airway smooth muscle [12,13]. Accordingly, aerosol of hydrogen sulfide prevented FGF2 and IL-13 upregulation, that are known to activate lung fibroblasts in humans and in

experimental animals [22,23]. In addition there is increasing evidence in humans for a strong correlation between sub-epithelial fibrosis and airway hyper-reactivity [24,25] as suggested by the increased numbers of fibroblasts/myofibroblasts found in the airways of asthmatic patients [26,27]. In order to address this issue we harvested fibroblasts from OVA-sensitized mice. These fibroblasts displayed an increased proliferation rate in vitro as well as a significant increase in the percent of fibroblasts converted in myofibroblasts. When fibroblasts were harvested from mice treated in vivo with hydrogen sulfide aerosol, the increased fibroblast proliferation rate as well as their differentiation into myofibroblasts were significantly inhibited. These data suggest that the hydrogen sulfide beneficial effects involves also modulation of mast cell-mediated fibroblast activation. This hypothesis is supported by the finding that in mast cell deficient mice FGF-2 as well as IL-13 up-regulation are not induced by OVA-sensitization. This is also in tune with the finding that IL-13+ cells, present within the airway smooth muscle of asthmatic patients, are predominantly mast cells [27]. IL-13 has been also shown to elicit many of the features of human asthma as well as to regulate production of several pro-fibrogenic cytokines including FGF-2 [23,28].

In conclusion, here we demonstrate that exposure of sensitized mice to hydrogen sulfide inhalation reverses airway hypereactivity. The hydrogen sulfide effect stems from the modulation operated by hydrogen sulfide on mast cell as demonstrated by the lack of effect of NaHS in Kit^{W-sh/W-sh} mice. The beneficial effect on bronchial hypereactivity involves the mast cell-induced fibroblast activation and in turn inhibition of IL-13 and FGF-2 expression. This is particular interesting on the clinical side since recently it has been demonstrated that FGF-2 levels in induced sputum significantly correlate with pulmonary function and have been proposed to be a possible biomarker of asthmatic airway remodeling [28]. These data also suggest that aerosol treatment with H₂S donors could be exploited as therapeutic complementary therapeutic approach in lung diseases such as asthma as well as other respiratory fibrotic-related diseases such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease.

Conflict of interest

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phrs.2015.07.032>

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