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Hydrogen sulfide inhibits apoptosis and protects the bronchial epithelium in an allergic inflammation mice model

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ABSTRACT

Studies suggest that hydrogen sulfide (H_2S) plays a relevant and beneficial role in the pathophysiology of pulmonary allergic diseases, such as asthma. These diseases may be triggered by changes in airway epithelium caused by repeated exposure to environmental allergens. This study aimed to investigate whether H_2S protects against bronchial epithelium apoptosis in allergic inflammation in mice. The effects of H_2S on the production of Th2 cytokines and on the infiltration of pulmonary inflammatory cells were also studied. Female BALB/c mice previously sensitized with ovalbumin (OVA) were treated with H_2S donor (sodium hydrosulfide [NaHS]) 30 min prior to OVA challenge. After euthanasia (48 h post challenge), the right lung was homogenized to study apoptosis protein expression and to analyze cytokine levels in lung tissue. The left lobe was fixed in formalin for morphological analysis of lung tissue and verification of apoptosis *in situ* by the TUNEL assay. Histological results showed that NaHS reduced the airway inflammatory infiltrate and prevented an increase in the IL-4, IL-5 and IL-25 levels caused by OVA challenge. Activation of caspase 3 and FasL in response to the allergen was also fully prevented by NaHS treatment. TUNEL staining showed that the challenge from OVA significantly increased the rate of apoptosis in the bronchiolar epithelium, and that this incremental apoptosis was abolished by NaHS treatment. In conclusion, our results showed that H_2S donor has a protective effect against airway epithelium damage caused by an allergic reaction, and represents a potential agent in treating allergic lung disorders, such as asthma.

1. Introduction

Asthma is a chronic inflammatory disease that causes characteristic structural changes in the airways, resulting in airway smooth muscle hyperplasia, fibrosis beneath the airway epithelium, greater number of goblet cells and increased vascularity [1]. Repeated injury, repair and regeneration of the airway epithelium, following exposure to environmental factors and inflammation, result in histological changes and functional abnormalities in the airway mucosal epithelium. These changes contribute to the pathophysiology of asthma, because damage to the barrier functions of the airway epithelium enhances the mucosal permeability of foreign substances [2]. In allergic asthma, the inflammation largely depends on the production of IgE by B cells, and the degranulation of mast cells, along with eosinophil infiltration dependent on T helper 2 (Th2) cytokines, such as interleukin (IL)-4 IL-5, IL-9 and IL-13 [3], which are target to the inflammatory treatment given to patients with severe airway disease [1]. These cytokines promote important changes in the airways and the lung parenchyma associated with asthma. Among the changes, the following stand out in particular: increase in the number of pulmonary eosinophils, and activation of lymphocytes and mast cells [3]. In addition, IL-25 (also known as IL-17E) is a distinct member of the IL-17 cytokine family, which promotes and augments Th2 responses locally and systemically [4]. IL-25 also can delay eosinophil apoptosis, leading to eosinophilia during allergic inflammation [5].

The persistence of eosinophils in the airways is related to the severity of disease symptoms, which may depend not only on the number of airway eosinophils, but also on their survival mechanism after recruitment [6]. There is an inverse correlation between the number of apoptotic eosinophils and the severity of symptoms of asthma,

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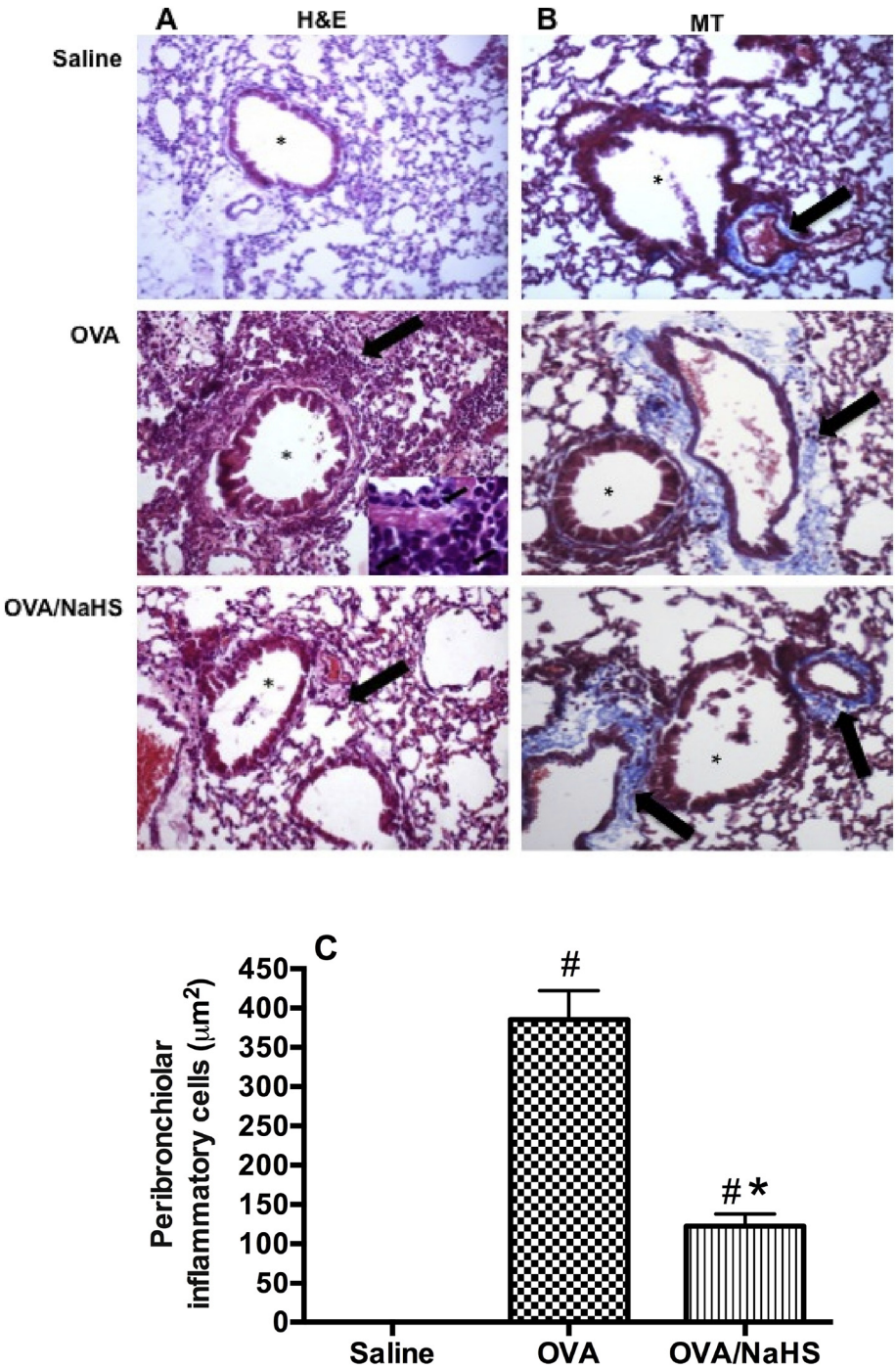

Fig. 1. Morphological analysis of inflammatory cell infiltration and collagen content in lung tissue of allergic mice. Panel A (H&E) and Panel B (MT) is representative of H&E staining and Masson's Trichrome staining, respectively, of a lung section. Arrows indicate peribronchiolar leukocyte infiltrate or perivascular collagen content, and asterisks indicate bronchiole; original magnification x200. Panel C: Quantitative evaluation of cell infiltration. The bars represent the mean ± S.E.M. of 8 mice/group. #$p < 0.05$ compared to saline. *$p < 0.05$ compared to OVA.

suggesting that apoptosis may be an important mechanism triggering the allergic inflammation [6]. Apoptosis can be initiated by two pathways, namely extrinsic (through cellular or cytoplasmic death receptors, such as FasL) or intrinsic (mitochondrial). When a death receptor is activated, a protein adapter is recruited and activates caspases initiators, such as caspase 8, which, in turn, activates caspase effectors, such as caspase 3, leading to cell death [6,7]. The mitochondrial pathway is initiated when the p53 protein activates an accessory pathway that results in the release of cytochrome *c* from mitochondria, with subsequent activation of caspase 9 [6]. Homeostasis is maintained by controlling the amount of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins. DNA damage increases the expression of pro-apoptotic proteins, inducing apoptosis [6,7].

H_2S is generated endogenously from l-cysteine by the action of enzymes such as cystathionine ϒ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (MST) [8,9]. Studies have shown that H_2S is involved in various physiological activities [8,9]. Treatment of mice with the H_2S donor (sodium hydrosulfite [NaHS]) has had a positive effect on asthma markers, by reducing airway eosinophil infiltration and lung oxidative stress [10,11].

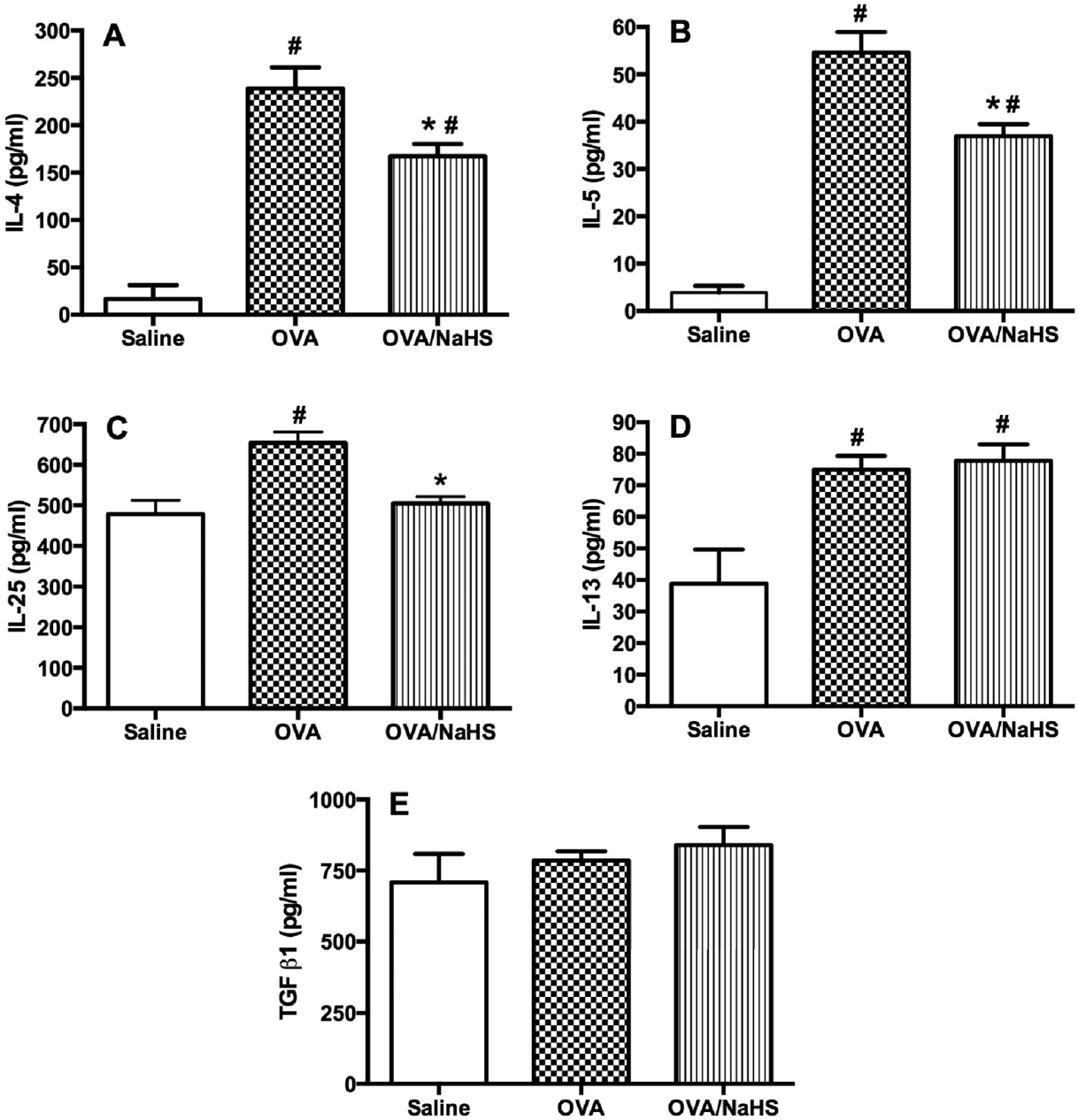

Fig. 2. NaHS pretreatment reduces cytokine levels in the lungs of OVA-sensitized and challenged mice. Panels A–E: cytokine levels. The bars represent the mean ± S.E.M. of 8 mice/group. #$p < 0.05$ compared to saline. *$p < 0.05$ compared to OVA.

The reduction in oxidative stress is due to decreased levels of oxygen-derived free radicals (ROS) and increased activity of glutathione reductase (GR) and of glutathione peroxidase (GPx), indicating that H_2S has an important antioxidant effect on the lungs, by modulating the enzymes responsible for the maintenance of GSH levels [11,12]. Since ROS are important inducers of apoptosis and their excessive production is involved in cell damage in asthma [13], we decided to investigate whether treatment with NaHS protects against bronchial epithelium apoptosis in allergic inflammation in mice. More specifically, we studied the effects of NaHS treatment on Th2 cytokine production and lung inflammatory cell infiltration.

2. Material and methods

2.1. Materials

Ovalbumin (OVA grade V), sodium hydrosulfide (NaHS), hydroxymethyl aminomethane (Tris), protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), protein assay kit, hematoxylin-eosin staining solution, Trichrome Stain (Masson) Kit and Trilogy™ reagents were purchased from Sigma Chemical (St. Louis, MO, USA). SDS-PAGE, nitrocellulose membranes and reagents used for Western blotting were acquired from Bio-Rad Laboratories (CA, USA), and SuperSignal West Pico Chemiluminescent-IL and β-actin antibody were obtained from Thermo Scientific (IL, USA). Secondary antibody IgG conjugated with peroxidase was purchased from Upstate Biotechnology (NY, USA), anti-caspase 3 antibody was acquired from Millipore (Burlington, MA, USA), and caspase 9, Bax, and FasL antibodies were obtained from Santa Cruz (TX, USA). According to the suppliers, both caspase antibodies recognize the intact caspase proteins as well as the proteolytic fragments. The *In Situ* Cell Death Detection Kit and the Millipex® MAP Immunoassay Kit were purchased from Roche Diagnostics (GmbH, Germany) and Millipore (Burlington, MA, USA), respectively.

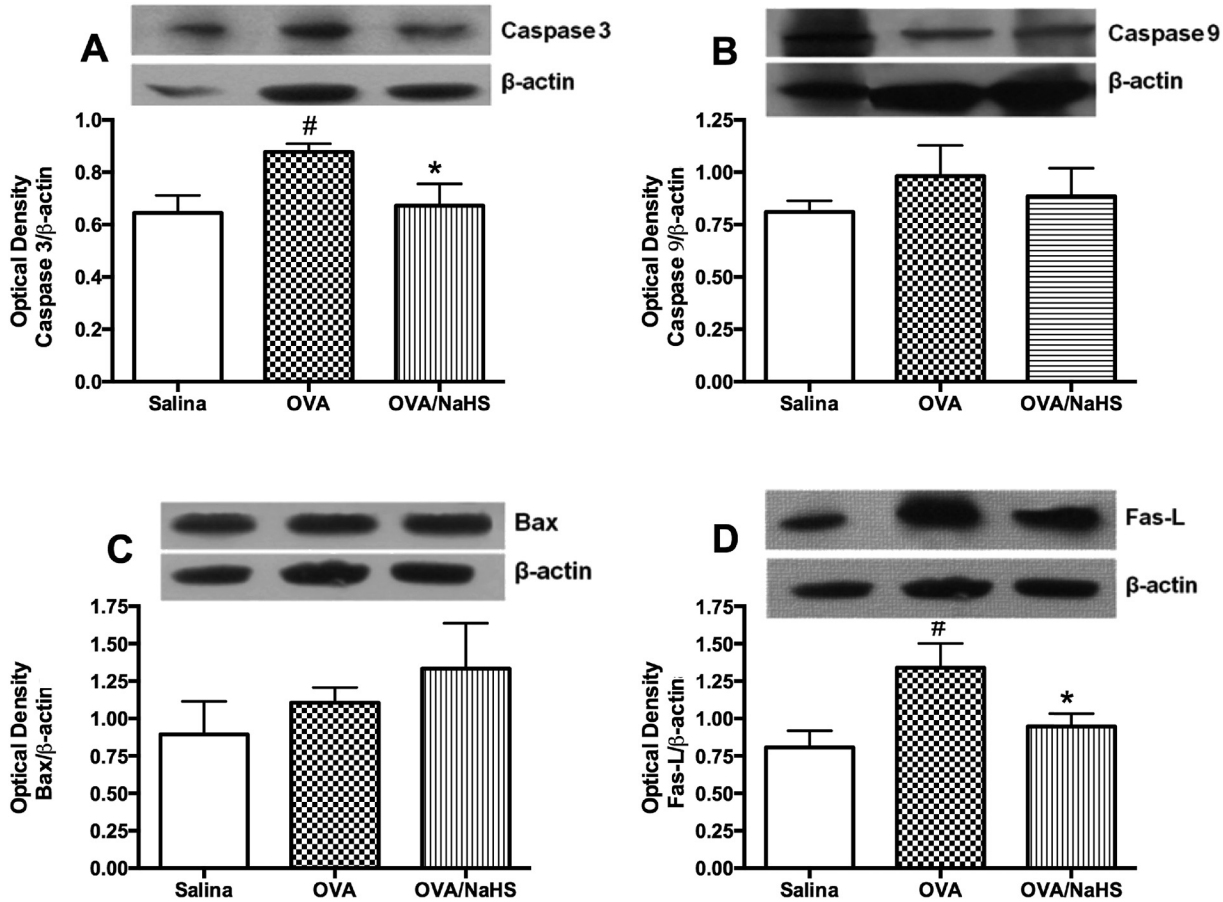

Fig. 3. Western blots and quantitative evaluation of the expression of caspase (A), caspase 9 (B), Bax (C) and FasL (D) in lung tissue with β-actin as protein load control. The bars represent the mean ± S.E.M. of 8 mice/group. Values of #$p < 0.05$ compared to saline. *$p < 0.05$ compared to OVA.

2.2. Animal handling and care

Female BALB/c mice, 5 to 8 weeks old, were obtained from the Multidisciplinary Center for Biological Research in Laboratory Animal Science (CEMIB/UNICAMP). The mice were maintained in propylene cages (five per cage) under standard controlled conditions (23 °C, a 12 h light/dark cycle) with food and water *ad libitum*. All experimental procedures were approved by the local animal ethics committee (San Francisco University, Brazil; number 0021108), and were in accordance with the Brazilian Society for Laboratory Animal Science (SBCAL/COBEA).

2.3. Sensitization, airway challenge and treatment

We used an experimental model of OVA-induced lung allergic inflammation and NaHS treatment as previously described [12]. Briefly, the mice were sensitized subcutaneously (s.c.) at days 0 and 7 with 400 μL of a suspension of 100 μg ovalbumin (OVA grade V) bound to 4 mg of aluminum hydroxide in sterile saline. Seven days after the second sensitization, the animals were challenged intranasally (i.n.) with 10 μg of OVA in 50 μL of sterile saline or with saline alone. The OVA or saline exposure were performed twice a day for two consecutive days. A set of animals from the OVA-challenged group received intraperitoneal (i.p.) injections of freshly prepared NaHS (14 μmol/kg dissolved in 300 μL sterile saline) twice a day, 30 min prior to OVA challenge. The untreated challenged mice received the same volume of sterile saline alone. Thus, the study design comprised 3 groups: OVA-sensitized and unchallenged (saline; 8 mice); untreated OVA-sensitized and challenged (OVA; 8 mice); and NaHS-treated OVA-sensitized and challenged (OVA/NaHS; 8 mice).

After euthanasia, the lungs were removed, and the right lung was homogenized in 50 mM hydroxymethyl aminomethane (Tris) buffer, pH 7.4, containing 1% protease inhibitor with 0.5 M phenylmethylsulfonyl fluoride (PMSF). The lung homogenate was centrifuged at 800*g* for 10 min at 4 °C. The supernatant was aliquoted, rapidly frozen in liquid nitrogen and then stored in a freezer at −80 °C, until performing Western Blotting analysis and measuring the cytokines. The left lung was fixed with 10% formaldehyde, subsequently embedded in paraffin (Merck, USA) and divided into sagittal sections of 5 μm to perform lung histology and TUNEL staining.

2.4. Morphological analysis of lung tissues

After fixation and deparaffinization using Trilogy reagent, the histological sections were stained with hematoxylin and eosin (H&E) for evaluation of cellular infiltration and Masson's Trichrome (MT) to verify the presence of peribronchiolar collagen. Using an optical microscope with a capture system (Nikon Instruments Inc., USA), three bronchioles were photographed on each slide. The area of inflammatory cell infiltration was evaluated considering only the cells around the bronchiole. The measurements were blindly assessed by three independent investigators on the digitalized images, with magnification of x200, using the ImageJ 1.47v program (Wayne Rasband National Institutes of Health, USA). The measurement in μm^2 was obtained by multiplying the value of the $pixel^2$ by 0.0025. The presence of collagen deposition was identified by visual analysis in the sections stained with MT.

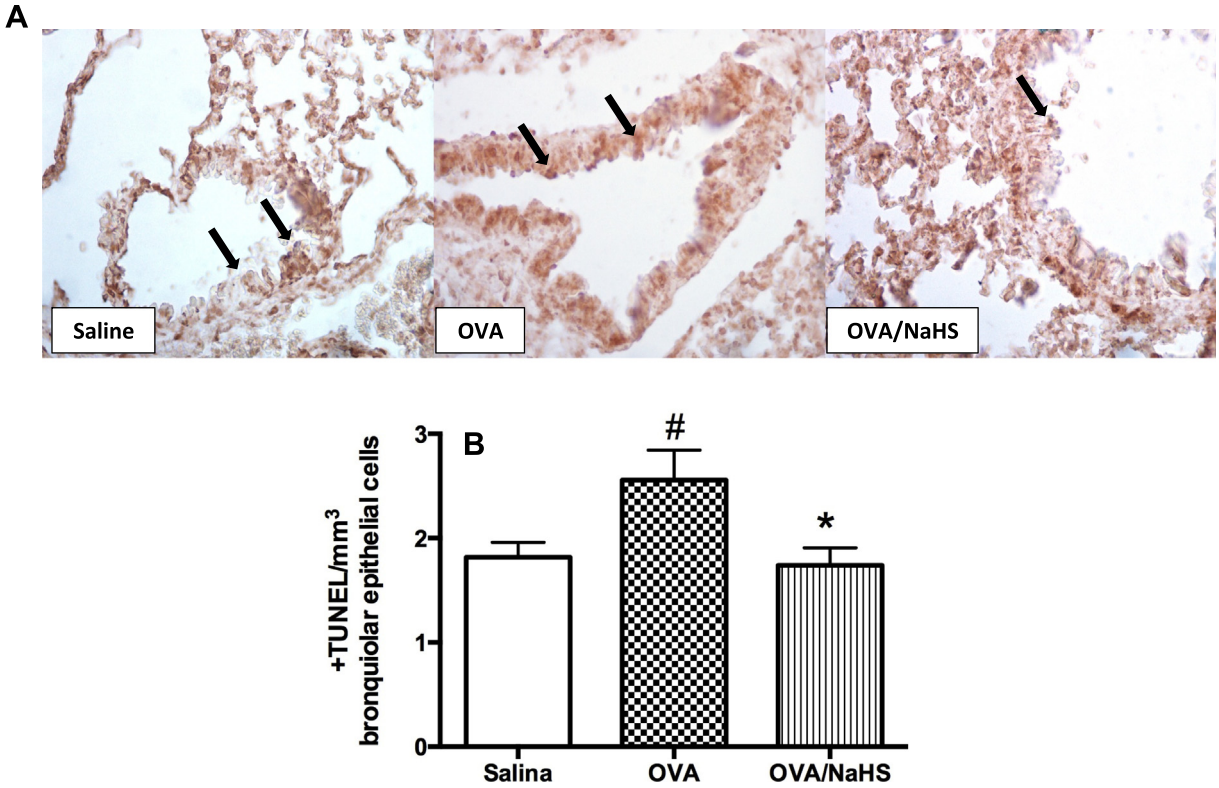

Fig. 4. Effect of NaHS pretreatment on lung apoptosis *in situ*. Panel A is representative of TUNEL staining. Arrows and asterisks indicate apoptotic cells and bronchiole, respectively; original magnification ×200. Panel B: quantitative evaluation of apoptotic cells. The bars represent the mean ± S.E.M. of 8 mice/group. Values of #$p < 0.05$ compared to saline. *$p < 0.05$ compared to OVA.

2.5. Western blotting

The lower lobe of the left lung was removed and homogenized in 300 μL of 1% protease inhibitor cocktail in 50 mM Tris buffer, pH 7.4. The homogenate was centrifuged (800*g*, 10 min, 4 °C), and the supernatant was aliquoted, frozen in liquid nitrogen and subsequently stored at −80 °C. Protein concentration was measured using the Bradford method, and 50 μg were applied on SDS-PAGE. The proteins were separated by electrophoresis and transferred to nitrocellulose membranes (Trans-Blot Transfer Medium). Non-specific protein blockage was performed using a 5% milk solution in TBS-T buffer. Membranes were incubated overnight at 4 °C with primary antibody, washed with TBS-T buffer, incubated with secondary antibody IgG conjugated with peroxidase at room temperature for 1 h. All primary antibodies (anti-caspase 3, caspase 9, Bax, FasL and β-actin) were used at a final concentration of 1 μg/mL in TBS-T. Binding of the specific antibodies was visualized by exposure to photographic film (Kodak-Medica X-ray Film, NY, USA). Densitometric analysis of the bands corresponding to β-actin (MW: 43 kDa), the intact caspases 3 (MW: 32 kDa) and 9 (MW: 46 kDa), FasL (MW: 40 kDa) and Bax (MW: 23 kDa) was performed with the aid of the software ImageJ 1.47 (US National Institute of Health, USA).

2.6. TUNEL

DNA fragmentation was analyzed by TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling) reaction in the lung cross section, using the *In Situ* Cell Death Detection Kit. The assay was performed according to the manufacturer's instructions. Lung paraffin slices were dewaxed in xylene and hydrated in ethanol series (100%, 95%, 90%, 80%, 70%), hydrated and pretreated with Trilogy® reagent. Endogenous peroxidase was blocked with 3% H_2O_2 for 20 min. The sections were rinsed in PBS and permeabilized in a solution with 0.1% Triton X-100 and 0.1% sodium citrate. Then, they were incubated with TUNEL reaction mixture for 1 h at room temperature in a dark-room. Slices were incubated in converter-POD reagent for 30 min, at 37 °C in a humid chamber, and washed again before adding 100 μL DAB substrate for signal conversion. Positive controls were obtained by 10 min pre-incubation with DNAse DNase I before applying the TUNEL method, whereas the negative controls were incubated with the label solution (nucleotide mixture) in the proportion of 1:9, respectively. The sections were dehydrated, mounted and analyzed; nuclei stained in brown-brownish were considered TUNEL positive. The quantification was performed using NIS Element D Microscope Imaging Software (Nikon Instruments Inc., USA).

2.7. Cytokine levels

Cytokine concentrations in the mice pulmonary homogenate were quantified using the Millipex® MAP Immunoassay Kit. The assay was performed according to the manufacturer's instructions. Six cytokines (IL-4, IL-5, IL-13, IL-25 and TGF-β1) were estimated.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M., and were analyzed by one-way ANOVA, followed by the Tukey test for multiple comparisons. Statistical significance was established at $p < 0.05$.

3. Results

3.1. NaHS treatment reduces inflammatory cell infiltration in lungs of OVA-challenged mice

Fig. 1A and C shows that the OVA challenge caused significant inflammatory cell infiltration, as detected around the bronchioles, compared with unchallenged mice (saline group). Pretreatment with NaHS

significantly reduced the cell infiltration into lungs (Fig. 1A, C).

Fig. 1B shows that there was no evidence of peribronchiolar collagen formation in mice challenged with OVA (Fig. 4A). Perivascular collagen was observed. This is a characteristic of blood circulation vessel structure, and is not related to airway remodeling.

3.2. NaHS treatment modulates cytokine levels in OVA-challenged mice

Fig. 2 shows that the OVA challenge significantly increased the concentration of IL-4, IL-5 IL-25 and IL-13, but not TGF-β1 in the lung tissue (Fig. 2A–E). Pretreatment with NaHS significantly reduced the levels of IL-4, IL-5 and IL-25 (Fig. 1A–C). NaHS treatment did not affect IL-13 or TGF-β1 levels (Fig. 1D, E).

3.3. NaHS attenuates lung apoptosis in OVA-challenged mice

Since Th2-cytokines could be involved in cell viability, we then investigated the effects of H_2S treatment on protein expression, which is part of the apoptosis process, and includes caspase 3, caspase 9, Bax and FasL in the lung homogenate (Fig. 3A–D). The increases in caspase 3 and FasL, in response to the OVA challenge, was fully prevented by NaHS treatment (Fig. 3A, D), whereas no changes were seen in the caspase 9 or Bax expression by OVA challenge or NaHS treatment (Fig. 3B, C).

Considering that the process of apoptosis could occur in some of the airway structures, we performed the apoptosis assay *in situ* in lung tissue, using TUNEL staining. Fig. 4(A, B) shows that OVA challenge significantly increased the TUNEL index in the bronchiolar epithelium, which was eliminated by NaHS treatment.

4. Discussion

Because of the relevance of H_2S as a possible alternative for use as a therapeutic agent in asthma and other immediate hypersensitivity reactions, we verified the mechanisms by which it could protect the airways against damage caused by allergic inflammation.

Previous studies have shown that H_2S may be beneficial to allergic respiratory diseases, by decreasing the migration of eosinophils and neutrophils to the lungs of sensitized mice after antigen challenge [10,11]. H_2S was also able to prevent the increase in lipid peroxidation that causes cellular damage in asthma, and exert a protective effect by increasing the activity of antioxidant enzymes (superoxide dismutase, glutathione peroxidase and glutathione reductase) in the lungs [11,12]. In the present study, we checked whether the ability of H_2S donor (NaHS) to reduce pathological changes in lung tissue triggered by the allergen in the sensitized mice was related to lung cytokine levels, especially those changes that can modulate apoptosis of airway cells. We observed that the level of cytokines involved in the allergic process was reduced by NaHS. The consequence of this reduction may promote bronchiolar epithelial cell protection against apoptosis, because of the decrease in caspase 3 and FasL expression in the airways.

Cytokines produced by Th2 lymphocytes are participants in the mechanisms associated with the development of lung allergic diseases, such as differentiation of immunoglobulins into IgE (IL-4), maturation of eosinophils and basophils (IL-5), mucus overproduction and allergic airway hyperresponsiveness, such as IL-4 and IL-13 [14]. In turn, the response of Th2-cytokines in lung allergic pathogeneses is intensified by IL-25 (IL-17E) secreted by airway epithelial cells and innate immune cells [15]. In our study, we observed that the increase in IL-4, IL-5 and IL-25 levels in the lungs of sensitized mice, in response to allergen exposure, was prevented by treatment with NaHS. The decrease in cytokines levels, concomitant with the reduction in peribronchiolar cell infiltration, suggests that NaHS might influence inflammatory cell migration to the lung.

On the other hand, TGF-β released by the epithelium in response to IL-13 may play a key role in driving the repair process that takes place after damage to the lung tissue. The production of a pathogenic fibrotic response is closely related to the progression of airway hyperreactivity and severity of asthma [16]. In our model of acute allergic lung inflammation, no change was observed in the TGFβ1 levels; this finding is in agreement with the absence of peribronchiolar collagen. Although IL-13 was increased by OVA challenge, the presumable cytokine level was insufficient to induce TGF-β augmentation. Neither the levels of IL-13 nor of TGF-β1 were influenced by the NaHS-treatment.

The pathogenesis of asthma is closely related not only to inflammatory cell infiltration and Th2-cytokines, but also to oxidative stress and cell apoptosis. Yan et al. (2017) showed that IL-25 increases both lung oxidative stress and apoptosis in the asthma model in mice, as well as in the *in vitro* human epithelial cell line 16HBE culture [13]. As in our previous studies, we demonstrated that NaHS treatment was effective in reducing oxidative stress of the airways by increasing the activity of antioxidant enzymes [11,12]. In this next step, we decided to investigate the apoptosis in the lungs of NaHS-treated allergic mice.

Knowing that activation of apoptosis can be initiated by two different routes [6], we verified the expression of effector caspase 9 and the Bax protein involved in the mitochondrial pathway, as well as effector caspase 3 and the receptor ligand (FasL) that initiates the extrinsic pathway. Our data showed that OVA challenge in sensitized mice was able to induce an increase in caspase 3 and FasL expression in lung tissue, both prevented by NaHS treatment. This suggests that the extrinsic pathway is the target of H_2S action in the lung apoptosis process after allergen challenge. These data corroborate the findings by Liu et al., 2013 [17], who demonstrated that NaHS treatment prevented oleic acid-induced acute lung injury in rats by reducing the FasL expression in alveolar epithelial cells [17].

Furthermore, the *in situ* apoptosis in lungs tissue verified by using TUNEL experiments in our study indicated that the allergenic challenge promoted an increase in bronchiolar epithelial cell apoptosis, which led to the destruction of the bronchial epithelium. Moreover, NaHS treatment prevented this effect, thus showing that H_2S can protect against lung epithelial injury.

These results lead us to conclude that H_2S promotes a protective effect against the damage caused by allergic reaction in the lung, by decreasing IL-25 and preventing bronchial epithelium apoptosis. As such, it represents a therapeutic potential for allergic lung diseases, such as asthma.

Authors' contributions

All the authors made substantial contributions to the conception and design of the study, and to the acquisition, analysis and interpretation of the data. All the authors were involved in drafting the manuscript or revising it, and all read and approved the final manuscript.

Declaration of Competing Interest

None.

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