



Chronic exposure to sulfur dioxide enhances airway hyperresponsiveness only in ovalbumin-sensitized rats

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HIGHLIGHTS

- ▶ SD rats, either OVA-sensitized or not, were exposed to sulfur dioxide (SO₂).
- ▶ The rats were examined for changes in structure and function of the airways.
- ▶ SO₂ promoted airway inflammation in both OVA-sensitized and normal rats.
- ▶ SO₂ enhanced airway hyperresponsiveness in OVA-sensitized, but not normal rats.
- ▶ SO₂ may be a specific risk factor to worsen airway function of the asthmatics.

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ABSTRACT

Sulfur dioxide (SO₂) is a common air pollutant that triggers asthmatic symptoms, but its toxicological mechanisms are not fully understood. Specifically, it is unclear how airborne SO₂ affects airway hyperresponsiveness (AHR) – a hallmark feature of asthma. To this end, we investigated the effects of chronic exposure to SO₂ on AHR, airway inflammation, tissue remodeling, cell stiffness (*G'*) and contractility of the airway smooth muscle cell (ASMC). Newborn Sprague–Dawley (SD) rats sensitized to ovalbumin (OVA) was used as the model to mimic asthmatic symptoms. The experimental results show that exposure to SO₂: (1) significantly increased Penh (an indicator of AHR) in the OVA-sensitized rats ($p < 0.01$) but not in the normal rats ($p > 0.05$), which correlated with the increase of airway smooth muscle mass; (2) increased IL-4 production in BALF of both the normal ($p < 0.05$) and OVA-sensitized rats ($p < 0.001$), but decreased IFN- γ in BALF of only the normal rats, and in serum only increased IL-4 production of the OVA-sensitized rats ($p < 0.001$); (3) increased ASMC stiffness (*G'*) and contractility only in the OVA-sensitized rats ($p < 0.001$, $p < 0.05$, respectively). Taken together, these results demonstrate that SO₂ may be a universal airway inflammatory factor, but more importantly, specific to exacerbating AHR in asthmatics only. These findings uncover a potential mechanism of SO₂-induced health effects and may provide a basis for therapeutic targets.

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1. Background

Asthma is an increasingly prevalent respiratory disease and represents a major global health issue with significant economic and social burden (Wegmann et al., 2005). Asthma prevalence is particularly high among children in more developed countries (Asher et al., 1995). It has been previously shown that asthma is

characterized by airway hyperresponsiveness (AHR), presence of inflammatory cells in bronchoalveolar lavage fluid (BALF) and in the bronchial wall, and airway wall tissue remodeling (Gueders et al., 2009; Li et al., 2008; Wegmann et al., 2005). However, asthma etiology is still not fully understood. Specific factors that may contribute to the pathogenesis of asthma include environmental conditions, such as the level of ambient air pollution. These airborne impurities are generally associated with the initiation, progression and exacerbation of the disease (Lin et al., 2004; Schneider and Freeman, 2001; Tarlo et al., 2001; Tsai et al., 2003; Watts, 2006).

Sulfur dioxide (SO₂) is known as a major environmental risk factor for initiation and/or exacerbation of asthma. For instance, chronic exposure to low dose (2.8–14 mg/m³) SO₂ has been found

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to aggravate symptoms in asthmatic patients (Grella et al., 2002; Tarlo et al., 2001). Moreover, other studies have linked SO₂ exposure to impaired immune defense functions within the respiratory system, as well as induction of respiratory-tract inflammation (Meng, 2000). These cases are generally worsened among children and in the elderly (Kilic, 2003; Koken et al., 2003; Lin et al., 2004; Millman et al., 2008; Qian et al., 2004; Tarlo et al., 2001).

At the cellular level, SO₂ has been implicated in membrane damage, genetic toxicity, oxidative damage, DNA damage, alterations in signal transduction, and changes to ion channels. These outcomes indicate the toxicological effects of SO₂ to be complex and broad (Bai and Meng, 2005; Meng, 2003; Meng et al., 2005; Nie and Meng, 2005; Qin and Meng, 2005, 2006). However, while prior studies have attributed the detrimental effects of SO₂ to its role in inducing airway inflammation, few have examined its capacity to regulate biomechanics of the airway smooth muscle cell (ASMC). Moreover, ASMC mechanics is known to ultimately mediate airway hyperresponsiveness (AHR) – a hallmark feature of asthma. Beyond hyperresponsiveness, ASMC mechanics further affects the dilative capacity of the airways during deep inspiration (DI).

In this study, our goal was to therefore determine whether SO₂ exposure would alter airway function and in particular, modify ASMC biomechanics and AHR. To address these questions, we evaluated AHR, airway inflammation, and airway tissue remodeling using a murine model of SO₂ exposure. Additionally, we investigated ASMC biomechanics using *in vitro* measurements of cell stiffness and contractility. Asthmatic symptoms were simulated via ovalbumin (OVA) sensitization. Our experimental results highlight a potential pathway in which SO₂ may affect asthmatic and non-asthmatic individuals. These results have implications in further understanding the pathogenesis of airway diseases such as asthma as well as facilitating new prevention/treatment paradigms.

2. Materials and methods

2.1. Experimental animals

Breeding male and female Sprague–Dawley (SD) rats were purchased from the animal center of Chongqing Medical University (CQMU). All breeding male and female rats were housed separately in this center and received food and water *ad libitum*. After two weeks, the female rats were impregnated and the neonatal male rats of four weeks were used for experimentation. These animals were randomly divided into four groups of 10 animals each. These groups were: (1) Control, of which the animals were not exposed to OVA or SO₂, (2) SO₂, of which the animals were exposed to SO₂ alone, (3) OVA, of which the animals were exposed to OVA alone, and (4) OVA + SO₂, of which the animals were exposed to both OVA and SO₂.

All the animals were cared and treated in the same animal center of CQMU, and when not being treated, the animals had free access to food and water. All animal procedures were approved by the Animal Investigational Committee of CQMU, and performed in accordance with The Guide for Care and Use of Laboratory Animals issued by the Ministry of Health, People's Republic of China.

2.2. Allergen sensitization

The animals used to mimic asthmatic subjects, i.e. those in the groups of OVA, and OVA + SO₂, were first sensitized by allergen challenge following a standard protocol (Chapman et al., 2011; Elwood et al., 1992). Briefly, on the first day of the study (Day 0), each animal was injected intraperitoneally with 10 mg of OVA together with 100 mg Al(OH)₃ in 1 ml 0.9% NaCl solution. Then, on Day 7 each animal was injected a second dose of 10 mg OVA with 100 mg Al(OH)₃ in 1 ml saline solution as a booster sensitization. Subsequently, from Day 15, each animal was exposed to aerosolized OVA (1%) for 30 min everyday for 4 weeks to maintain its asthmatic phenotype.

The animals used to mimic non-asthmatic subjects for exposure to SO₂, i.e. (SO₂ group) were treated in the same manner as described above except that the animal was injected with only a vehicle of 100 mg Al(OH)₃ in 1 ml 0.9% NaCl solution, and exposed to aerosolized saline.

The animals in the group of Control were exposed to filtered fresh air and aerosolized saline throughout the duration of the experiment (from Day 0 to Day 42).

2.3. Exposure to SO₂

2.3.1. Exposure chamber

We designed an exposure chamber of SO₂ based on a model published previously (Bai and Meng, 2005; Li et al., 2008). SO₂ was administered with this custom-built equipment and the concentration of SO₂ was determined by a SO₂ sensor (SO₂/CF-20 SO₂ sensor, Shenzhen ASA Technology Co., Ltd., China).

2.3.2. Exposure protocol

In addition to the above treatment, each animal in the group of OVA + SO₂, and SO₂ was placed into the above described exposure chamber and challenged with 2 ppm (5.6 mg/m³) SO₂ for 4 h everyday from Day 15 to Day 42 (4 weeks).

2.4. Assessment of airway responsiveness

Lung function of the rat was tested at 18–24 h after the last exposure to challenge (Black et al., 2001; Hirota et al., 2009; Leigh et al., 2002; Mathew et al., 2001; Elwood et al., 1992), during which the respiratory pressure curves were measured and recorded by using whole body plethysmograph (EMKA Technology, France) while methacholine (MCh) was administered to the rat by ultranebulization for 3 min at stepwise increasing concentrations from 0 to 50 mg/ml. Airway responsiveness was expressed as enhanced pause (P_{enh}), a calculated unit-less value that correlates the airway resistance, impedance, and intrapleural pressure within the same animal. A total of six rats were measured in each group.

2.5. Assessment of inflammatory response

To assess the inflammatory response in the rat, the level of two cytokines known to be associated with asthma, namely, interleukin (IL) – (4), and interferon (IFN) – γ were measured in BALF and serum for each rat. Briefly, subsequent to the test of lung function, 5 ml blood was taken from each experimental rat by the method of enucleating eyeball, and then centrifuged at 2500 rpm and 4 °C for 5 min. The supernatant was packaged into 1.5 ml centrifuge tubes, and stored at –20 °C before use. Afterward, the left lung of the rat was lavaged with saline via a tracheal catheter. This process was repeated three times with 6 ml saline as the total volume. The lavage fluid was then centrifuged at 2500 rpm and 4 °C for 5 min, and the supernatant was collected and stored at –80 °C before use. Finally, the level of IL-4, or IFN- γ in the supernatant collected from either BALF or serum was measured with ELISA by following the manufacturer's protocols.

2.6. Assessment of airway tissue remodeling

The right lungs (which were not lavaged) were fixed with 4% formaldehyde, and tissue slices from different lobes were embedded in paraffin and cut into 2 μ m-thick sections. Each section placed on glass slide was stained with hematoxylin and eosin (H&E) and double-blindly evaluated under microscope for histological changes by a professional pathologist.

To further quantitatively evaluate the airway tissue remodeling, the cross-sections of lung tissue as prepared above were imaged under microscope at 50 $\times$ magnification, and the images were subsequently analyzed by using an image processing and analysis software (Image-Pro® Plus, Media Cybernetics, Inc., Rockville, MD) to calculate the area of airway smooth muscle (WAm) in the bronchial airway wall according to the methods reported before (Wang et al., 2006). Briefly, each image was processed to identify the layer of the airway smooth muscle and the bronchial basement membrane. Then, the perimeter of the basement membrane (Pbm), the bronchial area within the outer (AMe), and inner edge (AMi) of the airway smooth muscle layer were computed, respectively. Thus, the area of airway smooth muscle (WAm) was defined as the area of the muscle layer per unit length of the basement membrane (Pbm), i.e. $WAm = (AMe - AMi)/Pbm$. To minimize systematic error or subjective bias, only cross-sections of similar bronchi from the four experimental groups were compared.

2.7. Assessment of stiffness and contractility of the ASMC

To assess ASMC mechanics, we measured stiffness and contractility of airway smooth muscle (ASM) cells cultured *in vitro* using optical magnetic twisting cytometry (OMTC). OMTC is a high-throughput tool for quantifying mechanics of adherent cells with remarkable temporal and spatial resolution, of which the details have been extensively published previously (Deng et al., 2005, 2004; Fabry et al., 2001a,b; Maksym et al., 2000). Briefly, ASM cells were harvested from bronchial tissue explants from the experimental SD rats. The primary cells were then cultured in T25 cell culture flask (Costar, Corning, NY) and passaged when the cells grew to confluence. Cells of 2–5 passage were used for experiment. Before experiment, the cells were seeded into collagen (Type I, Sigma) coated 96-well polystyrene dishes (Costar; Corning, NY) at 15,000 cells/well, and cultured for 24 h in serum-free medium. Then, 4.5 μ m ferrimagnetic beads (kindly gifted by the laboratory of Dr. J.J. Fredberg of Harvard School of Public Health, Boston, MA) were added to the cell surface, approximately one bead per cell, and incubated for ~20 min. Because the beads were pre-coated with synthetic peptide (50 μ g peptide/mg beads) of Arginine-Glycine-Aspartic Acid (RGD) sequence that binds specifically to integrin

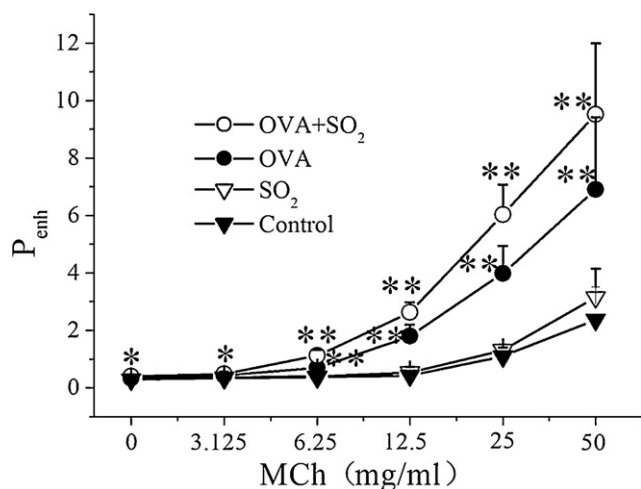


Fig. 1. Bronchial airway responsiveness to methacholine stimulation in different experimental animal models. The models were four groups of Sprague–Dawley (SD) rats that were either sensitized by ovalbumin (OVA) then exposed to sulfur dioxide (SO₂) (OVA + SO₂), OVA only (OVA), SO₂ only (SO₂), or unexposed (Control). Airway responsiveness of the animals was evaluated by the enhanced pause (Penh) in the pressure curves via whole body plethysmography following methacholine (MCh) challenge. The results are displayed as the mean (open circle for OVA + SO₂, filled circle for OVA, open triangle for SO₂, and filled triangle for Control, respectively) and the standard deviation (SD, the error bars) of Penh ($n=6$ for each group, and *, ** indicate $p < 0.05$, 0.01 , respectively).

receptors on the cell surface, they were tightly bound to the cells after incubation. To measure cell stiffness, a high intensity impulse (~100 ms) of magnetic field was first applied to the cell surface bound beads, which magnetized all the beads to the same direction horizontally. Then, a sinusoidal magnetic field was applied vertically, which generate a magnetic torque to the bead and thus oscillated the bead horizontally. The horizontal motion of the beads was optically recorded and quantified as bead displacement (nm). The cell stiffness (G) was defined as the ratio between the applied magnetic torque and the bead displacement with units of Pa/nm.

Typically, a few hundred cells were measured simultaneously during each experiment. ASMC stiffness was measured at multiple frequencies ranging from 0.1 to 100 Hz in order to characterize the dynamic behavior of the cells (Fabry et al., 2001b). To assess contractility of the ASMC, cell stiffness was measured continuously at a constant frequency bead oscillation (0.3 Hz) for up to 5 min, and at the time point after 50 s measurement either KCl (80 mM; iso-osmotic) or histamine (5 mM) was quickly added to the cells. KCl and histamine are well known contractile agonists for the ASMC, but the former is non-specific and the latter is specific to the G-protein coupled receptors (GPCRs). The addition of KCl or histamine would cause increase in cell stiffness due to cell contraction induced by the agent, thus the ratio of average cell stiffness measured after over that measured before drug addition was defined as contractility of the cell. More details regarding this method to assess ASMC contractility can be found in a previously published report (Fairbank et al., 2008).

2.8. Statistical analysis

All values were expressed as mean $\pm$ standard deviation (SD) except for those of cell stiffness that were expressed in median $\pm$ standard error (SE). Statistical analysis for multiple comparisons were performed with one-way analysis of variance (ANOVA) followed by Tukey test using SigmaPlot 12.0 (SysStat Software, San Jose, CA). A level of $p < 0.05$ was accepted as statistically significant.

2.9. Chemicals and reagents

SO₂ gas (purity: 99.95%) was purchased from South-China Special Gases Co., Ltd. (Guangdong, China), and OVA (grade V) was purchased from Sigma–Aldrich Chemical Co. (Poole, UK). ELISA kits for rat IFN- γ , and IL-4 were purchased from NeoBioscience Technology Co., Ltd. (Beijing, China), and Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China), respectively.

3. Results

3.1. SO₂ exposure and airway responsiveness

Fig. 1 shows the results of airway responsiveness as quantified by Penh in response to MCh challenge. In all cases, MCh challenge

caused dose-dependent increase of Penh, but the extent of Penh increase in response to same dose MCh stimulation was different in different subject group. In the absence of SO₂, the sensitized rats exhibited much greater extent of Penh increase in response to MCh stimulation as compared to the non-sensitized rats (OVA group vs. Control group, $p < 0.01$), indicating AHR as seen in asthmatics. In the presence of SO₂ exposure, the non-sensitized rats showed little change in Penh response to MCh challenge (SO₂ group vs. Control group, $p > 0.05$). However, the sensitized rats exhibited the greatest Penh increase in response to MCh challenge among all groups, even greater than the sensitized rats not exposed to SO₂ (Penh = 9.52 ± 2.27 in OVA + SO₂ vs. 6.91 ± 2.50 in OVA group at 50 mg/ml MCh, $p < 0.01$).

3.2. SO₂ induced airway inflammation and tissue remodeling

The H&E stained histological sections of experimental rat bronchial airways are shown in Fig. 2a–d. And the result of airway wall smooth muscle area (WAM) analysis is shown in Fig. 2e.

For the Control group, the alveolus and bronchiole structures were clear, with few infiltrated inflammatory cells and a thin layer of ASM cells (Fig. 2a and e). For the SO₂ group, hyperemia was found in the lung parenchyma, and there appeared to be an influx of inflammatory cells in the alveolus and bronchiole structures. However, there was little change to the ASM cell layer when compared to Control (Fig. 2b and e). For the OVA group, as compared to the Control group, there appeared to be more inflammatory cells residing around the bronchiole and capillary blood vessels. These cells were morphologically consistent with eosinophils and macrophages. The mucus plug could be seen inside the airway lumen, and the number of plicae around the inner wall of the bronchiole also appeared to have increased. Airway tissue remodeling was observed including damage to the cilia and epithelial layers, deposition of collagen fibers under the layer of epithelium, and thickening of the ASMC layer and its extracellular matrix (ECM) (Fig. 2c). Qualitatively, the OVA + SO₂ group exhibited similar pathological changes in the airway tissue to those observed in the OVA group (Fig. 2d). However, the extent of airway tissue remodeling in the OVA + SO₂ group seemed much more pronounced, in particular the increase of ASM layer (WAM) was significantly greater than that of the OVA group (Fig. 2e, $p < 0.001$).

3.3. SO₂ induced expression of IL-4, and IFN- γ

Fig. 3 shows the expression levels of IL-4 and IFN- γ , two inflammatory cytokines associated with asthma, as measured by ELISA in BALF and serum, respectively. In BALF, the measured concentration of IL-4 significantly increased, and IFN- γ significantly decreased in all cases as compared to the Control ($p < 0.001$). However, there was only a further increase of IL-4 production, but no change of IFN- γ in BALF of the OVA + SO₂ group when compared to the OVA group ($p < 0.001$). Interestingly, the concentration of IL-4 in BALF increased in the order of Control, SO₂, OVA, OVA + SO₂ group (9.34 ± 1.71 , 24.54 ± 2.93 , 44.99 ± 1.41 , 69.45 ± 7.08 pg/ml, $p < 0.001$) (Fig. 3a). In serum, the concentrations of IL-4 and IFN- γ appeared to be less influenced by SO₂ and/or OVA. As compared to the Control, IL-4 only increased significantly in the OVA + SO₂ group ($p < 0.001$), while IFN- γ decreased in the OVA and OVA + SO₂ groups ($p < 0.001$). However, there was no difference of the IFN- γ level between the OVA and OVA + SO₂ groups (Fig. 3b).

3.4. Effect of SO₂ on ASMC stiffness and contractility

Fig. 4 displays the log–log plot of ASMC stiffness (G) measured over a frequency (f) range of 0.1 and 100 Hz. Within this limit of measured frequencies, the cell stiffness exhibited typical

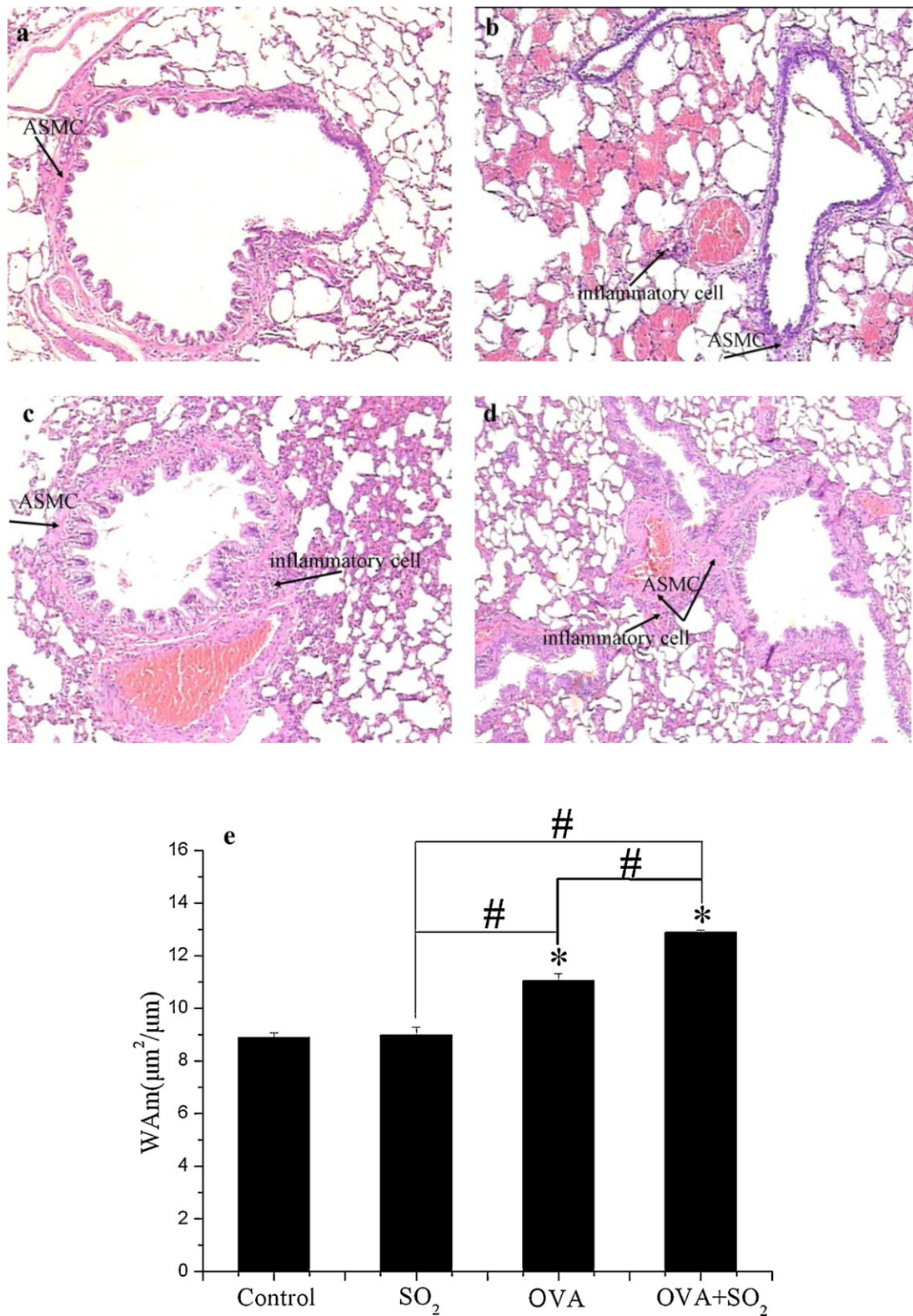


Fig. 2. Histological cross-sections of lung tissue and the airway wall smooth muscle area from the experimental animals. The lung tissue slices were fixed with formaldehyde and stained by hematoxylin and eosin (H&E), and then examined and imaged with optical microscopy (50 $\times$ mag.). The representative histology image from (a) Control group shows open clear airway lumen and a thin layer of ASM cells in the absence of inflammatory cells, (b) SO₂ group depicts narrowing of the airway lumen and infiltration of inflammatory cells, but no apparent changes to the ASM layer, (c) OVA group shows narrowed airway lumen and inflammatory cells in conjunction with an increase in ASM layer thickness and remodeling, (d) OVA + SO₂ group highlights severe narrowing of the airway lumen, inflammation and a marked increase in the ASM layer thickness. (e) Airway wall smooth muscle area (WAm) increased significantly in both OVA and OVA + SO₂ groups when compared to the Control, but the increase of WAm in the OVA + SO₂ group was even greater than in the OVA group ($n = 4$, * indicates $p < 0.001$ as compared to Control, and # indicates $p < 0.01$ as compared between the labeled groups, respectively).

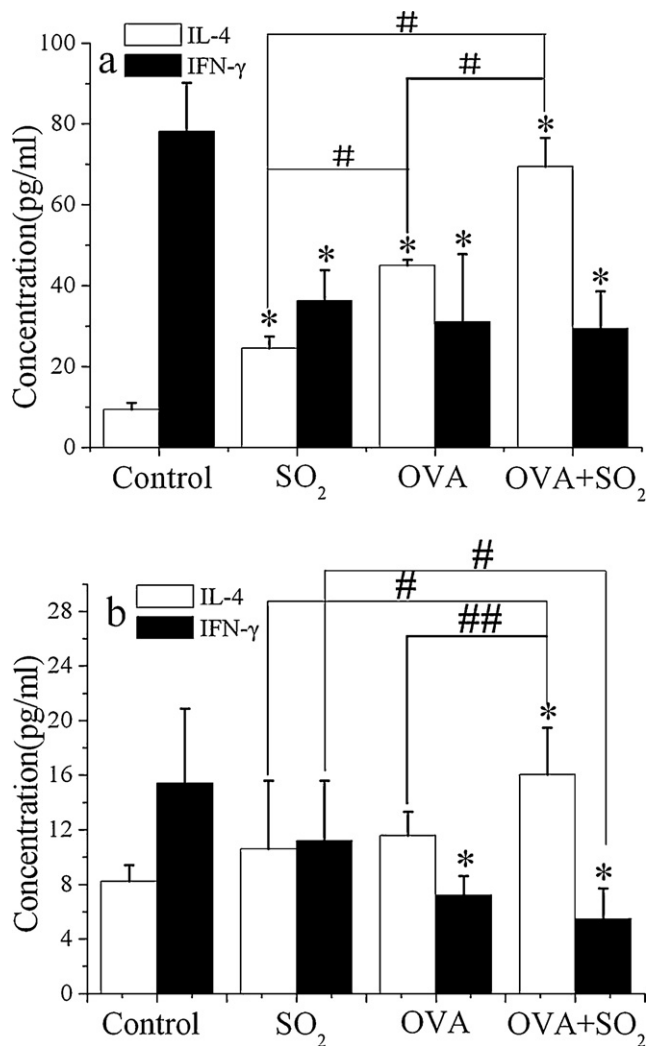


Fig. 3. Concentrations of inflammatory cytokines, IL-4, and IFN- γ in either BALF or serum obtained from different experimental animal models. The concentration of IL-4, and IFN- γ was measured by ELISA with corresponding antibodies, respectively. (a) The concentration of IL-4, and IFN- γ as measured in BALF. Results show IL-4 increased in the order of Control, SO₂, OVA, and OVA+SO₂, but IFN- γ decreased to similar extent in all cases as compared to the Control. (b) The concentration of IL-4 and IFN- γ as measured in serum. The serum level of IL-4 increased only in the OVA+SO₂ group, and IFN- γ decreased in the OVA and OVA+SO₂ groups as compared to the Control. Similar as in BALF, OVA+SO₂ exposure did not induce additional reduction of IFN- γ in serum as compared to the OVA group ($n=8$, * indicates $p < 0.001$ as compared to Control, and # indicates $p < 0.001$ as compared between the labeled groups, respectively).

power-law behavior. That is the stiffness value increased with increasing frequency (f) as a power-law function ($G \sim f^\alpha$). The magnitudes of cell stiffness were found to be in ascending order of Control, SO₂, OVA, and OVA+SO₂. The slope of the power-law (α) also increased slightly in the same trend.

A typical time course plot of the cell stiffness (G) is shown in Fig. 5. The measurements were sampled at 0.3 Hz before and after the addition of the contractile agonist. The baseline cell stiffness (G_0) is reported as the reading prior to the agonist. Following agonist application, G increased almost instantaneously but stabilized at this higher level within 10 s. The average value of G measured 10–50 s following agonist addition represents the post contraction cell stiffness (G'), while G'/G_0 was taken to be the ASMC contractility. Similar to the multi-frequency measurement results shown in Fig. 4, G_0 of ASMC in the groups of SO₂, OVA, and OVA+SO₂ increased in ascending order and were all significantly greater than

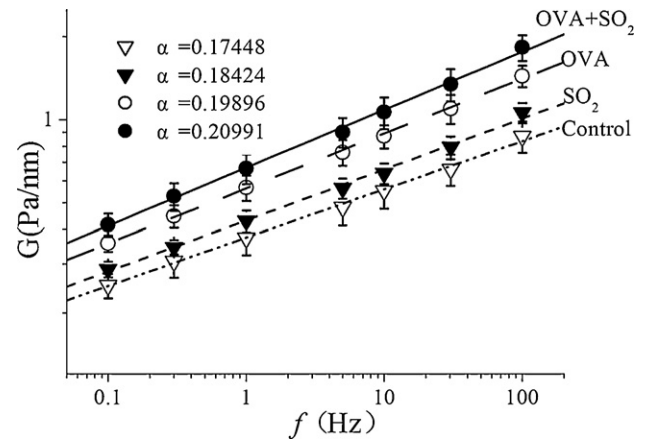


Fig. 4. ASMC stiffness as obtained from the experimental animal models. Cell stiffness (G , Pa/nm) was measured by optical magnetic twisting cytometry (OMTC) at a range of frequencies (f , Hz). The results are displayed as the median (open circle for OVA+SO₂, filled circle for OVA, open triangle for SO₂, and filled triangle for Control, respectively) and standard error (SE) of the measured G . Straight lines represent linear least-square fitting of G versus f in each group. Due to the log–log scale, the linear fit indicates a power-law behavior, i.e. the cell stiffness increased in proportional to the power (α) of frequency ($G \propto f^\alpha$). The value α demonstrates the slope of the fitted lines.

the Control ($p < 0.001$, $p < 0.001$, or $p < 0.01$, respectively) (Fig. 6a). In contrast, the contractility of ASMC (G'/G_0) was significantly increased only in the OVA+SO₂ group for both KCl and histamine stimulations when compared to the Control ($p < 0.05$) (Fig. 6b).

4. Discussion

Airborne pollutants pose significant risks to public health and our study was designed to elucidate the mechanism in which one of these components, SO₂ may contribute to the pathogenesis of respiratory diseases. SO₂ is of special interest because of its abundance in industrialized cities. For example, in Chongqing, China, high levels of SO₂ arise from burning sulfur rich coals. Geographically, the surrounding mountains further restrict airflow. This confluence of

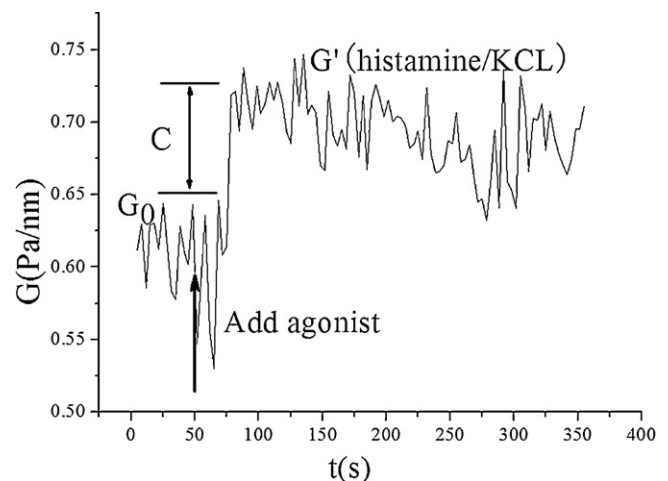


Fig. 5. Time course of the contractile and stiffness response in ASM cells following KCl or histamine stimulation. Cell stiffness (G) was measured continuously for up to 5 min. At a time point of 50 s following measurement start, the contractile agent (either histamine or KCl) was added to the cells. Although the measured G initially fluctuated, the average value steadily stabilized over time. Addition of histamine/KCl increased cell stiffness sharply, with a maximal contractile response obtained after ~10 s. The time averaged stiffness prior to drug addition was denoted G_0 (ASM cell baseline stiffness). Stiffness following drug exposure was taken to be G' while C indicated the scope of contractile response.

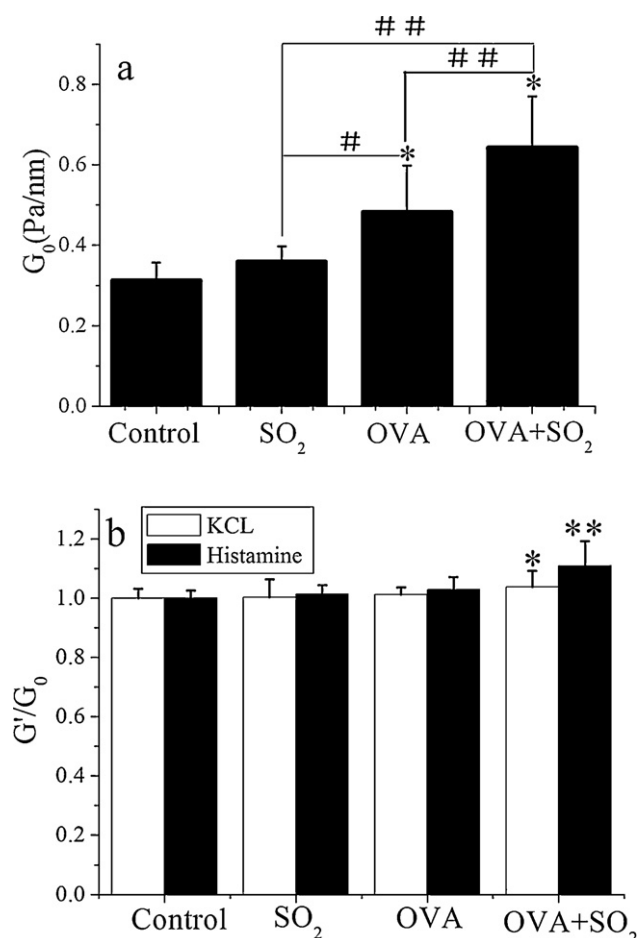


Fig. 6. Baseline stiffness and contractility of the ASM cells to different treatments. (a) Baseline stiffness, G_0 increased in the ascending order of Control, SO_2 , OVA, and OVA+ SO_2 and was similar to those measured at variable frequencies. (b) ASM cell contractility as defined by G'/G_0 . Only ASM cells of the OVA+ SO_2 group exhibited greater contractility as compared to the Control ($n=8$, *, ** indicate $p < 0.05$, 0.01, respectively as compared to Control, #, ## indicate $p < 0.05$, 0.01, respectively as compared between the labeled groups).

human and natural factors localizes SO_2 within the city, causing a potentially hazardous environment.

To investigate the effects of chronic SO_2 exposure on the structure and function of bronchial airways, we developed a murine model of asthma based on OVA sensitization. Newborn SD rats were used in order to simulate SO_2 -induced respiratory dysfunctions common to young children (Kilic, 2003; Millman et al., 2008; Qian et al., 2004). In particular we studied the in vivo effects on airway hyperresponsiveness as well as changes to ASMC biomechanics in vitro. A SO_2 concentration of 5.6 mg/cm^3 (2 ppm) was used to represent the ambient SO_2 levels found in industrialized cities such as Chongqing. In comparison, normal atmospheric values are on the order of 1.4 mg/cm^3 or less (Li et al., 2007).

Our results showed that SO_2 exposure resulted in universal inflammation of the bronchial airways regardless if the animals were sensitized to OVA. However, the magnitudes of the inflammatory responses were often higher in OVA-sensitized rats. SO_2 -induced inflammation was evident in the histological sections which depict infiltration of inflammatory cells (eosinophils or macrophages). Increased secretion of mucus was also observed. Higher levels of IL-4 were found while IFN- γ decreased in both the BALF fluid and serum. IL-4 and IFN- γ are two cytokines released from Th₁ and Th₂ cells and are characteristic to inflammatory diseases such as asthma. IL-4 is a factor for B-lymphocyte class

switching, and one of the primary cytokines for inducing naïve Th₀ cell differentiation into Th₂ cells. This in turn, positively regulates circulating IgE. In contrast, IFN- γ is known to induce Th₀ cells to differentiate into Th₁ cells, and negatively regulate IgE (Coffman and Carty, 1986). We found that exposure to SO_2 alone promoted IL-4 release but suppressed IFN- γ in BALF—a process indicative of airway inflammation. SO_2 alone, however, did not change the level of these cytokines in serum. Furthermore, these inflammatory biomarkers were to some extent mediated by OVA sensitization. In BALF, both OVA-sensitization and exposure to SO_2 alone led to an increase of IL-4 and a decrease in IFN- γ but OVA-sensitization seemed to be more potent for inducing IL-4 production. OVA+ SO_2 exposure resulted in an additive effect on IL-4 induction to SO_2 exposure or OVA-sensitization alone (24.54 ± 2.93 and 45.00 ± 1.41 vs. 69.45 ± 7.08 , $p < 0.001$) but no additional changes in IFN- γ . In serum, exposure to SO_2 alone had little effect on the two cytokines, and OVA-sensitization alone only induced reduction of IFN- γ but no change of IL-4 was detected. Nevertheless, OVA+ SO_2 exposure did increase the level of IL-4, but did not further reduce the level of IFN- γ . We speculate it may be possible that this broad airway inflammatory response in both OVA-sensitized and OVA-absent rats may be due to oxidative damage. Indeed, SO_2 has been demonstrated to induce systemic oxidative damage (Meng, 2003), which may further lead to inflammation (Bernstein et al., 2004) and tissue injury (Houtmeyers et al., 1999). The pathways for oxidative stress and oxidative damage have largely been discovered (Li et al., 2003; Meng and Zhang, 1992; Meng, 2000) but our results are consistent with this hypothesis. The results of the two cytokines in serum suggest that in our model the level of SO_2 exposure or OVA-sensitization could barely cause a systemic response, but OVA+ SO_2 exposure may potentiate the inflammatory effect of SO_2 on the systemic level of cytokines.

In addition to airway inflammation, AHR is recognized as a cardinal feature of asthma (Louis et al., 2002). We report that animals sensitized by OVA exhibited characteristic AHR in response to MCh stimulation. When the sensitized animals were further exposed to SO_2 the existing AHR was enhanced, with Penh increasing from 6.91 ± 2.50 to 9.52 ± 2.27 (50 mg/ml MCh, $p < 0.01$). In contrast, the airway response of non-sensitized animal subjects was negligibly impacted by SO_2 . This differential outcome in airway responsiveness between sensitized and non-sensitized animals was somewhat unexpected and suggests that SO_2 may have different impacts on the respiratory function of asthmatics vs. non-asthmatics. Exposure to SO_2 alone may not induce asthma, but may contribute to subsequent narrowing of the already hyperresponsive airways in asthmatic patients. This implies that SO_2 pollution could be a potential health risk that is selective to the asthmatic population.

Although the underlying mechanisms responsible for AHR may be multifaceted, its common final pathway may ultimately be correlated to ASMC mechanics (An et al., ERJ 2007). Alteration of various aspects of the ASMC has been associated with asthma, which in turn may contribute to AHR and airway narrowing (Deng et al., 2005, 2004, 2006; Fabry et al., 2001a,b; Fairbank et al., 2008). For example, it has been demonstrated that in asthma ASM cells undergo proliferation and hypertrophy—a phenomenon that eventually thickens the airway wall and obstructs airflow (Black et al., 2001; Deng et al., 2005; Zhai et al., 2004). When assessing cell biomechanics, we found that ASM cells exhibited the common power-law relationship between the cell stiffness and the measurement frequency (Deng et al., 2006; Fabry et al., 2001a,b). Exposure to SO_2 , OVA, and OVA+ SO_2 , however, increased both the magnitude of the ASMC stiffness and the power-law slope. The baseline stiffness also increased significantly in these groups ($p < 0.01$). These increases followed the same trend as the inflammatory cytokine and lung function responses.

The resultant inflammatory response as well as stiffening and possibly fluidization (i.e. the increase of power-law slope) of the ASMC are all possible contributors to enhanced airway narrowing. Moreover, cell stiffening and fluidization are correlated with increasing ASMC contractility (Deng et al., 2005, 2004, 2006; Fabry et al., 2001b; Fairbank et al., 2008). Indeed, exposure to SO₂ led to increased ASMC contractility in the OVA-sensitized animals when responding to either KCl or histamine ($p < 0.01$). Interestingly, ASM cells from the OVA-sensitized animals did not show increased contractility in the absence of SO₂. This may be due to the nature of the ASM cells used in this study as they were cultured and passaged in vitro. This subculturing may have degraded the contractile machinery of the ASM cells via loss of receptors and down regulation of contractile proteins.

There are several limitations to the current study. For example, we only established an indirect relationship between SO₂ and airway structure and function. Since inhaled SO₂ does not directly act upon the airway tissue, it is still unknown how SO₂ would affect ASM cells lining the airway walls. Furthermore, while our results corroborate studies that indicate a strong correlation between airway inflammation and responsiveness (Elwood et al., 1992), other study has concluded otherwise (Crimi et al., 1998). Asthmatic model and pathology differences (rat vs. human) as well as lack of causality between airway inflammation and hyperresponsiveness may be the source of these inconsistencies. Therefore, further studies are warranted to fully disclose the role in which SO₂ and other air pollutants affect respiratory health and disease.

Our findings suggest for the first time that chronic exposure to even low levels of SO₂, especially in asthmatic subjects, may exacerbate already compromised lung function. Exposure to SO₂ led to airway inflammation regardless if the subject exhibited asthma symptoms, but the outcomes were more dramatic with the OVA-sensitized subjects displaying asthma features. In contrast, exposure to SO₂ led to thickening of the ASM cell layer and airway hyperresponsiveness only in the OVA-sensitized subjects, suggesting that the adverse effects of SO₂ on ASM function were highly dependent on OVA-sensitization. The proposed mechanism is the alteration of the biomechanical properties in ASM cells, as evidenced by the increased cell stiffness and contractility in ASM cells of the subjects both OVA-sensitized and exposed to SO₂. Our work is consistent to the data of others which show that low level SO₂ may affect the respiratory system (Bethel et al., 1985; Park et al., 2001; Samukawa et al., 2003) and aggravate the symptoms of asthmatics (Grella et al., 2002). Therefore, SO₂ may be implicated as a possible health risk to pre-existing asthma patients. This information may prove useful in formulating better public health strategies for protecting asthmatics in locations where SO₂ pollution is high, or in developing therapeutic targets for respiratory conditions induced by SO₂ or other airborne pollutants.

Conflict of interest

None.

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