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Prior SO₂ exposure promotes airway inflammation and subepithelial fibrosis following repeated ovalbumin challenge

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Clinical and Experimental Allergy

Summary

Background Exposure to allergens or air pollutants often leads to asthma exacerbations associated with aggravation of airway inflammation. Although, repeated allergen challenge often induces chronic allergic airway inflammation (CAAI) and airway remodelling, yet, the effects of brief exposure to air pollutants such as SO₂ on development of CAAI and airway remodelling remain to be clarified.

Objective The aim of the experiment was to investigate the effects of acute neutrophilic airway inflammation induced by brief exposure to SO₂ on development of CAAI and subepithelial fibrosis (SEF) in a murine model of asthma.

Methods Acute airway inflammation was induced by brief exposure to 50 p.p.m. SO₂ (1 h/d, 3 days). CAAI and SEF in BALB/c mice were induced by repeated challenge with ovalbumin (OVA) for 5 or 9 weeks with or without prior exposure to SO₂. Bronchoalveolar lavage fluid (BALF) eosinophilia as index of CAAI, BALF endothelin-1 (ET-1) and TGF-β1 levels, morphometric evaluation of fibrotic area beneath subbasement membrane and lung hydroxyproline content (Hyp) as indexes of SEF were monitored.

Results Exposure to SO₂ led to acute neutrophilic inflammation and epithelial sloughing with profound elevation of BALF ET-1. Repeated OVA challenge resulted in CAAI and SEF along with elevation of Hyp, increase of fibrotic area beneath subbasement membrane and elevation of BALF TGF-β1. Preceding SO₂ exposure exaggerated BALF eosinophilia, facilitated and enhanced SEF with more significant elevation of BALF ET-1 and TGF-β1 levels compared with OVA-challenged mice without prior exposure to SO₂. The increase of Hyp was positively correlated with elevation of BALF TGF-β1 during CAAI ($r = 0.842$, $P < 0.01$).

Conclusion This data demonstrated that SEF developed in parallel with severity and time course of CAAI following repeated OVA challenge. SO₂-induced acute epithelial injury and neutrophilic inflammation could enhance CAAI and promote SEF, probably through overexpression of ET-1 and TGF-β1.

Keywords airway inflammation, growth factor, subepithelial fibrosis, sulphur dioxide

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Introduction

Bronchial asthma is characterized as a triplet of airway inflammation, airway remodelling and bronchial hyper-responsiveness. Inflammatory cells, airway resident cells and various cellular components form a complicated cellular network and are involved in the pathogenesis of asthma [1–3]. Epithelial injury and subepithelial fibrosis (SEF) are two prominent features of airway remodelling resulting in poor asthma control.

Although the pathogenesis of airway remodelling remains to be defined, repeated cycles of epithelial injury/abnormal repair and subsequent exaggeration of cytokine expression in the inflamed epithelium are believed to play pivotal roles in this complicated process [2–9]. Endothelin-1 (ET-1) and TGF-β1 are two pleiotrophic growth factors with potent profibrotic activity. Extensive clinical and animal studies have demonstrated the important links between up-regulation of these mediators by injured epithelium during airway inflammation and development

of SEF in asthma [8–11]. This study is to investigate whether airway epithelial injury from acute neutrophilic inflammation induced by brief exposure to SO₂ can enhance chronic allergic airway inflammation (CAAI) and promote the development of SEF following repeated allergen challenge, to assess the changes of airway expression of ET-1 and TGF-β1 during acute and chronic airway inflammation and to explore their roles in the development of SEF.

SO₂ is a major air pollutant and the toxic effect of SO₂ is dose and time dependent [12]. Exposure to high concentration of SO₂ caused significant epithelial damage, acute or chronic bronchitis with a predominantly neutrophilic inflammation, whereas lower concentration of SO₂ only resulted in decrease of mucociliary activity without significant morphological changes [12–15]. Epidemiological studies have demonstrated the association between air pollution by SO₂ and increased morbidity and exacerbations of asthma from aggravation of airway inflammation, induction of bronchospasm and worsening of airway obstruction in asthmatics [12, 16–18]. Airway mucosal damage and impaired mucociliary clearance may facilitate presentation of aeroallergens and enhance the eosinophilic airway inflammation [18–20], as recently demonstrated by Park et al. showing exposure to 0.1 p.p.m. SO₂ aggravated acute allergic inflammation and increased airflow resistance in ovalbumin (OVA)-challenged guinea pigs [21]. The present investigation on the impact of exposure to SO₂ to airway structural changes during CAAI may further our understanding on mechanisms of airway remodelling and toxicology of air pollutants like SO₂ in the pathogenesis of asthma.

To address this issue, first, animals were briefly exposed to 50 p.p.m. (132 mg/m³) SO₂ [22], resulting in reversible acute neutrophilic airway inflammation and epithelial injury without airway remodelling (our preliminary study determined the optimal concentration to be 50 p.p.m., unpublished data). Then, using a murine model of CAAI [23], airway eosinophilia, histological and biochemical parameters of SEF were monitored along with the levels of TGF-β1 and ET-1 in bronchoalveolar lavage fluids (BALF) in OVA-sensitized BALB/c mice following 5 or 9 weeks of OVA challenge with or without SO₂ exposure before the first allergen challenge.

Methods

All animal-use procedures were approved by the Beijing University of Medical Science Animal Care Committee. Female BALB/c mice (aged 6–8 weeks, 18–22 g; Center of Laboratory Animals, Guangzhou University of Medical Science, Guangzhou) were randomly allocated to seven groups: Vehicle (Group A, *n* = 15), Acute Injury (Group B, *n* = 10), 5 weeks OVA (Group C, *n* = 10), SO₂ + 5 weeks OVA (Group D, *n* = 10), 9 weeks OVA (Group E, *n* = 10), SO₂ + 9

weeks OVA (Group F, *n* = 10) and Healing (Group G, *n* = 10). Mice were housed in an animal facility, which was maintained under specific pathogen-free conditions at 22–26 °C with 40–69% humidity and a 12 h light/dark cycle.

Ovalbumin sensitization and challenge

Adopting Henderson's protocol [23], all mice in OVA groups (Groups C, D, E, F) and SO₂ inhalation groups (Groups B, G) received intraperitoneal injection of 100 µg of OVA (Grade V; Sigma, St Louis, USA) complexed with 0.2 mL alum (Serva, Heidelberg, Germany) on Days 0 and 14, followed with intranasal instillation of 500 µg OVA on Days 14, 27, 28, 29, 47, 61, 73, 74 and 75. The Vehicle group received normal saline with alum intraperitoneally on Days 0 and 14, and saline without alum intranasally on Days 4, 27, 28, 29, 47, 61, 73, 74 and 75.

SO₂ inhalation

In a modification of Min's model [22], all mice in SO₂ exposure groups (Groups B, D, F, G) were exposed to 50 ± 5 p.p.m. SO₂ (purity 99.98%) for 60 min per day on Days 7, 9 and 11 in an acrylic gas chamber, the concentration of SO₂ in the chamber was monitored using a gas analyser (Gastec, Tokyo, Japan). As negative control, the sham groups (Groups A, C, E) inhaled filtered air in the same exposure chamber for the same time period. The animals re-breathed ambient air when exposure time was fulfilled.

Killing of animals

At specified time points (Days 12, 48, 76), 24 h after the last SO₂ exposure or OVA challenge, mice were killed by a 0.2 mL intraperitoneal injection of pentobarbital sodium (60 mg/kg) [24]. Once deeply anaesthetized, the mice were used either for bronchoalveolar lavage (BAL; six mice) or for tissue collection (four mice). As the mice in Vehicle group killed on different time points displayed no significant variance in the observed parameters, the data were pooled together as a single control group [25].

Bronchoalveolar lavage and bronchoalveolar lavage fluid cytology

After anaesthesia, the trachea was cannulated, BAL was performed according to Tanaka's methods [24], by injecting 0.3 mL of calcium and magnesium-free phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.05 mM EDTA-2Na into the lung via the trachea. The fluid was withdrawn by syringe and stored on ice. This procedure was repeated three times (total volume: 1.2 mL, recovery >85%). Cells were stained with

Diff-Quik (Baxter Dade, Duding, Switzerland), and a differential count of 500 cells (magnification $\times 500$) was performed using standard morphological criteria. The remaining BALF was centrifuged (4 °C, 300 g for 10 min), and the supernatant was collected and stored at -80°C for cytokine measurements.

Measurement of bronchoalveolar lavage fluid growth factor levels

BALF ET-1 and TGF- β 1 levels were measured using commercially available ELISA kits: Endothelin-1 EIA kit (Biomedica GmbH, Vienna, Austria) and TGF- β 1 ELISA kit (Promega, Madison, WI, USA), the kit can measure both active and latent form of TGF- β 1 by acidification with 1 N HCl. The sensitivity of these assays was 15 and 5 pg/mL, respectively.

Histological observation of airway inflammation and subepithelial fibrosis

Tissue preparation: Adopting the method reported by Henderson et al. [23]. After anaesthesia, the lungs were inflated through the trachea with 4% buffered formalin solution in PBS (pH 7.4) under a constant pressure of 150 mm H₂O. After 2 h, the lungs were removed from the thoracic cavity, cleared of non-lung tissue and immersed in 4% formalin for 1 h. Lungs were routinely embedded in paraffin and 4 μm sections were cut and mounted on glass slides pre-coated with poly-L-lysine. For light microscopy, the lung sections were stained with haematoxylin and eosin to assess inflammatory cell infiltration, and Masson's trichrome to study collagen deposition in the sub-basement membrane region.

Morphometric study of SEF was performed using a method previously described by Komai et al. [26] using a Leica image analysis system (Leica Q550IW, Cambridge, UK). Briefly, the Masson's trichrome-stained histologic preparations of the right and left lobes each contributed two specimens for evaluation, in which the total length of the epithelial basement membrane of the bronchioles was 1.0–2.5 mm, and the fibrotic area (stained in blue) beneath the basement membrane in 20 μm depth was measured. The mean score of the fibrotic area divided by basement membrane length in four preparations from each mouse was calculated, then, the mean values of SEF were calculated in each group of four animals. The specimens were studied in random and the two reviewers (Profs Chen XD and Lai HW) were blinded to the grouping of the samples.

Measurement of lung hydroxyproline content

Whole lung collagen content was measured by determining lung hydroxyproline content (Hyp) as measured by

Tanaka et al. [24]. After BAL, the lungs were removed from the thoracic cavity, cleared of non-lung tissue, cut into sections (1-mm-thick) and weighed after drying with acetone. The sample was hydrolysed with 2 N HCl at 120 °C for 24 h. The amount of hydroxyproline in the hydrolysate was determined according to Kirivikko's method [27].

Statistical analysis

The data are reported as the mean $\pm$ SEM of the combined experiments. Differences were analysed for significance ($P < 0.05$) by either Student's two-tailed *t*-test for independent means or ANOVA using the protected least significant difference method as indicated. Pearson's correlation test was used to analyse for correlations between TGF- β 1 level in BALF and Hyp.

Results

Brief SO₂ exposure led to acute neutrophilic airway inflammation and epithelial sloughing, which healed in 5 weeks

Brief exposure to 50 p.p.m. SO₂ led to a sevenfold increase of BALF total leucocytes compared with normal controls ($P < 0.01$), of these cells, there was an overwhelming influx of neutrophils ($P < 0.01$), comprising 78.0% of all the leucocytes recovered from BAL fluid. Although there was a fivefold increase of eosinophil numbers ($P < 0.05$), the differential ratio (DR) of eosinophils remained the same as that of the Vehicle (Table 1).

Mice exposed to SO₂ manifested loss of cilia, epithelial sloughing, bronchial swelling and peribronchial infiltration of neutrophils without evidence of thickening of basement membrane (Figs 1a and b).

After brief exposure to 50 p.p.m. SO₂ and resumption of room air for 5 weeks since the last SO₂ exposure, there was no significant difference in BALF total leucocytes, eosinophil and neutrophil numbers and DR between the Healing and the Vehicle groups (Table 1). Histological study revealed absolute resolution of airway inflammation and reconstitution of airway epithelium (Fig. 1c).

Under Masson's trichrome stains, there was no evidence of increased subepithelial collagen deposition and thickening of basement membrane in the three groups (Fig. 2a). Consistent with the morphometric study, Hyp, biochemical index of collagen deposition in the airway [24], was similar among the three groups (Fig. 2b).

Repeated ovalbumin challenge caused chronic allergic airway inflammation and subepithelial fibrosis

Compared with the Vehicle, eosinophil number and DR increased profoundly in 5 weeks OVA and 9 weeks OVA

Table 1. Cellular features of bronchoalveolar lavage fluid during airway inflammation

Group (n = 6)	WBC (10 ⁵ /mL)	Eos DR (%)	Eos Total (10 ³ /mL)	Neu DR (%)	Neu Total (10 ³ /mL)
Vehicle	1.17 ± 0.27	0.17 ± 0.40	0.22 ± 0.53	4.43 ± 2.38	6.07 ± 2.58
SO ₂	8.05 ± 0.85**	0.17 ± 0.40	1.15 ± 2.81*	77.98 ± 5.87**	625.71 ± 92.03**
Healing	3.25 ± 0.51**	19.85 ± 2.97**	64.88 ± 14.13**	11.35 ± 2.54**	37.48 ± 13.25**
5 weeks OVA	5.04 ± 0.74***	20.80 ± 2.87**	112.76 ± 23.13***	9.93 ± 2.55**	52.97 ± 10.22**
SO ₂ -OVAs	4.68 ± 1.03**	23.80 ± 2.82**	115.67 ± 28.43**	6.13 ± 2.05	30.33 ± 10.12**
9 weeks OVA	6.60 ± 0.70***	29.02 ± 3.45***	195.75 ± 38.43***	9.87 ± 2.03***	65.03 ± 13.46***
SO ₂ -OVAI	1.12 ± 0.29	0.17 ± 0.40	0.15 ± 0.37	5.67 ± 0.92	6.48 ± 2.26

*, ***P* < 0.05, 0.01 vs. Vehicle group respectively.

#, ****P* < 0.05, 0.01 vs. 5 weeks OVA, 5 weeks OVA groups without SO₂ exposure respectively.

OVA, ovalbumin.

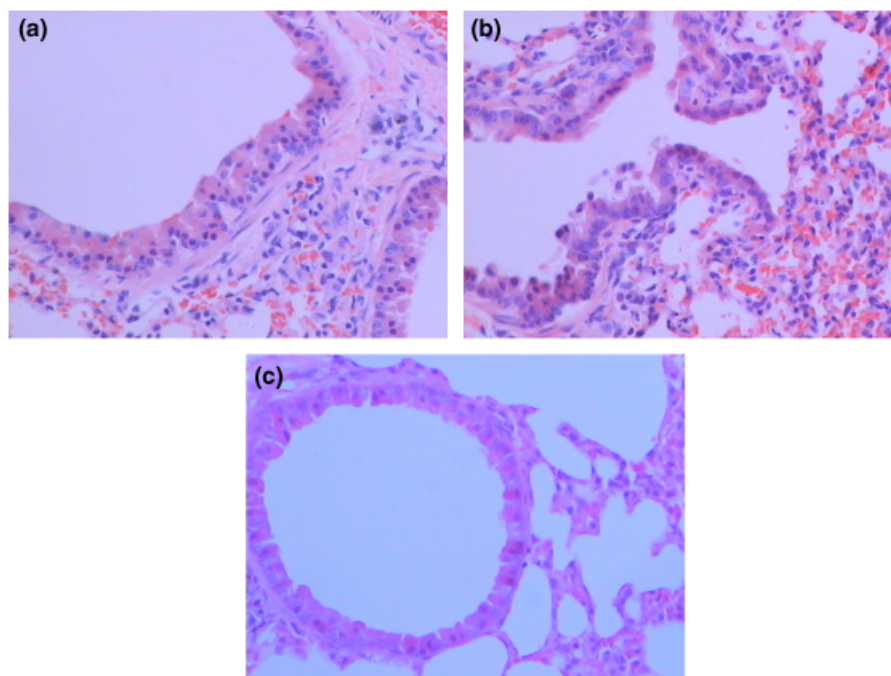


Fig. 1. Photomicrographs of haematoxylin- and eosin-stained lung sections of mice exposed to 50 p.p.m. SO₂. (a) Vehicle group; (b) loss of cilia, epithelial sloughing, bronchial oedema and peribronchial inflammatory cells infiltration following SO₂ exposure; (c) resolution of airway inflammation and restitution of injured epithelium after recovery in room air for 5 weeks. Sections observed at a magnification of × 200.

groups, respectively, whereas neutrophil number and DR increased only slightly, demonstrating a remarkable influx of BALF eosinophil (Table 1). BALF total leucocyte, eosinophil number and DR were higher in mice challenged periodically for 9 weeks than those challenged for 5 weeks (*P* < 0.05).

Corresponding to these findings, histological examination revealed peribronchial infiltration of inflammatory cells, and damage to epithelium in mice challenged with OVA, similar to the findings of Henderson and colleagues [23, 24], but only mice challenged for 9 weeks displayed significant thickening of basement membrane with more profound airway infiltration of inflammatory cells. The increased airway collagen deposition and thickening

of basement membrane were better demonstrated with Masson's trichrome stain and confirmed by morphometric study, proving only mice challenged for 9 weeks demonstrated significant increase of fibrotic area in the subbasement membrane region (Fig. 2a).

Prior SO₂ exposure enhanced bronchoalveolar lavage fluid eosinophilia, facilitated and aggravated subepithelial fibrosis

Similar to allergen-challenged mice without exposure to SO₂ (Groups 5 weeks OVA and 9 weeks OVA), OVA-challenged mice with prior exposure to SO₂ displayed allergic inflammation evidenced by BALF eosinophilia

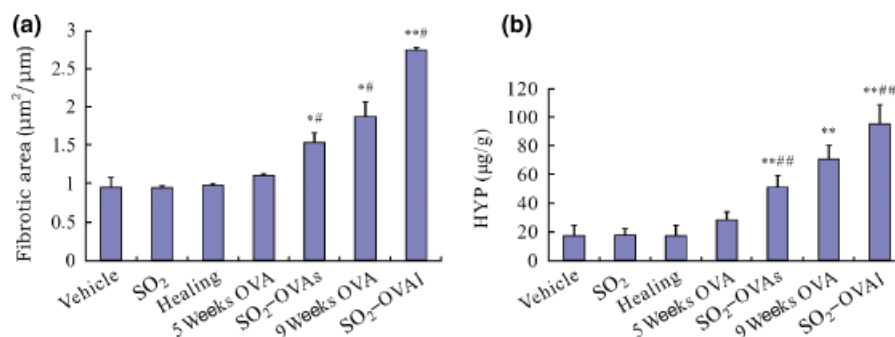


Fig. 2. (a) Morphometric study of subepithelial fibrosis. To evaluate the development of subepithelial fibrosis following repeated ovalbumin (OVA) challenge with or without prior exposure to SO₂, collagen deposition in subbasement membrane region was measured by studying the fibrotic area and thickness of subbasement membrane using Masson's trichrome stain in groups from Vehicle, acute airway inflammation following brief exposure to SO₂ (SO₂), recovery from SO₂ exposure (Healing), 5 weeks OVA challenge (5 weeks OVA), 5 weeks OVA challenge with prior exposure to SO₂ (SO₂-OVAs), 9 weeks OVA challenge (9 weeks OVA), 9 weeks OVA challenge with prior exposure to SO₂ (SO₂-OVAL). *, ** $P < 0.05$, 0.01 vs. Vehicle group, # $P < 0.01$ vs. 5 weeks OVA or 9 weeks OVA without SO₂ exposure. (b) Dynamic changes of lung hydroxyproline content. To evaluate the development of subepithelial fibrosis following repeated OVA challenge with or without prior exposure to SO₂, lung hydroxyproline content as index of lung collagen deposition was determined in groups from Vehicle, acute airway inflammation following exposure to SO₂ or repeated OVA challenge with or without prior exposure to SO₂, recovery from SO₂ exposure (Healing), 5 weeks OVA challenge (5 weeks OVA), 5 weeks OVA challenge with prior exposure to SO₂ (SO₂-OVAs), 9 weeks OVA challenge (9 weeks OVA), 9 weeks OVA challenge with prior exposure to SO₂ (SO₂-OVAL). ** $P < 0.01$ vs. Vehicle group, ## $P < 0.01$ vs. 5 weeks OVA or 9 weeks OVA without SO₂ exposure.

and submucosal infiltration of eosinophils, with increase of eosinophils predominating over increase of neutrophils (Table 1). Furthermore, for the same duration of OVA challenge (5 or 9 weeks), BALF total leucocyte count, eosinophil number and DR were higher in mice with prior exposure to SO₂ than those without prior SO₂ exposure ($P < 0.01$) (Table 1).

In parallel with the increased severity of CAAI, for the same duration of OVA challenge (5 or 9 weeks), fibrotic area beneath subbasement membrane and Hyp were higher in mice with prior exposure to SO₂ than those without prior SO₂ exposure ($P < 0.01$) (Figs 2a and b).

Changes of bronchoalveolar lavage fluid endothelin-1, transforming growth factor- β 1 levels following exposure to SO₂, repeated allergen challenge or both

Compared with the Vehicle, except for the Healing group, BALF ET-1 level in all the experimental groups increased ($P < 0.01$), with the most remarkable elevation in mice killed 24 h after exposure to 50 p.p.m. SO₂ (Fig. 3). For the same duration of OVA challenge (5 or 9 weeks), BALF ET-1 levels were higher in mice with prior exposure to SO₂ than those without prior SO₂ exposure, though the difference was significant only between Group 9 weeks OVA and Group 9 weeks OVA + SO₂ ($P < 0.05$) (Fig. 4).

Compared with the Vehicle, except for the Healing group, BALF TGF- β 1 levels in all the experimental groups increased significantly, with the most remarkable elevation in Group 9 weeks OVA + SO₂ (Fig. 4). For the same duration of OVA challenge (5 or 9 weeks), BALF TGF- β 1 levels were higher in mice with prior exposure to SO₂ than those without SO₂ exposure ($P < 0.01$) (Fig. 4).

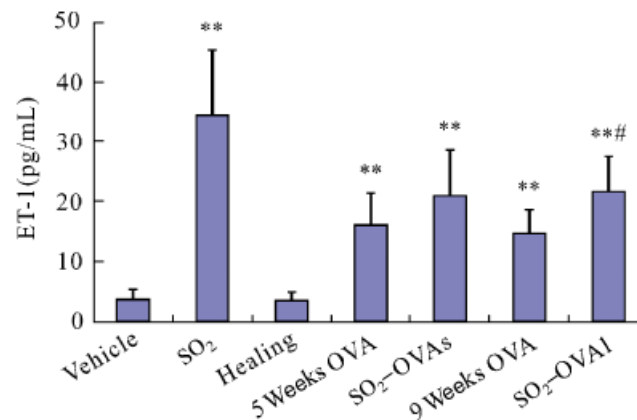


Fig. 3. Dynamic changes of bronchoalveolar lavage fluid (BALF) endothelin-1 level following exposure to SO₂ or repeated ovalbumin (OVA) challenge with or without prior exposure to SO₂. To evaluate the changes of airway secretion of endothelin-1 during the development and resolution of airway inflammation, endothelin-1 level in BALF was determined using ELISA. ** $P < 0.01$ vs. Vehicle group, # $P < 0.05$ vs. 9 weeks OVA without SO₂ exposure.

Pearson's test and linear regression showed a positive correlation between Hyp and BALF TGF- β 1 level during CAAI ($r = 0.842$, $P < 0.01$).

Discussion

In the present study, we first proved that brief exposure to 50 p.p.m. SO₂ could elicit acute neutrophilic airway inflammation and epithelial sloughing without consequent SEF. Then, we demonstrated that such exposure could aggravate CAAI and enhance SEF induced by

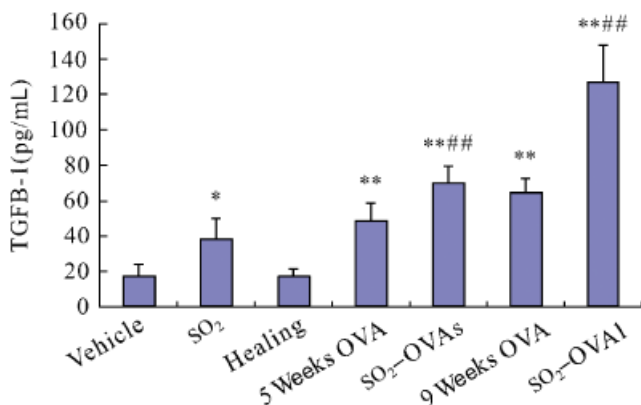


Fig. 4. Dynamic changes of bronchoalveolar lavage fluid (BALF) TGF- β 1 level following exposure to SO₂ or repeated ovalbumin challenge with or without prior exposure to SO₂. To evaluate the changes of airway secretion of endothelin-1 during the development and resolution of airway inflammation, TGF- β 1 level in BALF was determined using ELISA. *, ** P <0.05, 0.01 vs. Vehicle group (respectively), *** P <0.01 vs. 5 weeks OVA or 9 weeks OVA without SO₂ exposure.

repeated OVA challenge, possibly associated with elevation of BALF ET-1 and TGF- β 1 levels.

Similar to previous works by McMillan, Tanaka, Henderson [23–25], prolonged allergen challenge with OVA in our experiment resulted in CAAI with pronounced BALF eosinophilia and SEF with significant basement membrane thickening and increased subepithelial collagen deposition. To study the effect of different time course of inflammation on development of airway remodelling, BALF cellular profiles, lung histology and Hyp were monitored dynamically with comparison between mice challenged periodically for 5 or 9 weeks. Although both the groups displayed BALF eosinophilia, only mice with 9 weeks allergen provocation demonstrated histological and biochemical evidence of SEF. This result is similar to the findings of Trifilieff et al. demonstrating time-course dependent progression of airway remodelling following OVA challenge [28]. It is noteworthy that mice challenged for 5 weeks with prior SO₂ exposure demonstrated histological and biochemical evidence of SEF which was absent in those without SO₂ exposure, and the increased subepithelial collagen deposition was more profound in mice challenged for 9 weeks with prior SO₂ exposure than those without SO₂ exposure. This facilitation and enhancement of SEF by preceding SO₂ exposure was in parallel with the exaggeration of CAAI, demonstrating that development and progression of SEF during CAAI is in parallel with the progression and increased severity of allergic airway inflammation, rendering further evidence to support the suggestion that airway remodelling is the consequence of airway inflammation in asthma [1–3].

To our knowledge, this is the first study on the effect of SO₂ inhalation on the development of airway remodelling following allergen challenge, although there have been

several studies demonstrating the synergistic effects of exposure to 0.01–7 p.p.m. SO₂ and allergen sensitization on enhancement of acute allergic airway inflammation and increased airway hyperresponsiveness to OVA challenge [18, 29, 30]. Our findings may shed some light on the understanding of the association between air pollution due to SO₂ and increased morbidity and exacerbations of asthma [12, 16, 17], traditionally ascribed to enhanced bronchoconstriction mediated through parasympathetic reflex following SO₂ stimuli [20, 29], and recently supposed to be associated with enhanced inflammatory response due to compromise of epithelial function and loss of epithelium integrity following exposure to these hazardous pollutants [18, 19].

SO₂ is an important air pollutant, exposure to even 0.5 p.p.m. SO₂ could induce bronchoconstriction and asthma attacks in patients with mild asthma [12]. Acute exposure to SO₂ resulted in significant airway epithelial damage and ciliary dysfunction, acute inflammation along the tracheobronchial tree with epithelial sloughing, necrosis and activation of the residual epithelial cells [12, 16, 18, 31]. Similar to our study, restitution of damaged epithelium and absolute resolution of the neutrophilic airway inflammation has been documented following withdrawal of SO₂ exposure pending on the concentration of SO₂ and duration of exposure [12, 22].

In this study, brief exposure to 50 p.p.m. SO₂ caused acute neutrophilic airway inflammation with epithelial sloughing and loss of cilia. At this concentration and for this duration, airway inflammation and damage to airway structures was transient, reversible without consequent airway remodelling. This is essential because it proves that the promotion of CAAI and SEF during repeated allergen challenges is not a direct effect of acute neutrophilic airway inflammation, instead, the promotion of SEF is associated with the exaggeration of CAAI. Compromise of epithelial function, loss of epithelial integrity, exaggerated allergen presentation and priming or activation of injured epithelial cells during airway inflammation may be involved in the promotion of CAAI and development of airway remodelling [18, 32].

Recently, mounting evidence supports the hypothesis that airway epithelial damage from repeated endogenous and exogenous insults in asthma leads to activation of injured airway epithelial cells with overexpression of various proinflammatory and fibrogenic cytokines and growth factors such as ET-1 and TGF- β 1, orchestrating airway inflammation and remodelling through crosstalk with mesenchymal cells and inflammatory cells. Although the definitive process underlies airway inflammation, aberrant cycles of epithelial injury and repair remain to be elucidated [2–7].

Airway epithelium is a major source of ET-1 and TGF- β 1 during airway inflammation in asthma. Zhang and Morishima et al. respectively, demonstrated that

activation of injured bronchial epithelial cells following acute epithelial injury with increased release of ET-1 and other fibrogenic mediators, and the up-regulation of these pleiotrophic mediators were closely involved in increased subepithelial collagen synthesis [8, 9].

Brief exposure to SO₂ resulted in extensive epithelial injury and remarkable elevation of BALF ET-1. Although not studied in this investigation, activation of residual airway epithelial cells following inhalation of SO₂ was documented by Stratmann et al. [31], and overexpression of ET-1 by injured airway epithelial cells is regarded as a marker of epithelial activation in asthma [3, 4, 8–10]. Henceforth, the prominent elevation of ET-1 in the airway milieu may be a significant demonstration of activation of the residual epithelial cells following SO₂ insult, and these activated or primed epithelial cells may display an exaggerated allergic inflammatory response to the sensitized allergen exposed 3 days after the last insult by SO₂, similar to the exaggerated acute allergic inflammation following allergen challenge in other animal studies with prior or concurrent exposure to SO₂ or other oxidant pollutants [21, 29, 30, 32, 33]. In addition, as a fibrogenic and mitogenic growth factor [8, 10], overexpression of ET-1 during CAAI might be directly involved with the development of SEF.

TGF-β1 and its isoforms – TGF-β2 and TGF-β3 – have been implicated in the pathogenesis of SEF of asthma. Recent study by Alcorn et al. demonstrated the predominant role of TGF-β1 in the regulation of increased collagen synthesis and deposition in a murine model of asthma [34]. Immunoreactivity of TGF-β1 has been found to increase in BALF and airway epithelium in asthmatics with a positive correlation between the increase of airway mucosa TGF-β1 immunoreactivity and the severity of SEF [35]. Intratracheal instillation of TGF-β1 directly induced increased subbasement membrane deposition of collagen, suggesting a causal effect between elevation of TGF-β1 in airway microenvironment and development of SEF [36]. Similar to Tanaka et al. [24], we demonstrated that elevation of BALF TGF-β1 was positively correlated with increase Hyp during progression of CAAI and development of SEF.

In Tanaka's experiment [24], escalation of CAAI was achieved by increasing aeroallergen provocation concentrations. In this investigation, it was achieved by brief exposure to SO₂ before allergen challenge, with the possible result of activation and priming of airway epithelium, both as target of insult from SO₂ inhalation and as one of the important effectors in the inflammation/repair process following allergen challenge, which, along with the compromise of mucociliary activity and loss of epithelial integrity, resulted in the exaggeration of allergic airway inflammation and enhancement of epithelial damage following the first allergen challenge. This might be the initiation of the vicious cycle of epithelial injury

and abnormal repair. With repeated allergen provocations, the exaggerated allergic inflammation and epithelial damage might be passed along, resulting in the facilitation and enhancement of SEF.

Despite strenuous efforts, the mechanism of asthma remains unresolved because of the complexity of cells and cellular components involved in its pathogenesis. There are many limitations with this primary study, for example, the cellular and molecular mechanisms involved in the promotion of allergic inflammation and SEF with prior exposure to SO₂ require further elaboration, the suggested activation of injured epithelial cell during neutrophilic and allergic inflammations needs concrete evidence, dynamic changes of ET-1 and TGF-β1 expression by injured airway epithelium during these inflammations need illustration, the definitive roles of up-regulated ET-1 and TGF-β1 warrant further clarification by using antagonists or other special inhibitors, and the roles of other cytokines and cellular components indicated in the pathogenesis of CAAI and SEF should be explored.

In conclusion, our study demonstrated that brief exposure to 50 p.p.m. SO₂ led to acute neutrophilic airway inflammation and epithelial injury, which exaggerated CAAI and promoted the development of SEF following repeated allergen challenge with ovalbumin, and the overexpression of ET-1 and TGF-β1 in airway milieu during the inflammatory process might be involved in the promotion of SEF caused by brief exposure to SO₂.

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