



Effect of sulfur dioxide on inflammatory and immune regulation in asthmatic rats



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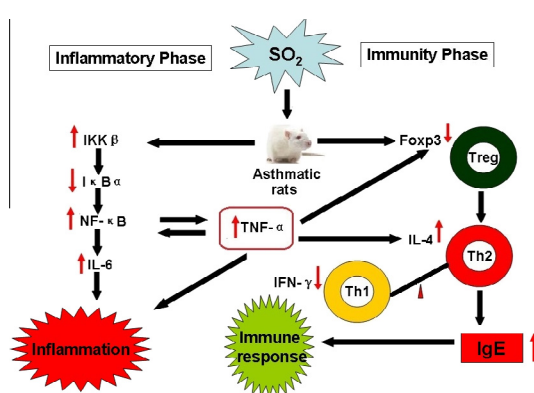
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HIGHLIGHTS

- Indicating NF- κ B nuclear translocation and activation after SO₂ and OVA exposure.
- Showing inflammatory responses accompanied with TNF- α and IL-6 high-expression.
- Revealing immune regulation related to IFN- γ , IL-4 and Foxp3 expression.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Exposure to sulfur dioxide (SO₂) increases asthma risk. Inflammatory and immune responses are typical in asthma disease. The exact effect of SO₂ on modulation of the inflammatory and immune responses in asthmatic rats remains unclear.

Objectives: Here we sought to investigate the molecular mechanisms underlying the NF- κ B inflammatory pathway and the Th1/Th2 imbalance in asthmatic rats exposed to SO₂.

Methods: Male Wistar rats were challenged by ovalbumin (OVA) or SO₂ alone or together, and then mRNA and protein levels of some inflammatory and immune genes were measured. NF- κ B nuclear translocation was analyzed. Bronchoalveolar lavage (BAL), inflammatory cell counts and histopathologic examination were performed.

Results: (1) OVA plus SO₂ induced abnormal pathological changes and inflammatory responses in lung relative to exposure to OVA alone; (2) showing NF- κ B nuclear translocation and activation through up-regulating IKK β mRNA and protein expression and down-regulating I κ B α expression in the presence of OVA or OVA plus SO₂; (3) OVA plus SO₂ significantly raised TNF- α and IL-6 levels in BALF compared with the OVA group; (4) SO₂ markedly elevated IL-4 levels and decreased IFN- γ levels in BALF in the

Abbreviations: BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; Foxp3, forkhead transcription factor 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HE, hematoxylin and eosin; IL, interleukin; IKK β , inhibitor of I κ B kinase β ; IFN- γ , interferon- γ ; I κ B α , inhibitor of NF- κ B α ; IgE, immunoglobulin E; LSD, least significant difference; NF- κ B, nuclear factor kappa B; OVA, ovalbumin; ANOVA, one-way analysis of variance; PBS, phosphate-buffered saline; Tregs, regulatory T cells; SO₂, sulfur dioxide; TNF- α , tumor necrosis factor alpha; Th, T helper.

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asthmatic rats, stimulating IgE generation which was closely related to inhibiting the expression of Foxp3, a specific marker of regulatory T cells.

Conclusions: SO₂ affects the airway inflammatory and immune responses of the asthmatic rats and enhances the susceptibility to OVA by aggravating inflammatory responses in lungs, up-regulating pro-inflammatory cytokine expression, and causing the Th1/Th2 imbalance, which might contribute to the increased risk of asthma disease.

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1. Introduction

Sulfur dioxide (SO₂) is a harmful air pollutant derived from combustion of fossil fuels. It is present in community and workplace air. As a strong irritant, SO₂ may trigger inflammation through epithelial injury, airway hyperresponsiveness (AHR) and neurogenic inflammation (Lin et al., 2009). Researchers have demonstrated that SO₂ can compromise pulmonary function, aggravate respiratory tract diseases such as chronic bronchitis and asthma, and is closely related to lung cancer (Smargiassi et al., 2009; Tseng et al., 2012; Chang et al., 2012).

Asthma is a chronic inflammatory disease characterized by AHR and mucus secretion. About 300 million people world-wide are afflicted by asthma (Pruitt and Lawson, 2011). In 1960s, the influence of SO₂ on asthmatic patients had been studied (Nakazawa, 1969). Afterwards Jaeger et al. (1979) found that SO₂ resulted in an almost immediate decrease of mid-maximal expiratory flow rate which lasted throughout the exposure in asthmatics. During 1980s to 1990s, some epidemiology data showed that many individuals with asthma were experiencing significantly clinical airway constriction and symptoms exposed to SO₂ (Bethel et al., 1983; Hunt and Holman, 1987; Walters et al., 1994). Recently, more and more researchers have focused on the effects of SO₂ on asthma and confirm that repeated exposure to SO₂ can increase the asthma incidence among workers or children (Andersson et al., 2006; Smargiassi et al., 2009; Deger et al., 2012; Kara et al., 2013). On the other hand, using asthmatic rats challenged by ovalbumin (OVA), Park et al. (2001) found lung function changes and infiltration of inflammatory cells in the OVA-sensitized guinea pigs exposure to SO₂, and Li et al. (2007a, 2007b, 2008) reported that SO₂ could elevate asthma-related gene expression in airway of rats and in human bronchial epithelial cells, inducing mucus secretion and inflammatory cell infiltration.

It has been demonstrated that inflammatory cell infiltration (e.g., eosinophils), mast cell degranulation, airway smooth muscle contraction, and T helper (Th) type 1(Th1)/Th2 cell imbalance play a key role in the initiation and development of asthma (Deo et al., 2010; Shao and Wang, 2011). Also, nuclear factor kappa B (NF-κB) activation, release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and secretion of Th2 cytokines including IL-4, IL-13 and IL-5 may promote the inflammatory responses (Mazzarella et al., 2000; Tak and Firestein, 2001; Ngoc et al., 2005; Tomasiak-Lozowska et al., 2010).

In allergic airway inflammation, T lymphocyte and its secretion of various cytokines play an important role in this process. Th1/Th2 imbalance exerts a key role in the pathogenesis of asthma (Mazzarella et al., 2000). Among these cells, Th1 cells secrete interferon-γ (IFN-γ) and IL-2, while Th2 cells produce IL-4, IL-5 and IL-13. Cytokines like IL-4 generally stimulate immunoglobulin E (IgE) production (Deo et al., 2010), which is a crucial characteristic of the asthma pathology. For the past few years, the general knowledge regarding the regulation of asthma by CD4⁺CD25⁺ T-regulatory (Treg) cells has rapidly increased. CD4⁺CD25⁺ Tregs are not only the regulator of the Th1/Th2 network balance, but also possibly play

an important role in the pathogenesis of asthma diseases (Ling et al., 2004). Forkhead transcription factor 3 (Foxp3) is the most vital marker for CD4⁺CD25⁺ Tregs and has been shown to govern the development and function of Tregs and Th1/Th2 cells (Ochs et al., 2005; Shi et al., 2011). In recent years, the effect of Th1/Th2 and Treg cells on asthma has been paid more and more attention, becoming a new way of shedding light on revealing the pathogenesis of asthma. SO₂ is a non-antigenic substance, but it may enhance sensitization to allergens, and promote the immune system's inflammatory responses once challenged in animals (Cai et al., 2008). However, so far limited data are available on the immunoregulation of SO₂ on Th1/Th2 balance and Treg function and SO₂-induced inflammatory responses mediated by NF-κB inflammatory pathway in asthmatic rat airway.

In this study, we analyzed the mRNA and protein expressions of both inflammation-related genes [such as NF-κB, inhibitor of NF-κB α (IκBα), inhibitor of IκB kinase β (IKKβ), TNF-α and IL-6] and immunization-related genes including IL-4, IFN-γ and Foxp3 in rats treated with OVA or SO₂ inhalation alone or associated using real-time RT-PCR assay, enzyme-linked immunosorbent assay (ELISA) method and western blot analysis, respectively. In addition, the serum total IgE levels, numbers of inflammatory cells, NF-κB DNA binding activity and the pathological characteristics in rat lung were detected.

2. Materials and methods

2.1. Animals and treatment protocols

Male Wistar rats, weighing 180–200 g, were purchased from Animal Center of Hebei Medical University. The rats were divided randomly into 4 equal groups of 6 animals each: (1) SO₂ group, (2) OVA group, (3) OVA + SO₂ group, and (4) control group. The treatment protocol was performed as described previously (Li et al., 2007a). The rats in the OVA group and in the OVA and SO₂-exposed group were sensitized by an intraperitoneal (i.p.) injection of 100 mg OVA (Sigma, grade II) in 1 mL saline mixed with 100 mg of Al(OH)₃ on the first day. Simultaneously, each rat received an i.p. injection of 1 mL Bordetella pertussis (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) containing 6 × 10⁹ heat-killed bacilli as the second adjuvant. A booster sensitization was performed 7 d later, and the second injection of 10 mg OVA in 1 mL saline mixed with 100 mg of Al(OH)₃ was given. After sensitization (on the 15th d), the sensitized rats were challenged by an aerosol of 1% OVA for a period of 30 min for each challenge on 7 consecutive days. The rats exposed to SO₂ alone or to OVA plus inhaled SO₂ were administered using the technique of SO₂ dynamic inhalation for 1 h d⁻¹ (9:00 AM–10:00 AM) for 7 d. After SO₂ inhalation, the rats in OVA plus SO₂ group were challenged with aerosolized 1% OVA (30 min d⁻¹). The mean concentration of SO₂ was 5.6 ± 1.5 mg m⁻³ (about 2 ppm). The dose of SO₂ was selected on the basis of the Threshold Limit Value (TLV) in the US American Conference of

Governmental Industrial Hygienists (ACGIH) for SO₂ in the industrial environment and the occupational exposure limit values (OELs) for SO₂ with a respiratory critical effect in workplace air in Sweden (Hansson and Rudén, 2006). Sham immunizations were done with saline.

All animal procedures were approved by the Shanxi University Animal Investigational Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by Ministry of Health People's Republic of China.

2.2. Bronchoalveolar lavage, Wright-Gemsa staining and hematoxylin and eosin (HE) staining

Under anesthesia, the blood samples were obtained via cardiac puncture. The serum was isolated from the samples after centrifuging at each stage of phlebotomy to determine serum IgE levels in the various groups. Then, the right lung was ligated and a tracheal catheter was inserted into the trachea. The left lung was immediately lavaged with phosphate-buffered saline (PBS pH7.4) by gently instilling PBS solution into the left lung via the tracheal catheter. Such process was repeated three times with 5 mL PBS as total volume. After the collection of bronchoalveolar lavage fluid (BALF), the inflammatory cells in BALF were counted with Wright-Gemsa staining. Then, a piece of middle lobe of the right lung was used to extract nuclear and cytoplasmic protein. Another one was fixed in 4% paraformaldehyde in PBS for the HE staining analysis, and the rest of the lung and the trachea tissues were frozen in liquid nitrogen for total RNA and total protein extraction.

2.3. Real time quantitative RT-PCR

The frozen lung and trachea tissue samples were thawed, and mRNA was extracted using the Transzol reagent (Transgen, Beijing, China) according to the manufacturer's protocol. First-strand cDNA was synthesized using AMV RT First Strand cDNA Synthesis Kit (Transgen, Beijing, China) according to the manufacturer's protocols. cDNA product was stored at –80 °C until use.

Expression levels were assessed by real-time PCR in a iCycler iQ Real Time PCR Detection System (Bio-Rad, Richmond, CA, USA) with the Quantitect SYBRGreen I PCR kit (Qiagen, Valencia, CA, USA). The PCR amplification was performed in a 20 µL reaction solution containing 7 µL DDH₂O, 10 µL of SYBR Green qPCR Super-Mix, 1 µL each of the forward and reverse primers and 1 µL cDNA using the iCycler iQ Real Time PCR Detection System. Thermal cycling was carried out as follows: initial denaturation at 95 °C

for 30 s, followed by 40 cycles of 94 °C for 20 s, 55 °C (for TNF-α and glyceraldehyde-3-phosphate dehydrogenase GAPDH) or 60 °C (for NF-κB, IKKβ, IκBα, IL-6, IL-4, IFN-γ, and Foxp3) for 20 s and 72 °C for 20 s. Melting curve analysis was also performed using 81 cycles of 15 s increasing from 55 °C to 95 °C. The relative quantification of the expression of the target genes was measured using the housekeeping gene GAPDH mRNA as an internal control. The copy numbers of target gene/GAPDH mRNA ratio was measured in all samples. The GenBank accession numbers and the primer sequences of the tested genes (NF-κB, IKKβ, IκBα, IL-6, TNF-α, IL-4, IFN-γ and Foxp3) and GAPDH together with the PCR product amplified fragments are listed in Table 1.

2.4. Western blotting

Cellular plasma and nuclear protein were extracted from fresh lung tissue with a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China). Total protein was extracted from frozen lung tissue with protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Protein concentrations were determined by Bradford Protein Assay Kit (Beyotime, Shanghai, China). Samples were mixed with loading buffer and boiled for 5 min. Western blot analysis was performed as described previously (Li et al., 2008). The rabbit polyclonal antibodies specific for rat NF-κB and IκBα (Santa Cruz, CA, USA), goat polyclonal antibodies specific for rat IKKβ, or mouse monoclonal antibodies for Foxp3 (Santa Cruz, CA, USA) and for β-actin (Onco-gene, Boston, MA) were incubated at a concentration of 1:100 (for NF-κB, IKKβ, IκBα and Foxp3), or 1:5000 (for β-actin) in 1 × PBS-0.1% Tween and 5% BSA overnight at 4 °C. The infrared-labeled anti-rabbit, anti-goat or anti-mouse secondary antibodies (Li-COR Biosciences, USA) at a concentration of 1:5000 were added to membranes and incubated for 1.5 h at room temperature. The membranes were scanned and the band densities were quantified using the Odyssey Infrared Imaging System (Li-COR Biosciences, USA).

2.5. Electrophoretic mobility shift assay

NF-κB DNA binding activity was examined with electrophoretic mobility shift assay (EMSA). EMSA was performed using a light shift chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Briefly, 10 µg of the nuclear proteins and 1 µL of biotin-labeled NF-κB probe were incubated for 20 min. Samples were separated on a 6% non-denaturing

Table 1
Primer sequences and the PCR product amplified fragments used in real-time RT-PCR.

Genes	Accession No.		Sequence
NF-κBp65	NM_199267	Forward primer	5'-CCACGATCTGTTCCCTCAT-3'
Products	68bp	Reverse primer	5'-TGATCTCCACATATGGCCAG-3'
IKKβ	AF115282	Forward primer	5'-CCACCACAGAAGCACACAATG-3'
Products	142bp	Reverse primer	5'-CCCGCAGCAGTCCAAAGG-3'
IκBα	NM_001105720	Forward primer	5'-ACCTGGTCTCGCTCTGTG-3'
Products	173bp	Reverse primer	5'-GCTCTCTCATCTCACTCTCG-3'
IL-6	NM_012589	Forward primer	5'-TCTACCCCACTTCAATGCTC-3'
Products	79bp	Reverse primer	5'-TTGGATGGTCTTGGTCTTAGCC-3'
TNF-α	NM_012675	Forward primer	5'-ACAGAAAGCATGATCCGAGA-3'
Products	148bp	Reverse primer	5'-TCAGTAGAGAGAAGAGCGTGG-3'
IL-4	NM_201270	Forward primer	5'-AACACCACGGAGAACGAGCTCATC-3'
Products	152bp	Reverse primer	5'-AGTGAGTTCAGACCGCTGACACCT-3'
IFN-γ	NM_138880	Forward primer	5'-CAACCCACAGATCCAGCACAAAG-3'
Products	110bp	Reverse primer	5'-CCCAGAAATCAGACCGACTCC-3'
Foxp3	NM_001108250	Forward primer	5'-GTGGCCTCAGTGGACAAGAGC-3'
Products	119bp	Reverse primer	5'-TGTCTCCGACAGCAACAAG-3'
GAPDH	NM_017008	Forward primer	5'-ATG TAT CCG TTG TGG ATC TGA C-3'
Products	78bp	Reverse primer	5'-CCT GCT TCA CCA CCT TCT TG-3'

acrylamide gel, pre-run for 45 min at 100 V, in $0.5 \times$ Tris boric acid-EDTA buffer at room temperature for 2 h at 100 V. Gels were transferred to positively charged nylon membranes where the gel shifts were visualized by a chemiluminescent detection system (Pierce Biotechnology, USA). The chemiluminescent signals were recorded by exposure to X-ray film for 40–60 min, and the levels of NF- κ B DNA binding activity was quantified by gray scale scanning.

2.6. ELISA

Levels of TNF- α , IL-6, IL-4 and IFN- γ in BALF and total IgE in serum of different groups were measured using commercially available ELISA kits (R&D systems, USA) according to the test protocols. Absorbance was measured at 450 nm using a Multifunctional Microplate Reader (Thermo Scientific Varioskan Flash, USA). The protein concentration of BALF (mg protein mL⁻¹) was measured using Bradford Protein Assay Kit (Beyotime, Shanghai, China).

2.7. Statistical analysis

Statistical analyses were performed by the use of one-way analysis of variance (ANOVA) with SPSS10.0 packed programs for Windows. All data was tested for homogeneity of variances by Levene's Test for Equality of Variances. In instances of equal variance, data was subjected to one-way ANOVA and post hoc tests were conducted to determine the difference between groups, followed by Fisher's least significant difference (LSD) test. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Morphological changes and inflammatory cell counts in BALF

The results of lung histopathologic examination and inflammatory cell counts in BALF of rats were shown in Fig. 1. There was no abnormality in lungs of rats in the control (Fig. 1A), while some slight pathological changes, such as the diminished alveolar spaces and bronchial epithelial cell proliferation, were observed in lungs of the rats exposed to SO₂ (Fig. 1B). OVA-sensitized animals showed leukocyte and eosinophil cell infiltration in the surrounding peribronchiolar areas, the diminished alveolar spaces, and thickened subepithelial cell layers (Fig. 1C). In the SO₂ + OVA group, the histopathologic changes were severe with inflammatory cell infiltration, prominent airway mucus secretion, thickened bronchial epithelial cell layers and basement membrane, and a pronounced airway obstruction (Fig. 1D). In Fig. 1E, the changes of numbers of macrophages, lymphocytes, eosinophils, and neutrophils in BALF exposed to SO₂ were not significant compared with the control. The counts of the inflammatory cells were significantly increased exposed to OVA or to OVA plus SO₂ ($P < 0.05$, $P < 0.01$ respectively), versus the control. Furthermore, exposure to OVA plus inhaled SO₂ significantly increased the numbers of eosinophils, lymphocytes and macrophages compared with exposure to OVA alone ($P < 0.05$).

3.2. Effects of SO₂ inhalation on mRNA expression of pro-inflammatory genes and immune-related genes in the lungs and tracheas from rats

Real-time PCR was used to analyze the levels of NF- κ B, IKK β , I κ B α , TNF- α , IL-6, IL-4, IFN- γ , and Foxp3 mRNA in lungs and tracheas of rats exposed to SO₂ and/or OVA (Fig. 2).

In Fig. 2A and B, the mRNA expressions of NF- κ B and IKK β were significantly increased in lungs and tracheas of rats treated with

OVA or with OVA plus inhaled SO₂ compared with the control ($P < 0.05$, $P < 0.01$ respectively). The significant change of NF- κ B mRNA level in the tracheas was observed in rats exposed to SO₂ alone ($P < 0.05$). Furthermore, the NF- κ B mRNA levels from rats exposed to OVA and SO₂ were higher than those of rats exposed to OVA alone. However, significant decreases in the mRNA levels of I κ B α were observed in rats treated with OVA or with OVA plus inhaled SO₂, relative to the control ($P < 0.05$, $P < 0.01$ respectively). The mRNA expression of I κ B α was markedly lower in the SO₂ + OVA group than that in the OVA group ($P < 0.05$, $P < 0.01$ respectively).

Besides, OVA exposure caused significant increases of TNF- α and IL-6 mRNA levels in lungs and tracheas of rats ($P < 0.05$, $P < 0.01$ respectively) compared with the control, and OVA plus inhaled SO₂ also induced such effect. The TNF- α and IL-6 mRNA levels exposed to OVA and SO₂ were increased more strongly than those of lungs and tracheas of rats exposed to OVA alone ($P < 0.05$, $P < 0.01$ respectively). No significant change of TNF- α and IL-6 mRNA levels was observed in rats exposed to SO₂ alone (Fig. 2A and B).

As for mRNA expression of IL-4, IFN- γ and Foxp3, OVA exposure and OVA plus SO₂ exposure markedly elevated IL-4 mRNA levels in lungs and tracheas from rats compared with the control ($P < 0.05$, $P < 0.01$ respectively), whereas IL-4 mRNA levels exposed to OVA and SO₂ were higher than those of lungs and tracheas of rats exposed to OVA alone (Fig. 2A and B). However, the IFN- γ and Foxp3 mRNA levels were decreased in the rats treated with OVA or with OVA plus SO₂ compared with the control ($P < 0.05$, $P < 0.01$ respectively), while Foxp3 mRNA levels exposed to OVA and SO₂ in tracheas of rats were lower than those of rats exposed to OVA alone ($P < 0.05$) (Fig. 2B).

3.3. Effects of SO₂ inhalation on NF- κ B, IKK β , I κ B α and Foxp3 protein expression in the lungs

We demonstrated that the changes of NF- κ B, IKK β , I κ B α and Foxp3 protein expression in lungs from rats exposed to SO₂ or/and OVA. In Fig. 3, exposure to OVA or OVA plus inhaled SO₂ significantly caused increase of nucleus NF- κ B and IKK β protein levels in rat lungs compared with the control ($P < 0.05$, $P < 0.01$ respectively), but the increase of protein levels after SO₂ inhalation was not statistically significant. Moreover, exposure to OVA plus inhaled SO₂ significantly induced NF- κ B and IKK β protein expression compared with exposure to OVA alone ($P < 0.05$) (Fig. 3).

Fig. 3A and C also presents I κ B α and Foxp3 protein levels in lungs from rats exposed to SO₂ and/or OVA. I κ B α and Foxp3 protein levels were significantly decreased in lungs from rats exposed to OVA or to OVA plus SO₂ ($P < 0.05$). No significant increases of I κ B α and Foxp3 protein levels were observed in rats exposed to SO₂ alone. The protein expressions of I κ B α and Foxp3 were significantly reduced in rats exposed to OVA plus inhaled SO₂ ($P < 0.05$), versus OVA group (Fig. 3A and C).

Furthermore, cytoplasm and nucleus protein expression of NF- κ B was shown in Fig. 3B. Nucleus NF- κ B expression was obvious more than cytoplasm NF- κ B expression in the presence of OVA or OVA plus SO₂. Besides this, The EMSA data (Fig. 4A and B) showed that DNA binding activity of NF- κ B was very low in lung tissues in rats from the control and SO₂ group. In the presence of OVA stress, while NF- κ B activity was significantly increased 1.8-fold as compared with the control ($P < 0.01$). NF- κ B binding activity in rats exposed to OVA plus inhaled SO₂ was higher than the control ($P < 0.01$), and even increased versus OVA group ($P < 0.01$). It suggests nuclear translocation of NF- κ B in rat lung in the presence of OVA or OVA plus SO₂.

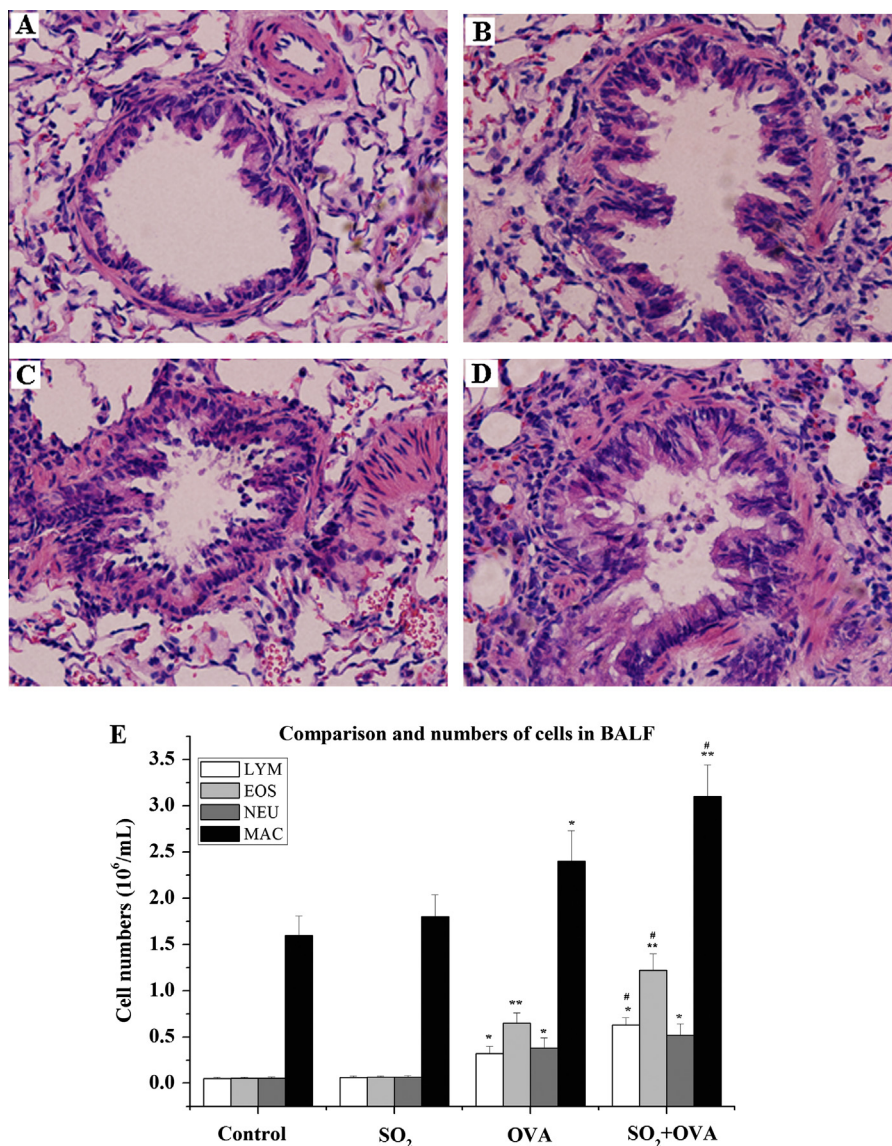


Fig. 1. HE staining of morphological characteristics of sections in the lungs of rats from control (A), SO₂ (B), OVA (C) and OVA + SO₂ (D) group, 400 × magnification. Cell numbers in BALF (E), Key to abbreviations: MAC, macrophages; LYM, lymphocytes; EOS, eosinophils; NEU, neutrophils. Data were expressed as mean ± SD (n = 6); *P < 0.05, **P < 0.01 versus control; #P < 0.05, ##P < 0.01 versus the OVA group.

3.4. Effects of SO₂ inhalation on IL-4, IFN-γ, IL-6 and TNF-α protein in BALF and total IgE levels in serum from rats

Figs. 5 and 6 present protein concentrations of IL-4, IFN-γ, IL-6 and TNF-α in BALF and total IgE levels in serum from rats treated with SO₂ or/and OVA.

In Fig. 5, OVA or OVA plus SO₂ exposure markedly increased IL-4 protein levels in BALF, whereas IFN-γ protein levels reduced compared with the control ($P < 0.01$). The significant change of IL-4 and IFN-γ protein levels in BALF of rats exposed to OVA plus SO₂ were observed compared to the rats exposed to OVA alone ($P < 0.05$). Also, OVA exposure caused significant increase of the protein levels in BALF compared with the control ($P < 0.05$). The protein expression of IL-6 and TNF-α was significantly elevated in BALF from rats exposed to OVA or to OVA plus inhaled SO₂ ($P < 0.05$, $P < 0.01$ respectively) versus controls. Furthermore, the IL-6 and TNF-α levels in BALF from rats exposed to OVA and SO₂ were induced more strongly than those of BALF from rats exposed to OVA ($P < 0.05$, $P < 0.01$ respectively). The protein level changes of IL-6 and TNF-α in rats exposed to SO₂ were not significant relative to the control.

In Fig. 6, the total IgE levels in serum were increased in the rats treated with OVA or with OVA plus SO₂ relative to the control ($P < 0.01$), while the increase of total IgE levels in serum of rats exposed to OVA plus SO₂ were significant compared with the rats exposed to OVA alone ($P < 0.05$).

4. Discussion

The epidemiological studies have concluded that people with asthma and children are particularly sensitive to SO₂ exposure (Hunt and Holman, 1987; Andersson et al., 2006; Smargiassi et al., 2009; Deger et al., 2012). The animal studies revealed that SO₂ may enhance the asthmatic reactions by inflammatory response and immediate-type hypersensitivity reaction (Riedel et al., 1988; Park et al., 2001; Su et al., 2010). However, the detailed mechanisms of SO₂ on regulation of inflammation and immunization in asthmatic rats have not been fully established. On the basis of our previous work about asthma related gene expression caused by SO₂ in the asthmatic rats (Li and Meng, 2007; Li et al., 2007a,b, 2008), we further studied the inflammatory and immune

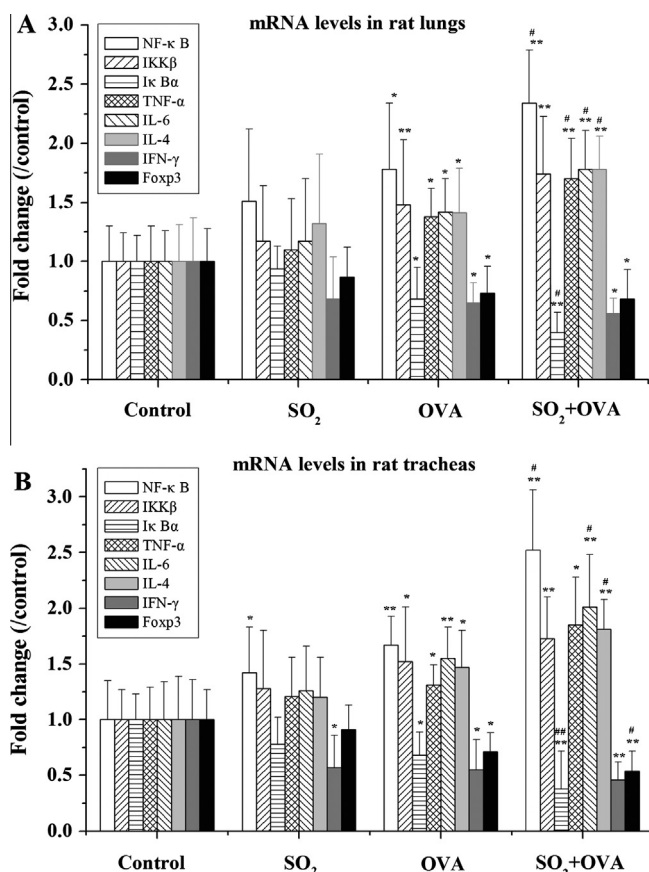


Fig. 2. mRNA levels of NF- κ B, IKK β , I κ B α , TNF- α , IL-6, IL-4, IFN- γ and Foxp3 in lungs and tracheas of rats in the different groups. Value in each treated group was expressed as a fold change compared to mean value in vehicle control group, which has been ascribed as an arbitrary value of 1. Data were expressed as mean $\pm$ SD ($n = 6$); * $P < 0.05$, ** $P < 0.01$ versus control; # $P < 0.05$, ## $P < 0.01$ versus the OVA group.

responses of SO₂ on the asthmatic rats from a novel angle underlying the NF- κ B inflammatory pathway and Foxp3 mediation in the Th1/Th2 imbalance.

The NF- κ B dimers (p50/p65 heterodimer) are retained in the cytoplasm in an inactive form in unstimulated cells as a consequence of their association with members of another family of proteins called I κ B (Napetschnig and Wu, 2013). The activated IKK β can cause I κ B α degradation, which induces the NF- κ Bp65 subunit phosphorylation, leading to NF- κ B degradation and the nuclear translocation of NF- κ Bp65 (Napetschnig and Wu, 2013). The activation of NF- κ Bp65 further can modulate the transcription of numerous cytokines including TNF- α (Barnes, 2008). In turn, these induced cytokines can further activate the NF- κ B pathway (Kips et al., 1993), thus establishing a positive autoregulatory loop that can amplify the inflammatory response, increase the duration of chronic inflammation, and aggravate asthma symptoms. OVA, used as an allergen of murine asthmatic model, may induce unique histological characteristic of inflammatory response in lung. Our present studies showed that activated NF- κ Bp65 migrated into the nucleus and raised the DNA binding activity of NF- κ Bp65 (In Figs. 3A, B and 4) in the OVA-sensitized rats, accompanying the up-regulation of IKK β , IL-6 and TNF- α as well as down-regulation of I κ B α , whereas SO₂ could enhance these effects. IKK β /I κ B α /NF- κ Bp65 signal pathway activation, NF- κ Bp65 nuclear translocation and IL-6 and TNF- α over-expression may be the crucial causes that SO₂ raised the levels of pro-inflammatory mediators in the asthmatic rats. It suggested that SO₂ could affect the inflammatory

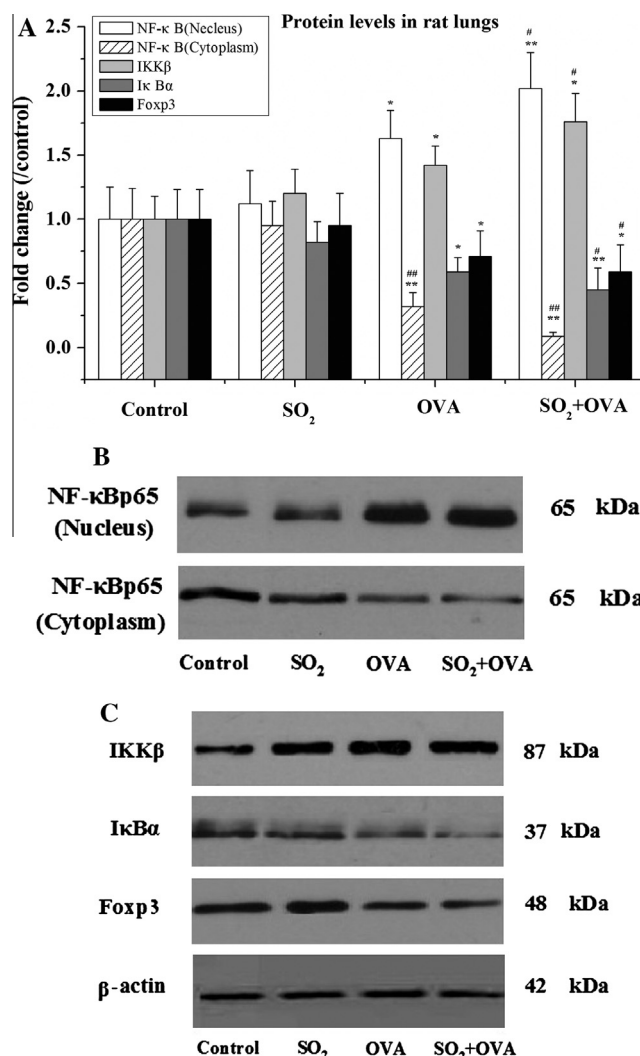


Fig. 3. NF- κ B, IKK β , I κ B α and Foxp3 protein levels of in lungs (A), the nucleus and cytoplasm protein bands of NF- κ B (B) and IKK β , I κ B α and Foxp3 protein bands of rat lung in the different groups (C). Value in each treated group was expressed as a fold change compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as mean $\pm$ SD ($n = 6$); * $P < 0.05$, ** $P < 0.01$ versus control; # $P < 0.05$ versus the OVA group.

responses in the asthmatic rats by increasing the transcription and translation levels of NF- κ B, TNF- α and IL-6, consequently enhancing the susceptibility allergen such as OVA. Notably, some novel inhibitors of NF- κ B and TNF- α are thought as promising anti-inflammatory therapeutics and have received growing attention (Ivanenkov et al., 2011; Qureshi et al., 2011), implying that NF- κ B and TNF- α may be potential key targets for intervention in treating inflammatory conditions in asthma disease.

It has been shown that Th1/Th2 imbalance and IgE level increase are the important mechanism of asthma (Mazzarella et al., 2000; Shi et al., 2011). IL-4 causes a switch to IgE production by differentiating B lymphocytes, while IFN- γ can inhibit that switch and prevent the production of specific IgE (Deo et al., 2010). Decreased IFN- γ and increased IL-4 production can enhance IgE secretion and generating a large number of inflammatory factors to mediate an asthma attack (Fang et al., 2005). Relatively, Tregs can affect Th2 cell responses, reduce the IgE generation and suppress allergic inflammation directly though inhibiting IL-4 levels produced by Th2 cells (Taylor et al., 2001). The research revealed that Foxp3 expression within CD4⁺CD25⁺ Treg cells was

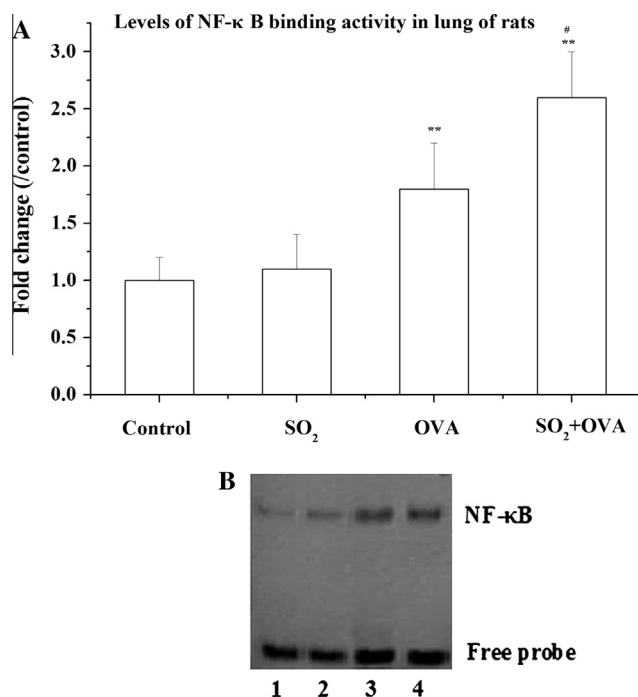


Fig. 4. Results of EMSA assay of NF-κB activity (A) and levels of NF-κB binding activity in lung of rats in different groups (B). Lane 1, control; Lane 2, SO₂ group; Lane 3, OVA group; Lane 4, SO₂ + OVA group. Value in each treated group was expressed as a fold change compared to mean value in control group, which has been ascribed as an arbitrary value of 1. Data were expressed as mean ± SD (n = 6); *P < 0.05, **P < 0.01 versus control; #P < 0.05 versus the OVA group.

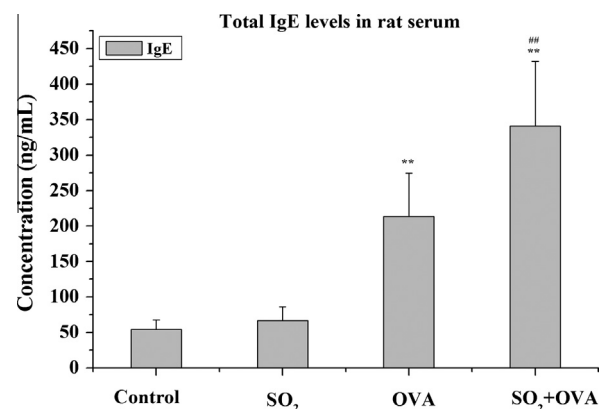


Fig. 6. Total IgE levels in serum in the different groups. Data were expressed as mean ± SD (n = 6); **P < 0.01 versus control; ##P < 0.01 versus the OVA group.

Th1/Th2 balance, may be a possible avenue for targeted therapy for asthma disease.

Here, three possible causes of SO₂-induced the significantly pathological change and inflammatory responses of the lungs from asthmatic rats were discussed. First, SO₂ may generate sulfur- and oxygen-centered free radicals, such as SO₃[•], SO₄[•] and SO₅[•] (Shi, 1994). The one electron oxidation of bisulfite, a known derivative of SO₂, produces the sulfur trioxide radical anion and superoxide anion radical, which reacts rapidly with molecular oxygen to form a peroxy radical. Reactive oxygen species (ROS) including superoxide anion radical and peroxy radical appear to play a central role in the pathophysiological cellular responses including inflammation and proliferation (Dröge, 2002), but also that ROS production and its triggering biologic or toxicological effects are involved in pro-inflammatory cytokines (Yanagisawa et al., 2008). The free radicals generated by SO₂ can induce oxidative stress and lung damage, participate in the pathogenesis of inflammation and elevate pro-inflammatory cytokines including IL-6 and TNF-α (Meng et al., 2003, 2005). Second, SO₂ may enhance sensitization to allergens (Cai et al., 2008). The data in this study showed that there was an increasing trend in TNF-α, IL-6 and IL-4 levels in rats exposed to SO₂, although the levels were not significant differences compared to the control. Specially, SO₂ exposure significantly increased NF-κB and IFN-γ mRNA expression, suggesting SO₂ may have a potential regulatory capacity to stimulate transcription of pro-inflammatory factors, and may enhance the susceptibility to allergen such as OVA. Indeed, SO₂ significantly raised the levels of pro-inflammatory mediators in the asthmatic rats compared with the OVA group, implying that SO₂ may impact on OVA response though increasing the transcription and translation levels of NF-κB, TNF-α and IL-6. Third, SO₂ is clearly an important irritant gas, and it may reduce physiological function of respiratory system and affect lung function, related to asthma (Smargiassi et al., 2009; Tseng et al., 2012; Chang et al., 2012). The studies revealed that SO₂ can irritate sensory nerve in airway and cause airway neurogenic inflammation by increasing plasma contents of substance P (SP) (Lin et al., 2009), which may induce the airway mucus secretion and promote airway smooth muscle contraction (Wagner et al., 1995; Li and Shang, 2012). Taken together, the mechanisms of SO₂ aggravate asthma symptom are very complex, accordingly many research work in depth is required to do further.

What is more, normal tissue structure is one of the important material bases that the body may exert normally physiological functions. Trachea conducts inhaled air into the lungs through its tubular branches. The finding reveals that asthma may affect the trachea construction change and lung inflammatory responses (Teramoto et al., 2013; Manni et al., 2014). In this study, OVA

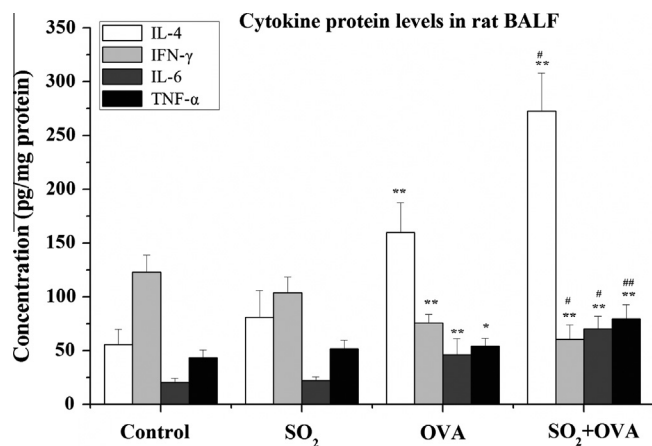


Fig. 5. Protein levels of IL-4, IFN-γ, IL-6 and TNF-α in rat BALF in the different groups. Data were expressed as mean ± SD (n = 6); *P < 0.05, **P < 0.01 versus control; #P < 0.05, ##P < 0.01 versus the OVA group.

significantly lessened in asthmatic patients compared to the normal control group and had a positive correlation with IFN-γ level decline and IL-4 production increase (Provoost et al., 2009), suggesting low Foxp3 expression of Tregs under the asthmatic condition cannot inhibit the Th2 cell proliferation effectively and Foxp3 is operative on Th1/Th2 balance in asthma development. In this study, the mRNA and protein levels of IL-4 and the serum IgE levels were increased, whereas the IFN-γ levels and Foxp3 expression were reduced in the rat's exposure to OVA plus SO₂ compared to the OVA-sensitized rats, indicating that SO₂ could elevate serum IgE levels in asthmatic rats by increasing IL-4 level and decreasing IFN-γ level, which were closely related to Foxp3 expression decline. Foxp3, as a "star molecule" in the upstream regulation of

induced the pro-inflammatory factor expressions and caused IL-4 level increase/IFN- γ level decrease in tracheas and lungs of rats compared with the control, while OVA plus SO₂ had the similar effects. Considering that SO₂ raised significantly NF- κ B levels and inhibited IFN- γ levels in tracheas from rats compared with the control ($P < 0.05$) whereas did not cause significant changes of the asthma related gene expressions in lungs, the trachea tissue might be more sensitive to SO₂ than lung. The detail mechanism needs to investigate further.

5. Conclusion

The results of this study provide evidences that SO₂ affects the inflammation and immune responses in the airway of the asthmatic rats. SO₂ could enhance the susceptibility to OVA, along with the aggravating inflammatory responses in lungs, activating NF- κ B and up-regulating the transcription and translation levels of TNF- α and IL-6, increasing IL-4 and IgE levels and decreasing IFN- γ , which might contribute to the increased risk of asthma disease. The mechanism might be associated with the oxidative stress, pro-inflammatory cytokine expression, Th1/th2 imbalance and the regulation of NF- κ B and Foxp3. NF- κ B and Foxp3 may be the attractive target for novel therapeutic intervention to treat and prevent asthma.

Conflict of interest statement

The authors declare they have no competing financial interests.

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