

RESEARCH ARTICLE

Effects of sulfur dioxide on expressions of p53, bax and bcl-2 in lungs of asthmatic rats

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Abstract

Inhibition of cell apoptosis is an increasingly important factor in modulating airway inflammation in asthma, which is related to environmental pollutants. To investigate the effects of sulfur dioxide (SO₂) on the mRNA and protein expressions of apoptosis-related genes in lungs from asthmatic rats, male Wistar rats were challenged by ovalbumin (OVA) or SO₂ (2 ppm) inhalation alone or together. Examinations were performed 24 h after the last treatment. The mRNA and protein levels of p53, bax, and bcl-2 were analyzed in lungs using real-time reverse transcription-polymerase chain reaction (RT-PCR) assay and Western blot analysis, respectively. The results indicated that increases of bcl-2 or decreases of p53 and bax mRNA and protein levels were not significant in lungs of rats exposed to SO₂ alone, compared with controls, but elevated or reduced levels of these genes appeared in lungs of asthmatic rats exposed to SO₂ plus OVA, compared with controls, suggesting that SO₂ exposure could result in OVA-induced increases or decreases of transcription and translation levels of these apoptosis-related genes in rat lungs, and may have relations to airway inflammation in asthma. The regulation mechanism of apoptosis in asthma disease exposure to SO₂ needs further study.

Keywords: sulfur dioxide; asthmatic rats; lungs; bax; bcl-2; p53

Introduction

Sulfur dioxide (SO₂) is a common air pollutant. It exists in low concentrations in the atmosphere and high concentrations in densely populated areas (Amdur, 1969). The main pollution is from the combustion and disposal procedure of sulfur fuels. SO₂ exposure has been a widespread problem in coal-fired power plants, smelters, sulfuric acid factories, and so on. Previous studies showed that long-term exposure to a certain amount of SO₂ might lead to airway mucosa thickening, pneumonia, nasopharyngitis, gastritis, and changes to smell and taste (Ferris Jr et al., 1967). Epidemiological surveys have shown that SO₂ inhalation could increase morbidity and mortality in humans, including the occurrence of cancer (Glasser et al., 1967; Lave & Liskin, 1970; Nafstad et al., 2003), especially in people who have respiratory, heart, and lung diseases.

Asthma is a chronic airway inflammatory disease characterized by infiltration and activation of various inflammatory cells, including eosinophils, neutrophils, lymphocytes,

macrophages, and so on (Hamid et al., 2003). A recent study has shown that the development of asthma involves many genes and environmental factors (Blumenthal, 2005). Low doses of SO₂ could affect human airway hyperresponsiveness, with changes to functions of the lungs and the immune system (Mehiman 1983; Samukawa et al., 2003). It also could increase asthma morbidity and aggravate asthma symptoms by enhancing the sensitivity of asthma patients to antigen (Grella et al., 2002). Although the pathogenesis of asthma is still not clear, inhibition of cell apoptosis might play an important role in the development of asthma (Jang et al., 2000).

Apoptosis, also called programmed cell death, represents the culmination of naturally occurring or highly programmed mechanisms. It plays a key role in the physiological control of development and growth and in the immune function. Now, apoptosis is implicated in the pathogenesis of a number of diseases and considered to be involved in pathological cell death as well (Thompson, 1995). Some recent observations

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suggest that there may be a distinct genetic program involving selective transcription of certain critical genes required for apoptosis to proceed, including p53, bax, and bcl-2. Bax and p53 play a positive role in the process of cell apoptosis, while bcl-2 will inhibit apoptosis. However, few studies have focused on the effects of SO₂ on the expression of apoptosis-related genes in the asthmatic rat.

The asthmatic rat model challenged by ovalbumin (OVA) has been applied in asthma experiments as well as therapies (Wang & Li, 2003; Pilegaard & Madsen, 2004; Wu et al., 2006) for its advantages of genetic homogeneity of the study subjects as well as ready availability (Hylkema et al., 2002). In this study, we analyzed the expressions of p53, bax, and bcl-2 in the lungs of rats exposed to OVA plus inhaled SO₂ using real-time reverse transcription-polymerase chain reaction (RT-PCR) assay and Western blot analysis.

Material and methods

Animals and treatment protocols

Male Wistar rats were used as described previously (Qin & Meng, 2006). 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 the Ministry of Health, People's Republic of China.

The rats were divided randomly into four equal groups of six animals each. They were treated by intraperitoneal (i.p.) injection, and 1 ml of *Bordetella pertussis* vaccine containing 6×10^9 heat-killed organisms was also given intraperitoneally as adjuvant. Exposure to SO₂ alone or to OVA plus inhaled

SO₂ was performed using the technique of SO₂ dynamic inhalation as described previously (Qin & Meng, 2006). The treatment protocol is shown in Table 1.

Detection of SO₂ concentration

The SO₂ within the chambers was measured every 30 min by pararosaniline hydrochloride spectrophotometry (Goyal, 2001). Briefly, 5 L of SO₂ contaminated atmosphere was collected in 10 ml formaldehyde absorption buffer solution by an air sampler (KC-24; Qingdao, China), and 0.5 ml formaldehyde absorption solution was added to the above. Then 0.5 ml H₂NSO₃Na was added, and the solution was incubated for 10 min in order to avoid the influence of NO_x. Finally, 1.0 ml pararosaniline hydrochloride (PRA, 0.5 g/L) was added, the solution was incubated at room temperature for 20–30 min, and samples were read at 577 nm in a spectrophotometer (U-3010; Hitachi, Tokyo, Japan).

According to experimental measurement, the mean concentration of SO₂ was 5.60 ± 1.12 mg/m³ (2 ppm).

Real-time quantitative RT-PCR

Rats were killed 24 h after the last exposure, and the lungs were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

The frozen tissue samples were homogenized in Trizol reagent using 1 ml of Trizol per 50 mg of tissue. RNA isolation, cDNA synthesis, and PCR analysis were performed as described previously (Qin & Meng, 2006). Sequences of primers and probes are listed in Table 2. Each treatment included six samples and each PCR was carried out in duplicate. The conditions of PCR were as follows: 3 min at 95°C, 45 cycles of

Table 1. Rat treatment protocols for gene expression measurements.

Group	Treatment protocol		
	Day 1	Day 8	Days 15–21
Exposure to SO ₂	Injection of 1 ml saline per rat	Non-treatment	Exposure to SO ₂ (about 2 ppm, 1 h/day)
Exposure to OVA (Sigma, grade II)	Sensitization with 100 mg OVA in 1 ml saline mixed with 100 mg of Al(OH) ₃ per rat	Sensitization with 10 mg OVA in 1 ml saline mixed with 100 mg of Al(OH) ₃ per rat	Challenge with aerosolized 1% OVA (0.5 h/day)
Exposure to OVA plus inhaled SO ₂	Sensitization with 100 mg OVA in 1 ml saline mixed with 100 mg of Al(OH) ₃ per rat	Sensitization with 10 mg OVA in 1 ml saline mixed with 100 mg of Al(OH) ₃ per rat	After SO ₂ inhalation (about 2 ppm, 1 h/day), the rats were challenged with aerosolized 1% OVA (0.5 h/day)
Control	Injection of 1 ml saline per rat	Non-treatment	Fresh air inhalation

Table 2. Primer and probe sequences used in real-time RT-PCR.

p53	NM_030989	Forward primer	5'-CAGCTTTGAGGTTTCGTGTTTGT-3'
		Reverse primer	5'-ATGCTCTTCTTTTTCGCGAAA-3'
		Taqman probe	FAM5'-CCTGTCCTGGGAGAGACCGTCGG-3'TAMRA
Bcl-2	NM_016993	Forward primer	5'-GGAGCGTCAACAGGGAGATG-3'
		Reverse primer	5'-GATGCCGGTTCAGGTACTCAG-3'
		Taqman probe	FAM5'-TCCACAGAGCGATGTTGTCCACCA-3'TAMRA
Bax	NM_017059	Forward primer	5'-CCA AGA AGC TGA GCG AGT GTC TC-3'
		Reverse primer	5'-AGT TGC CAT CAG CAA ACA TGT CA-3'
		Taqman probe	FAM5'-CCACCCGGAAGAAGACCTCTCGGGG-3'TAMRA
GAPDH	NM_017008	Forward primer	5'-ATGTATCCGTTGTGGATCTGAC-3'
		Reverse primer	5'-CCTGCTTACCACCTTCTTG-3'
		Taqman probe	FAM5'-ACTCACTCTTGTCTCTCTGGCGA-3'TAMRA

20 s at 94°C, 20 s at 55°C, and 20 s at 72°C. Fluorescence data were acquired at the 72°C step. The relative quantification of expressions of the target genes was measured using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control.

Western blot analysis

Western blot analysis was performed as described previously (Qin & Meng, 2006). Briefly, proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE; 12% separating, 4% stacking) and transferred to nitrocellulose (NC) membranes. After the membranes were blocked in 5% nonfat dry milk in phosphate buffered saline (PBS) containing 0.1% Tween 20, immunostaining was performed with goat polyclonal antibody specific for rat p53 (Sc-6243; Santa Cruz, CA, USA), bcl-2 (Sc-783; Santa Cruz), and bax (Sc-526; Santa Cruz) at a concentration of 1:500, and mouse monoclonal antibody for actin (Oncogene, Boston, MA) at a concentration of 1:5000, overnight at 4°C. Anti-goat or anti-mouse secondary antibody (Jackson Immuno Research Laboratories and Amersham Pharmacia Biotech, Inc.) at a concentration of 1:5000 was added to membranes which were then incubated for 1.5 h at room temperature. The protein signal was amplified and visualized via chemiluminescence using the ECL Western blotting detection system and Hyperfilm ECL autoradiography (Amersham Pharmacia Biotech, Inc.). Images were quantified using Labworks v3.0.2 image scanning and analysis software (Ultra-Violet Products Ltd.).

Statistical analysis

Statistical analyses were performed by the use of one-way analysis of variance (ANOVA) with SPSS 10.0 packed programs for Windows. Post hoc tests were conducted to determine the difference between groups, followed by Fisher's least significant difference (LSD) test and Duncan's test. Differences were considered to be significant if $p < 0.05$.

Results

In order to study the effects of SO₂ and OVA on the body weights of the rats, these were quantified during the experiment. Although the results showed that the final weights of both the control group and other groups were significantly increased relative to the starting values (data not shown), no significant differences were found in the mean body weight gains of rats exposed to SO₂ and/or OVA compared with controls. Moreover, at the end of treatment the condition of the control group rats appeared normal, and the condition of the SO₂ treated group appeared similar to that of the control group. However, other rats exposed to OVA alone or to OVA plus inhaled SO₂ showed abnormal symptoms such as rapid breathing and sluggish movement, etc.

In the course of establishment of the OVA-sensitized rat model, the numbers of total cells, eosinophils, lymphocytes, macrophages, and neutrophils in bronchoalveolar lavage fluid (BALF) exposed to OVA alone were increased, and a

significant difference was found compared with controls. Upon examination using hematoxylin and eosin (H&E) staining, abnormal pathological changes were observed in rats exposed to OVA alone compared with controls. These histopathological or BALF data in OVA-sensitized rats have been described previously (Li et al., 2007); thus, they are not repeated in this article. These data demonstrated that the OVA-sensitized rat model was successful.

Real-time RT-PCR analysis

p53 mRNA expression

Real-time PCR was used to analyze the levels of p53 mRNA in the lungs of rats exposed to OVA and/or SO₂. The mRNA levels of p53 in lungs are shown in Figure 1a. With exposure to OVA or to SO₂ alone, the decrease in p53 mRNA levels was not statistically significant. The mRNA levels of p53 in lungs was significantly decreased 1.57-fold with exposure to SO₂ with OVA, compared to the control group ($p < 0.05$). However, compared to the OVA group, the decrease was not statistically significant ($p > 0.05$).

bax mRNA expression

Figure 1b presents bax mRNA levels in the lungs of rats exposed to OVA and/or SO₂. We demonstrated that OVA or OVA plus SO₂ inhalation caused statistically significant decreases of bax mRNA levels (0.71-fold, $p < 0.05$; 0.65-fold, $p < 0.01$) in lungs from rats compared with controls. However, bax mRNA levels were not significantly decreased in lungs from rats treated with SO₂ alone.

bcl-2 mRNA expression

The mRNA levels of bcl-2 in rat lungs are shown in Figure 1c. OVA or OVA plus SO₂ inhalation caused statistically

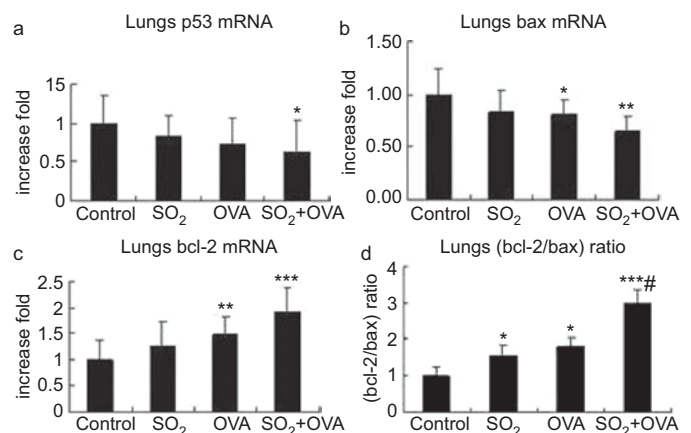


Figure 1. p53 (a), bax (b), and bcl-2 (c) mRNA levels in lungs of rats treated with ovalbumin (OVA) or SO₂ inhalation alone or associated, and bcl-2/bax ratios (d). Each column and bar represent the mean + SD of six individual samples. The mean expression in each treated group is shown as an x-fold increase compared to the mean expression in the control group, which has been ascribed an arbitrary value of 1. Statistical significance was analyzed using one-way ANOVA compared with control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Exposure to OVA plus inhaled SO₂ resulted in a larger increase of mRNA expression versus exposure to OVA alone (# $p < 0.05$).

significant increases of bcl-2 mRNA levels (1.47-fold, $p < 0.01$; 1.93-fold, $p < 0.001$) in rat lungs, whereas bcl-2 mRNA levels were not significantly increased in lungs from rats treated with SO₂ alone. Compared to the group treated with OVA, the increase was not statistically significant ($p > 0.05$) in the group exposed to OVA plus SO₂.

bcl-2/bax mRNA expression

Figure 1d shows that the ratios of bcl-2/bax in the lungs of asthmatic rats were increased, compared with the control group. OVA and/or SO₂ inhalation caused statistically significant increases of the bcl-2/bax ratio (1.52-fold, $p < 0.05$; 1.8-fold, $p < 0.05$; 2.99-fold, $p < 0.001$) in the rat lungs. Compared to the group treated with OVA, the increase was statistically significant ($p < 0.05$) in the group exposed to OVA plus SO₂.

Western blot analysis

p53 protein expression

Figure 2 shows that exposure to OVA plus inhaled SO₂ caused a significant decrease of p53 protein levels in the lungs of rats, compared with controls ($p < 0.05$), but the decrease of protein levels after SO₂ inhalation or treatment with OVA was not statically significant ($p > 0.05$). The results are in line with the above results for p53 mRNA expression in rat lungs.

bax protein expression

The protein expression levels of bax in the lungs are shown in Figure 3. We demonstrated that exposure to OVA or to OVA plus inhaled SO₂ caused statistically significant decreases of bax protein expression (0.68-fold, $p < 0.05$; 0.62-fold, $p < 0.01$) in rat lungs, but the decrease of protein levels after SO₂ inhalation was not statically significant ($p > 0.05$). Compared to exposure to OVA, the decrease for rats treated with OVA plus inhaled SO₂ was not statically significant ($p > 0.05$).

bcl-2 protein expression

In Figure 4, it can be seen that exposure to OVA alone or to inhaled SO₂ caused a significant increase of bcl-2 protein levels in the lungs of rats (1.65-fold, $p < 0.05$; 1.68-fold, $p < 0.05$) compared with controls. Also, the protein levels of bcl-2 in rat lungs were significantly increased (2.05-fold) after exposure to OVA plus inhaled SO₂ compared with the control group ($p < 0.01$). However, compared to exposure to OVA alone, the increase was not statistically significant ($p > 0.05$).

Discussion

During the last 20 years, it has been estimated that about 155 million people worldwide are affected by asthma, which is associated with airway hyperresponsiveness, chronic inflammation, and mucus overproduction (Wills-Karp, 2000; Duncan, 2004; Wegmann et al., 2005). The number with asthma is still increasing particularly seriously in developed countries. Recent study has shown that the incidence of asthma presents an increasing trend in many countries and regions, year by year. It has become a widespread social health issue which arouses great concern worldwide.

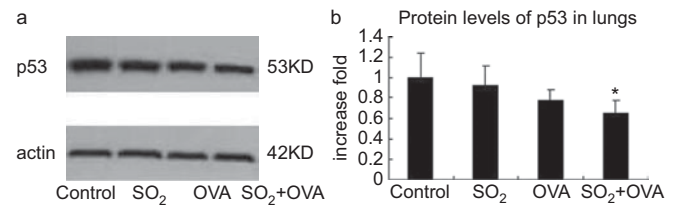


Figure 2. p53 protein levels in lungs of rats treated with OVA or SO₂ inhalation alone or associated. (a) Western blot analysis was performed with antibody against p53 and with actin. Each image corresponds to a representative experiment from six experiments. (b) Each column and bar represent the mean + SD of six individual samples. The mean expression in each treated group is shown as an x-fold increase compared to the mean expression in the control group, which has been ascribed an arbitrary value of 1. Statistical significance was analyzed using one-way ANOVA compared with control, * $p < 0.05$.

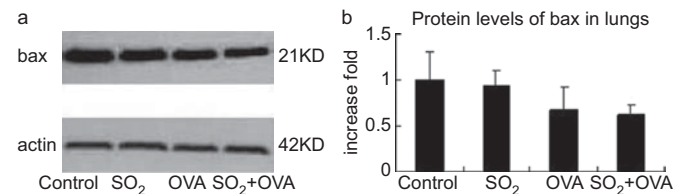


Figure 3. bax protein levels in lungs of rats treated with OVA or SO₂ inhalation alone or associated. (a) Western blot analysis was performed with antibody against bax and with actin. Each image corresponds to a representative experiment from six experiments. (b) Each column and bar represent the mean + SD of six individual samples. The mean expression in each treated group is shown as an x-fold increase compared to the mean expression in the control group, which has been ascribed an arbitrary value of 1. Statistical significance was analyzed using one-way ANOVA compared with control, * $p < 0.05$, ** $p < 0.01$.

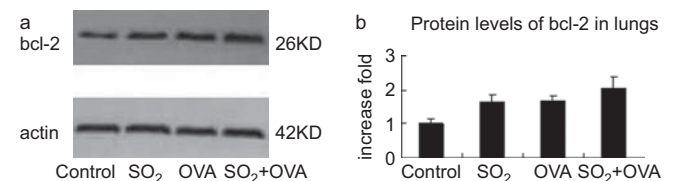


Figure 4. bcl-2 protein levels in lungs of rats treated with OVA or SO₂ inhalation alone or associated. (a) Western blot analysis was performed with antibody against bcl-2 and with actin. Each image corresponds to a representative experiment from six experiments. (b) Each column and bar represent the mean + SD of six individual samples. The mean expression in each treated group is shown as an x-fold increase compared to the mean expression in the control group, which has been ascribed an arbitrary value of 1. Statistical significance was analyzed using one-way ANOVA compared with control, * $p < 0.05$, ** $p < 0.01$.

SO₂, one of the most important air pollutants, could affect the respiratory system in children and elderly people (Tseng & Li, 1990; Derya, 2003). In particular, low-dose exposure to SO₂ might aggravate symptoms in asthmatic subjects (Grella et al., 2002), while SO₂ at dose levels of 1.5 and 2 ppm could induce respiratory tract inflammation and an increase of airway resistance in experimental animals (Mehiman 1983; Samukawa et al., 2003). SO₂ at 2 ppm was used in this study to examine the effects of its exposure. Although the experiment may be viewed as beyond the normal atmosphere encountered in the human environment (<0.5 ppm), there are four important considerations to be made. First, the animals were subjected to regular periods of extended exposure, with relief periods between protocols (i.e. 6 h/day, for 7 days, with 18 h between exposures). This may provide a corollary to individuals exposed to the gas in an occupational setting. Second, the rat is an obligate nose breather and approximately 95% of inhaled SO₂ is trapped in the nasal passages. The actual concentrations of gas reaching the lungs may therefore have been significantly lower than that in the exposure chamber. Third, it was reported that symptoms could be aggravated in asthmatics at 1- and 5-ppm concentrations of SO₂ (Sheppard et al., 1980; Horstman et al., 1988). In an animal experiment, lung function changes were observed in asthmatic guinea pigs challenged by OVA following exposure to 5 ppm SO₂ on 5 consecutive days over 8 h per day (Riedel et al., 1992). Recently, Park et al. (2001) reported that repeated exposure even to low levels of SO₂ (<1 ppm) could enhance the development of OVA-induced asthmatic reactions in guinea pigs. Fourth, the threshold limit value (TLV) from the US American Conference of Governmental Industrial Hygienists (ACGIH) for SO₂ in an industrial environment is 2 ppm, and occupational exposure limit values (OELs) for SO₂ with a respiratory critical effect in workplace air in Sweden is also 2 ppm (Hansson & Rudén, 2006). Therefore, the dose of SO₂ (2 ppm) used in this study may have actual meaning in terms of occupational exposure.

The rat model induced by OVA in this study was used to investigate the molecular mechanism between SO₂ exposure and the pathogenesis of asthma and expressions of p53, bax, and bcl-2 in the lungs of asthmatic rats exposed to SO₂. The results indicated that increases of bcl-2 or decreases of p53 and bax mRNA and protein levels were not significant in rat lungs exposed to SO₂ alone compared with controls, but elevated or reduced levels of these genes were observed in the lungs of rats exposed to OVA and/or to SO₂ plus OVA compared with controls.

How do we explain the relationship between changes in apoptotic gene expression in lungs from asthmatic rats and exposure to SO₂? On the one hand, inflammatory cells, especially eosinophils, are known to play an important role in the onset and maintenance of asthma (Vignola et al., 1999; Druilhe et al., 2000), while the inhibition of inflammatory cell apoptosis in acute asthma is associated with the expression of pro- and antiapoptotic genes (Jang et al., 2000). bcl-2 is a type of apoptosis suppression gene, while bax or p53 are examples of proapoptotic genes. As to the functions of

apoptosis regulation, p53 exerts control over cell cycling by regulating progression through the G1 phase, and is reported to be involved in the induction of apoptosis (Van Gijssel et al., 1997); bax, a 21-kDa protein co-immunoprecipitated with bcl-2, was identified as a promoter of cell death whose proapoptotic function was directly antagonized by bcl-2 through the formation of bax/bcl-2 heterodimers (Oltvai et al., 1993; Sato et al., 1994; Yin et al., 1994). The ratio of bax to bcl-2 determines the susceptibility of a cell to apoptosis. Alteration of these gene expressions affects cell proliferation, growth, metabolism, and differentiation and regulates the expression of a number of proteins important in proliferation. To some extent, bax/bcl-2 heterodimers predominate in cells that overexpress bcl-2, and the susceptibility of these cells to apoptosis is reduced; in turn, cell proliferation is observed (Oltvai et al., 1993; Yang & Korsmeyer, 1996). The previous study showed that the levels of inflammatory cells (such as eosinophils, macrophages, and T lymphocytes) were increased in BALF from asthmatic rats after exposure to OVA plus SO₂, compared with controls (Li et al., 2007). In this study, the ratios of bcl-2/bax were significantly increased after exposure to OVA or OVA plus SO₂. OVA could significantly induce bcl-2 mRNA and protein expression, and inhibit p53 and bax mRNA and protein expression. Significant changes of gene expression were observed in the lungs of rats exposed to OVA plus SO₂ inhalation compared with either alone or controls. Combining these data with changes to inflammatory cells induced by OVA or OVA plus SO₂ according to the previously published article, it is suggested that inhibition of inflammatory cell apoptosis might be induced by up-regulation of bcl-2 and down-regulation of bax or p53. These results might explain the increase of inflammatory cells in the development of asthma, and might provide more useful evidence to understand the possible mechanism via which SO₂ pollution aggravates asthma disease.

On the other hand, the increase of the ratio of bcl-2/bax was statistically significant ($p < 0.05$) after exposure to OVA plus SO₂ inhalation compared to the group treated with OVA. This indicates that SO₂ could exacerbate changes to apoptotic gene expression in lungs of asthmatic rats. Because asthma syndromes are related to insufficient expression of apoptotic genes or to the inhibition of inflammatory cell apoptosis, these results again might provide useful evidence to understand the possible mechanism via which SO₂ pollution aggravates asthma disease.

In addition, SO₂ at 10 and 20 ppm was able to regulate changes to apoptosis genes (down-regulation of bcl-2 and up-regulation of bax or p53) in rat lungs, while SO₂ at 5 ppm could not induce significant changes to apoptosis genes (Bai & Meng, 2005). In this study, SO₂ at 2 ppm increased the levels of bcl-2 mRNA and protein expression and decreased the expression of bax or p53. Although changes to apoptosis gene expression were not significant compared with controls, this indicated an opposite tendency compared to the result above. Perhaps high levels of SO₂ can induce cell apoptosis, while low levels of SO₂ promote cell multiplication. However,

understanding the complex role of this different regulation of the apoptotic mechanism induced by different levels of SO_2 needs further investigation.

In conclusion, SO_2 can inhibit proapoptotic gene expression such as p53 and bax, and induce antiapoptotic gene expression such as bcl-2 in asthmatic rats, and may have relations to the inhibition of inflammatory cell apoptosis in asthma. These results provide more useful evidence to understand the possible development mechanism of asthma due to low-level repeated exposure to SO_2 . However, the mechanism of regulation of apoptosis in asthma disease following exposure to SO_2 still needs further study.

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