

Repeated exposure to low levels of sulfur dioxide (SO₂) enhances the development of ovalbumin-induced asthmatic reactions in guinea pigs

Jae-Kyoung Park MD*; Yoon-Keun Kim, MD*†‡; Sang-Rok Lee, MD*; Sang-Heon Cho, MD*†‡; Kyung-Up Min, MD*†‡; and You-Young Kim, MD*†‡

Background: Sulfur dioxide (SO₂) is one of the major air pollutants. It is known to aggravate asthma symptoms in human beings, but few studies have focused on the effects of SO₂ upon the development of bronchial asthma in animal models.

Objective: This study was undertaken to evaluate the role of SO₂ upon the development of ovalbumin (OA)-induced asthmatic reactions in guinea pigs.

Methods: Guinea pigs were divided into four groups: (1) OA- and SO₂-exposed group (n = 12), (2) SO₂-exposed group (n = 12), (3) OA-exposed group (n = 11), and (4) saline-exposed group (n = 7). Guinea pigs of the first and second groups were exposed to 0.1 ppm SO₂ for 5 hours a day on 5 consecutive days. Guinea pigs in the first and third groups inhaled 0.1% OA aerosols for 45 minutes a day on days 3, 4, and 5. One week after the sensitization procedure, all the guinea pigs underwent bronchial challenge with 1.0% OA aerosols, using unrestricted whole-body plethysmography. Bronchoalveolar lavage and histopathologic examination were performed 24 hours after the bronchial challenge.

Results: Increases in enhanced pause (P_{enh}), as an index of airway obstruction, after the bronchial challenge was significantly higher in OA- and SO₂-exposed group (group 1) than the other groups (*P* < .05, respectively). Eosinophil counts in bronchoalveolar lavage fluids were also significantly higher in group 1 than in the other groups (*P* < .05, respectively). Histopathologic findings of bronchial and lung tissue in the group 1 showed an infiltration of inflammatory cells, bronchiolar epithelial damage, and mucus and cell plug in the lumen, but no significant abnormalities were observed in the other groups.

Conclusions: These results indicate that repeated exposure to low levels of sulfur dioxide may enhance the development of ovalbumin-induced asthmatic reactions in guinea pigs.

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INTRODUCTION

It is well recognized that bronchial asthma and other allergic diseases of the respiratory system are increasing worldwide.¹ Reasons for the increased

incidence of asthma are likely to be attributable to the increased exposure to common aeroallergens as a result of changing lifestyles. However, environmental air-borne pollutants are believed to bear a measure of this responsibility.^{2,3}

Sulfur dioxide (SO₂) is one of the major air pollutants. It is derived mainly from the combustion of fossil fuels. Korean air quality standard maximum mean exposure levels for SO₂ are as follows: 1 year, 0.03 ppm; 24 hours, 0.14 ppm; and 1 hour, 0.25 ppm. The 24-hour SO₂ level has often been exceeded in several Korean cities. It is well established that SO₂ can

aggravate asthma symptoms in human beings.^{4–8} A number of epidemiologic studies have revealed that ambient SO₂ levels are associated with the increased prevalence of asthma.⁹ Controlled studies also have shown that an exposure to 1.0 ppm SO₂ causes bronchoconstriction in subjects with asthma.¹⁰ Animal studies have reported that previous exposure to high levels of SO₂ with ovalbumin (OA) could enhance the development of allergen-induced anaphylaxis in guinea pigs.^{11,12} However, it has been unknown whether repeated exposure to low levels of SO₂ could enhance the development of asthma.

This study was undertaken to evaluate whether repeated exposure to low levels (0.1 ppm) of SO₂ could enhance the development of OA-induced asthmatic reactions in guinea pigs. To test this hypothesis, bronchial obstruction was measured using single-chamber whole-body plethysmography, and inflammatory changes in the airways were evaluated by examining bronchoalveolar lavage (BAL) fluids and the lung tissue of guinea pigs with a sensitization to OA caused by low levels of sulfur dioxide.

MATERIALS AND METHODS

Materials and Study Protocol

Dunkin-Hartley guinea pigs, maintained on OA-free diets, were used throughout this study. They were all males and weighed between 250 and 350 g during the sensitization period. The guinea pigs were divided into four groups: (1) OA- and SO₂-exposed (n = 12); (2) SO₂-exposed (n = 12); (3) OA-exposed (n = 11); and (4) saline-exposed (n = 7) groups. Guinea pigs

* Department of Internal Medicine, Seoul National University College of Medicine.

† Institute of Allergy and Clinical Immunology, Seoul National University College of Medicine.

‡ Clinical Institute of Seoul National University Hospital, Seoul, Korea.

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of the first and the second groups were exposed to 0.1 ppm SO₂ for 5 hours a day on 5 consecutive days. Guinea pigs in the first and the third groups inhaled 0.1% of OA aerosols for 45 minutes a day on days 3, 4, and 5. Guinea pigs of the fourth group were sensitized with phosphate-buffered saline (PBS) instead of OA. One week after the sensitization procedure, all the guinea pigs underwent bronchial challenge with 1.0% OA aerosols using single-chamber whole-body plethysmography (Buxco Electronics, Inc, Troy, NY). BAL and histopathologic examination were performed 24 hours after the bronchial challenge. The study protocol was approved by the Ethics Committee of Seoul National University Hospital.

Development of an Asthma Model in Guinea Pigs: Preliminary Study

As a preliminary study, the asthma model in guinea pigs was developed as follows. Four groups of guinea pigs (three to four guinea pigs from each group) were sensitized to 0.1, 0.5, 1.0, and 2.0% OA aerosols for 45 minutes over 3 consecutive days. Seven days after the sensitization procedure, guinea pigs were placed in a bias-flow-ventilated single-chamber whole-body plethysmograph, and were subjected to bronchial challenge using 1.0% OA aerosols. In the chamber, box pressure signals were obtained from the pressure differential between the main chamber and the reference chamber. This pressure difference was caused by the respiratory cycle of the guinea pig in the main chamber. Pressure signals were analyzed using a computed analyzer and visualized on a monitor. Airway resistance was expressed as follows: $P_{\text{enh}} = ([T_e/0.3 T_r] - 1) \times [2P_{\text{ef}}/3P_{\text{if}}]$, where P_{enh} equals enhanced pause, T_e equals expiratory time (in seconds), T_r equals relaxation time (in seconds), P_{ef} equals peak expiratory flow (in mL/sec), and P_{if} equals peak inspiratory flow (mL/sec). BAL was performed 24 hours after bronchial challenge, and inflammatory cells were

counted under the light microscope. Histopathologic changes in bronchial and lung tissues were examined after lung dissection. In this preliminary study of the asthma model in guinea pigs, bronchoconstriction after OA bronchial challenge and histopathologic changes indicative of asthma were demonstrated after sensitization with 0.5, 1.0, and 2.0% OA, but not with 0.1% OA.

Exposure to SO₂ and OA

Because the preliminary study showed that exposure to 0.1% OA did not induce asthma in guinea pigs, guinea pigs were sensitized with 0.1% OA. A modification of Riedel's procedure¹² was adopted for SO₂ and OA exposure. Guinea pigs were placed in a 40-L exposure box, and exposed to 0.1 ppm SO₂ for 5 hours a day over 5 consecutive days (day 1 to 5). SO₂ was supplied with a constant velocity of 3 L/min, and the concentration of SO₂ was measured by test tubes (Gastec, Japan), at 1-hour intervals to maintain SO₂ levels at 0.1 ppm. Temperature and humidity within the exposure chamber were kept at 20° C to 24° C and 60%.

For sensitization with OA, guinea pigs were placed in the main chamber, and inhaled 0.1% OA aerosols for 45 minutes a day on days 3, 4, and 5.

Bronchial Challenge with 1.0% OA Aerosols

Lung functions of the guinea pigs were measured using single-chamber whole-body plethysmography. Before the lung function measurement, the guinea pig was placed in the main chamber repeatedly to familiarize it with the surroundings.

On day 7 after the sensitization period, guinea pigs were placed in a single chamber and challenged with 1.0% OA aerosols. After waiting longer than 10 minutes for an animal to stabilize in the chamber, baseline lung function was recorded for 3 minutes. PBS and 1.0% OA aerosols were nebulized and passed into the main chamber for 5 minutes. P_{enh} was then recorded for 3 minutes after exposure to PBS for 5 minutes, and after exposure to 1.0% OA for 5 minutes, 10

minutes, 20 minutes, 30 minutes, 45 minutes, 1 hour, and 24 hours.

Analysis of BAL Fluid

After the intraperitoneal injection of sodium pentothal (50 mg/kg), the trachea was exposed and a polyethylene tube inserted. Fifteen milliliters of sterile saline was instilled twice. When 80% to 90% of the instilled saline was recovered, BAL fluid was passed through gauze and centrifuged at 200 *g* for 10 minutes at 4° C. The supernatant was discarded, and the cell pellet was washed. After Diff-quick staining, two investigators counted blindly more than 300 white blood cells under a light microscope and classified them as macrophages, lymphocytes, neutrophils, and eosinophils.

Histopathologic Examination of Bronchial and Lung Tissue

After BAL, 10% formalin solution was instilled into the trachea through a polyethylene tube, and the lung was dissected and fixed in a 10% formalin solution and embedded. Random sections of 3- μ m thickness were stained with hematoxylin and eosin. Two investigators examined blindly histopathologic changes such as the epithelial damage and the infiltration of inflammatory cells under light microscope. The severity of inflammatory reactions was evaluated by semiquantitative grading as shown in Table 2 (see "Results").

Statistical Analysis

Statistical significance among the four groups was evaluated using the Mann-Whitney *U* test, and results were considered significant when $P < .05$.

RESULTS

Compared with the PBS challenge, the percentage increases (mean $\pm$ SEM) of P_{enh} at 30 minutes after the bronchial challenge with 1.0% OA aerosols were $57.4 \pm 10.9\%$ for the OA- and SO₂-exposed group (group 1); $10.0 \pm 4.2\%$ for the SO₂-exposed group (group 2); $14.5 \pm 4.9\%$ for the OA-exposed group (group 3); and $4.0 \pm 4.1\%$ for the saline-exposed group (group 4; Fig 1). Figure 1 indicates that the severity

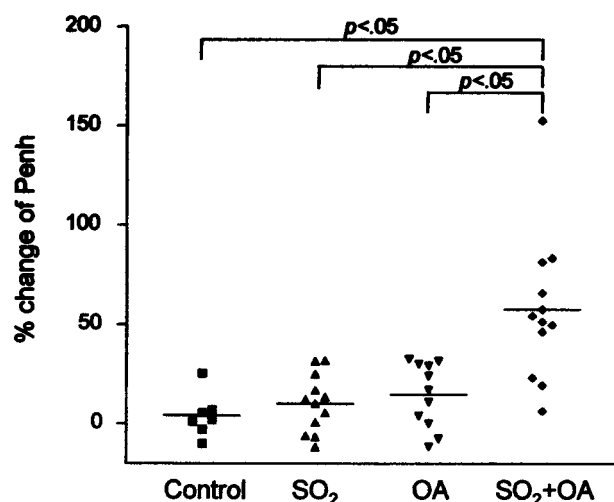


Figure 1. Percent change of P_{enh} 30 minutes after bronchial challenge with 1.0% OA. SO_2 - and OA-exposed (SO_2 +OA) guinea pigs show a significant increase in P_{enh} as compared with saline-exposed, SO_2 -exposed (SO_2) or ovalbumin-exposed (OA) guinea pigs ($P < .05$, respectively).

of bronchial obstruction after 1.0% OA challenge was significantly higher in the first group than in the other groups ($P < .05$), but no significant differences were found among the other groups. In 8 of 12 guinea pigs exposed to both SO_2 and OA, the percentage increases of P_{enh} 30 minutes after the bronchial challenge were more than 50%. Figure 2 shows a typical box-pressure waveform displayed on the monitor in the case of a guinea pig belonging to group 1. In contrast, no animal in the other groups showed a

P_{enh} increase $> 50\%$ at any time after the bronchial challenge.

BAL fluids from groups 2, 3, and 4 showed no significant infiltration of inflammatory cells. In contrast, the eosinophil counts of BAL fluids were significantly increased in the first group ($P < .05$; Table 1, Fig 3). In the first group, bronchial examinations showed an infiltration of inflammatory cells, bronchiolar epithelial damage, and mucus and cell plug in the bronchiolar lumen (Fig 4). However, bronchial and lung tissue from groups 2 to

4 revealed no significant abnormalities on histopathologic examination. Table 2 shows the severity of the inflammatory reactions for the four groups by semiquantitative grading. The grade [median (range)] for the infiltration of inflammatory cells was 2 (0 to 3) for group 1, and 0 (0 to 0) or 0 (0 to 1) for the other groups. The grade [median (range)] for epithelial damage was 2 (1 to 3) for group 1, and 0 (0 to 1) or 0 (0 to 2) for the other groups.

DISCUSSION

The relationship between bronchial asthma and SO_2 has been evaluated by epidemiologic studies, and exposure experiments with both animals and mild asthmatic patients.^{8,13,14} Epidemiologic studies have demonstrated that asthma symptoms are aggravated by transient exposure to SO_2 . Several human studies have reported that mild asthmatic patients developed bronchoconstriction by inhaling SO_2 at much lower concentrations than the average person.^{6,8} However, few studies have evaluated that repeated exposure to low levels of SO_2 increases the asthmatic reactions in animal models.¹² The aim of this present study was to evaluate whether repeated exposure to low levels of SO_2 , at concentrations that are often reached in urban areas, enhance the development of OA-induced asthmatic reactions in guinea pigs.

Because 0.1% OA did not cause an asthmatic response in the preliminary study, a 0.1% OA solution was chosen for the sensitization of the guinea pigs. Guinea pigs sensitized to only 0.1% OA, 0.1 ppm SO_2 , or PBS, showed no apparent change of the bronchial obstruction after 1.0% OA bronchial challenge. Further, no significant infiltration of inflammatory cells was found in BAL fluid and no apparent damage of the bronchial epithelium on histopathologic examination was observed in these groups. However, guinea pigs exposed to both SO_2 and OA showed an increased P_{enh} compared with the other groups and pathologic findings compatible with asthmatic airway inflammation. These

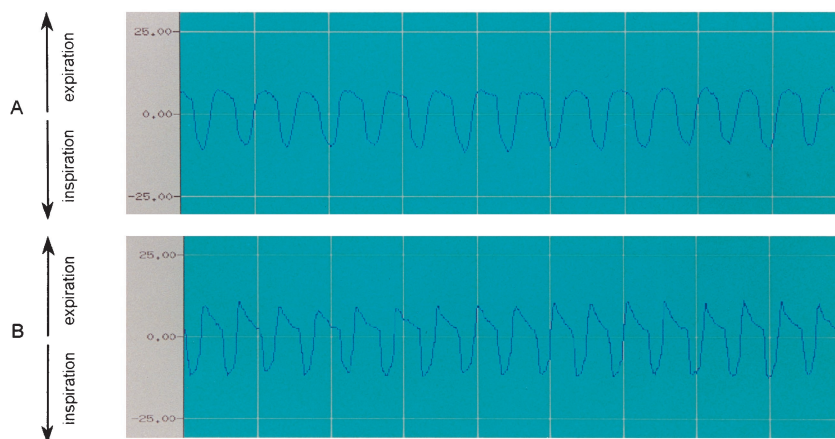


Figure 2. Changes in box-pressure waveform after OA bronchial challenge. Waveform of the box-pressure signal derived from a SO_2 - and OA-exposed guinea pig after 3 minutes of nebulization with aerosolized PBS (A) or aerosolized OA (1.0%; B).

Table 1. Differential Leukocyte Counts in Bronchoalveolar Lavage Fluids Obtained 24 Hours after Bronchial Challenge with 1.0% OA

	Macrophage (%)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)
Saline	79.2 ± 3.7	13.0 ± 4.3	5.0 ± 0.7	2.8 ± 1.0
SO ₂	75.0 ± 10.5	12.6 ± 6.2	8.0 ± 3.4	4.8 ± 1.9
OA	84.0 ± 3.1	9.8 ± 1.5	3.8 ± 0.9	2.8 ± 1.2
SO ₂ +OA	76.1 ± 6.5	7.0 ± 1.6	5.9 ± 1.8	10.8 ± 4.7*

OA: ovalbumin. Data represent mean values ± SEM.

Statistical analysis was done with Mann-Whitney *U* test for unpaired samples.

* Significantly increased as compared with control, OA-exposed, and SO₂-exposed guinea pigs (*P* < .05, respectively).

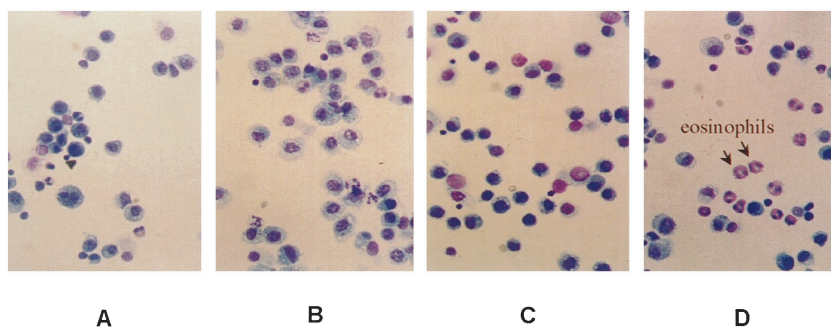


Figure 3. Cells in BAL fluids from saline-exposed (A), SO₂-exposed (B), OA-exposed (C), and SO₂- and OA-exposed (D) guinea pigs 24 hours after OA bronchial challenge. Many eosinophils are observed from the SO₂- and OA-exposed (D) guinea pigs (Diff-quick stain, × 100).

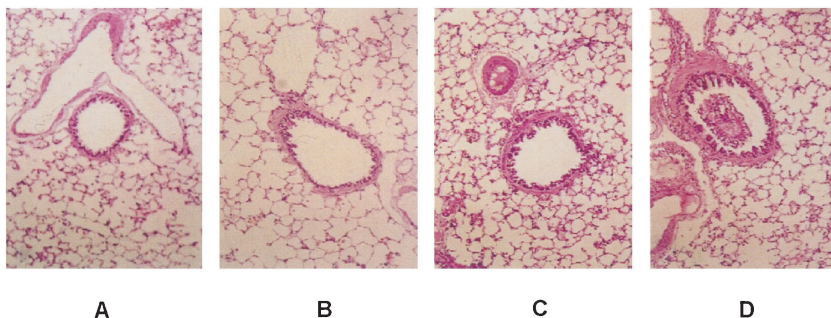


Figure 4. Sections through bronchiolar regions of lung tissue from saline-exposed (A), SO₂-exposed (B), OA-exposed (C), and SO₂- and OA-exposed (D) guinea pigs 24 hours after 1.0% OA bronchial challenge. A to C: Bronchiolar epithelium is well preserved. D: Epithelial desquamation is observed. An inflammatory cells/mucus plug is found in the lumen (hematoxylin-eosin stain, × 100).

results are in accord with epidemiologic studies, which suggest that the prevalence of asthma was correlated with the levels of outdoor air pollutants.⁹

Many investigators have measured the lung functions of small animals by invasive procedures, such as anesthesia and tracheotomy.¹⁵ Recently, noninvasive whole-body plethysmography had been intro-

duced, but lung functions were measured using two-chamber plethysmography.^{12,16} However, using this technique, it was very difficult to measure lung functions for either an extended time or repeatedly because of the need for neck fixation. To study the mechanisms and kinetics underlying the development of increased airway responsiveness after allergen challenge, unrestrained ani-

mal models have been invaluable. The single-chamber whole-body plethysmograph allows a small animal to move freely and allows the uninhibited measurement of lung functions.^{17,18} Using whole-body plethysmography, increases in *P*_{enh} values after bronchial challenge have been known to be well correlated with increased intrapleural pressure and pulmonary resistance, measured in anesthetized animals.¹⁸ Using the single-chamber whole-body plethysmograph and increases in *P*_{enh} as an index of airway obstruction, we measured airway responsiveness in conscious, unrestrained guinea pigs after sensitization and airway challenge with OA.

In this study, BAL was performed 24 hours after the bronchial challenge because other investigators had demonstrated that eosinophil counts in BAL fluids reached peak levels 24 hours after OA inhalation.^{19,20} The results of this study showed that histopathologic changes of bronchial and lung tissue in guinea pigs, after the OA challenge, were compatible with allergic inflammation.²¹

Although the mechanism by which SO₂ contributes to the development of asthma is not clear, direct irritant effects to hyperresponsive airway, bronchial epithelial damage or inflammatory cell infiltration in the bronchus, or enhanced sensitization to allergens may be responsible.²² Jackson and Eady¹³ reported that bronchial responsiveness to histamine increased and persisted for 2 hours in dogs after inhalation of 400 ppm SO₂. In an animal experiment, airway inflammation was induced by SO₂.¹⁵ In this study, however, the bronchial mucosa in guinea pigs exposed only to SO₂ revealed no significant inflammatory change. Therefore, it is unlikely that airway inflammation induced directly by low levels of SO₂ plays a role in the development of bronchial asthma. Mucosal inflammation resulting from upper respiratory tract infection or exposure to toxic substances can increase the permeability of the mucosal barrier, which increases the

Table 2. Inflammatory Changes in Bronchial and Lung Tissue after Bronchial Challenge with 1.0% OA

	Infiltration of inflammatory cells	Epithelial damage
Saline	0 (0–0)	0 (0–0)
SO ₂	0 (0–1)	0 (0–1)
OA	0 (0–1)	0 (0–2)
SO ₂ +OA	2 (0–3)	2 (1–3)

Data are presented as median, with range in parentheses.

Inflammatory changes were graded by semiquantitative scoring system as below.

Grade	Infiltration of inflammatory cells	Epithelial damage
0	None	None
1	Mild	Mild cell loss
2	Moderate	Moderate cell loss
3	Severe	Severe cell loss, hyperplasia, or plug

likelihood of contact between an aeroallergen and the immune system, and enhanced allergenic sensitivity. Several epidemiologic studies have suggested that air pollutants increase the sensitization to aeroallergens. For example, Japanese cedar pollinosis tends to be more prevalent in areas of high automobile pollution than in the forests where the pollen count is higher.²³ This raises the possibility that air pollutants derived from the automobile are responsible for the increased sensitization to Japanese cedar pollens. In this study, bronchial obstruction after bronchial challenge was demonstrated using single-chamber plethysmographic measurements, and asthmatic airway inflammation was confirmed by histopathologic changes, such as the infiltration of inflammatory cells, bronchiolar epithelial damage, and mucus and cell plug in guinea pigs sensitized to both OA and SO₂. These findings suggest that repeated exposure to low levels of SO₂ with causative allergens may develop asthmatic reactions in guinea pigs by enhanced sensitization to allergen.

CONCLUSION

In conclusion, this study demonstrates that low levels of SO₂ enhance the development of OA-induced asthmatic reactions in guinea pigs. The results

suggest that repeated exposure to low levels of SO₂ with causative allergens may enhance the development of asthma in susceptible individuals.

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- Request for reprints should be addressed to:*
You-Young Kim, MD
Department of Internal Medicine
Seoul National University College of Medicine
28 Yongon-dong, Chongno-gu
Seoul, 110–744, Korea.
E-mail: youyoung@plaza.snu.ac.kr
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