
Increased oxidative stress in the airway and development of allergic inflammation in a mouse model of asthma

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Background: The exact pathogenic role of oxidative stress in the development of allergic airway inflammation is still largely unknown.

Objective: To investigate a possible link between increased pulmonary oxidative stress and the pivotal features of asthma during the mounting of an allergic inflammatory response.

Methods: To determine the relationship between oxidative stress and allergic inflammatory responses, we evaluated the sequential kinetics of oxidative stress in the lung, the development of airway inflammation, mucin hypersecretion, and airway hyperresponsiveness (AHR) in an ovalbumin (OVA)–sensitized and challenged mouse with and without antioxidant. Parameters were measured at 9 points for more than 28 days, starting from the first day of OVA challenge with or without antioxidant treatment. The ratio of reduced to oxidized glutathione in the lungs and levels of intracellular reactive oxygen species (ROS) in the bronchial epithelium were serially measured. Bronchoalveolar lavage fluid cells, histopathologic features, and AHR were analyzed at the same time points.

Results: The reduced to oxidized glutathione ratio was reduced from immediately after OVA challenge to day 1, remained at this level until day 1, and rapidly recovered to the normal level after more than 2 days. Intracellular ROS levels in the bronchial epithelium followed similar kinetics. The inflammatory cells in bronchoalveolar lavage fluid reached a maximum of 3 days and decreased progressively thereafter. Histopathologic examination revealed that substantial airway inflammation persisted through day 28. The proportion of mucin-producing epithelial cells significantly increased after day 1, reached a maximum at day 3, and remained at this level until day 5. The AHR peaked on day 1 and normalized within 5 days. The pretreatment of antioxidant significantly reduced not only the increased ROS levels but also development of other phenotypes of asthma.

Conclusion: These results indicate that increased oxidative stress in the lung precedes other pivotal phenotypes of allergic airway disease, suggesting a critical role for increased oxidative stress in the induction of allergic airway inflammation.

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INTRODUCTION

The important underlying pathophysiological features of bronchial asthma, which is characterized by episodic wheezing, dyspnea, and sputum production, are T_H2-dominant inflammation, mucous hypersecretion, and airway hyperresponsiveness (AHR).^{1,2} There is overwhelming evidence that airway inflammation plays a crucial role in bronchial asthma. However, the exact pathogenic mechanism that causes the T_H2-dominant inflammation is not clearly defined. Furthermore, the correlation of AHR and mucin production with the

degree and/or kinetics of airway inflammation has not been thoroughly investigated, although inflammation has been known to be closely associated with the induction of AHR and mucous hypersecretion.

Oxidative stress is primarily induced by various free oxygen radicals known as reactive oxygen species (ROS). ROS have received considerable attention in the pathogenesis of chronic inflammatory immunological diseases, including atherosclerosis, diabetes mellitus, and neoplasms. Accumulating evidence has also implicated oxidative stress in the pathogenesis of asthma. Enhanced oxidative stress in asthmatic patients has been consistently observed; oxidative stress not only increases but is also correlated with disease activity.^{3,4} ROS have been shown to strongly evoke bronchial hyper-reactivity and to enhance mucous secretion.⁵ In addition, ROS can reportedly result in direct oxidative damage to lung cells and trigger the release of various inflammatory mediators and cytokines.^{6–8}

Despite abundant evidence to support a close link between the development of asthma and increased oxidative stress, the precise role of ROS in the pathoetiology of asthma is not

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clearly defined. Although oxidative stress could simply be a consequence of ROS production in recruited inflammatory cells, substantial evidence suggests otherwise, pointing to a role for oxidative stress as an initiating factor in the generation of airway inflammation.

This study was undertaken to fill the unmet need for a systemic evaluation of the role and kinetics of ROS in the inflammatory cascade during the initial period of airway inflammation. To accomplish this, we investigated the sequential changes in oxidative stress that occur in the lung after allergen challenge using an ovalbumin (OVA)-sensitized mouse model with or without antioxidant treatment. In addition, the association between oxidative stress and airway inflammation, mucin production, and AHR (all pivotal phenotypes of asthma) was also evaluated.

METHODS

Study Animals

Female BALB/c mice (aged 6–8 weeks) housed under pathogen-free conditions in the animal facility of the Asan Institute for Life Science, Seoul, Korea, were used. None of the mice were exposed to OVA before the experiment. All animal experiments were approved by the Animal Research Ethical Review Board of the Asan Institute for Life Science.

Experimental Design

Allergic inflammation was studied in a murine asthma model generated as follows. Twenty-eight days before allergic challenge (day –28), each mouse was immunized via intraperitoneal injection with 20 μ g of chicken lipopolysaccharide-free OVA (Sigma Chemical Co, St Louis, Missouri) and 2 mg of aluminum oxide, followed by an identical booster injection on day –14. On days –2, –1, and 0, the mice were challenged with inhaled allergen (5% OVA in phosphate-buffered saline [PBS]) for 30 minutes per day. For this procedure, mice were placed in a plastic chamber (30 $\times$ 30 $\times$ 15 cm) and administered aerosolized OVA solution (particle size, 2–3 μ m) via a jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment, Richmond, Virginia) driven by compressed air at a flow rate of 6 L/min. Beginning just after the first inhalation challenge (day –2), the degree of oxidative stress in the lung and airway was measured at different time points (day –1, 0, 1, 2, 3, 5, 9, and 28). *N*-acetylcysteine (NAC) was used as an antioxidant in this study. NAC-treated mice underwent the same sensitization and challenge schedule with OVA. NAC was given intraperitoneally at 200 mg/kg body weight at 1 hour before each challenge of OVA, as previously described.^{9,10}

There were animal groups, each containing 5 mice per group. For controls, sham-treated mice underwent the same sensitization and challenge schedule and received vehicle (PBS) rather than OVA. PBS-sensitized mice, followed by OVA-challenged and OVA-sensitized with PBS-challenged mice, were also added.

Measurement of Oxidative Stress in the Lung

The degree of oxidative stress was evaluated by measuring the ratio of reduced to oxidized glutathione (GSH:GSSG) in lung tissue and evaluating *in situ* 2',7'-dichlorofluorescein (DCF) fluorescence in airway epithelium. GSH and GSSG levels were measured by a modification of a previously described method.^{11,12} Briefly, mouse lungs were removed, washed with cold saline, weighed, and stored at –80°C until assay. The lungs were homogenized in 10 mL of ice-cold buffer (50-mmol/L phosphate buffer containing 1-mmol/L EDTA) per gram tissue. After centrifugation at 10,000g for 15 minutes at 4°C, the supernatants were removed and deproteinated. Total GSH and GSSG levels were determined using a glutathione assay kit (Cayman Chemical Company, Ann Arbor, Michigan) according to the manufacturer's protocol.

Oxidative stress in the airway was detected by measuring intracellular ROS in bronchial epithelium using a modification of a previously described method.¹³ Immediately after extraction, whole tracheas were soaked in 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (Invitrogen, Corp, Carlsbad, California), incubated for 20 minutes, and washed. The tracheas were promptly embedded and stored at –80°C until ready to assay. Immediately after sectioning, oxidized products of redox-sensitive probes were analyzed using confocal microscopy (Leica TCS SP2; Leica Microsystems Heidelberg GmbH, Mannheim, Germany).

Evaluation of Airway Inflammation

Airway inflammation was investigated through analysis of bronchoalveolar lavage fluid (BALF) cells and histopathologic examination results. BALF was obtained by twice instilling and withdrawing 1 mL of saline via a tracheal cannula and centrifuging at 400g for 5 minutes; cell pellets were resuspended in RPMI 1640 medium (Gibco-BRL, Grand Island, New York), and the total number of BALF cells was determined. Slides for differential cell counts were prepared using the cytopspin technique and stained with a kit (HEMA 3; Biochemical Sciences Inc, Swedesboro, New Jersey). For each sample, 2 blinded investigators performed differential cell counts of 300 cells under a light microscope.

For histopathologic evaluation, the lungs from each mouse were removed, fixed in 10% buffered formalin overnight, and embedded in paraffin. The tissues were sliced into 4- μ m sections and stained with hematoxylin-eosin. The inflammation score was graded by 2 independent, blinded, board-certified pathologists. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. The degree of inflammation was evaluated on a subjective scale of 0 to 3, as previously described.¹⁴ Briefly, 0 indicates no detectable inflammation; 1, occasional cuffing with inflammatory cells; 2, most bronchi or vessels surrounded by a thin

layer (1–5 cells); and 3, most bronchi or vessels surrounded by a thick layer (5 cells deep).

Measurement of Mucin Production

The quantity of mucin production in the airway was evaluated by measuring the mucous-secreting goblet cells stained with periodic acid–Schiff (PAS). The percentage of PAS-positive cells was determined in the largest visible airway, which, in these horizontal sections through the hilum, was usually the left main bronchus. Mucin production was graded by 2 independent, blinded, board-certified pathologists. We categorized each animal according to a semilogarithmic scale as grade 0 ($\leq 1\%$ positive cells), 1 (1%–3% positive cells), 2 (4%–10% positive cells), 3 (11%–30% positive cells), and 4 ($\geq 31\%$ positive cells).

Determination of Airway Responsiveness

After the final aerosol challenge, AHR was evaluated in conscious, unrestrained mice by whole-body plethysmography. Individual mice were placed in a plastic chamber and exposed to increasing concentrations of aerosolized methacholine solution (0, 2.5, 5, 10, and 20 mg/mL) (Sigma Chemical Co) for 3 minutes. After each exposure to methacholine, the mean enhanced pause (Penh) value was calculated.

Statistical Analysis

The data are expressed as mean (SEM). Analysis of variance was used to determine the levels of difference between groups. Statistical significance was defined as $P < .05$.

RESULTS

Sequential Changes in the Degree of Oxidative Stress in the Lungs

We found that the GSH:GSSG ratio was reduced on day –2, immediately after OVA challenge. The decreased levels were apparent until day 1; levels rapidly recovered to the normal level after day 2. Although a mildly decreased GSH:GSSG ratio was observed in NAC-pretreated groups, there was no statistical significance vs control groups (Fig 1A). Intracellular ROS levels in the bronchial epithelium, determined by in situ DCF fluorescence, were observed on day –2, maintained during challenges, and decreased continuously until day 3 (when they reached a minimum value that was sustained until the end of the experiment [day 28]); this pattern was similar to that of the GSH:GSSG ratio. The mildly increased in situ DCF fluorescence in the NAC-pretreated group was observed on days –2, –1, and 0. However, the signals were weak compared with those in asthmatic mice. In bronchial epithelial cells from all groups, little fluorescence signal was detected later than 3 days after the last inhaled challenge (Fig 1B). All results obtained for the OVA/sham group were similar to those of the sham/sham group. Therefore, we have described the results of the sham/sham and sham/

OVA groups as controls. In every control group, no significantly increased fluorescence was detected.

Sequential Changes in BALF Cells

A significant difference in the total number of BALF cells was observed in asthmatic mice and control groups at all time points, except on day –2. The total BALF cell count peaked on day 2 and decreased with time thereafter (Fig 2A). An analysis of differential cell counts showed distinct patterns of leukocyte infiltration into the airway lumen. Macrophage counts started to increase after the first OVA challenge, significantly increased after day 0 and all time points, and showed the highest levels at 2 days. Eosinophils gradually increased, reaching a maximum between 3 and 5 days, and then began to decline after 9 days. Neutrophils substantially increased after allergen challenge; the largest number of neutrophils was observed at 3 days. Lymphocyte counts increased to a maximum at 9 days, and elevated numbers persisted to 28 days. Total and different cell counts, especially eosinophils and lymphocytes, were significantly decreased by pretreatment of NAC (Fig 2B).

Sequential Changes in Histopathologic Findings

The degree of histopathologic airway inflammation was gradually increased and peaked 5 days after OVA challenge, but identifiable inflammation persisted through day 28 (Fig 3A). Inflammation scores in asthmatic mice were significantly higher than for control mice at all time points after day 0 ($P < .05$). The inflammation score in NAC-pretreated mice was significantly reduced compared with that in asthmatic mice after day 5 (Fig 3B).

Sequential Changes in Mucin Production in the Bronchial Epithelium

Percentages of airway epithelial cells stained positively for PAS sharply increased after the first OVA challenge, significantly increased after day –1, reached a maximum at day 3, and remained at this level until day 5 ($P < .05$). After day 9, the percentage of PAS-positive cells started to decrease, and by day 28, there was no difference compared with control groups. The increased levels of PAS-positive airway epithelium were decreased significantly by the pretreatment of NAC (Fig 4A and B).

Sequential Changes in AHR

AHR was most highly enhanced 1 day after the last OVA challenge and decreased with time. Penh values were significantly greater for asthmatic mice than for controls on days 1, 2, and 3, when the highest concentration of methacholine (20 mg/mL) was administered. The value of Penh returned to near-normal levels 5 days after the last OVA challenge. The increased levels of Penh were significantly reduced by the pretreatment of NAC (Fig 5).

DISCUSSION

The results of this study demonstrate that increased oxidative stress in the lung preceded other characteristics of asthma, such as the accumulation of inflammatory cells into the

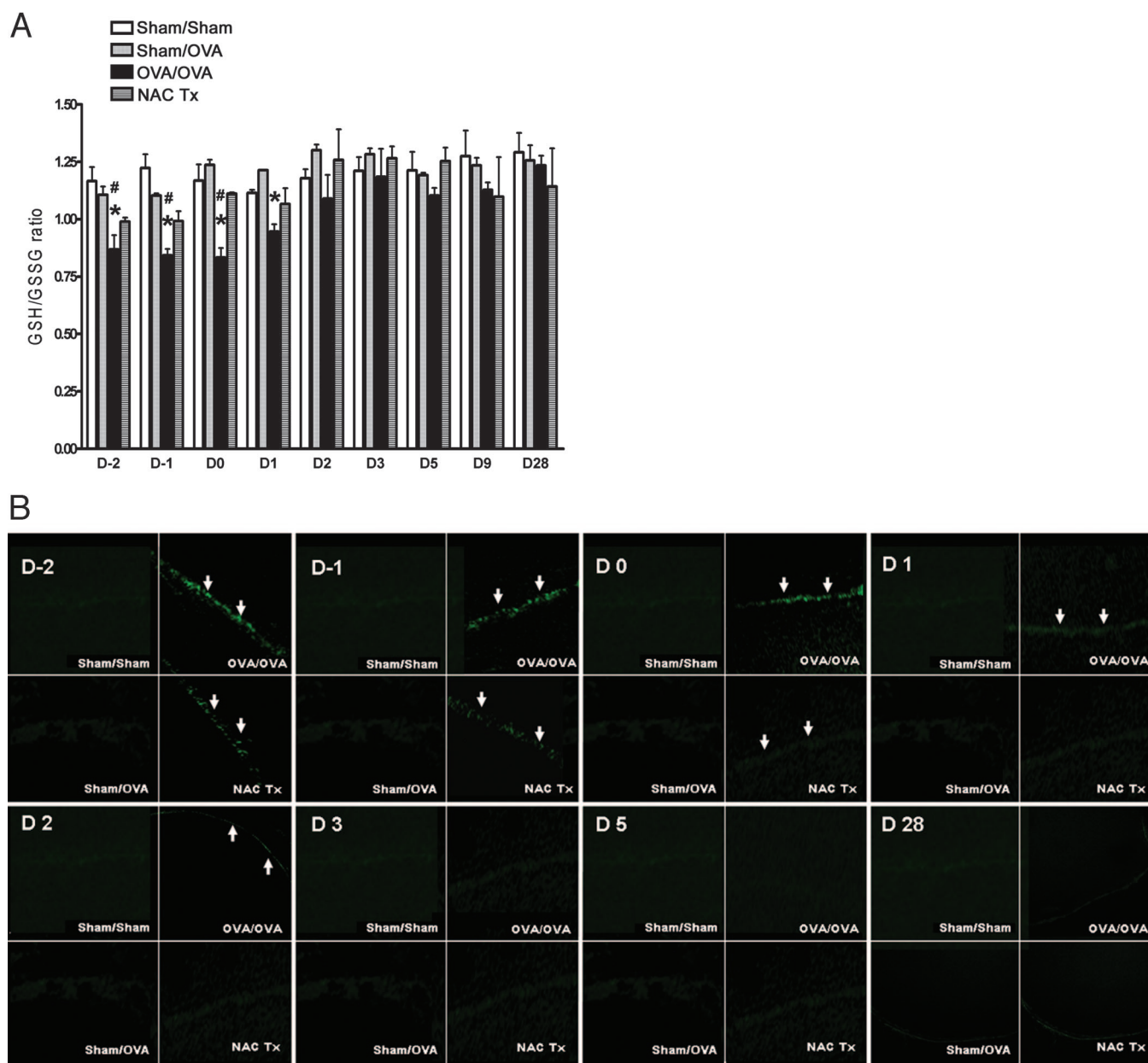


Figure 1. Changes in intracellular reactive oxygen species (ROS) levels after allergen challenge. Sampling was performed from immediately after last challenge until day (D) 28 from the sham-sensitized and sham-challenged (sham/sham), sham-sensitized and ovalbumin (OVA)–challenged (sham/OVA), OVA-sensitized and OVA-challenged (OVA/OVA), and OVA-sensitized and *N*-acetylcysteine (NAC) pretreated before OVA challenged (NAC Tx) mice. A, Intracellular reduced to oxidized glutathione (GSH:GSSG) ratios were determined by enzyme-linked immunosorbent assay. Each group contained 5 mice. Data are given as mean (SEM). #*P* < .05 vs sham/sham, and **P* < .05 vs sham/OVA. B, Changes in airway epithelium ROS levels as determined by 2',7'-dichlorofluorescein (DCF) fluorescence. Arrows indicate the increased DCF fluorescence in the bronchial epithelium (original magnification $\times 100$).

airway, mucin production, and AHR, during the initial phase of an airway inflammatory response. Furthermore, the pretreatment of antioxidant significantly reduced not only the increased ROS levels but also development of other pheno-

types of asthma. These results suggest that enhanced oxidative stress may play a critical role as an initiating factor, rather than acting as a coexisting aggravating factor, in the pathogenesis of bronchial asthma.

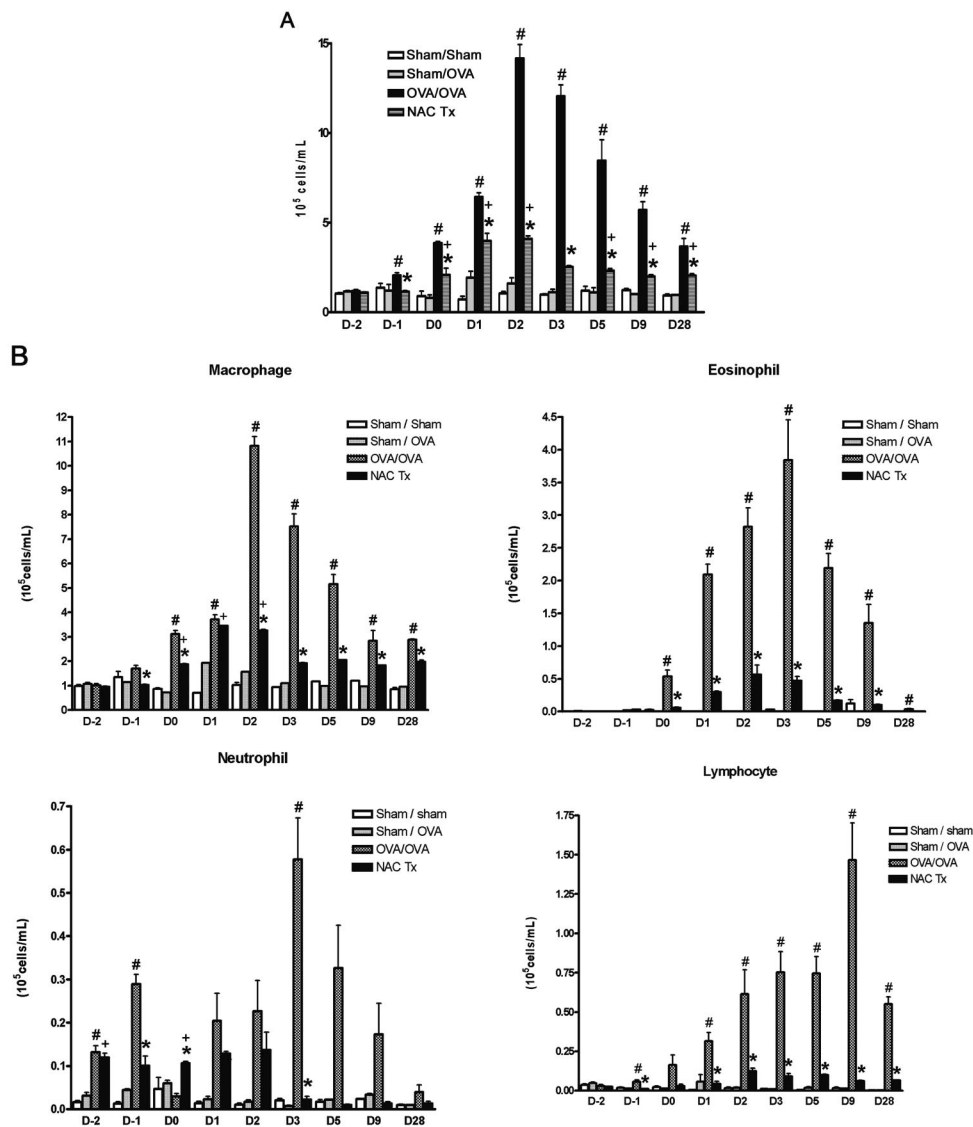


Figure 2. Leukocyte kinetics in bronchoalveolar lavage fluid (BALF) after ovalbumin (OVA) challenge. BALF samples were taken from immediately after OVA challenge to day (D) 28 in sham-sensitized and sham-challenged (sham/sham), sham-sensitized and OVA-challenged (sham/OVA), OVA-sensitized and OVA-challenged (OVA/OVA), and OVA-sensitized and *N*-acetylcysteine (NAC) pretreated before OVA challenged (NAC Tx) mice. A, Total counts of BALF cells at different time points after allergen challenge were determined. B, The absolute counts of different cell types were determined. Each group contained 5 mice. Data are given as mean (SEM). #*P* < .05 vs sham/sham and sham/OVA, **P* < .05 between the NAC Tx group and the OVA/OVA group, and +*P* < .05 between the NAC Tx group and both the sham/sham and sham/OVA groups.

A growing body of evidence implicates oxidative stress in the pathogenesis of bronchial asthma.^{15,16} ROS are also known to directly stimulate histamine release from mast cells and cause excessive mucin secretion.¹⁷ In addition, ROS can contribute to enhance AHR by increasing vagal tone, damaging oxidant-sensitive β -adrenergic receptors.^{18,19} Finally, oxidative stress might activate various intracellular signaling pathways, leading to secretion of various proinflammatory cytokines and chemokines in the asthmatic airway.²⁰

Although all the studies previously mentioned have demonstrated an important role for oxidative stress in the pathogenesis of asthma, it is difficult to establish a clear cause-and-effect relationship between airway inflammation and ROS generation because asthmatic patients have been exposed for a long time to allergens and have preexisting, persistent, airway inflammation. A comprehensive understanding of the role of ROS in allergic airway inflammation would be aided by an analysis of the sequential *in vivo* events of each

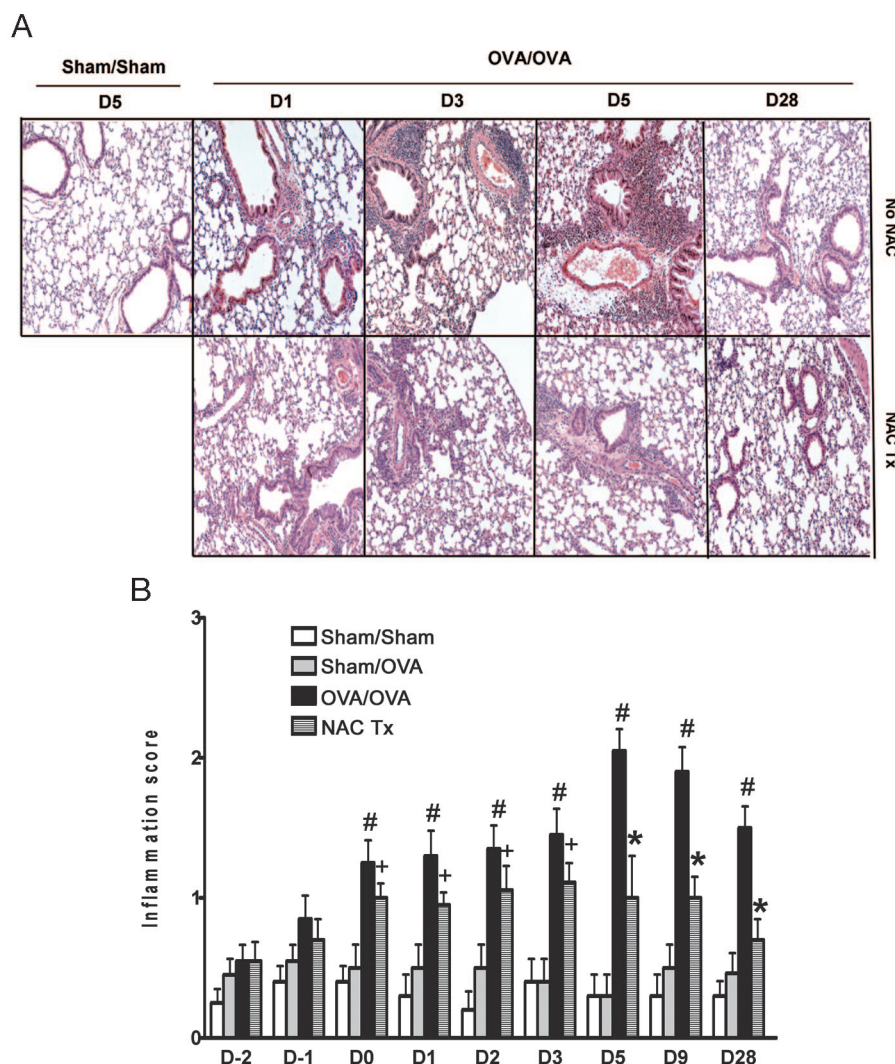


Figure 3. Sequential changes in inflammation scores after allergen challenge. Lungs were sampled from immediately after ovalbumin (OVA) challenge to day (D) 28 in sham-sensitized and sham-challenged (sham/sham), sham-sensitized and OVA-challenged (sham/OVA), OVA-sensitized and OVA-challenged (OVA/OVA), and OVA-sensitized and *N*-acetylcysteine (NAC) pretreated before OVA challenged (NAC Tx) mice. A, Histopathologic airway inflammation was gradually increased and peaked 5 days after OVA challenge, but identifiable inflammation persisted through day 28. B, Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Each group contained 5 mice. Data are given as mean (SEM). # $P < .05$ vs both the sham/sham and sham/OVA groups, * $P < .05$ between the NAC Tx group and the OVA/OVA group, and + $P < .05$ between the NAC Tx group and both the sham/sham and sham/OVA groups.

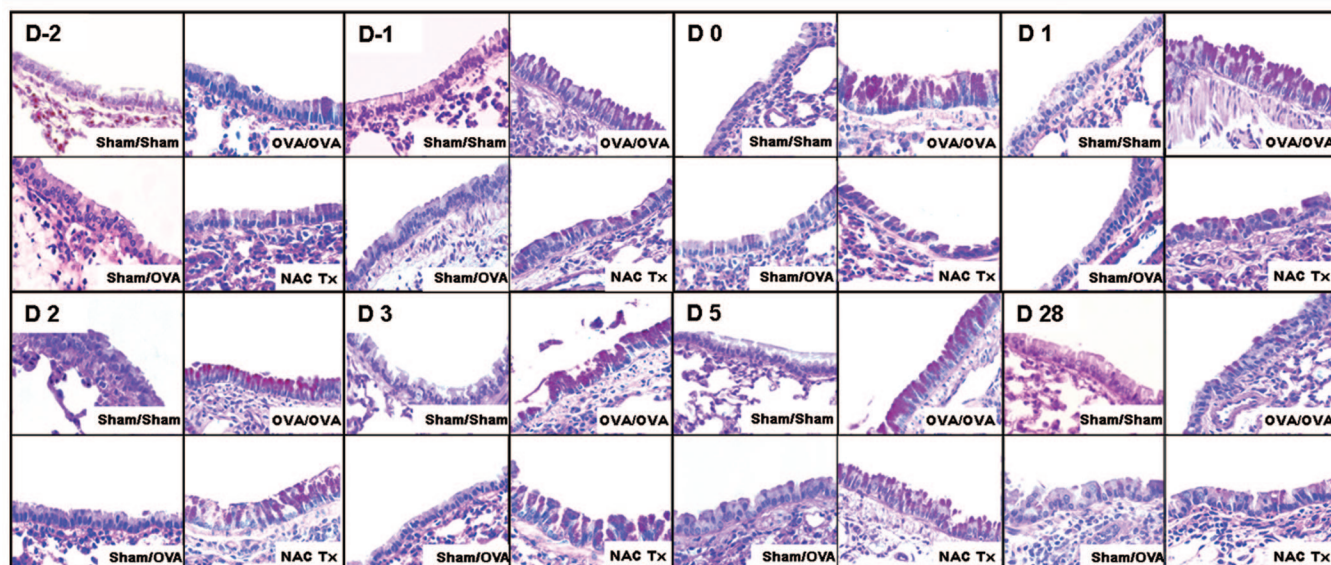
characteristic feature of asthma and ROS kinetics after experimental allergen challenge.

Although there are many animal studies that demonstrated that ROS generation occurs in the early period after OVA challenge, the analyses of end points have been examined in the short time (24–72 hours after last challenge) when major phenotypes of asthma, such as airway inflammation and AHR, could be already observed. To our knowledge, this is the first study to evaluate sequential changes in both airway oxidative stress and other pivotal characteristics of asthma during the critical initial period when an allergic airway inflammatory

response is being mounted. We found that ROS levels in the lung were the first to show an increase immediately after allergen challenge and quickly decreased to normal levels, despite persistent airway inflammation. These results indicate that ROS production is an early preceding event that might be fundamental to the development of an inflammation cascade. Although early appearance does not necessarily imply causation, these data at least preclude the possibility that ROS are merely a simple consequence of airway inflammation.

The increased intracellular ROS levels observed herein, especially in bronchial epithelium, were evident just after

A



B

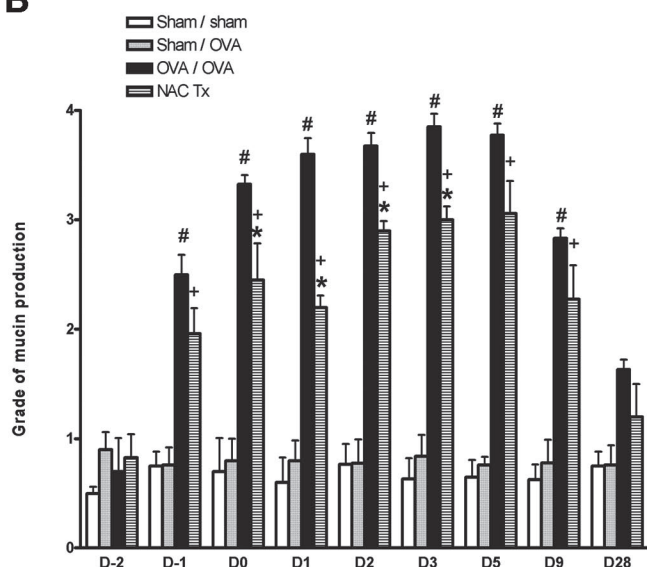


Figure 4. Changes in mucin production after allergen challenge. Lungs were sampled from immediately after last challenge to day (D) 28 in sham-sensitized and sham-challenged (sham/sham), sham-sensitized and ovalbumin (OVA)-challenged (sham/OVA), OVA-sensitized and OVA-challenged (OVA/OVA), and OVA-sensitized and *N*-acetylcysteine (NAC) pretreated before OVA challenged (NAC Tx) mice. A, Periodic acid-Schiff (PAS)-stained sections of lung. The violet color indicates PAS-positive mucus expression. B, Mucin production was measured by grading. Each group contained 5 mice. Data are given as mean (SEM). #*P* < .05 vs both the sham/sham and sham/OVA groups, **P* < .05 between the NAC Tx group and the OVA/OVA group, and +*P* < .05 between the NAC Tx group and both the sham/sham and sham/OVA groups.

allergen challenge. Bronchial epithelial cells are known to secrete various cytokines and chemokines that are involved in the pathogenesis of asthma.²¹ In addition, other studies have shown that epithelial lining cells are induced to produce mucin by a process in which ROS are critical factors.²² Consistent with this, the results of the present study showed

that enhanced mucin production followed the initial increase of intracellular ROS levels in bronchial epithelium.

In the present study, substantial airway inflammation was observed at 28 days despite the absence of continued exposure to antigen. Increased oxidative stress seems to be rapidly ameliorated by intrinsic defensive antioxidant systems, not-

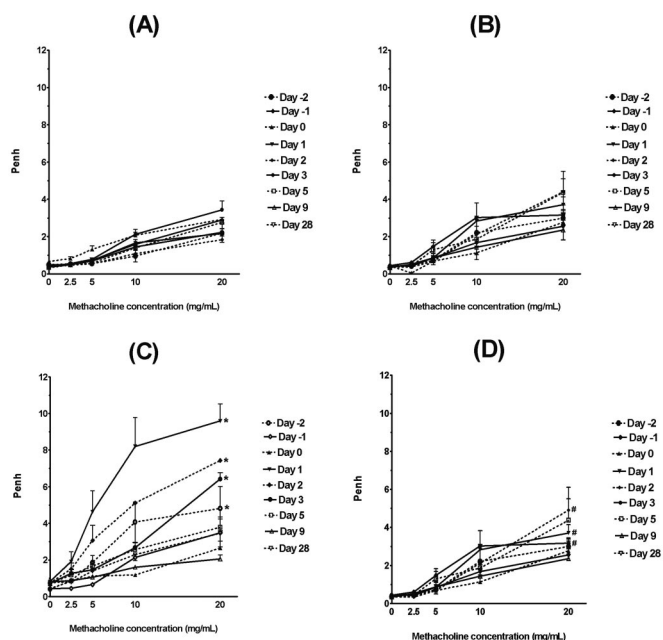


Figure 5. Kinetics of the development and resolution of airway hyper-responsiveness (AHR) after ovalbumin (OVA) challenges. Mean enhanced pause (Penh) values were evaluated at the indicated time points after last antigen challenge in sham-sensitized and sham-challenged mice (A), sham-sensitized and OVA-challenged mice (B), OVA-sensitized and OVA-challenged mice (C), and OVA-sensitized and *N*-acetylcysteine (NAC) pretreated before OVA challenged (NAC Tx) mice (D). * $P < .05$ vs both the sham/sham and sham/OVA groups, and # $P < .05$ between the NAC Tx group and the OVA/OVA group.

withstanding the existence of inflammation. One possibility is that increasing ROS is closely associated with the presence of antigen in the airway, whereas the maintenance of inflammation may be controlled by other factors. Interestingly, the other features of asthma, such as mucin hypersecretion and AHR, also did not correlate precisely with the degree of airway inflammation. Some other studies have revealed varying degrees of disjunction between eosinophil infiltration and AHR.^{23,24} One study using a chronically allergen-challenged mouse model of asthma found a distinct pattern of AHR that developed independently of enhanced eosinophilic inflammation.²⁵ The results of human studies on the relationships between AHR and airway inflammation are similarly inconsistent, although some transient weak correlations have been noted.²⁶ Collectively, these data indicate that airway inflammation is not an essential prerequisite for AHR or mucin production.

In this context, recent data have shown that oxidative stress can polarize the differentiation of T cells toward a T_H2 phenotype.^{27,28} There are several reports suggesting that the redox disequilibrium between reductive and oxidative properties of antigen-presenting cells plays a critical role in regulating T_H1 vs T_H2 dependence of the immune response,^{12,29,30} with the generation of oxidative stress at

the antigen-presenting cell level reportedly skewing the immune response in favor of T_H2 .^{28,31,32} Furthermore, ROS generated by intrinsic pollen nicotinamide adenine dinucleotide phosphate (NADPH) oxidase were shown to provide a critical signal for the initiation and augmentation of allergic inflammation.^{13,33} Related experiments conducted in NF-E2-related factor 2 (Nrf2)-disrupted mice, which are intrinsically susceptible to oxidative stress, showed an amplified inflammatory response after allergen challenge.³⁴ Finally, mice deficient for the tyrosine kinase, SHP-1, also exhibit intrinsic sensitivity to oxidative stress and break immune tolerance when exposed to OVA without any adjuvant.⁹ Taken together, our results provide additional evidence to support the view that increased oxidative stress could be a critical contributor to the induction of T_H2 -dominant airway inflammation. The exact mechanisms by which airway ROS levels and inflammation are controlled, however, are not clear from this study and will require further investigation.

In addition, there is growing interest in developing sophisticated strategies for the prevention of asthma, and the search for novel therapeutic agents based on an understanding of the pathogenesis of asthma is intensifying. The findings presented herein suggest that reducing airway oxidative stress with antioxidants may represent an attractive strategy. In the present study, NAC was used as a prototype of antioxidants, which appeared to mitigate pivotal phenotypes of the asthmatic mice model. There has been, however, controversy over the therapeutic effect of antioxidants in the murine model of asthma. In some studies, NAC administered systemically by the intraperitoneal or oral route was reported to effectively suppress the allergic inflammation and AHR,^{9,10,34,35} whereas aerosolized NAC in an OVA/OVA model demonstrated that NAC failed to modulate the hyper-responsiveness of murine airways.³⁶ Furthermore, human studies with not only NAC³⁷ but also other antioxidants, such as vitamin C or B, found that those agents had little effect on any spirometric variable, lung mechanic, or symptoms in the asthmatic patients. At the present time, the exact reasons for those discrepancies about possible therapeutic efficacy are not clear. The differences in tissue distribution or cellular penetration of the drug according to the selected routes and the timing of the drug administration might result in the differences in the findings of the cellular and lung mechanics in various studies. Further studies are required to clarify the exact role and functioning mechanism of the antioxidants in asthma. Although few antioxidants have been found to be effective in the management of established airway inflammation, a critically timed intervention to reduce ROS production in certain clinical contexts, such as steroid-refractory asthma or an acute exacerbation because of viral infection or allergen exposure, may be a key in the effective management of asthma.

The fact that the experimental system used in this study was artificially constructed to facilitate the dissection of the kinetics of airway inflammation needs to be taken into

consideration. In fact, a persistent increase in ROS levels has been observed in human asthmatic airways. Accordingly, the model may not fully recapitulate the situation found in human asthmatic patients. Further studies will be needed to establish the origins of ROS and their contribution to the pathogenesis of allergic inflammation in humans. Although the use of Penh as a calculation of airway resistance can be found in numerous publications using murine models, its physiologic relevance has been questioned in terms of measuring AHR in this mouse model of asthma.^{38–40} Thus, Penh may or may not be correlated with airway resistance in this study.

In conclusion, our results provide evidence that increased oxidative stress in the lung precedes other pivotal phenotypes of allergic airway disease, including airway inflammation, mucin production, and AHR. The work also provides insight into the pathogenesis of the disease and highlights potential novel strategies for preventing and treating bronchial asthma.

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