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Effects of acute ozone exposure on lung peak allergic inflammation of mice

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1. ABSTRACT

Asthma exacerbations are often triggered by air pollution, including O_3, whereas how patients with asthma exacerbations react to high levels of ambient ozone remain unknown. Here, we investigated the manner in which acute ozone exposure affects the pathophysiological characteristics of an asthma model on the premise of culminated allergic airway inflammation. The asthma model was constructed in mice, and enhanced pause (P_{enh}), total and differential cell number, soluble mediator concentration, histopathology, and *Muc5ac* mRNA expression in the mice were observed. The results showed that ozone could induce airway hyperresponsiveness (AHR) in controls and an additional enhancement of preexisting AHR in asthmatic mice. When exposed to ozone, the asthmatic mice expressed more neutrophils, TNF-α, IL-13, and hyaluronan in bronchoalveolar lavage than controls. The mice with asthma and the controls both showed decreased epithelial cell density in the proximal and distal airways. Ozone aggravated the increased mucus production and mucin gene expression in mice with asthma. These results show that subjects with asthma may react differently to the same high level of ambient ozone, especially for those with asthma exacerbations.

2. INTRODUCTION

Asthma is a clinical syndrome characterized by airway hyperresponsiveness (AHR), airway inflammation, and mucus hypersecretion. Acute exacerbation of asthma is a common but economically consumptive clinical problem, of which the main feature is airflow obstruction, which is often caused by hypersensitivity and hypercontractility of airway smooth muscle, airway wall edema, and luminal obstruction with mucus (1). Asthma exacerbations are often triggered by environmental allergens or air pollution (2). Ozone (O_3) is a ubiquitous, potent oxidant that participates in photochemical air pollution, and adversely affects human health by irritating the mucosa and harming the respiratory system because of its potential toxic effects related to its oxidant properties (3). Acute O_3 exposure is known to decrease pulmonary function (4), increase AHR (5), and induce airway inflammation (6) in humans. Ozone has been proved to be associated with not only the onset (7), but also the exacerbation (8) of asthma, and even the mortality of asthmatic patients (9). Furthermore, it has been reported that the effect of O_3 exposure differs between subjects with asthma and without asthma (10), with atopy and without atopy (4), and with atopic asthma and atopic patients without asthma (11), indicating that different backgrounds

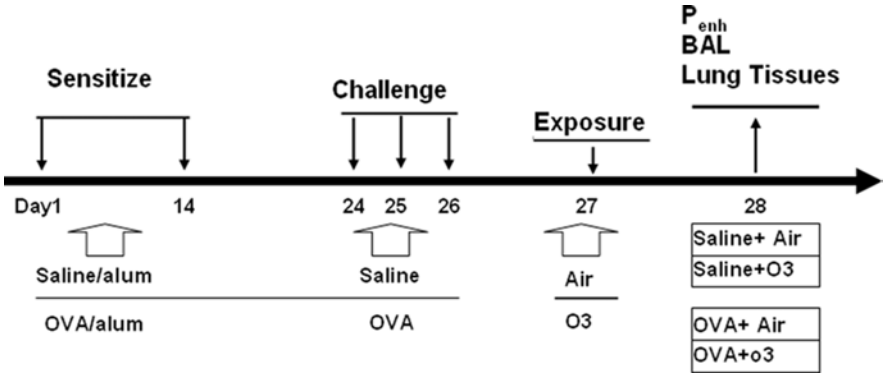

Figure 1. Schematic diagram of the experimental protocol. Twenty-eight mice were randomly divided into two groups: control and asthma model. Different substances were administered intraperitoneally to sensitize the mice on day 1 and day 14, and the mice were challenged with the substances via aerosol administration on days 24, 25 and 26. On day 27, equal numbers of mice (n = 7) from each group were selected randomly and exposed to filtered air or 2.0 ppm ozone for 3 hours.

of airway inflammation cause different reactions to O_3. Patients with asthma exacerbations have distinct pulmonary inflammation and equal chances of exposure to high levels of ambient O_3. However, how these patients react to acute O_3 exposure remains unknown. For ethical reasons, this problem can be experimentally investigated only in animal models.

Animal models of allergic asthma are used frequently to mimic human responses to various stimuli in asthmatic patients. Previous studies in which the effects of O_3 on allergic asthma models were studied placed their emphases primarily on how O_3 affects the immune processes of asthma by exposing the animals to certain doses of O_3 at different stages of the establishment of the animal model (12-15). A limited number of authors have administered O_3 exposure after the entire procedure had been completed, or at different intervals, such as 48 h (16) or 82 h (17) after the onset of the induction of asthma, and reported some damage caused by O_3 in their laboratory models without mentioning whether there were interactions between O_3 and allergic inflammation. Because of the delay in the administration of O_3, however, none of these studies established a model exhibiting the culminated pulmonary inflammation caused by both an allergic response and O_3 exposure. The latter type of model might enhance our understanding of how acute O_3 inhalation affects patients with asthma exacerbations. This will be the center of our focus in the present study.

In this study, we established a murine model of allergic asthma and exposed the animals to 2 ppm O_3 for 3 consecutive hours 24 h after the last challenge, at which time the AHR and airway inflammation of these animals were at their peak (18), mimicking the airway status of acute exacerbation of atopic asthma (19). The aim of this study was to find out how O_3 exposure affects AHR, pulmonary inflammation, and mucus production of the already asthmatically inflamed airway, and further, to discuss whether there are additional effects caused by O_3 on these pathophysiological features of asthma models versus their control littermates.

3. MATERIALS AND METHODS

3.1. Animals

Six- to eight-week-old female Balb/c mice weighing 18~20 g were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and bred under specific pathogen-free conditions in our own facility which was approved by the Shanghai Committee for Accreditation of Laboratory Animal. The animals were kept on an ovalbumin (OVA)-free diet. Experiments were conducted under a protocol approved by the State Science and Technology Commission.

3.2. Experimental protocol

Twenty-eight animals were randomly divided into 2 groups according to their sensitization and challenge protocol, and each group was further separated into 2 equal subgroups according to their exposure protocol, which are both outlined in Figure 1. Mice were sensitized by i.p. injection of 20 μg OVA (Grade V, Sigma Aldrich, St. Louis, MO, USA) emulsified in 2.0 mg of alum (Shanghai No.4 Reagent & H.V. Chemical Industries, Ltd, Shanghai, China) in a total volume of 100 μL of 0.9% sterile saline on days 1 and 14. Non-sensitized mice only received 2.0 mg of alum in 0.9% saline. On days 24, 25, and 26, mice were challenged via the airway using an aerosol of 5% OVA (Grade II, Sigma Aldrich) in 0.9% saline (non-sensitized mice received saline only) for 30 min daily. On day 27, all mice were exposed to either 2 ppm O_3 or filtered air for 3 h. On day 28, the animals were tested for enhanced pause (P_{enh}), and then sacrificed to harvest bronchoalveolar lavage fluid (BALF) and lung tissue.

3.3. Aerosol exposure

Using a sealed Perspex box (50 × 30 × 40 cm^3) as an exposure chamber, different groups of animals were placed in different stainless steel cages with irradiated food and acidified water provided *ad libitum*. A solution of 5% OVA in normal saline was aerosolized by delivering compressed air to a sidestream jet nebulizer (PARI BOY, Germany) and injected into the airstream entering the chamber via a plastic pipe connected to the upper vent hole

at the top of the sidewall of the chamber, with a lower one at the bottom of the opposite sidewall.

3.4. Ozone generation and exposure

Mice were placed in stainless steel cages inside the exposure chamber with free access to food and water, and exposed to 2 ppm O_3 for 3 h. Ozone was generated by directing an air stream with a micro air pump (SC3601PM; Skoocom Electronic Co. Ltd., Shenzhen, China) through an Aqua Medic ozone generator (model 300; AB Aqua Medic GmbH, Bissendorf, Germany). The O_3–air mixture was metered into the inlet air stream, and the chamber O_3 concentration was monitored continuously and maintained at a level of approximately 2 ppm using an Ozone Switch (OS-4; Eco Sensors Division, KWJ Engineering Inc., Newark, NJ, USA), with its sampling probe placed on the chamber wall at the nose level of the mice. According to a preset concentration range, the Ozone Switch controlled the current of the air pump by feeding back to the instantaneously measured value of the O_3 concentration. In the present experiment, we set the range at 1.75~2.25 ppm, which had been proved by our preliminary experiment to be able to maintain a comparatively stable average O_3 concentration at 2.02 ± 0.03 ppm. To avoid an excessively high peak concentration value, the intensity of the voltage within the O_3 generator was adjusted according to the number of laboratory animals. The room temperature was maintained at 20~23 °C, and the relative humidity at 50~60%. The control mice were exposed to filtered air for 3 h.

3.5. Measurement of airway responsiveness

Airway responsiveness was assessed noninvasively using a whole-body plethysmograph (Buxco, Troy, NY, USA) on conscious, unrestrained mice as described previously (20). Briefly, at day 28, 24 h after the last exposure, the mice were placed in individual chambers. After 10 min of adaptation, they were exposed to aerosolized PBS (to establish baseline), followed by increasing concentrations of aerosolized methacholine (MCh) (1.5625, 3.125, 6.25, 12.5, 25, 50 mg/mL). Each dose of MCh was delivered for 30s and respiratory measurements were recorded and averaged over a 7-min period from the beginning of nebulization. Airflow obstruction was expressed as enhanced pause (P_{enh}), which correlates well with airway resistance (20), and calculated as: P_{enh} = [Te (expiratory time)/Tr (relaxation time)] × [Pef (peak expiratory flow)/Pif (peak inspiratory flow)]. The P_{enh} obtained at each concentration was expressed as the percentage change from baseline. The concentrations of MCh required to increase P_{enh} by 50%, 100%, and 200% from baseline were calculated by polynomial fits ($LogPC_{50}$, $LogPC_{100}$, and $LogPC_{200}$, respectively).

3.6. Bronchoalveolar lavage and measurements of BAL soluble mediators

Immediately after the assessment of airway reactivity, mice were sacrificed by an overdose of pentobarbital (100 mg/kg i.p.) and lavaged with 3 0.3-mL aliquots via the endotracheal tube, which was retrieved as the bronchoalveolar lavage fluid (BALF). The BALF was then centrifuged at 1000 ×g for 10 min at 4 °C. The supernatant was aliquoted and stored at −80 °C until the samples were assayed. The remaining cell pellet was resuspended in 1 mL PBS. Total cell counts were determined using a hemocytometer by adding 100 μL of the cell suspension to 100 μL trypan blue stain. Differential cell counts were done by the same inspector on cytocentrifuge preparations (Cytospin 2; Shandon, UK) stained with Wright-Giemsa stain by counting 200 cells from each mouse under ×400 magnification in a blinded manner. Cells were identified by standard morphology and differentiated into neutrophils, eosinophils, lymphocytes, and macrophages.

Concentrations of soluble mediators in BALF supernatants were determined by enzyme-linked immunosorbent assay as described previously (21). Measurements of TNF-α, IL-5, IL-13, and hyaluronan (HA) were performed by using commercial kits (R&D Systems China Co. Ltd., Shanghai, China) according to the manufacturer's protocol. The detection limits were 3 pg/mL for IL-5 and IL-13, and 5 pg/mL for TNF-α and HA.

3.7. Processing of lung tissues and histological scoring of lung inflammation

After BAL, the left lung lobe was removed and placed in 10% neutral-buffered formalin solution and subsequently processed and embedded in paraffin. The remains were micro-dissected, placed in liquid nitrogen, and then kept at -80 °C until further processing for quantitative real-time PCR (qPCR). Lung sections of 2~3-μm thickness were cut from the anterior surface of tissue blocks and then stained with hematoxylin and eosin for routine histology, or with periodic acid–Schiff (PAS) to detect mucosubstance secretion.

Slides were coded and graded by 2 independent investigators in a blinded fashion, using a reproducible scoring system as described elsewhere (22). A value from 0 to 3 per criterion was assigned to each scored tissue section. Two criteria were scored to document pulmonary inflammation: peribronchial (PB) and perivascular (PV) inflammation. A value of 0 was assigned when no inflammation was detectable; a value of 1 for occasional cuffing with inflammatory cells; a value of 2 for most bronchi or vessels surrounded by a thin layer (1~5 cells) of inflammatory cells; and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than 5 cells) of inflammatory cells. Total lung inflammation was defined as the average of the PB and PV inflammation scores. Two to three tissue sections per mouse were scored, and the inflammation scores were expressed as a mean value of 15~20 sections per subgroup.

3.8. Morphometry of stored mucosubstances of the epithelial surface, % PAS+ cells, and epithelial cell density

To estimate the amount of stored mucosubstance of the epithelial surface in mice airways, the volume density (Vs) of the PAS-stained mucosubstance was quantified using computerized image analysis and standard morphometric techniques following a slightly modified method as described previously (23). Briefly, using the

public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health, and available on the Internet at http://rsb.info.nih.gov/nih-image/) at a final magnification of ×400 and resolution of 0.26 μm, the length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The total area of the epithelium on the entire tissue face was outlined with a light pen, the PAS-positive area within this region was determined automatically by staining the intensity, and both areas were then calculated. The Vs of the epithelial surface mucosubstances was calculated according to the following equation: [(stained area/total explored area) × π]/(4 × length of basal lamina/explored area) and expressed as nanoliters of epithelial surface mucosubstances per square millimeter of basal lamina (nL/mm^2).

The epithelial cell densities were then calculated manually by counting the total epithelial cells per millimeter epithelium basal lamina in the proximal and distal airway segments, and the proportions of PAS-positive stained cells were measured by first counting the cells with a red-purple stained area within or at the outer edge of their profiles, and then dividing the number of these cells by the number of total epithelial cells. Two to three segments per mouse in normal control and 5~9 segments per mouse in the other subgroups were calculated. Data were expressed as the mean value of all segments per mouse or per subgroup.

3.9. Real-time reverse transcription-PCR of Muc5AC mRNA

Total RNA was extracted using the TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA (2 μg per sample) was used to synthesize the single-stranded complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). The cDNA generated was used as a template in subsequent real-time PCR analysis. Transcript levels were determined by the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and using the SYBR Green PCR Master Mix Reagent (Qiagen). The sequences of the murine forward and reverse primers used were as follows: β-actin, forward primer 5′-ATTGCTGACAGGATGCAGA-3′ and reverse primer 5′-GAGTACTTGCGCTCAGGAGGA-3′; Muc5ac forward primer 5′-CCATGCAGAGTCCTCAGAACAA-3′ and reverse primer 5′-TTACTGGAA AGGCCCAAGCA-3′ (designed by Invitrogen). The qPCR thermal cycling program was as follows: Step 1, 15 min at 95 °C; step 2, 15 s at 95 °C; step 3, 30 s at 60 °C; step 4, 30 s at 72 °C, with step 2 to step 4 repeated for 40 cycles. Relative gene expression was normalized to β-actin. Data were expressed as the fold increase in RNA expression compared with that in a particular mouse from the normal control group, whose value was set as 1.

3.10. Statistical analysis

Data were expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used for comparisons among all 4 subgroups, and the unpaired *t*-test for comparisons within each group. The additional effects of O_3 on the asthma model were evaluated by factorial analysis using univariate 1-way ANOVA. The criterion for significance was $p \leq 0.05$. The SPSS program was used for the statistical analysis (SPSS Inc., Chicago, IL, USA).

4. RESULTS

4.1. Influence of O_3 exposure on AHR

The P_{enh} was measured 24 h after O_3 or air exposure, a time when the airway inflammation and AHR developed in response to MCh induced by O3 is most prominent, as shown by a recent report (24). Exposure to 2 ppm O_3 significantly increased the baseline P_{enh} in both control mice and asthma model (Figure 2, a). The AHR was assessed by calculating the percentage change of P_{enh} after each dose of MCh over the baseline value, as shown by the concentration–response curves (Figure 2, b), where the curves all shifted to the left at different degrees from the curve representative of naive mice (air-exposed, saline-pretreated). To quantify AHR, the log provocative concentrations of MCh required to increase P_{enh} from baseline by 100% ($LogPC_{100}$ P_{enh}) were calculated (Figure 2, c). The allergic animal models had higher airway reactivity to MCh (lower $LogPC_{100}$ P_{enh}) than normal controls when exposed to air (0.498 ± 0.072 vs. 1.209 ± 0.056, $p < 0.001$). Exposure to O_3 increased the airway responsiveness to the agonist both in the asthma model and in control mice (0.217 ± 0.034, $p < 0.05$; 0.402 ± 0.087, $p < 0.001$; respectively). Furthermore, factorial analysis demonstrated that the interaction effect of O_3 exposure and OVA-pretreatment on mice airway reactivity to MCh was statistically significant ($F = 15.649$, $p < 0.01$). Ozone exposure of the asthma model caused a significant decrease of $LogPC_{50}$ P_{enh}, but not of $LogPC_{200}$ P_{enh} (Figure 2, d).

4.2. Influence of O_3 exposure on pulmonary inflammation

To determine the effects of O_3 exposure on airway inflammation, we first quantified the total cells and the differential cellular components in BALF of 7 mice in the O_3-exposed, OVA-pretreated subgroup, and 6 mice in the other subgroups immediately after the measurement of AHR (the loss of samples in these groups was the result of mislabeling). The BALF of mice in the allergic model group contained increased total cells, lymphocytes, eosinophils, and neutrophils (Figure 3, a). Exposure to O_3 had increased the total cells, macrophages, lymphocytes, and neutrophils in control mice, whereas in the asthma model, O_3 inhalation significantly increased only the number of neutrophils.

Subsequently, lung histological analyses were performed to evaluate additional inflammatory parameters (see Methods) (Figure 3, b). As expected, mice in the asthma model had higher scores of PB, PV, and total (T) inflammation [1.917 ± 0.229 (PV, $p < 0.001$), 1.250 ± 0.250 (PC, $p < 0.01$), 1.583 ± 0.212 (T, $p < 0.001$)] than naive mice [0.412 ± 0.123 (PV), 0.353 ± 0.1193 (PC), 0.382 ± 0.101 (T)]. Exposure to O_3 caused a further

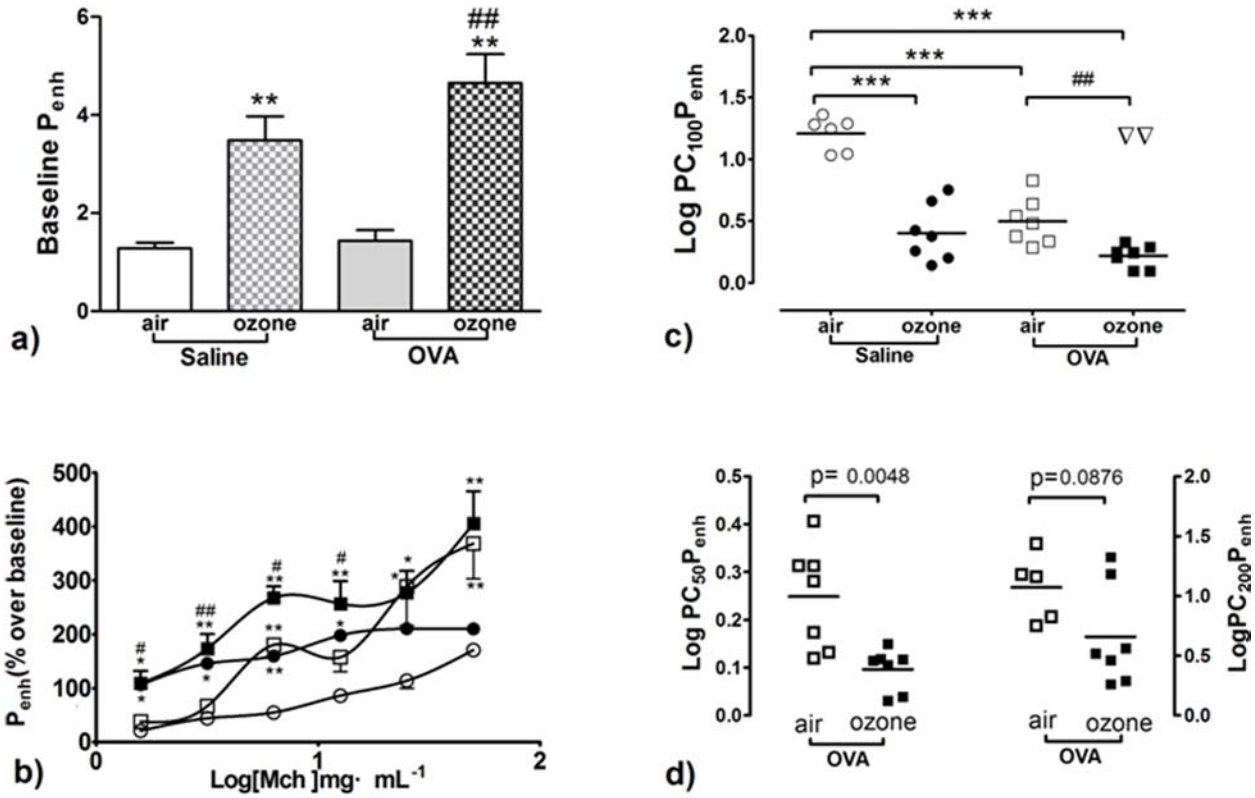

Figure 2. Ozone-induced changes in airway hyperresponsiveness. Mice sensitized and challenged with ovalbumin (OVA group) or saline (saline group) were exposed to 2 ppm ozone versus filtered air. Penh were measured at 24 hours post-exposure in conscious, unrestrained mice using whole body plethysmography. (A) The baseline airway status was determined by averaging the Penh measured at initial circumstances and after saline inhalation. (B) Airway responsiveness was assessed by inhalational challenge with nebulized saline, 1.56, 3.13, 6.25, 12.5, 25 and 50 mg/ml of Methacholine (MCh). The data are presented as the log of the provocative concentration of MCh required to increase Penh from baseline by 100% (PC_{100}) (C), by 50% (PC_{50}) and by 200% (PC_{200}) (D). The data are expressed as the mean ± SEM from seven mice per group. *: $p < 0.05$; **: $p < 0.01$, compared with air-exposed mice in the saline group; #: $p < 0.05$; ##: $p < 0.01$, compared with air-exposed mice from the OVA group. ### $p < 0.01$. Factorial analysis of the interaction effect of ozone-exposure and OVA-pretreatment of Balb/c mice

increase in the grades of PV inflammation and in T inflammation in allergic asthma models [2.789 ± 0.096 (PV, $p < 0.01$), 2.211 ± 0.117 (T, $p < 0.05$)]. In naive mice, however, O_3 inhalation only increased the grades of PV inflammation (0.947 ± 0.162, $p < 0.05$). Representative examples of histological findings are shown in Figure 3, c.

4.3. Influence of O_3 exposure on airway soluble mediators

In an attempt to find out how acute O_3 exposure influences the production of airway Th1 and Th2 cytokines, we measured the concentrations of TNF-α (Th1), IL-5, and IL-13 (Th2) in BALF. Hyaluronan is an important component of the extracellular matrix involved in allergic airway inflammation, and also contributes much to O_3-induced AHR. Taking this into account, we also measured the BAL levels of HA. Mice with asthma had higher BAL levels of HA, TNF-α, IL-5, and IL-13 (Figure 4) compared with normal controls when exposed to air [34.560 ± 7.463 vs. 11.500 ± 1.711, $p < 0.05$ (HA); 85.740 ± 7.910 vs. 30.890 ± 7.087, $p < 0.01$ (TNF-α); 197.700 ± 25.140 vs. 68.08 ± 8.450, $p < 0.01$ (IL-5); 32.620 ± 8.059 vs. 9.475 ± 1.252, $p < 0.05$ (IL-13)]. Exposure to O_3 on allergic models dramatically increased the BAL concentration of HA, TNF-α, and IL-13 (111.5 ± 16.70, $p < 0.001$; 155.0 ± 29.69, $p < 0.01$; 65.02 ± 11.17, $p < 0.05$, respectively), while in naive mice, the same exposure increased all 4 mediators [51.24 ± 6.203, $p < 0.001$ (HA); 115.8 ± 29.91, $p < 0.05$ (TNF-α); 117.4 ± 15.17, $p < 0.05$ (IL-5); 30.13 ± 4.324, $p < 0.01$ (IL-13)]. Although O_3-exposed asthma models had the highest levels of all these soluble proteins in BAL, no statistically significant effects of O_3 and allergy were found on any of these mediators.

4.4. Influence of O_3 exposure on mucus production and epithelial cell density

To find out whether and to what extent O_3 exposure could affect airway mucus secretion, we measured the stored mucosubstances of the epithelial surface and calculated the percentage of cells positively stained with PAS in PAS-stained lung tissue sections. Asthma models had far more stored mucosubstances at the epithelial surface than control mice when exposed to air (2720 ± 450.6 vs. 1.055 ± 0.1924, $p < 0.001$) (Figure 5, a).

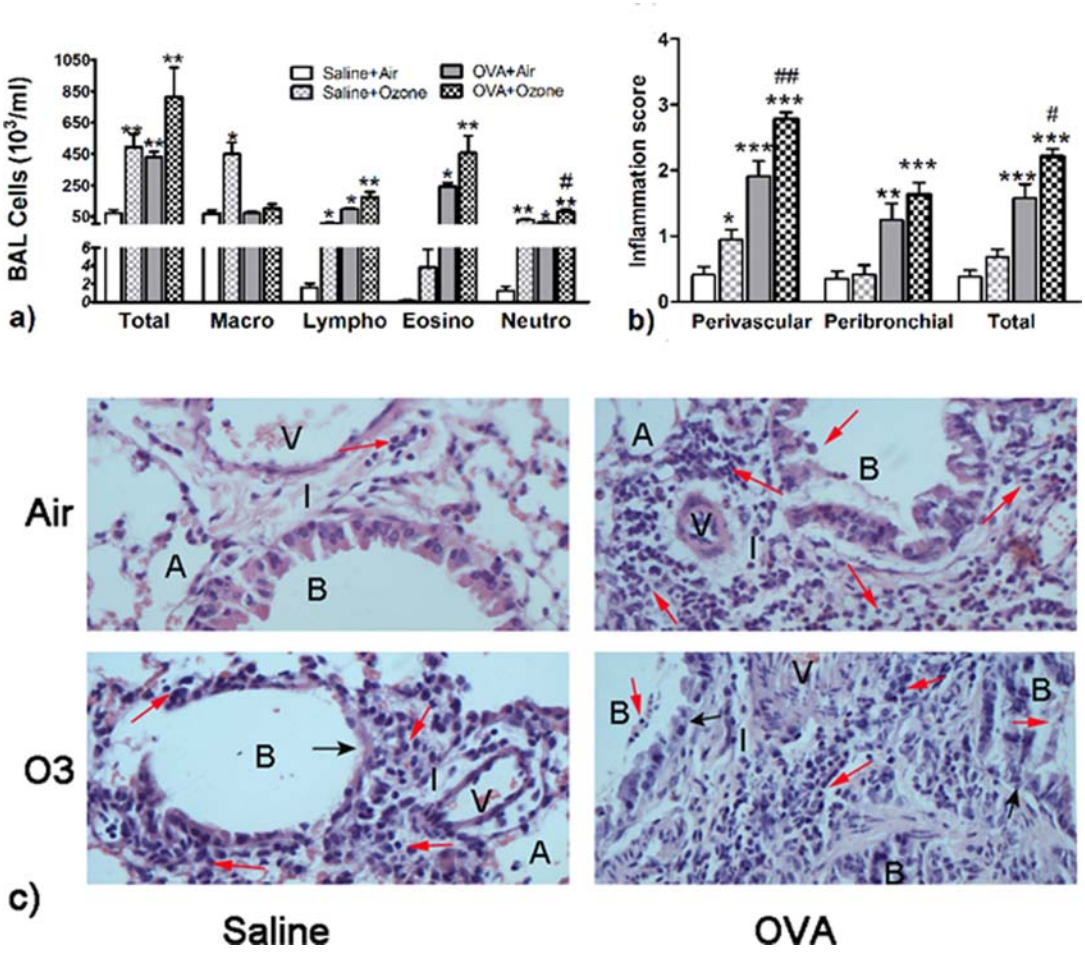

Figure 3. Ozone-induced changes in pulmonary inflammation. a) Changes in total cells and differential cellularity. b) Inflammation grades in peribronchial and perivascular inflammation (2~4 sections per mouse). c) Haematoxylin staining of lung sections in mice (40×); the red arrow marks inflammatory cells, and the black arrow points to epithelial damage. A: alveolar, B: bronchiole, I: interstitial space, V: vascular. One histological sample is representative of typical changes of each group. Data expressed as the mean ± SEM for seven mice in the ozone-exposed OVA-pretreated group, and from six mice in the other groups. *: $p < 0.05$, **: $p < 0.01$, compared with air-exposed mice from the saline group; #: $p < 0.05$; ##: $p < 0.01$, compared with air-exposed mice from the OVA group.

Exposure to O_3 slightly increased the amount of mucus in the control mice (20.32 ± 4.435, $p < 0.05$), but significantly increased that in the asthma group (7214 ± 871.3, $p < 0.001$). There was a synergistic effect of O_3 exposure on airway mucus production in the asthma model, demonstrated by the statistically significant interaction between O_3 exposure and mucus production ($F = 11.844$, $p = 0.001$). Similarly, the percentages of PAS-positive cells were higher in the asthma model than in control mice inhaling air (55.83 ± 2.203 vs. 10.05 ± 1.281, $p < 0.001$) (Figure 5, b). Ozone inhalation increased the proportion of PAS-positive cells of mice both from the asthma model and from the normal control (76.19 ± 1.365, $p < 0.001$; 28.63 ± 1.642, $p < 0.001$; respectively) at similar magnitude.

To confirm these histological observations, we tested the expression of Muc5ac mRNA in the lungs of 3~4 mice per group in an independent experiment. As expected, mice with asthma showed a higher expression of Muc5ac at the mRNA level than those of control mice when exposed to air (7.037 ± 1.096 vs. 0.797 ± 0.102, $p < 0.01$) (Figure 5, c). Ozone exposure caused a further increase in the Muc5ac mRNA of mice pretreated with OVA (15.64 ± 2.630, $p < 0.05$), but not in saline-pretreated mice (0.602 ± 0.179, $p > 0.05$). The interactive effect of O_3 exposure and OVA-pretreatment on Muc5ac mRNA expression was statistically significant ($F = 10.66$, $p = 0.01$).

We also calculated the number of total epithelial cells per mm basal lamina from selected proximal or distal airway segments of lung sections stained with PAS in each mouse to evaluate the epithelial injury that might have been caused by O_3 (Figure 5, d). Mice in the asthma group had a higher epithelial cell density in the distal, but not the proximal, airway than naive mice (112.77 ± 2.856 vs. 94.24 ± 6.124, $p < 0.05$, distal; 120.76 ± 2.88 vs. 122.21 ± 4.30, $p = 0.77$, proximal). Ozone exposure decreased the epithelial cell density both in the proximal and distal airways of the

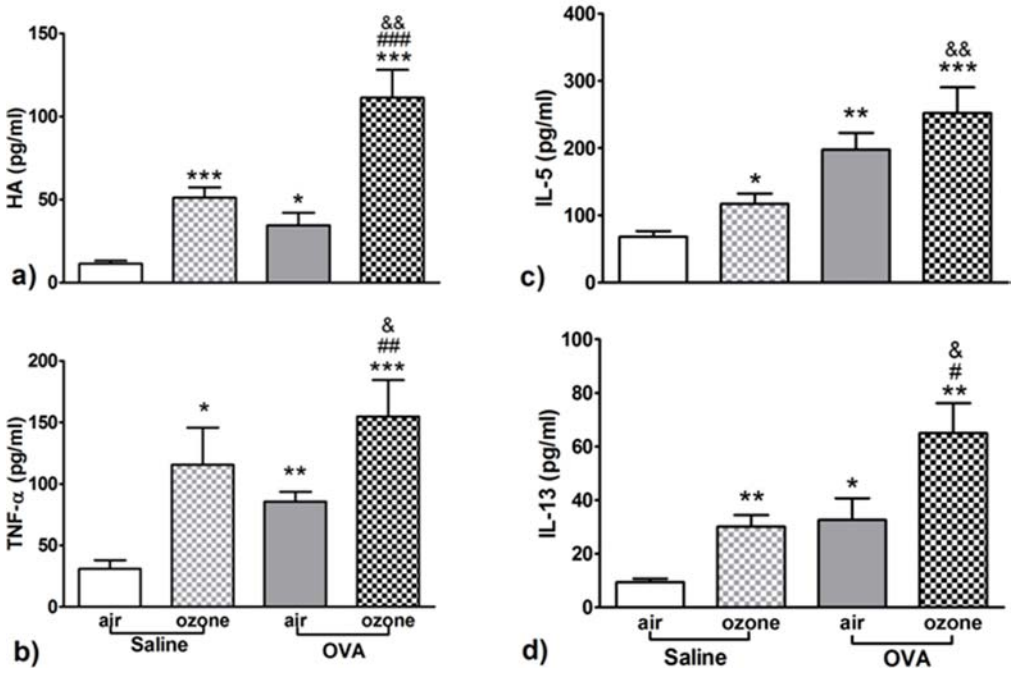

Figure 4. Ozone-induced changes in airway soluble mediators. Airway soluble mediators in the supernatant of the BAL of each mouse were assessed by ELISA. a) Hyaluronan, b) tumor necrosis factor-α (TNF-α), c) interleukin-5 (IL-5), and d) IL-13. Data expressed as the mean ± SEM from seven mice in the ozone-exposed OVA-pretreated group, from six mice in the other groups. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, compared with naive mice; #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$, compared with air-exposed OVA-pretreated mice; $: $p < 0.05$; $$: $p < 0.01$ compared with ozone-exposed, saline-pretreated mice.

control mice (53.23 ± 2.50, $p < 0.001$, proximal; 51.17 ± 2.141, $p < 0.001$, distal) and the asthma model (84.04 ± 3.82, $p < 0.001$, proximal; 78.01 ± 5.129, $p < 0.001$, distal). The magnitude of the O_3-induced decrease in epithelial cell density of the proximal airways was attenuated in mice by pre-treatment with allergen, and the interaction effect between the cell density decrease and allergen pre-treatment was also statistically significant ($F = 20.583$, $p < 0.001$). Representative examples of PAS-stained segments of the proximal and distal airways are shown in Figure 5, e.

4.5. Correlation analyses

With the aim to probe the mechanisms underlying O_3-induced pathophysiological abnormalities in our experimental model, we further explored the correlations between them by regression analysis. Airway inflammatory cellularity is also involved in AHR. Statistical analysis revealed that there was a significant negative correlation between the decreased extent of $LogPC_{100}$ P_{enh} and the increased numbers of BAL eosinophils ($r^2 = 0.93$, $p = 0.0017$, not shown) in air-exposed asthmatic mice, whereas in the O_3-exposed asthma model, the extent of AHR correlated positively with the numbers of BAL neutrophils (Figure 6, a) but not eosinophils ($p = 0.0609$, not shown). Ozone-induced AHR in control mice was also positively correlated with airway neutrophils ($r^2 = 0.69$, $p = 0.0408$, not shown). Airway soluble mediators contribute substantially to the development of AHR and the recruitment of airway inflammatory cells. However, no significant correlations were observed between any of the detected mediators and AHR in the asthma model when the animals were exposed to air. In O_3-exposed control mice, the BAL level of IL-13, but not the other mediators, correlated positively with AHR ($r^2 = 0.83$, $p = 0.0122$, not shown), whereas in the O_3-exposed asthma model, only the BAL level of HA had a positive correlation with AHR (Figure 6, b). In the air-exposed asthma model, there was a significant positive correlation between the increased number of BAL eosinophils and the elevated BAL level of IL-5 ($r^2 = 0.69$, $p = 0.0496$, not shown). In the O_3-exposed allergic animals, however, a positive correlation was observed between the BAL level of TNF-α and the number of airway neutrophils ($r^2 = 0.70$, $p = 0.0188$, not shown). In O_3-exposed control mice, none of the soluble mediators was found to correlate significantly with any changes of airway cellularity. Epithelial integrity plays important roles in airway function. The O_3-induced decrease of epithelial cell density in both the proximal and distal airways correlated negatively with AHR in the asthma model (Figure 6, c) and in naive mice ($r^2 = 0.70$, $p = 0.0188$, proximal; $r^2 = 0.60$, $p = 0.0403$, distal; not shown). There were no significant correlations between decreased epithelial cell density and inflammatory cellular components or soluble mediators. Increased mucus production and mucin gene expression may be attributed to some inflammatory cells or mediators. Regression analyses also indicated a significant positive correlation between the increased BAL levels of HA and the elevated content of stored mucosubstances of the epithelial surface in the O_3-exposed asthma model (Figure 6, d).

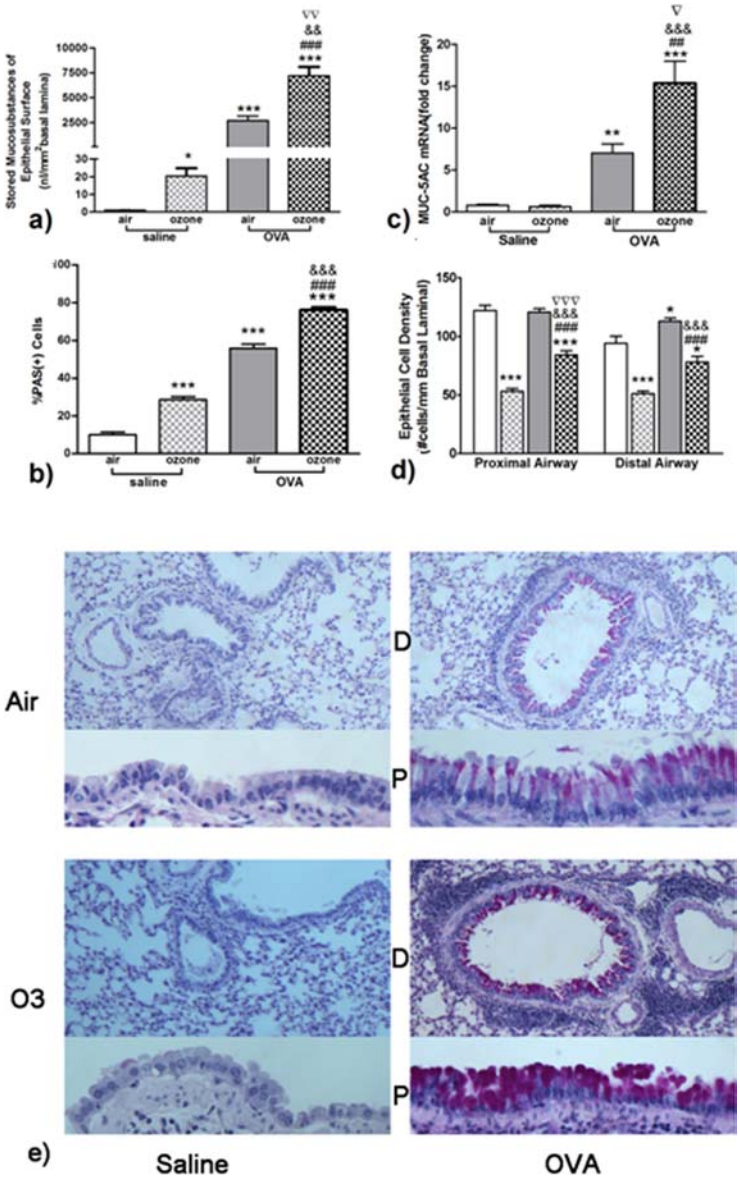

Figure 5. Ozone-induced changes in mucous metaplasia and epithelial cell density. a) Stored mucosubstances of the epithelial surface; b) the percentage of PAS-positive cells in the epithelia; c) quantitative PCR of Muc5ac mRNA, expressed as the fold change of one certain naive mouse; d) epithelial cell density of proximal or distal airways; and e) PAS staining of lung sections are shown. The quantitative morphometric assessments were based on the selected segments, with 2~3 segments of each individual, and a total of 21 segments in naive mice, and 4~8 segments per animal and a total of 41~53 segments in the other groups, and calculated separately between proximal and distal airways. Stored mucus on epithelial surfaces demonstrated by the red-purple area of the proximal (P, 40×) or the distal (D, 10×) airway. The data are expressed as the mean ± SEM for seven mice in each group (except for data from qPCR of Muc5ac mRNA, which are representative of 4 mice in the ozone-exposed, saline-pretreated group, and 3 in the other groups of an independent experiment). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, compared with naive mice; #: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$, compared with air-exposed OVA-pretreated mice; $: $p < 0.05$; $$: $p < 0.01$; $$$: $p < 0.001$, compared with ozone-exposed, saline-pretreated mice. ∇: $p < 0.05$; ∇∇: $p < 0.01$; ∇∇∇: $p < 0.001$, factorial analysis of the interaction effect of ozone-exposure and OVA-pretreatment of Balb/c mice.

5. DISCUSSION

This study was designed to promote our understanding of how acute O_3 exposure affects the main pathophysiological features of an established murine model of allergic asthma, with the aim of mimicking the human condition, in that exposure to high levels of ambient O_3 can lead to exacerbations of asthma. Our results indicate that O_3 (i) enhances AHR, probably by increasing airway sensitivity in particular; (ii) alters pulmonary inflammation;

(iii) elicits a certain degree of epithelial damage in both the proximal and distal airways; and (iv) promotes mucus production and mucin gene expression in mice of the asthma model.

Ozone can increase the baseline P_{enh} of mice regardless of the pretreatments (Figure 2, a). All the O_3-exposed mice in the present study had far more oronasal secretions during the period of P_{enh} measurement than air-exposed mice. However, the morphometric analysis revealed inconsistent changes of airway mucus production in contrast to those of baseline P_{enh} in O_3-exposed control mice. We therefore considered that this increment of baseline P_{enh} might be the consequence of O_3-induced alterations of nasal epithelia, such as mucous metaplasia and/or epithelial edema (25, 26). To eliminate this interference, we used the percentage changes over baseline P_{enh} to reflect the airway reactivity to MCh.

Ozone caused different degrees of change in the airway response to MCh in the asthma model and in the control mice. As also reported previously (27-30), O_3 induced AHR in the normal female Balb/c mice of the present study. The magnitude of the change of AHR quantified by LogPC100 P_{enh} and induced by O_3 in the asthma model was far greater than that in the control mice. This phenomenon may pertain to the synergistic effect of O_3 exposure and the allergic response, as revealed by their significant interaction with respect to their effect on AHR in this study. In addition, this conspicuous aggravation of AHR in the asthma model induced by O_3 may actually arise from increased airway sensitivity, rather than from excessive airway narrowing. This is because there were similar apparent leftward shifts (i.e., hypersensitivity) in the concentration–response curves, but no apparent alterations in the slope height (i.e., hyperreactivity) of O_3-exposed mice, and a lower LogPC50 P_{enh} in the asthma model, but a similar LogPC200 P_{enh} could be observed in their O_3-exposed littermates. The increased airway sensitivity to an agonist caused by O_3 has been reported in humans (31) and laboratory animals (32). The mechanisms underlying this phenomenon are also documented in the literature, including O_3-induced elevated oxidative products (33, 34); increased cholinergic neurotransmission (30, 35); and direct stimulation of the airway sensory nerve (36). Although worldwide agreement has been reached in that O_3 can increase the airway smooth muscle contraction of various species (37-39), it is not clear whether O_3 can similarly influence an "irritated" airway smooth muscle in an experimental asthma model. Kierstein, S. and colleagues exposed their *Aspergillus fumigatus*-sensitized/-challenged mice to 3 ppm O_3 for 3 h, 82 h after the last challenge, and found that O_3 increased the P_{enh} reaction to the agonist in both the control and asthma groups, but exclusively enhanced the contractility of the tracheal smooth muscle ring of the latter. The authors emphasized that only following prior allergen challenge can O_3 enhance the contractility of airway smooth muscle, without mentioning the underlying mechanisms. Despite the discrepancy of their results with that of others about whether O_3 can increase airway smooth muscle contraction under normal conditions, there might be an element of truth in the conclusion of their study, where O_3 was administered 3 days after the last challenge, when the pulmonary inflammation and activation of smooth muscle contraction apparatus were at least partly resolved. However, in unresolved conditions, like those in patients with asthma exacerbations and in the laboratory asthma model of the present study, it makes sense that airway smooth muscle might be maximally activated, and hence generate no more static force after the O_3 exposure. Nevertheless, this speculation is worth further *ex vivo* investigation involving biophysical characteristics of airway smooth muscle. Furthermore, the protective role of fluctuations of the load against which the airway smooth muscle shortens, as generated by tidal breathing or deep inspiration, is impaired in asthmatic subjects (40). Whether or not O_3 could deteriorate this impairment, and hence also increase the excessive airway narrowing requires further investigation.

Although eosinophilic airway inflammation induced by O_3 exposure has been reported by a number of previous studies (17, 41), we observed a conspicuous neutrophilic airway inflammation induced by O_3 in our experimental animals, in agreement with other researchers (42, 43). Furthermore, this accumulation of neutrophils in O_3-exposed asthmatic mice may partly be attributed to the airway TNF-α, which was dramatically increased and correlated positively with the number of BAL neutrophils in this subgroup (not shown). The accumulation of neutrophils might have some involvement in O_3-induced aggravation of AHR in the asthma model, as a significant positive correlation between neutrophil accumulation and O_3-induced AHR aggravation was observed in the present study. Despite causing neutrophilic inflammation in the airway, O_3 altered the lung inflammation in a pattern inducing or deteriorating inflammation in the PV regions in preference to the PB regions, which indicated that the PV area might be more sensitive to O_3-induced damage in Balb/c mice. In the present study, in addition to the alterations in lung parenchyma, the change of epithelial cell density was also induced by O_3 exposure or OVA pretreatment, either alone or together. The larger epithelial cell density exhibited in the asthma model, in contrast to that of the control group, was probably the result of the enhanced mucus cell metaplasia, demonstrated by the higher percentage of PAS-positive cells in the epithelia of this group. Ozone exposure caused a decrease in epithelial cell density of both groups, which could be a consequence of epithelial cell shedding, and hence may indirectly reflect epithelial injury (44, 45). This decrease was attenuated in the asthma model, probably owing to the hyperproduced mucus in the airway lumen, which forms a thicker liquid layer over the epithelial surface and subsequently impedes the direct damaging effect of O_3 on epithelial cells. The negative correlation between epithelial cell density and LogPC100 P_{enh} revealed contributory roles of epithelial destruction in the aggravation of AHR in the asthma model and the development of AHR in the control mice induced by O_3 (Figure 6, b; in O_3-exposed control mice, data not shown). However, the epithelial damage we observed in the present study was not observed by Wagner and coworkers (45); in their study, exposure of rats to 1 ppm O_3 for 8 h did not affect their epithelial cell density. We propose that this

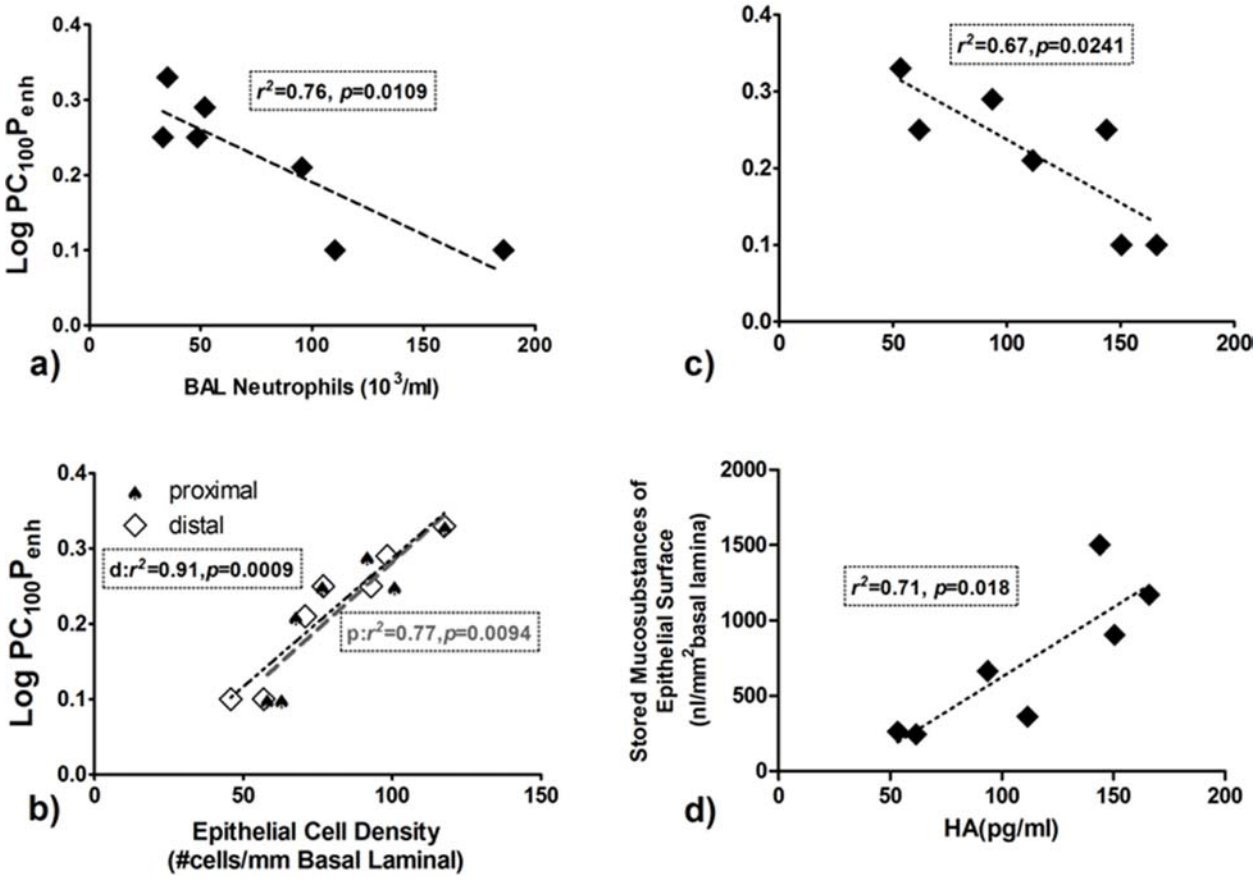

Figure 6. Correlation analyses. Correlations of a) BAL neutrophils and AHR; b) epithelial cell density of the proximal bronchus or distal bronchiole and AHR; c) BAL levels of HA and AHR; and d) BAL levels of HA and the content of stored mucosubstances of the epithelial surface are shown. Each spot is representative of an individual from the ozone-exposed, OVA-pretreated group. Morphometric data are expressed individually by averaging the calculations of 2~3 segments per mouse in naive or 5~8 segments per mouse in other groups.

divergence resulted mainly from differences between the laboratory animals and exposure protocols in the 2 studies. Nevertheless, extending our research by employing more accurate methods to measure epithelial damage, such as detecting the permeability of epithelia or airway clearance (46), and by further investigating the molecular mechanisms, such as the altered expression of proteins composing an epithelial cell–cell junctional complex (47, 48), may promote our understanding of how a high level of ambient O_3 affects the bronchial epithelia of patients with asthma exacerbations.

Except for TNF-α, the O_3 exposure in the asthma model also caused an increase in the concentrations of IL-13 and HA in BAL. IL-13 has been reported to be involved in O_3-induced AHR and airway inflammation (43). This observation is supported in the present study by the significant positive correlation between the increased BAL levels of IL-13, and the extent of AHR in O_3-exposed control mice (not shown). However, in the asthma model, though BAL levels of IL-13 were elevated after O_3 exposure, we did not find any significant correlations of IL-13 with AHR, or with airway cellular components. These results suggest that IL-13 might be less likely to play an important role in O_3-induced synergistic enhancement of AHR in our murine model of asthma. The increased HA in the BAL of mice were mainly of low molecular weight (LMW), which was confirmed by previous studies using gel electrophoresis (49). Based on the negative correlation between the content of BAL HA and LogPC100 P_{enh} observed in this subgroup (Figure 6, c), LMW-HA might play important roles in the formation of O_3-induced aggravation of the AHR of asthmatic mice. Although LMW-HA was reported to contribute greatly to the O_3-induced development of AHR in mice (49), the underlying pathway is not well understood. We previously showed that the p38 mitogen-activated protein kinase (MAPK) pathway is involved in O_3-induced AHR (38). Accordingly, whether p38MAPK is involved in the cascade of LMW-HA-mediated development of AHR of normal mice and aggravation of O3-induced AHR in asthmatic mice remains an interesting issue to be studied.

Ozone exposure also caused different changes of mucin production and mucin gene expression between the control mice and the asthma model, with a larger

magnitude of augmentation in the latter. The conspicuously high value of the morphometric measurement of the airway mucus content and the increase in Muc5ac mRNA expression in the O_3-exposed asthma model may have arisen from the synergistic action of O_3 and the allergic response, which factorial analysis revealed. LMW-HA may also have been involved in the additional promotion effect of O_3 on mucus metabolism, as indicated by the positive correlation between the BAL level of HA and the amount of stored mucus on the epithelial surface in the O_3-exposed asthma model (Figure 6, d). The binding of LMW-HA to its receptor, CD44, which heterodimerizes with the epidermal growth factor receptor (EGFR), facilitates the activation of tissue kallikrein, which cleaves EGF precursors into mature EGF, and hence activates the EGFR pathway, one of the 2 important and well-documented pathways involved in mucus production (50-52). It has also been confirmed that IL-13 (53), TNF-α (54), and neutrophils (55) play roles in mucus production. The present study, however, provides no further evidence of their contribution to O_3-induced aggravation of the upregulated expression of the mucin gene and protein in the asthma model, except for their coincidentally high value exhibited in this subgroup. It has also been reported that low levels of acute O_3 exposure in mice can induce mucus cell metaplasia, whereas high levels of O_3 exposure (>500 ppb) inhibit the metaplasia because of its direct toxic effect (42). In the present study, 2 ppm O_3 caused a very small increment of mucus secretion, but the extensive decrease of epithelial cell density in the control mice may have been caused by its highly toxic properties. Therefore, the protective role of a thick fluid layer covering the surface of the airways in mice with asthma may also contribute to the O_3-induced, intensified mucus production. The discordant changes of mucin production and mucin gene expression in control mice after O_3 exposure may presumably be a consequence of O_3-induced enhancement of the mucus secretion process, during which the physically synthesized baseline intracellular mucus is secreted into the extracellular space through exocytotic machinery (56). This is most likely induced by the oxidative stress-mediated promotion of phosphorylation of the myristoylated, alanine-rich C-kinase substrate (57, 58), which plays a pivotal role in orchestrating the process of mucus secretion (59, 60). This hypothesis needs to be confirmed by further investigations.

This study has a few limitations. We used P_{enh}, but not an invasive, and hence more accurate method to measure AHR.

6. CONCLUSION

This study investigated the different effects of acute ozone exposure on a murine model of asthma and normal mice. Exposure to 2 ppm ozone for 3 hours induced neutrophilic airway inflammation and epithelial injury on both of them. Compared with normal mice, the same exposure of the asthma model engenders additional increments in airway hyperresponsiveness and mucus production, both of which may involve hyaluronan. Given the synergistic effects of ozone and allergic inflammation, it is important to realize that subjects with asthma, especially those with asthma exacerbations, may react differently to the same level of ambient ozone compared to normal subjects.

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Abbreviations: AHR: airway hyperresponsiveness; BAL(F): bronchoalveolar lavage (fluid); EGF(R): epidermal growth factor (receptor); ELISA: enzyme-linked immunosorbent assay; FEV1: forced expiratory volume in 1 s; HA: hyaluronan; H&E: hematoxylin and eosin; IL: interleukin; LMW: low molecular weight; MAPK: mitogen-activated protein kinase; MARCKS: myristoylated alanine-rich C-kinase substrate; MCh: methacholine; mRNA: messenger ribonucleic acid; O_3: ozone; OVA: ovalbumin; PAS: periodic acid–Schiff; PB: peribronchial; PBS: phosphate buffered saline; P_{enh}: enhanced pause; ppb: parts per billion; ppm: parts per million; PV: perivascular; TK: tissue kallikrein; TNF: tumor necrosis factor.

Key Words: Airway Hyperresponsiveness, Epithelial Injury, Hyaluronan, Inflammation, Mucus Hyperproduction, Ozone

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