Effect of ozone on bronchial mucosal inflammation in asthmatic and healthy subjects

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Abstract Epidemiological studies suggest that asthmatics are more affected by ozone than healthy people. This study tested three hypotheses (1) that short-term exposure to ozone induces inflammatory cell increases and up-regulation of vascular adhesion molecules in airway lavages and bronchial tissue 6 h after ozone exposure in healthy subjects; (2) these responses are exaggerated in subjects with mild allergic asthma; (3) ozone exacerbates pre-existent allergic airwaysinflammation.

We exposed 15 mild asthmatic and 15 healthy subjects to 0.2 ppm of ozone or filtered air for 2 h on two separate occasions. Airway lavages and bronchial biopsies were obtained 6 h post-challenge.

We found that ozone induced similar increases in bronchial wash neutrophils in both groups, although the neutrophil increase in the asthmatic group was on top of an elevated baseline. In healthy subjects, ozone exposure increased the expression of the vascular endothelial adhesion molecules P-selectin and ICAM-1, as well as increasing tissue neutrophil and mast cell numbers. The asthmatics showed allergic airways inflammation at baseline but ozone did not aggravate this at the investigated time point.

At 6 h post-ozone-exposure, we found no evidence that mild asthmatics were more responsive than healthy to ozone in terms of exaggerated neutrophil recruitment or exacerbation of pre-existing allergic inflammation. Further work is needed to assess the possibility of a difference in time kinetics between healthy and asthmatic subjects in their response to ozone.

Keywords asthma, pollution, ozone, airways inflammation, neutrophil.

INTRODUCTION

Ozone (O₃) is an important component of modern day air pollution and is linked to increased respiratory morbidity in healthy and asthmatic subjects. Exposure to ozone impairs lung function, increases bronchial reactivity and is associated with increased hospital admissions (1).

In healthy subjects, ozone induced inflammatory responses in bronchoalveolar lavage (BAL) have been detected across the first 24 h post-challenge (2). We have in earlier studies shown exposure to ozone to increase the expression of the vascular endothelial adhesion molecules P-selectin and ICAM-1 in bronchial mucosa as early as 1.5 h post-exposure. At this very early time point, no neutrophilic infiltration occurred in the airway tissue, despite evidence of oxidative stress demonstrated in BAL fluid (3,4). At a later time point, 18 h post-ozone-exposure a neutrophilic infiltration in bronchial mucosa was observed (5).

Asthma is considered an airway mucosal inflammatory disease, but to the best of our knowledge, no previous data are available on the early bronchial mucosal tissue responses of asthmatic subjects exposed to ozone. A few experimental exposure studies using BAL have reported greater increases in neutrophils, IL-6, IL-8 and protein 18 h after ozone challenge in asthmatics as compared to healthy volunteers (6–8). Importantly however, these previous experimental studies comparing airway inflammatory responses between healthy and asthmatic subjects have used quite high ozone burden during exposures. Such exposure conditions are very rare even in the...
Additionally, the scientific literature has been lacking information regarding the early ozone induced responses in bronchial mucosa and BAL in asthmatics. This study was therefore designed to evaluate the early airway responses of healthy and mild asthmatic subjects to a brief exposure to 0.2 ppm ozone, giving an ozone burden more environmentally relevant than those used in previous studies of ozone effects in asthmatics. Bronchoscopy was performed at 6 h after the end of the exposure period to obtain bronchial mucosal biopsies and airway lavage samples. We hypothesised that short-term exposure to ozone would induce neutrophilic airways inflammation in healthy subjects, with up-regulation of relevant vascular adhesion molecules, and that either these responses would be exaggerated in subjects with mild allergic asthma, or ozone would exacerbate pre-existent allergic airways inflammation.

**METHODS**

**Subjects**

Fifteen healthy, non-atopic, subjects (six males, nine females; mean age 24, range 19–31 yrs) and 15 asthmatic subjects (nine males, six females; mean age 29, range 21–48 yrs) with intermittent to mild persistent disease were included (9). The asthmatics were hyper-responsive to methacholine (geometric mean PC20 2–3 mg ml⁻¹) with mean FEV1 90% (range 75–114) of predicted and at least one positive skin prick test to common allergens. Apart from inhaled β₂-agonists on demand, they needed no anti-asthma therapy. All subjects were never-smokers and free of airway infection for at least 6 weeks before and throughout the study. No anti-inflammatory drugs or antioxidant supplements were allowed during the study.

**Study design**

Subjects were exposed to filtered air or 0.2 ppm of O₃ for 2 h in random order in an exposure chamber (4). Exposures were at least 3 weeks apart. Bronchoscopy was performed 6 h after each exposure as previously described (10). Lung function tests (FEV₁ and FVC) were performed before and immediately after each exposure.

**Methods**

During exposures, subjects exercised on a bicycle ergometer (V₂=20 l min⁻¹) alternating with rest for 15-min periods. The O₃ concentration remained stable at 0.20±0.01 ppm (mean±SEM). The study was approved by Umeå University Ethics Committee; all subjects gave informed consent. Lung function was measured using a conventional spirometer (Vitalograph-COMPACT; Vitalograph Ltd., Buckingham, UK). The asthmatic subjects inhaled salbutamol, 0.2 mg dry powder, prior to bronchoscopy. Mucosal biopsies were processed into glycolmethylacrylate resin, stained using monoclonal antibodies (Table I) and assessed as previously described (3,4,11,12). Bronchial wash (BW) was performed by instilling 2×20 ml sterile saline (pH 7.4, 37°C). The first BW sample was used for total and differential cell counts and analysis for soluble mediators. The second BW fraction was used for antioxidant determinations (13).

Bronchoalveolar lavage (BAL, 3×60 ml saline) was then performed. BW and BAL samples were collected separately and immediately placed on ice. Lavage fluid was filtered to remove mucus (pore diameter 100 μm, Syntab, Malmö, Sweden) and centrifuged at 400 g for 15 min to remove cellular components. Cell pellets were re-suspended in PBS at 10⁶ cells ml⁻¹ for total and differential cell counts. Cytocentrifuged specimens were prepared with 5×10⁴ non-epithelial cells per slide (Cytospin 3, Shandon Southern Instruments Inc., Sewickley, PA, USA). Differential cell counts were performed after staining with May–Grünwald Giemsa (400 cells slide⁻¹). Mast cells were counted in ≥10 visual fields at ×160 magnification on slides stained with acid toluidine blue, and counterstained with Mayer’s acid haematoxylin. BW and BAL myeloperoxidase, methyl-histamine and eosinophil cationic protein concentrations were analysed by RIA (Kabi Pharmacia Diagnostics, Uppsala, Sweden).

**Table I.** Monoclonal antibodies used for immunohistochemical staining of bronchial biopsies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope stained</th>
<th>Source</th>
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<tbody>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>AA1</td>
<td>Mast cell tryptase</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>EG2</td>
<td>ECP</td>
<td>Pharmacia-Upjohn, Uppsala, Sweden</td>
</tr>
<tr>
<td>CD3</td>
<td>CD3+ T-cells</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>EN4</td>
<td>Endothelium</td>
<td>Monosan, Uden, the Netherlands</td>
</tr>
<tr>
<td>CD62P</td>
<td>P-selectin</td>
<td>Serotec, Kidlington, Oxford, UK</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>Dako, Glostrup, Denmark</td>
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</tbody>
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Analyses

Statistics were performed using the Unistat (Unistat Ltd, London, UK) and SPSS version 10-05 (SPSS inc., Chicago, USA) software. Wilcoxon’s nonparametric signed-rank test was used to assess paired observations. Between-group comparisons were assessed by Mann-Whitney U-test, except for lung function data that were analysed using a repeated measures two-way ANOVA. The degree of association between neutrophil responses to ozone in different compartments were assessed by Spearman’s rank correlation test. *P-values < 0.05 were considered significant.

RESULTS

Lung function responses

Baseline FEV1 and FVC values did not differ significantly between the healthy and asthmatic subjects. Exposure of healthy subjects to ozone resulted in significant decreases in FEV1 and FVC (P < 0.05). Significant decrements in FVC were observed in the asthmatics (P < 0.05). There was no difference between the two groups in the magnitude of the FEV1 and FVC decrements (Table 2).

Cellular data

Post-air total leukocyte numbers were lower in the BW of asthmatics relative to healthy subjects (median [interquartile range]: 6.7 [4.0–10.7] × 10⁴ vs. 11.1 [7.30–20.0] × 10⁴ cells ml⁻¹, P < 0.05). At baseline, the asthmatic group had higher neutrophil counts in BW, BAL, bronchial epithelium and bronchial submucosa compared to the healthy group (Fig. 1) but BW–MPO was lower in the asthmatic group (1.7 vs. 8.3 μg l⁻¹). Ozone exposure did not alter total leukocyte counts in BW or BAL in either group, but in both groups there was a significant and similar increase in neutrophils in BW, but not in BAL (Fig. 1). In the asthmatic group, there was also evidence of neutrophil activation, as shown by an increased BW–MPO concentration after ozone, increasing by 1.8-fold (P < 0.01) (Fig. 2).

In the healthy controls, ozone exposure increased neutrophil numbers in bronchial epithelium and submucosa (respectively 0.0 [0.0–1.4] cells mm⁻¹ post air vs. 2.0 [0.0–8.0] cells mm⁻¹ post ozone, P < 0.05; and 21.0 [13.6–54.2] cells mm⁻² post air vs. 97.3

![Figure 1](image1)

**FIG. 1.** Bronchial airway neutrophil responses to ozone and control air challenges in mild asthmatic and healthy control subjects. Individual responses and group median values are illustrated. *P < 0.05, **P < 0.01, ***P < 0.001.

| TABLE 2. Lung function responses in healthy and mild asthmatic subjects exposed to air and 0.2 ppm ozone |
|---------------------------------|----------------|-----------------|-----------------|-----------------|
|                                | Healthy Controls |               | Mild Asthmatics |               |
|                                | Pre-Air          | Post-Air        | Pre-Ozone       | Post-Ozone      |
| FVC (l)                        | 5.12 ± 1.16      | 5.02 ± 1.19     | 5.02 ± 1.22     | 4.76 ± 0.97*    |
| FEV1 (l sec⁻¹)                 | 4.05 ± 0.90      | 4.00 ± 0.92     | 3.99 ± 0.91     | 3.89 ± 0.85*    |
|                                | Pre-Air          | Post-Air        | Pre-Ozone       | Post-Ozone      |
| FVC (l)                        | 5.03 ± 1.01      | 5.04 ± 0.99     | 5.07 ± 1.00     | 4.96 ± 1.05*    |
| FEV1 (l sec⁻¹)                 | 3.01 ± 0.75      | 3.70 ± 0.72     | 3.76 ± 0.75     | 3.89 ± 0.75     |

Analysed using a repeated measures two-way ANOVA, with expressions for time and treatment. *P < 0.05.
Lymphocyte cell numbers in the epithelium (10^3–15^4 cells mm^-1 post ozone, P < 0.01). There was no change in tissue neutrophil numbers after ozone in the asthmatic group. (Figs I and 3). Expression of P-selectin and ICAM-1 on vascular endothelium was upregulated after ozone in healthy controls (P < 0.01; P < 0.05 respectively) but no significant changes in adhesion molecule expression were seen in the asthmatic group, although they did start from a higher baseline level of ICAM-1 expression (P < 0.01) (Table 3 and Fig. 3).

Mast cell numbers were elevated in the BW and BAL of asthmatics compared with healthy subjects, (respectively 5.0 [4.0–10.0] % vs. 0.4 [0.3–1.1] %, P < 0.0001; and 6.0 [4.0–9.0] % vs. 1.0 [0.3–1.7] %, P = 0.0001). The asthmatics also showed higher mast cell counts than healthy subjects in epithelium and submucosa (respectively 2.1 [0.0–4.0] cells mm^-1 vs. 0.0 [0.0–4.0] cells mm^-1, P < 0.05; and 36.2 [13.7–58.6] cells mm^-2 vs. 8.0 [3.5–15.4] cells mm^-2, P = 0.001). Mast cell numbers did not change in BW or BAL in either group after ozone, but submucosal mast cell numbers increased in healthy subjects (8.0 [3.5–15.4] cells mm^-2 post air vs. 18.3 [11.7–25.2] cells mm^-2 post ozone, P < 0.05) while epithelial mast cell numbers decreased significantly in the asthmatics (0.4 [0.0–2.1] cells mm^-1 post ozone, P < 0.05).

Lymphocyte numbers in BAL fluid were increased at baseline in the mild asthmatics compared with healthy controls (13.9 [8.8–15.6] % vs. 3.5 [3.0–8.0] % respectively, P < 0.05). Asthmatics also had higher baseline lymphocyte cell numbers in the epithelium (10.56 [5.2–24.3] cells mm^-1 vs. 0.0 [0.0–1.2] cells mm^-1; P < 0.001) and submucosa (81.6 [39.7–180.6] cells mm^-2 vs. 19.8 [5.3–41.0]; P < 0.001). Ozone exposure did not affect lymphocyte numbers in either group.

Eosinophils were present in BW, BAL and submucosa of asthmatic subjects but did not change after ozone. No eosinophils were detected in the healthy controls. BW/ BAL methyl−histamine and ECP concentrations were not significantly affected by ozone.

In healthy subjects, where increases in submucosal, epithelial and BW neutrophils were observed, only a weak association was apparent between submucosal and BW increases (R=0.46, P=0.05), with no significant association noted between the epithelial and BW response (Rs=0.30, P=0.19) but the increases in epithelial and submucosal neutrophils were strongly correlated (Rs=0.84, P<0.001). In asthmatic subjects, there was no correlation between changes in neutrophil numbers in any pair of compartments.

**DISCUSSION**

When exposed to an environmentally relevant concentration of ozone for 2 hours, both healthy and asthmatic subjects showed a neutrophilic airway response, but there was no discernible change in eosinophil or lymphocyte numbers. In the healthy group, ozone also induced up-regulation of the vascular endothelial adhesion molecules P-selectin and ICAM-1 together with increases in tissue neutrophil and mast cell numbers, responses which were not seen in the asthmatic group. The effects of ozone on lung function were small and similar in both groups, in line with earlier work (1.8,14–16).

Previous bronchoscopy studies of the effect of ozone on asthmatics airways have used higher ozone burden, i.e. higher concentration of ozone, longer exposure time and higher total ventilation, than were employed in the present study (6–8). The present study confirms that the neutrophilic responses seen after higher ozone burden are also present at environmentally relevant ozone concentrations. However, the eosinophilic response reported in one high dose study was not apparent here (17), neither was there any evidence of the exaggerated neutrophilic response in asthmatics seen after high ozone exposure (6–8).

We have recently reported increased expression of the vascular endothelial adhesion molecules P-selectin and ICAM-1 in the bronchial mucosa of healthy subjects 1-5 h after ozone exposure, at which time there was no neutrophilic inflammation in the bronchial mucosa (3,4). The up-regulation of P-selectin and ICAM-1 in the present study confirms the importance of these vascular endothelial adhesion molecules in the recruitment of inflammatory cells into the airways of healthy subjects following exposure to ozone.
Six hours after ozone exposure, BW MPO was elevated in the asthmatic group. Similar responses have previously been seen at 18 h post-exposure (8) and in sputum 24 h post-ozone-exposure (18). The fact that increased neutrophil numbers and MPO levels were found in BW of the asthmatic subjects, without any overt change in the biopsies, indicates that their acute response to ozone may be occurring more distal than the proximal biopsies examined here. The neutrophil influx was also more pronounced in the proximal BW sample.
This is consistent with the reported deposition characteristics and dosimetry of ozone (19), which suggest that ozone mainly deposits in the terminal conducting airways.

To our knowledge, this is the first study evaluating the early bronchial tissue responses in asthmatic subjects after exposure to ozone. The asthmatic subjects recruited for this study all had a pre-existent allergic airways inflammation with elevated numbers of eosinophils, mast cells, T-lymphocytes and neutrophils, cells that are considered to play important roles in the pathophysiology of asthma (20). No signs of aggravation of the mucosal allergic status were detected 6 h post-exposure. In contrast, Pedersen et al. reported increases in BAL eosinophils 18 h after exposure to 0·16 ppm ozone for 7·6 h. These subjects were said to be mild allergic asthmatics without inhaled steroid therapy but their baseline BAL eosinophil numbers were quite high, indicating that they had more severe asthma than the group studied here (17).

The absence of any ozone-induced inflammatory responses in bronchial tissue of the asthmatic subjects could be due to differences in basal inflammatory airway status or in the time-kinetics of inflammatory responses to ozone. The asthmatic group showed a more marked tissue and lavage neutrophilia at baseline compared to the healthy subjects. This baseline mucosal inflammation could have activated counter-inflammatory mechanisms (21), which might damp the inflammatory response to ozone. This damping might affect both the magnitude and kinetics of the response and hence delay or attenuate the mucosal response to ozone in asthmatics. Six hours post-exposure has previously been reported as the peak period of ozone-induced neutrophil influx into the airways of healthy subjects (2) with the response persisting up to 24 h post-challenge (5, 22, 23). Given that many epidemiological studies show a time lag of days between exposure and negative airway effects (24–26), it remains possible that inflammatory responses may be seen in the bronchial tissue of asthmatics at a later time point post-exposure.

In the present study, the only significant tissue response after ozone in the asthmatic group was a decrease in epithelial mast cell numbers post ozone exposure. This could possibly represent cell migration into the airways, although no corresponding increase in mast cell numbers was observed in the BAL or BW. Alternatively, this change could have been due to apoptosis or degranulation, although there was no increase in methyl-histamine in BAL or BW to support this latter explanation.

In conclusion, at 6 h post-ozone-exposure we found no evidence that mild asthmatics were more responsive to ozone than healthy controls in terms of exaggerated neutrophil recruitment or exacerbation of pre-existing allergic inflammation. Further work is needed to assess the possibility of a difference in time kinetics between healthy and asthmatic subjects in their response to ozone.

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