

## Effect of ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures of non-asthmatic and asthmatic subjects

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### Summary

**Background** Although epidemiological as well as *in vivo* exposure studies suggest that ozone (O<sub>3</sub>) and nitrogen dioxide (NO<sub>2</sub>) may play a role in airway diseases such as asthma, the underlying mechanisms are not clear.

**Objective** Our aim was to investigate the effect of O<sub>3</sub> and NO<sub>2</sub> on the permeability of human bronchial epithelial cell (HBEC) cultures obtained from non-atopic non-asthmatic (non-asthmatics) and atopic mild asthmatic (asthmatics) individuals.

**Methods** We cultured HBECs from bronchial biopsies of non-asthmatics and asthmatics, and exposed these for 6 h to air, 10 to 100 parts per billion (p.p.b.) O<sub>3</sub>, or to 100 to 400 p.p.b. NO<sub>2</sub>, and assessed changes in electrical resistance (ER) and movement of 14C-BSA across the cell cultures.

**Results** Although exposure to either O<sub>3</sub> or NO<sub>2</sub> did not alter the permeability of HBEC cultures of non-asthmatics, 10 to 100 p.p.b. O<sub>3</sub> and 400 p.p.b. NO<sub>2</sub> significantly decreased the ER of HBEC cultures of asthmatics, when compared with exposure to air. Additionally, 10, 50 and 100 p.p.b. O<sub>3</sub> led to a significant increase in the movement of 14C-BSA across asthmatic HBEC cultures, after 6 h of exposure (medians = 1.73%;  $P < 0.01$ , 1.50%;  $P < 0.05$  and 1.53%,  $P < 0.05$ , respectively), compared with air exposed cultures (median = 0.89%). Similarly, exposure for 6 h to both 200 and 400 p.p.b. NO<sub>2</sub> significantly increased the movement of 14C-BSA across asthmatic HBEC cultures, when compared with air exposure. A comparison of data obtained from the two study groups demonstrated that 10 to 100 p.p.b. O<sub>3</sub>- and 200 to 400 p.p.b. NO<sub>2</sub>-induced epithelial permeability was greater in cultures of asthmatics compared with non-asthmatics.

**Conclusion** These results suggest that HBECs of asthmatics may be more susceptible to the deleterious effects of these pollutants. Whether in patients with asthma the greater susceptibility of bronchial epithelial cells to O<sub>3</sub> and NO<sub>2</sub> contributes to the development of the disease, or is a secondary characteristic of this condition, remains to be determined.

**Keywords** atopic asthma, epithelial permeability, nitrogen dioxide, ozone

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### Introduction

Although it has been suggested that air pollutants such as ozone (O<sub>3</sub>) and oxides of nitrogen (NO<sub>x</sub>) may play a role in respiratory morbidity, particularly asthma, the underlying mechanisms are not clear. Recent studies suggest that airway epithelial cells may play a role, as they form the first line of defence against toxic and infectious agents in the inspired air and they can synthesize pro-inflammatory mediators that influence the function of inflammatory cells known to be involved in the pathogenesis of asthma.

*In vivo* exposure studies have demonstrated that exposure to O<sub>3</sub> leads to significant cell damage as indicated by the increased release of protein, albumin, lactate dehydrogenase (LDH) and an increase in the levels of IL-6 and IL-8, granulocyte

macrophage-colony stimulating factor (GM-CSF), growth-related oncogene  $\alpha$  (Gro $\alpha$ ),  $\alpha_1$ -antitrypsin, PGE<sub>2</sub>, fibronectin, and inflammatory cells such as polymorphonuclear leucocytes (PMNs), in proximal airway lavage (PAL) and bronchoalveolar lavage (BAL) fluid of healthy volunteers [1–3]. More recently, it has been reported that exposure of exercising individuals to 70 parts per billion (p.p.b.) O<sub>3</sub> for 2 h enhanced airway permeability, as indicated by increased levels of Clara-cell protein (CC16 or CC10) in serum [4]. Furthermore, studies of asthmatics have reported that exposure to 160 to 200 p.p.b. O<sub>3</sub> led to increased levels of PMNs, eosinophils, LDH, total protein, myeloperoxidase, fibronectin and IL-8 in both PAL and BAL from asthmatics, and that the increase in PMNs and total protein were greater in asthmatics when compared with non-asthmatics [5,6].

More recent studies reported that exposure to 200 p.p.b. O<sub>3</sub> for 2 h with intermittent exercise led to increased levels of PMNs in induced sputum recovered from mild atopic asthmatic patients 6 h after exposure [7]. Additionally, an increase in albumin, total protein, myeloperoxidase and eosinophilic cationic protein (ECP) was detected 24 h following O<sub>3</sub> exposure. Studies

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of nitrogen dioxide (NO<sub>2</sub>) have also demonstrated that exposure for 20 to 240 min to higher concentrations of 2 to 5.5 parts per million (p.p.m) of this pollutant increases the proportions of lymphocytes, mast cells, PMNs, IL-6, IL-8  $\alpha_1$ -antitrypsin and tissue plasminogen activator in bronchial wash (BW) and BAL of non-asthmatic subjects [8–10]. A recent study of asthmatics and non-asthmatic subjects has reported that exposure to 1 p.p.m. NO<sub>2</sub> for 3 h led to an increase in PGD<sub>2</sub> in BAL recovered from asthmatic patients, but not in BAL from non-asthmatic subjects [11].

*In vitro* studies of primary cultures of human bronchial epithelial cells (HBECs) obtained from surgical explants have reported that exposure to 400–800 p.p.b. NO<sub>2</sub> for 20 min to 6 h, or to 10–500 p.p.b. O<sub>3</sub> for 6 h can increase the permeability and/or release of <sup>51</sup>Cr from these cells [12–14]. More recently, we have demonstrated that incubation of HBECs cultured from both asthmatic and nonasthmatic individuals with diesel exhaust particles (DEP) leads to decreased ciliary beat frequency (CBF) and increased release of inflammatory mediators from these cells [15]. However, the inflammatory mediator release from HBECs of asthmatics is greater, as compared with non-asthmatics. Furthermore, our recent studies of O<sub>3</sub> and NO<sub>2</sub> have shown that exposure of HBEC cultures of asthmatic and non-asthmatic subjects to 50–100 p.p.b. O<sub>3</sub> or 200–400 p.p.b. NO<sub>2</sub> increases the release of inflammatory mediators from these cells, and that the release from HBECs of asthmatics is greater than the release from cells of non-asthmatics [16]. To date, however, there is no information on the effects of exposure to O<sub>3</sub> or NO<sub>2</sub> on the permeability of HBEC cultures from asthmatic patients, who are generally thought to be at a greater risk from the adverse effects of these pollutants. Therefore, the aim of this study was to investigate the effect of various concentrations of O<sub>3</sub> and NO<sub>2</sub> on the permeability of HBEC cultures from well characterized groups of non-atopic non-asthmatic subjects (non-asthmatics) and atopic patients with mild asthma (asthmatics).

## Methods

### *Non-atopic non-asthmatic subjects and atopic asthmatic patients*

Twenty-eight volunteers (12 women and 16 men), with a mean age of 29 years (range 19 to 45 years), took part in the study. Of these, 13 were non-atopic non-asthmatic subjects (control subjects) and 15 were subjects with atopic mild asthma. There were no significant differences in the age and sex ratios of asthmatic and non-asthmatic volunteers. Atopy was confirmed by positive skin prick reactions (weals with a mean diameter of > 3 mm with associated flare and itching, in the absence of any reaction to the negative control) to house dust mite (*Dermatophagoides pteronyssinus*) with or without other allergens such as grass pollen, *Aspergillus fumigatus* and dog or cat dander (ALK, Allergolist Laboratorium, Horsholm, Denmark). All asthmatic patients had a resting forced expiratory volume in 1 s (FEV<sub>1</sub>) of greater than 70% predicted for their age and height and a provocative concentration of 2 mg/mL histamine or less, leading to a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub>FEV<sub>1</sub>).

Before entry into the study, the patients were clinically stable and had not required systemic antihistamines, non-steroidal

anti-inflammatory agents,  $\beta$  blockers, long acting  $\beta_2$ -agonists or oral bronchodilators for 6 weeks or inhaled steroid, nedocromil sodium or sodium cromoglycate for 6 months. During the study, only inhaled salbutamol or terbutaline, as required, was permitted. None of the volunteers entering into the study were smokers or ex-smokers and had not suffered from upper or lower respiratory tract infections within 1 month of the study. The study was approved by the Ethics Committee of St Bartholomew's Hospital, and all volunteers provided written informed consent on entry.

### *Reagents*

All reagents were of tissue culture grade and purchased from Sigma Chemical Company (Poole, Dorset, UK), unless stated otherwise.

### *Bronchial tissue*

Bronchial biopsy specimens were obtained from the right segmental and subsegmental carinae of each volunteer during bronchoscopy performed under light sedation with midazolam and local anaesthesia with lignocaine, according to the American Thoracic Society guidelines [17]. A maximum of six specimens of approximately 1 to 2 mm<sup>3</sup> in size were obtained from each individual by using rotatable Olympus FB-19KR cup-biopsy forceps (KeyMed Ltd, Southend-on Sea, UK) and immediately placed in ice-cold medium 199, prior to further processing for culture of HBECs.

### *Culture of bronchial epithelial cells*

HBECs were cultured by modification of an explant cell culture technique developed by Devalia and colleagues [18]. Each biopsy specimen was examined with a dissecting microscope and the epithelium was dissected from the underlying tissue. The epithelium was then cut further into smaller sections, approximately 0.5 mm<sup>3</sup> in size, and two to three sections were processed and explanted into untreated 6-cm diameter Falcon® 'Primaria<sup>TM</sup>' plastic culture dishes (Becton Dickinson Ltd, Oxford, UK) and incubated at 37 °C in a 5% CO<sub>2</sub> in air atmosphere in 2 mL freshly prepared and micropore filter-sterilized culture medium, as previously described [15, 16]. These 2- to 3-week-old cultures were used for some other studies [15]. The tissue explants were then transferred into 9-mm diameter Falcon® Cell Culture Inserts, with 0.45- $\mu$ m pore size microporous membranes (Becton Dickinson Ltd). Medium (0.2 mL and 0.4 mL) was added into the insert and the insert well, respectively, and treated as for the cultures established in dishes, as was described before [16]. When cells reached confluence, all explants were removed to allow the cultures to grow to complete confluence before use in further studies.

The purity and identity of the cells were checked in randomly selected cultures and confirmed by using (i) light microscopy, (ii) electron microscopy and (iii) indirect immunoperoxidase staining techniques with specific monoclonal antibodies (mAbs) directed towards cytokeratin and specific ciliated epithelial cell antigens, as described previously [15, 16, 18]. Similarly, randomly selected cultures were also analysed for the presence of contaminating cell types by staining with specific mAbs for T and B lymphocytes, endothelial cells, eosinophils, fibroblasts, mast cells, neutrophils and macrophages, as previously explained [15, 16].

### *Exposure of bronchial epithelial cell cultures to O<sub>3</sub> and NO<sub>2</sub>*

HBEC cultures from both non-asthmatic and asthmatic subjects were exposed to O<sub>3</sub> and NO<sub>2</sub>, as described previously [12–14,16].

Gas mixtures containing concentrations of 0 to 100 p.p.b. O<sub>3</sub> in air were generated by use of a Dasibi Model 1108 O<sub>3</sub> generator/monitor (Quantitec Ltd, Milton Keynes, UK) and supplied at a flow rate of 3 L/min through Teflon tubing connected to a rocking airtight polycarbonate chamber of 5.5 L volume (Billups Rothenberg, Del Mar, CA, USA), containing the HBEC cultures.

Similarly, gas mixtures containing concentrations of 0 to 400 p.p.b. NO<sub>2</sub> in 5% CO<sub>2</sub> in air were generated by diluting 20 p.p.m. NO<sub>2</sub> in air Spectraseal grade stock gas mix (BOC Special Gases, London, UK) with a 5% CO<sub>2</sub> in air gas mix by using a Quantitec Model QC 101 gas blender (Quantitec Ltd). Study concentrations of NO<sub>2</sub> were supplied into the polycarbonate chamber through Teflon tubing, at a flow rate of 1.5 L min<sup>-1</sup>. The concentration of NO<sub>2</sub> in the final gas mix was continuously measured by the use of a CEA Instruments Inc. Model TGM 555 NO<sub>2</sub> monitor (Quantitec Ltd). Exposure of HBEC cultures to O<sub>3</sub> or NO<sub>2</sub> gas mixtures was achieved by alternately tilting the chamber to an angle of 10° from horizontal every 90° in the horizontal plane, on a Luckham Model 4RT rocking table (Luckham Ltd, Burgess Hill, UK), thereby eliminating gas or mediator diffusion gradients.

Using this system, sets of 2 to 3 weeks old primary HBEC cultures of non-asthmatic and asthmatic individuals grown in cell culture inserts were exposed for 6 h to either (i) air, (ii) 10, 50 and 100 p.p.b. O<sub>3</sub> or (iii) 100, 200 and 400 p.p.b. NO<sub>2</sub>. Following exposure for 6 h to air, O<sub>3</sub> or NO<sub>2</sub>, the cultures were incubated further for 18 h under standard culture conditions, before termination of the experiment.

### *Effect of O<sub>3</sub> and NO<sub>2</sub> on permeability of bronchial epithelial cell cultures*

Changes in permeability of HBEC cultures from both non-asthmatic and asthmatic individuals were investigated by measuring changes in both the electrical resistance (ER) of and movement of <sup>14</sup>C-bovine serum albumin (<sup>14</sup>C-BSA) (Amersham International plc, Amersham, UK) across HBEC cultures. Prior to investigation, HBECs were grown to confluence, followed by removal of the explants. The epithelial cells were then allowed to grow over the area of the insert membrane left barren by the removal of the explant, and when completely confluent were used further. Immediately before exposure to air, O<sub>3</sub> or NO<sub>2</sub>, the cultures were washed gently with fresh culture medium and then incubated for 30 min in the presence of 0.025 µCi <sup>14</sup>C-BSA. At the end of this incubation the medium in each insert well was collected and analysed for total radioactivity, by liquid scintillation counting in a Beckman LS6500 scintillation counter (Beckman-RIIC Ltd, High Wycombe, UK). When the total radioactivity passing through the culture was found to be less than 0.5% of the total <sup>14</sup>C-BSA added to the inserts at the beginning of the experiment, the culture was deemed to be fully confluent and the experiment was proceeded further. In this manner, sets of at least six separate HBEC cultures of non-asthmatic or asthmatic subjects, each from different individuals, were exposed to different concentrations of O<sub>3</sub> ranging from 0 to 100 p.p.b. or 0 to 400 p.p.b.

NO<sub>2</sub>. ER was measured immediately prior to exposure and then again after 2 h, 6 h and 24 h, using an EVOM™ micro volt-ohm meter (World Precision Instruments, Owslebury, UK).

Immediately following measurement of the ER at each specific time-point during incubation, the medium was collected from each insert well. Fresh medium was replaced into the insert well and the culture was incubated further at 37 °C in a 5% CO<sub>2</sub> in air atmosphere; 25 µl aliquots of each sample were collected and analysed for radioactivity, and after correction for the total amount of radioactivity passing across the epithelial cultures at each time-point, expressed as a percentage of the total added to the culture at the beginning of the experiment.

### *Statistical analysis*

All data were tested for skewness and then analysed further using non-parametric statistical tests. The Mann–Whitney *U*-test was applied to test for differences in the age of non-atopic non-asthmatic subjects and atopic asthmatic patients. Firstly, the Kruskal–Wallis one-way analysis of variance by ranks test was applied to determine the significance of any differences between all the sets of cultures investigated. This was followed by further analysis with the Mann–Whitney *U*-test, to assess the significance of any differences between the individual treatment groups. Bonferroni's correction was employed for multiple comparisons and results were expressed as medians and inter-quartile ranges (Q1–Q3). All statistical tests were performed using a standard computer package (Minitab Data Analysis Software Release 6.1.1, 1987; Minitab Inc., State College, PA, USA), and all *P*-values of less than 0.05 were considered to be significant.

## **Results**

The cells reached confluence after 2 to 3 weeks in culture and demonstrated typical polygonal morphology, characteristic of epithelial cells. The epithelial nature of the cells was confirmed by positive staining of the cells for cytokeratin. Staining with specific mAbs against contaminating cell types, including fibroblasts, muscle cells, endothelial cells, macrophages, T and B lymphocytes, mast cells, eosinophils and neutrophils, did not show any indication of these cells.

### *Effect of exposure to ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures*

The permeability of HBEC cultures from both non-asthmatics and asthmatics was investigated by measurements of ER and the movement of <sup>14</sup>C-BSA across these cultures as described above.

Under air exposure, changes in ER of non-asthmatic and asthmatic HBEC cultures from baseline were not significantly different over a period of 24 h of incubation (Tables 1 and 2, Fig. 1). Similarly, changes in the movement of <sup>14</sup>C-BSA across these cultures under the same culture conditions were not found to be significantly different (Tables 3 and 4, Fig. 2).

Exposure to O<sub>3</sub> (10 to 100 p.p.b.) or NO<sub>2</sub> (100 to 400 p.p.b.) did not significantly alter the ER or the movement of <sup>14</sup>C-BSA across HBEC cultures of non-asthmatics, compared with exposure to air at any of the time-points investigated (Tables 1 and 3, Figs 1a, 2a, 3a and 4a).

**Table 1.** Median and interquartile values of 10 to 100 p.p.b. O<sub>3</sub>-induced and 100 to 400 p.p.b. NO<sub>2</sub>-induced percentage changes in the ER of non-asthmatic HBEC cultures, compared with air

|                            | Changes from baseline (%) |                    |                  |
|----------------------------|---------------------------|--------------------|------------------|
|                            | t2                        | t6                 | t24              |
| Air                        | 21<br>(7, 49)             | 36<br>(23, 113)    | 95<br>(67, 330)  |
| O <sub>3</sub> 10 p.p.b.   | 16<br>(- 10, 32)          | - 22<br>(- 32, 22) | 97<br>(66, 208)  |
| O <sub>3</sub> 50 p.p.b.   | 11<br>(3, 84)             | 33<br>(29, 148)    | 119<br>(68, 602) |
| O <sub>3</sub> 100 p.p.b.  | - 7<br>(- 20, 46)         | 9<br>(- 16, 59)    | 200<br>(27, 267) |
| NO <sub>2</sub> 100 p.p.b. | 19<br>(10, 43)            | 39<br>(24, 116)    | 192<br>(57, 321) |
| NO <sub>2</sub> 200 p.p.b. | 17<br>(4, 46)             | 9<br>(3, 72)       | 93<br>(41, 202)  |
| NO <sub>2</sub> 400 p.p.b. | 12<br>(- 6, 29)           | 28<br>(18, 49)     | 159<br>(58, 191) |

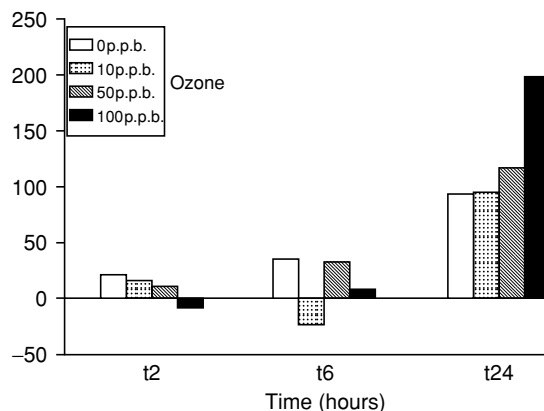
**Table 2.** Median and interquartile values of 10 to 100 p.p.b. O<sub>3</sub>-induced and 100 to 400 p.p.b. NO<sub>2</sub>-induced percentage changes in the ER of asthmatic HBEC cultures, compared with air

|                            | Changes from baseline (%) |                        |                   |
|----------------------------|---------------------------|------------------------|-------------------|
|                            | t2                        | t6                     | t24               |
| Air                        | 11<br>(- 0.7, 17)         | 12.3<br>(- 1.3, 47)    | 99<br>(11.2, 191) |
| O <sub>3</sub> 10 p.p.b.   | - 21*<br>(- 35, - 1.5)    | - 21*<br>(- 44, - 1)   | 39<br>(30, 46)    |
| O <sub>3</sub> 50 p.p.b.   | - 4.8<br>(- 16, 39)       | - 12<br>(- 19, 37)     | 51<br>(38, 211)   |
| O <sub>3</sub> 100 p.p.b.  | - 12*<br>(- 32, - 4)      | - 29**<br>(- 45, - 16) | 58<br>(- 12, 100) |
| NO <sub>2</sub> 100 p.p.b. | 7<br>(2, 15)              | 17<br>(10, 26)         | 49<br>(35, 99)    |
| NO <sub>2</sub> 200 p.p.b. | 3<br>(- 2, 13)            | 13<br>(- 5, 30)        | 41<br>(9, 176)    |
| NO <sub>2</sub> 400 p.p.b. | - 5<br>(- 27, 6)          | - 31**<br>(- 42, - 2)  | 21<br>(4, 42)     |

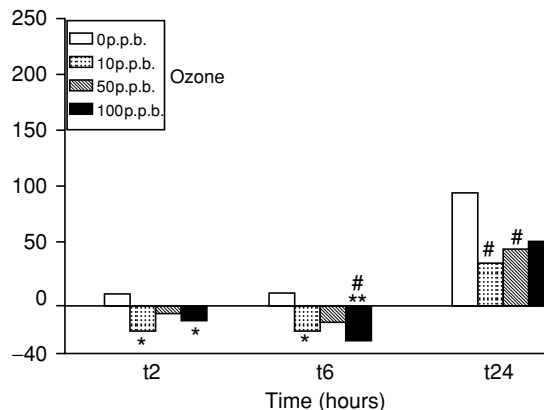
\**P* < 0.05 and \*\**P* < 0.01 vs. air.

In contrast, exposure to 10 p.p.b. O<sub>3</sub> significantly decreased the ER of HBEC cultures of asthmatic patients after 2 h (*P* < 0.05) and 6 h (*P* < 0.05), as compared with air. Similarly, exposure to 100 p.p.b. O<sub>3</sub> led to a significant decrease in the ER of asthmatic HBEC cultures after exposure for 2 h (*P* < 0.05) and 6 h (*P* < 0.01), compared with exposure to air at the same time-points (Table 2, Fig. 1b). Furthermore, exposure of asthmatic HBEC cultures to 10 p.p.b., 50 p.p.b. and 100 p.p.b. O<sub>3</sub> led to significantly increased movement of <sup>14</sup>C-BSA across these cultures, after 2 h (*P* < 0.01 for each concentration of O<sub>3</sub>, respectively) and 6 h of exposure (*P* < 0.01, *P* < 0.05, *P* < 0.05 for 10, 50 and 100 p.p.b., respectively), as compared with exposure to air (Table 4, Fig. 2b). The 10 and 100 p.p.b. O<sub>3</sub>-induced

(a) % change in ER



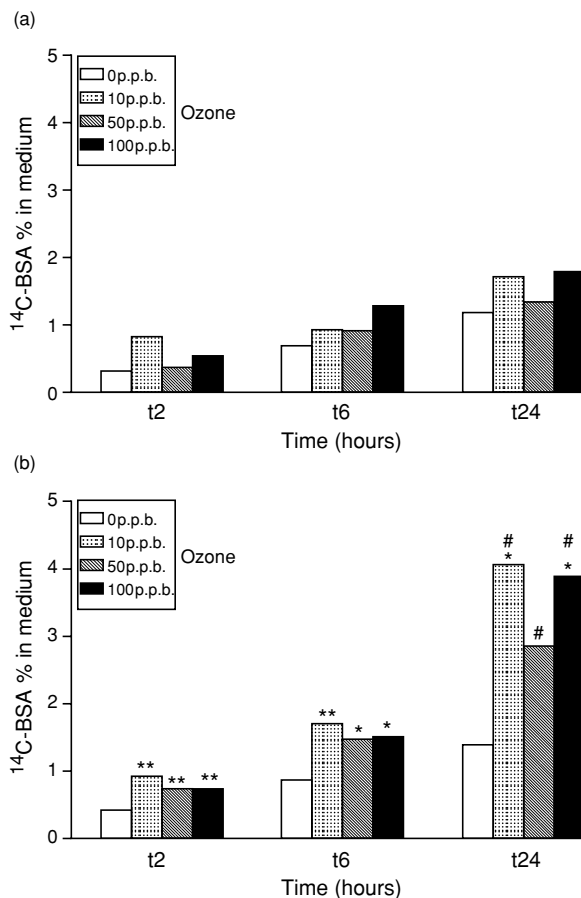
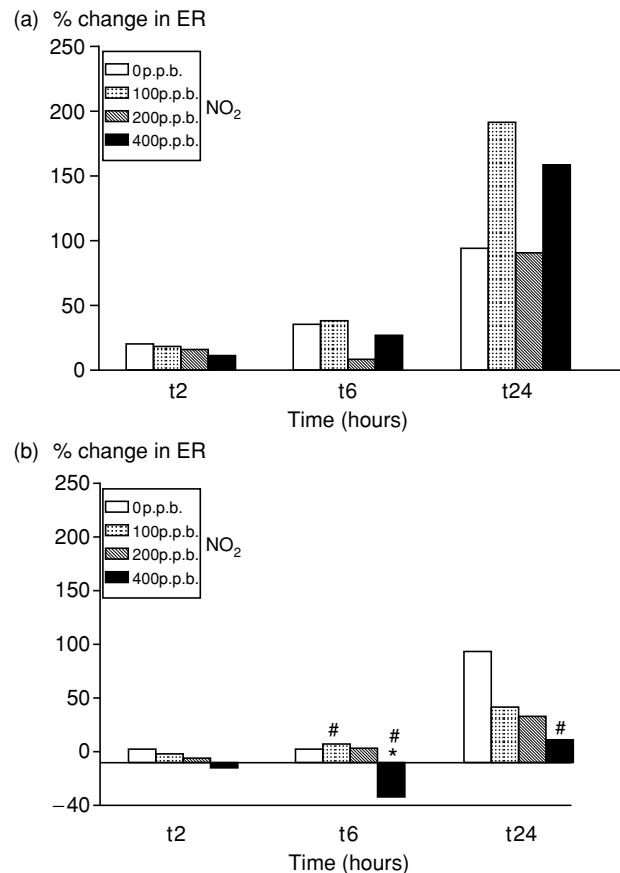
(b) % change in ER

**Fig. 1.** Effect of exposure for 6 h to 10 to 100 p.p.b. O<sub>3</sub> on the electrical resistance (ER) of bronchial epithelial cell cultures of (a) non-atopic non-asthmatics, and (b) atopic patients with mild asthma (results are expressed as median values; *n* ≥ 6 at each time-point; \**P* < 0.05, \*\**P* < 0.01 vs. asthmatic cultures exposed to air; #*P* < 0.05 vs. non-atopic non-asthmatics).**Table 3.** Median and interquartile values of 10 to 100 p.p.b. O<sub>3</sub>-induced and 100 to 400 p.p.b. NO<sub>2</sub>-induced movement of <sup>14</sup>C-BSA across non-asthmatic HBEC cultures, compared with air

|                            | <sup>14</sup> C-BSA percentage in the culture medium |                      |                      |
|----------------------------|------------------------------------------------------|----------------------|----------------------|
|                            | t2                                                   | t6                   | t24                  |
| Air                        | 0.30<br>(0.13, 0.39)                                 | 0.68<br>(0.3, 1.0)   | 1.2<br>(0.63, 1.51)  |
| O <sub>3</sub> 10 p.p.b.   | 0.82<br>(0.66, 1.06)                                 | 0.95<br>(0.46, 1.63) | 1.73<br>(1.17, 2.28) |
| O <sub>3</sub> 50 p.p.b.   | 0.37<br>(0.30, 0.73)                                 | 0.92<br>(0.59, 1.74) | 1.36<br>(0.88, 2.49) |
| O <sub>3</sub> 100 p.p.b.  | 0.53<br>(0.26, 0.88)                                 | 1.28<br>(0.44, 1.61) | 1.80<br>(0.68, 3.24) |
| NO <sub>2</sub> 100 p.p.b. | 0.53<br>(0.07, 0.97)                                 | 1.06<br>(0.28, 1.92) | 2.08<br>(0.49, 2.85) |
| NO <sub>2</sub> 200 p.p.b. | 0.58<br>(0.13, 0.85)                                 | 1.09<br>(0.25, 1.55) | 2.17<br>(0.52, 3.29) |
| NO <sub>2</sub> 400 p.p.b. | 0.41<br>(0.35, 0.50)                                 | 1.24<br>(0.8, 1.67)  | 2.19<br>(1.44, 2.62) |

**Table 4.** Median and interquartile values of 10 to 100 p.p.b. O<sub>3</sub>-induced and 100 to 400 p.p.b. NO<sub>2</sub>-induced movement of <sup>14</sup>C-BSA across asthmatic HBEC cultures, compared with air

|                            | <sup>14</sup> C-BSA percentage in the culture medium |                        |                       |
|----------------------------|------------------------------------------------------|------------------------|-----------------------|
|                            | t2                                                   | t6                     | t24                   |
| Air                        | 0.44<br>(0.33, 0.53)                                 | 0.89<br>(0.65, 1.37)   | 1.43<br>(1.06, 3.95)  |
| O <sub>3</sub> 10 p.p.b.   | 0.94**<br>(0.81, 0.98)                               | 1.73**<br>(1.62, 1.93) | 4.11*<br>(3.53, 4.41) |
| O <sub>3</sub> 50 p.p.b.   | 0.77**<br>(0.56, 0.90)                               | 1.50*<br>(1.26, 1.99)  | 2.91<br>(2.51, 4.04)  |
| O <sub>3</sub> 100 p.p.b.  | 0.75**<br>(0.60, 0.79)                               | 1.53*<br>(1.39, 1.94)  | 3.92*<br>(3.17, 5.04) |
| NO <sub>2</sub> 100 p.p.b. | 0.51<br>(0.11, 0.64)                                 | 1.23<br>(0.74, 1.51)   | 2.66<br>(2.39, 3.33)  |
| NO <sub>2</sub> 200 p.p.b. | 0.61<br>(0.5, 0.84)                                  | 1.38*<br>(1.3, 2.03)   | 2.94<br>(2.66, 5.88)  |
| NO <sub>2</sub> 400 p.p.b. | 0.53<br>(0.40, 0.64)                                 | 1.47*<br>(1.26, 1.73)  | 2.96<br>(2.88, 4.01)  |

\**P* < 0.05 and \*\**P* < 0.01 vs. air.**Fig. 2.** Effect of exposure for 6 h to 10 to 100 p.p.b. O<sub>3</sub> on the movement of <sup>14</sup>C-BSA across bronchial epithelial cell cultures of (a) non-atopic non-asthmatics, and (b) atopic patients with mild asthma (results are expressed as median values; *n* ≥ 6 at each time-point; \**P* < 0.05, \*\**P* < 0.01 vs. asthmatic cultures exposed to air; #*P* < 0.05 vs. non-atopic non-asthmatics).**Fig. 3.** Effect of exposure for 6 h to 100 to 400 p.p.b. NO<sub>2</sub> on the electrical resistance (ER) of bronchial epithelial cell cultures of (a) non-atopic non-asthmatics, and (b) atopic patients with mild asthma (results are expressed as median values; *n* ≥ 6 at each time-point; \**P* < 0.01, vs. asthmatic cultures exposed to air; #*P* < 0.05 vs. non-atopic non-asthmatics).

movement of <sup>14</sup>C-BSA was maintained during subsequent incubation for 18 h, under standard culture conditions.

Exposure of asthmatic HBEC cultures to 400 p.p.b. NO<sub>2</sub> significantly decreased the ER of asthmatic HBEC cultures after 6 h over baseline (*P* < 0.01) (Table 2, Fig. 3b), whereas exposure to both 200 (*P* < 0.05) and 400 p.p.b. NO<sub>2</sub> (*P* < 0.05) led to significantly increased movement of <sup>14</sup>C-BSA across these cultures after 6 h (*P* < 0.05), compared with exposure to air (Table 4, Fig. 4b).

A comparison between O<sub>3</sub>-induced changes in asthmatic and non-asthmatic HBEC cultures demonstrated that 10 p.p.b. (*P* < 0.01) and 50 p.p.b. (*P* < 0.05) O<sub>3</sub>-induced decreases in the ER of cultures of asthmatics were significantly greater than the decreases in ER of non-asthmatic cultures at 24 h after exposure. Similarly, 100 p.p.b. O<sub>3</sub>-induced attenuation of the ER of HBEC cultures of asthmatics was significantly greater than that observed in non-asthmatic cultures after 6 h exposure to 100 p.p.b. O<sub>3</sub> (*P* < 0.05) (Tables 1 and 2, Fig. 1). Moreover, O<sub>3</sub>-induced movement of <sup>14</sup>C-BSA across HBEC cultures was significantly greater in asthmatic HBEC cultures exposed to all concentrations of O<sub>3</sub>, after 24 h, as compared with the cultures of non-asthmatics (*P* < 0.05) (Tables 3 and 4, Fig. 2).

Analysis of NO<sub>2</sub>-induced changes in HBEC cultures of non-asthmatics and asthmatics has also demonstrated that 100 and 400 p.p.b. NO<sub>2</sub>-induced decreases in ER of asthmatic HBEC

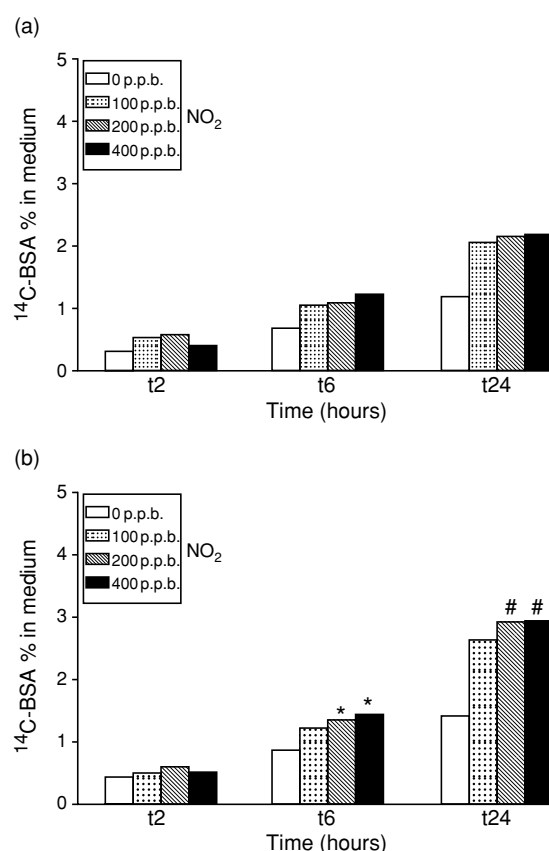


**Fig. 4.** Effect of exposure for 6 h to 100 to 400 p.p.b. NO<sub>2</sub> on the movement of <sup>14</sup>C-BSA across bronchial epithelial cell cultures of non-atopic non-asthmatics, and atopic patients with mild asthma (results are expressed as median values;  $n \geq 6$  at each time-point; \* $P < 0.05$  vs. asthmatic cultures exposed to air; # $P < 0.05$  vs. non-atopic non-asthmatics).

cultures were significantly greater than the decrease in ER of non-asthmatic cultures after 6 h exposure ( $P < 0.05$ ). Additionally, 400 p.p.b. NO<sub>2</sub>-induced decrease in ER of HBEC cultures of asthmatics was significantly greater, as compared with non-asthmatic cultures after 24 h of exposure ( $P < 0.05$ ) (Tables 1 and 2, Fig. 3). Similarly, 200 and 400 p.p.b. NO<sub>2</sub>-induced movement of <sup>14</sup>C-BSA across HBEC cultures of asthmatics was significantly greater, after 24 h ( $P < 0.05$ ), compared with the cultures of non-asthmatics (Tables 3 and 4, Fig. 4).

## Discussion

In the present studies, we have investigated the effect of exposure to O<sub>3</sub> and NO<sub>2</sub> on the permeability (as indicated by changes in the ER and the movement of <sup>14</sup>C-BSA across these cultures) of HBEC cultures from asthmatic patients and non-asthmatic subjects. Our studies have demonstrated that although there were no differences between the permeability of asthmatic and non-asthmatic HBEC cultures under air exposure, HBEC cultures of asthmatics appeared to be significantly more sensitive to the effects of O<sub>3</sub> and NO<sub>2</sub> than the cultures of non-asthmatics. Our studies of non-asthmatics have demonstrated that exposure to O<sub>3</sub> or NO<sub>2</sub> did not have any effect on the permeability of HBEC cultures obtained from these individuals. In contrast,

exposure for 2 and 6 h to 10 p.p.b. and 100 p.p.b. O<sub>3</sub>, and for 6 h to 400 p.p.b. NO<sub>2</sub> led to a significant decrease in the ER of asthmatic HBEC cultures. Similarly, exposure for 6 h to all concentrations of 10 p.p.b., 50 p.p.b. and 100 p.p.b. O<sub>3</sub> studied, and 200 and 400 p.p.b. NO<sub>2</sub> significantly increased the movement of <sup>14</sup>C-BSA across HBEC cultures obtained from asthmatics.

Analysis of differences between pollutants-induced decreases in the ER of HBEC cultures of asthmatics and non-asthmatics have demonstrated that exposure to 10 to 100 p.p.b. O<sub>3</sub> and 400 p.p.b. NO<sub>2</sub> led to a greater decrease in the ER of asthmatic HBEC cultures than the ER of HBEC cultures obtained from non-asthmatics, after 6 h exposure and 24 h incubation. Similarly, pollutants-induced movement of <sup>14</sup>C-BSA across HBEC cultures was significantly greater in asthmatic cultures exposed to 10 to 100 p.p.b. O<sub>3</sub> and 200 to 400 p.p.b. NO<sub>2</sub> after 24 h, compared with non-asthmatic cultures. Taken together, the findings from our studies suggest that the epithelial membrane integrity of HBEC of asthmatics is likely to be compromised more readily by exposure to gaseous air pollutants than that of cells of non-asthmatics, and this may be of particular significance in exacerbation of asthma, especially if it leads to increased access of allergens to the intra- and subepithelial tissue, where encounter with cells of the immune system can occur.

To our knowledge, these studies are the first of their kind. Our findings complement the findings of Calderon from our laboratory who has shown that exposure for 6 h to 10 to 100 p.p.b. O<sub>3</sub> and 400 p.p.b. NO<sub>2</sub> did not significantly alter the ER of nasal epithelial cell cultures of non-atopic non-rhinitics, atopic non-rhinitics or atopic rhinitics out of the pollen season, whereas exposure to 100 p.p.b. O<sub>3</sub> or 400 p.p.b. NO<sub>2</sub> significantly decreased the ER of nasal epithelial cell cultures of atopic rhinitics during the pollen season, after 6 h of exposure to these pollutants, respectively [19]. This raises the possibility that increased sensitivity of the rhinitics to allergen exposure, especially during the pollen season when the quality of air deteriorates, may at least in part be a consequence of the increased susceptibility of their nasal epithelial cells to O<sub>3</sub> and NO<sub>2</sub>, and possibly other oxidizing air pollutants.

Investigating the effects of common atmospheric pollutants, we previously demonstrated that exposure of HBEC cultures to 10 to 500 p.p.b. O<sub>3</sub> led to significant epithelial cell damage as indicated by increased release of <sup>51</sup>Cr from pre-labelled HBECs [14]. Similarly, we also showed that exposure for 20 min to 400 to 800 p.p.b. NO<sub>2</sub> led to significant cell damage and increased permeability of HBEC cultures established from surgical explants [12]. Additionally, we have demonstrated that exposure of HBEC cultures obtained from surgical explants for 6 h to 400 p.p.b. NO<sub>2</sub> significantly reduced the ER of these cultures [13]. More recently, our studies of O<sub>3</sub> and NO<sub>2</sub> have shown that although exposure for 6 h to 50 to 100 p.p.b. O<sub>3</sub> and 200–400 p.p.b. NO<sub>2</sub> led to a significant increase in the release of IL-8 and sICAM-1 from HBEC of non-asthmatics, the same concentrations of both pollutants significantly increased the release of IL-8, GM-CSF, RANTES and sICAM-1 from HBEC cultures of asthmatics. Moreover, 100 p.p.b. O<sub>3</sub>-induced release of GM-CSF and sICAM-1 was significantly greater in HBEC of asthmatics as compared with non-asthmatics [16].

Indeed, the *in vitro* findings of increased susceptibility of HBEC/NEC of atopic asthmatic/rhinitic individuals to O<sub>3</sub> and

NO<sub>2</sub>, is complemented by several *in vivo* studies. Studies have demonstrated that exposure for 4 h to 0.2 p.p.m. O<sub>3</sub> induces a significant increase in levels of PMNs and total protein in BAL derived from asthmatics, compared with non-asthmatic individuals [5]. Peden and colleagues have reported that exposure of atopic asthmatic patients to 0.16 p.p.m. O<sub>3</sub> leads to a significant increase in airway eosinophils recovered from bronchial lavage fluid (BLF) of asthmatics [6]. More recently, Newson and colleagues found that exposure to 0.2 p.p.m. O<sub>3</sub> for 2 h caused an increase in the concentrations of PMNs, albumin, total protein, myeloperoxidase and ECP in induced sputum recovered from mild atopic asthmatic patients [7]. Furthermore, it has been reported that although exposure to 1 p.p.m. NO<sub>2</sub> increases the levels of thromboxan (Tx) B<sub>2</sub> in BAL from both asthmatics and non-asthmatics, levels of PGD<sub>2</sub> were found to be increased in only BAL of asthmatics [11].

Several studies have suggested that prior exposure to individual pollutants such as O<sub>3</sub>, NO<sub>2</sub> and SO<sub>2</sub> or to a combination of these may increase the airway response to inhaled allergen in atopic asthmatics. Studies of allergic asthmatics and atopic seasonal rhinitis have demonstrated that prior exposure for 1 and 3 h to 120 and 250 p.p.b. O<sub>3</sub> leads to increased bronchial responsiveness to inhaled allergen, when compared with prior exposure to air [20,21]. Similarly, it has been reported that exposure to 400 p.p.b. NO<sub>2</sub> for 1 h or to a combination of 400 p.p.b. NO<sub>2</sub> and 200 p.p.b. SO<sub>2</sub> for 6 h, significantly increased the airway response of asthmatics to inhaled allergen, when compared with exposure to air [22,23]. Nasal lavage studies have shown that exposure to 400 p.p.b. O<sub>3</sub> or NO<sub>2</sub> prior to allergen challenge significantly increased the allergen-induced release of ECP in nasal lavage fluid of both perennial allergic asthmatics and seasonal allergic rhinitis, respectively [24,25], suggesting that acute exposure to O<sub>3</sub> and NO<sub>2</sub> may 'prime' the eosinophils to subsequent activation by inhaled allergen.

Whilst to date there are no *in vitro* studies of assessment of epithelial permeability for cultured asthmatic epithelial cells, *in vivo* studies have produced conflicting results. Several studies have suggested that the permeability of the bronchial epithelium of asthmatics is increased, compared with non-asthmatics [26,27]. It has also been suggested that bronchial epithelial cells of asthmatics are aberrantly permeable to airborne allergens [28], and that exposure to allergen can cause an acute increase in bronchovascular permeability to serum proteins [29]. Indeed, recent studies by Broeckaert and colleagues have reported that exposure of exercising individuals to 70 p.p.b. O<sub>3</sub> for 2 h enhanced airway permeability as indicated by increased levels of Clara-cell protein (CC16- or CC10) in serum [4]. In contrast, others have not been able to show differences in epithelial permeability of asthmatics and non-asthmatics, either under basal conditions [30] or following histamine inhalation, which increased lung permeability equally in asthmatic and non-asthmatic individuals [31].

In conclusion, these results suggest that NO<sub>2</sub> and O<sub>3</sub> may modulate airway disease, such as asthma, by adversely influencing the integrity of bronchial epithelial cells of these patients, and that the bronchial epithelium of asthmatics may be more sensitive to the deleterious effects of O<sub>3</sub> and NO<sub>2</sub> than the epithelium of non-asthmatics.

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