UC Davis
UC Davis Previously Published Works

Title
Fluticasone propionate protects against ozone-induced airway inflammation and modified immune cell activation markers in healthy volunteers.

Permalink
https://escholarship.org/uc/item/45c9j0c7

Journal
Environmental health perspectives, 116(6)

ISSN
0091-6765

Authors
Alexis, Neil E
Lay, John C
Haczku, Angela
et al.

Publication Date
2008-06-01

DOI
10.1289/ehp.10981

Peer reviewed
Ozone is a commonly encountered environmental air pollutant. In epidemiologic investigations, exposure to increased levels of ambient air O₃ has been associated with exacerbations of asthma, chronic obstructive pulmonary disease (COPD), and pneumonia, generally 24–48 hr after exposure occurs (Bernstein et al. 2004; Peden 2001). Controlled chamber exposures to O₃ cause an influx of neutrophils to the airway and a decrease in lung function, although these two effects do not correlate with each other, indicating that separate mechanisms account for these effects (Bernstein et al. 2004). O₃ exposure also causes increased responsiveness to allergen in allergic asthmatics (Peden 2001). We have recently observed that O₃ exposure can also result in increased expression of CD11b, CD14, CD16, CD80, CD86, and HLA-DR on airway dendritic cells (DCs), monocytes, and macrophages (Alexis et al. 2004b). It has been suggested that the action of O₃ on airway neutrophils, monocytes, and macrophages accounts for much of the disease outcomes associated with O₃ exposure. These inflammatory events also mimic the type of inflammation that occurs with acute viral and bacterial infection and exacerbations of asthma and COPD (Maneechotesuwan et al. 2007; Pauwels 2004).

Together, these observations suggest that O₃ challenge may be a useful controlled human disease model for screening novel anti-inflammatory pharmaceutical agents in phase I proof-of-concept trials. Holz et al. (2005) tested the utility of a 0.25-ppm O₃ challenge as a drug efficacy screen, using a single pretreatment dose of the established anti-inflammatory agents fluticasone propionate (FP) and oral prednisolone as test agents in a randomized three-arm crossover study in 18 healthy subjects comparing the effect of these two treatments with that of placebo on O₃-induced airway inflammation. Holz et al. (2005) reported that, compared with placebo, pretreatment with 2 mg inhaled FP and 50 mg oral prednisolone resulted in a significant reduction in post-O₃ sputum neutrophils per milliliter (by 62% and 64%, respectively) and myeloperoxidase (MPO; by 55% and 42%, respectively). These results demonstrated that corticosteroids do inhibit the proinflammatory actions of O₃.

In the present study, we sought to extend these observations by comparing the effect of a single administration of a high dose of inhaled FP (2 mg) with a dose that is employed in clinical practice for asthma and COPD (0.5 mg) and placebo. Given the importance that monocytes, macrophages, and DCs likely have in the pathophysiology of O₃-induced exacerbations of disease, we also examined the effect of these treatments on expression of CD11b/CR3, mCD14, CD16/FcγRII, CD64/FcγRI, CD86/B7, and HLA-DR on monocytes, macrophages, and DCs recovered from airway sputum. Clara cell protein 16 (CCP16) and surfactant protein D (SP-D) are innate immune molecules and products of airway epithelial cells (Haczku 2006) that can be released to the circulation during lung injury (Holz et al. 2005). CCP16 is induced in the serum of subjects exposed to O₃ challenge (Blomberg et al. 2003). We have previously shown that SP-D levels in the lung are significantly altered after O₃ inhalation in mice (Kiernstein et al. 2006), but whether similar changes can be detected in the human serum is not known. Thus, we evaluated CCP16 and SP-D for their potential utility as serum biomarkers for assessing the effects of inhaled corticosteroids on O₃ injury in the respiratory tract.
study. All subjects underwent a thorough physical examination and had no history of cardiovascular or chronic respiratory disease and were free of upper or lower respiratory tract infection at least 4 weeks before study participation. All subjects had a forced expiratory volume in 1 sec (FEV1) of at least 80% predicted for a normal population of similar weight and height. A positive urine pregnancy test resulted in exclusion of female subjects from the study. The use of prescription drugs, over-the-counter medication (e.g., aspirin and nonsteroidal anti-inflammatory drugs), vitamins, antioxidants, and dietary supplements was not permitted for the duration of the study. All study participants were able to produce an adequate sputum sample (≥ 1 × 10^6 total cells, ≥ 50% cell viability, ≤ 20% squamous epithelial cells) as measured on their first baseline visit (sputum with no O3 exposure), and all were responsive to O3 (defined as ≥ 10% increase in total and percent sputum neutrophils) (Holz et al. 2005) after exposure to 0.25 ppm O3 for 3 hr with intermittent moderate exercise (ventilation_expiratory = 12.5 L/min/m2 body surface area) as measured on the second study visit. The study was approved by the Committee on the Protection of the Rights of Human Subjects, School of Medicine, University of North Carolina at Chapel Hill, and by the Institutional Review Board at the Rancho Los Amigos National Rehabilitation Center. Informed written consent was obtained from all subjects before their participation in the study.

Study design. This was a double-blind, placebo-controlled, single-dose, randomized, three-period crossover study conducted at two sites. Controlled O3 exposures were performed in comparable chamber setups at both the University of North Carolina, Chapel Hill and the Rancho Los Amigos facility (Alexis et al. 2004a; Gong et al. 1998). All subjects underwent 3-hr exposures to 0.25 ppm O3 with intermittent moderate exercise (15 min rest, 15 min exercise at 12.5 L/min/m2 body surface area) at screening visit 2 and each study session thereafter (visits 3–5). Based on FP half-life and washout of sputum neutrophils after O3 exposure (Holz et al. 2005), O3 exposures were separated by a minimum of 2 weeks to avoid carryover effects. FEV1 and forced vital capacity (FVC) were also measured for the purpose of assessing subject safety. Sputum induction was performed at screening visits and at 3 hr after the conclusion of each O3 exposure (i.e., post-exposure). Sputum was analyzed for total and differential leukocyte count and fluid-phase components and in a subset of subjects (n = 7) for cell-surface phenotypes and cell function by flow cytometry. The study design, including measurement time points, is depicted in Figure 1. FP was provided as a metered dry powder inhaler (Diskus; GlaxoSmithKline, Research Triangle Park, NC). Each Diskus device contained 60 × 0.5 mg doses of FP. A matching placebo Diskus was also provided. Subjects were randomized to receive one of the following treatment regimens: a) 0.5 mg FP (one inhalation of 0.5 mg FP plus three inhalations of placebo); b) 2 mg FP (one inhalation of 0.5 mg FP plus three inhalations of 0.5 mg FP); and c) placebo (one inhalation of placebo plus three inhalations of placebo). The study staff observed each subject using the Diskus during clinic visits to ensure that the device was used correctly.

Pulmonary function. We used both spirometry and impulse oscillometry (IOS) to assess lung function status in subjects. Spirometry was assessed at preexposure, immediately postexposure, and then at 1-hr intervals for 3 hr. IOS was assessed at preexposure, and then hourly for 3 hr beginning 1 hr postexposure. Airway resistance and airway reactance were determined by IOS (Jaeger MS-IOS and LAB Manager Software, version 4.53.2; Jaeger, Hoechberg, Germany) using the recommended techniques of the manufacturer and as previously described (Singh et al. 2006). Real-time recordings of mouth pressure and flow signals pulsed through 5- to 35-Hz spectrum were superimposed on tracings of tidal breathing and displayed on a computer screen. Measurements of total respiratory resistance, resonant frequency (FRec), reactance at 5 Hz, and low-frequency reactance area (area of reactance integrated from 5 Hz up to FRec) were recorded at the 5th, 10th, 15th, and 20th min time points after the IOS test challenge. Spirometry was performed according to current American Thoracic Society spirometry standards (Enright 2003).

Sputum induction and processing and fluid-phase analyses. Subjects provided an induced sputum sample during the screening visit and at 3 hr post-O3 exposure. The sputum induction and processing methods have been previously described in detail (Alexis et al. 2003, 2006). In brief, three 7-min inhalation periods of nebulized hypertonic saline (3%, 4%, 5%; Devilbiss UltraNeb 99 ultrasonic nebulizer; Sunrise Medical, Somerset, Somerset, PA) were followed by expectation of sputum into a sterile specimen cup. Sputum cell aggregates (cellular mucus plugs) were macroscopically identified and manually selected from their surrounding fluid and treated with 0.1% dithiothreitol (DTT; Sputolysin, Calbiochem, San Diego, CA). Total cell counts and cell viability were determined using a Neubauer hemacytometer and trypan blue (Sigma Chemical Co., St. Louis, MO) exclusion staining. Differential cell counts were analyzed using the Hema-Stain-3 kit (Fisher Diagnostics, Middletown, VA). Aliquots of DTT-treated sputum supernatant were immediately frozen and stored at −80°C for later analysis of MPO and total protein by multiplex assay (Pierce Biotechnology, Rockford, IL). All soluble factors (cytokines and chemokines) in sputum (MPO, total protein) were analyzed by a contract laboratory (HFL, Fordham, UK) using validated commercial

![Figure 1. Schematic of the study design. Abbreviations: PLA, placebo; Spiro, spirometry; V, visit. The study was a double-blinded, randomized, cross-over design with a 2-week washout period between visits. Except for the first visit (screen) and last visit (follow-up), all visits included an O3 exposure (0.25 ppm, 3 hr).](image-url)
enzyme-linked immunosorbent assay (ELISA) kits. All compounds were validated in the presence of DTT. The limits of detection after dilution (to minimize potential effects of DTT and to achieve sufficient volume for measurements) were 40 µg/mL for total protein (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) and 36 ng/mL for MPO (Immundiagnostik, Bensheim, Germany).

For a subset of samples, remaining cells were resuspended in Hank’s balanced salt solution and kept on ice for immediate use in flow cytometric assays for selected cell-surface molecules and phagocytosis.

**Systemic biomarkers.** Venipuncture was performed at 4 or 5 hr after O3 exposures to obtain serum for Multiplex systemic biomarker analysis of tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin-6 (IL-6), IL-1β, IL-1α, IL-17, eotaxin, and IL-12P40 using fluorometric custom-designed validated Multiplex kits (Pathway Diagnostics, Malibu, CA). CCP16 and SP-D were assayed using multiplex kits (Pathway Diagnostics, Malibu, CA). CCP16 and SP-D were assayed using multiplex kits (Pathway Diagnostics, Malibu, CA). CCP16 and SP-D were assayed using multiplex kits (Pathway Diagnostics, Malibu, CA). CCP16 and SP-D were assayed using multiplex kits (Pathway Diagnostics, Malibu, CA).

**Flow cytometry and immunofluorescent staining.** All flow cytometry acquisitions and analyses (surface markers, phagocytosis) were performed as previously described (Alexis et al. 2003, 2000a) using a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and CellQuest Pro v5.3 software (Becton Dickinson).

**Cell-surface phenotypes.** Immunofluorescent staining and flow-cytometry methodology have been described in detail in previous publications (Alexis et al. 2000a) using a FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and CellQuest Pro v5.3 software (Becton Dickinson).

**Immune function.** Monoclonal antibodies were purchased from Beckman Coulter Corporation (Miami, FL).

**Phagocytosis.** We analyzed phagocytosis using fluorescent isothiocyanate–labeled IgG-opsinized Saccharomyces cerevisiae zymosan-A BioParticles (Molecular Probes, Eugene, OR) as previously described (Alexis et al. 2003, 2006). All samples were analyzed by flow cytometry within 24–48 hr of fixation in 1% paraformaldehyde. Particle uptake was displayed on histograms and identified as a rightward shift in MFI of the phagocytic population versus autofluorescence of the unlabeled control cells.

**Exhaled nitric oxide.** We measured exhaled NO (eNO) levels preexposure, immediately after exposure, and then at 1-hr intervals for 4 hr according to standardized procedures jointly recommended by the American Thoracic Society and the European Respiratory Society (2005) using a NIOX NO analyzer (Aerocline AB, Solna, Sweden).

**Statistical analysis.** To determine the total number of neutrophils and fluid-phase markers (MPO, protein) in induced sputum 6 hr postchallenge, we analyzed data following a natural logarithmic transformation using a mixed effects model, with period and treatment fitted as fixed effects and subject as a random effect. The suitability of the transformation was assessed by examining the model residuals. Treatment effects were evaluated in terms of treatment ratios and were calculated as the antilog for the differences between the least squares means; 95% confidence intervals (CIs) were determined using pooled estimates of variance for the least squares means difference and then antilogged.

For assessment of differences between specific treatment conditions (postscreen O3 challenge vs. placebo vs. both doses of FP) for CCP16, SP-D, systemic cytokines, and cell-surface marker expression in the subset \((n = 7)\) of volunteers studied at University of North Carolina, Chapel Hill, we used nonparametric one-way analysis of variance for repeated measures (Friedman test) and Dunn’s post hoc analysis of specific pairs of variables. An overall significance level of \(p < 0.05\) was considered to be significant. All values are expressed as mean ± SE. We used GraphPad Prism 3.1 software (GraphPad Software, Inc., San Diego, CA) for statistical analysis.

**Results**

**Patient demographics and overall safety.** Seventeen volunteers participated in the study; patient demographics are outlined in Table 1. No serious adverse events were reported during this study.

**Effects of FP on 0.25 ppm O3-induced changes in pulmonary function.** O3 exposure caused decreases in FVC and FEV\(_1\) during all exposures. Decrements in FVC and FEV\(_1\) were evident immediately after O3 exposure during placebo, 0.5 mg, and 2 mg FP treatments but were subsiding by 1 hr post-exposure for each treatment condition (Table 2). Decrements in FVC and FEV\(_1\) were minimal by 3 hr postexposure (Table 2). Neither dose of FP had a statistically significant effect on O3-induced lung function changes compared with placebo. No consistent O3-induced changes were observed in IOS end points at any postexposure time point (Table 2).

**Effects of FP on 0.25 ppm O3-induced changes in sputum neutrophils and fluid-phase markers of neutrophil activation (MPO, total protein).** Analysis of percent neutrophil levels post-O3 challenge yielded evidence of a statistically significant difference for both active treatments (0.5 mg and 2 mg FP) relative to placebo. Mean ± SE levels of percent polymorphonuclear leukocytes (PMNs) for placebo and 0.5 mg and 2 mg FP were 54 ± 5.4%, 44 ± 4.5%, and 35 ± 3.6%, respectively (Figure 2), which reflect an 18% and 35% reduction in sputum percent PMNs for 0.5 mg and 2 mg FP, respectively. The data indicate a dose–response pattern.

FP also affected the relatively more variable total number of neutrophils/mL. The mean (95% CI) numbers of PMNs/mL were 66.05 × 10\(^4\) cells/mL (34.78–125.41 cells/mL), 56.87 × 10\(^4\) cells/mL (30.15–107.27 cells/mL), and 37.49 × 10\(^4\) cells/mL (19.89–70.68 cells/mL) for placebo, 0.5 mg FP, and 2 mg FP, respectively, 3 hr post-O3 exposure. These values reflected 14% fewer neutrophils in sputum when subjects were pretreated with 0.5 mg FP and statistically significantly \((p < 0.05)\) fewer neutrophils (43%) when pretreated with 2 mg FP, indicating a dose–response effect on the total number of neutrophils per milliliter. In terms of variability, the neutrophil responses on the O3/placebo visit versus the O3-only visit were very similar for both percent neutrophils (mean ± SE, 54 ± 5% vs. 55 ± 5%) and the absolute number of neutrophils per milligram sputum (mean ± SE, 66.05 × 10\(^4\) cells/mL (34.78 to 125.41 cells/mL) vs.

| Table 1. Subject demographics (n = 17). |
|----------------------------------------|
| Characteristic | Mean ± SE |
| Age (years) | 26.4 ± 1.8 |
| Sex | Male 8 Female 9 |
| Race | Caucasian 10 African American 3 American Hispanic 2 Asian Other 1 |
| Height (cm) | 170 ± 2.6 |
| Weight (kg) | 78 ± 3.9 |


**Effects of FP on 0.25 ppm O₃-induced changes in serum marker expression and phagocytosis on sputum monocytes, macrophages, DCs, and neutrophils.** Figure 3 shows the effect of the 0.5 and 2 mg pretreatments with FP on O₃-induced changes in the cell-surface markers CD11b, mCD14, CD64, CD16, HLA-DR, and CD86 on monocytes, macrophages, and DCs. Baseline (i.e., no O₃ exposure) sputum cell-surface marker values (MFI; mean ± SE) from a different cohort of healthy volunteers (n = 15) were as follows: for CD11b, 21 ± 8; mCD14, 65 ± 16; CD64, 59 ± 14; monocytes, 61 ± 11; DCs; for CD64, 5 ± 1; for CD16, 238 ± 44; macrophages, 195 ± 28; for HLA-DR, 31 ± 5; and for CD86, 62 ± 4 monocytes (Lay et al. 2007). Compared with the O₃-only condition in this study (data not shown), baseline expression of these surface markers was significantly (p < 0.05) lower, indicating that O₃ causes an up-regulation of these cell-surface phenotypes.

In general, 2 mg FP exerted a statistically significant effect on post-O₃ surface marker expression relative to placebo treatment. There was also a similar trend after the 0.5 mg dose, which suggests a dose–response effect of FP on O₃-induced changes in mononuclear cell-surface markers. We also observed a significant decrease in CD16/FcγRIII expression on neutrophils after 2.0 mg FP compared with placebo (MFI (mean ± SE): for phagocytosis for macrophages, 478 ± 76 and 606 ± 102 versus 400 ± 50; for monocytes, 348 ± 46 and 365 ± 55 versus 292 ± 39; and for neutrophils, 296 ± 48 and 418 ± 87 versus 270 ± 29.

**Effects of FP on 0.25 ppm O₃-induced changes in serum CCP16, SP-D, eNO, and other systemic biomarkers.** To determine whether serum levels of the airway epithelial cell products SP-D and CCP16 would reflect inflammatory airway changes after O₃ exposure, we measured the concentration of these molecules at baseline and after each O₃ inhalation session in a subset of seven subjects 5 hr after O₃ exposure. Our results showed that serum CCP16 levels were statistically significantly increased after O₃ inhalation and that pretreatment with 2 mg FP statistically significantly inhibited this effect compared with placebo (Figure 4). The effects of FP on CCP16 were dose dependent. SP-D levels were not statistically significantly altered pre–versus post-O₃ exposure (mean ± SE, 61 ± 6 ng/mL vs. 55 ± 4 ng/mL) and were not significantly affected by 0.5 mg FP (53 ± 5 ng/mL) or 2 mg FP (64 ± 5 ng/mL) compared with placebo (55 ± 5 ng/mL).

**Discussion**

Numerous laboratory studies of healthy young individuals exposed to O₃ at a dose comparable to that used in the present study have demonstrated decrements in spirometric lung function (Holz et al. 1999, 2005; McDonnell et al. 1997; Nightingale et al. 2000). A study similar to this one in terms of the cohort characteristics, O₃ concentration, and ventilation rate also reported similar postexposure decrements in FVC and FEV₁ (Holz et al. 1999).

In the present study we found that pretreatment with therapeutic doses of FP had no significant protective effect on spirometric response, which is in agreement with the finding of Nightingale et al. (2000). FP did,

**Table 2. Mean (± SE) pulmonary function, eNO, and IOS.**

| Pretreatment | FEV₁ (L) | FVC (L) | eNO (ppb) | R5 | IOS |
|--------------|---------|---------|-----------|----|-----|
| Placebo      | 3.76 ± 0.18 | 4.69 ± 0.21 | 12.54 ± 1.55 | 0.376 ± 0.008 | –0.112 ± 0.003 | 12.27 ± 0.30 |
| 0.5 mg FP    | 3.84 ± 0.01 | 4.70 ± 0.02 | 11.49 ± 1.26 | 0.409 ± 0.007 | –0.161 ± 0.002 | 12.03 ± 0.28 |
| 2 mg FP      | 3.72 ± 0.01 | 4.60 ± 0.06 | 12.41 ± 1.49 | 0.379 ± 0.008 | –0.099 ± 0.002 | 11.85 ± 0.27 |
| 0.5 mg FP    | 3.84 ± 0.02 | 4.75 ± 0.03 | 11.65 ± 1.91 | 0.383 ± 0.007 | –0.112 ± 0.002 | 12.10 ± 0.24 |
| 2 mg FP      | 3.51 ± 0.05 | 4.43 ± 0.05 | 14.80 ± 1.68 | 0.356 ± 0.007 | –0.168 ± 0.002 | 12.01 ± 0.25 |
| 0.5 mg FP    | 3.69 ± 0.04 | 4.60 ± 0.05 | 15.53 ± 1.70 | 0.362 ± 0.007 | –0.101 ± 0.01 | 12.26 ± 1.43 |
| 2 mg FP      | 12.16 ± 1.2 | 12.38 ± 1.2 | 0.393 ± 0.005 | 0.196 ± 0.08 | 12.99 ± 1.76 |

Abbreviations: R5, total respiratory resistance (cm H₂O/L/sec); X5, reactance (cm H₂O/L/sec). Statistical analysis of other systemic biomarkers or eNO yielded no clear changes induced by O₃ exposure. No significant effects on systemic cytokines (IL-6, IL-12, IL-4, IL-10, IL-17, IL-1α, IL-1β, IFN-γ, TNF-α), mediators (MPO, coX1), or CN (Table 2) were observed after 0.5 mg or 2 mg FP versus placebo. For eNO, levels at 1, 2, and 3 hr (Table 2) postexposure were not statistically significantly different from one another.
however, inhibit inflammatory cell (neutrophils, PMNs) influx to the airways induced by a 3-hr exposure to 0.25 ppm O₃ in a dose-dependent manner. The lack of correlation between spirometry and airway inflammation after O₃ has been well documented (Blomberg et al. 1999; Hazucha et al. 1996), so our finding with FP in this regard was not surprising.

We observed a significant inhibition of the percent PMNs present in airway sputum after O₃ challenge with either 0.5 mg or 2 mg FP pretreatment, and a significant reduction and a trend for a reduction in the number of sputum neutrophils per milliliter post-O₃ with 2 mg and 0.5 mg FP, respectively. Furthermore, we showed that serum CCP16 is a valuable systemic marker of the inflammatory state of the lung and is responsive to the effects of inhaled FP. We also observed evidence of diminished neutrophil activation with 2 mg FP, as it decreased the expression of CD16, a marker of neutrophil activation, compared with placebo. This observation coincided with a reduced MPO/total protein ratio with 2 mg FP, supporting the notion of reduced neutrophil activation. Taken together with previously published results (Holz et al. 2005), our results indicate that O₃ challenge in healthy individuals is a useful model for screening novel anti-inflammatory agents designed for treatment of airway diseases that have elevated neutrophils as a principal component of their airway inflammation. These include a subtype of severe asthma with minimal airway eosinophils (Louis et al. 2000; Wenzel 2003; Wenzel et al. 1999), as well as COPD during an exacerbation (Hill et al. 1999; Stockley 1998).

An important feature of our study design was that we limited volunteer recruitment to persons with documented responsiveness to O₃, defined as a minimum of a 10% increase in airway PMNs after a screening O₃ challenge, to enable the assessment of FP. Nightingale et al. (2000) failed to observe an effect when they examined the effect of 2 weeks of treatment with 800 µg inhaled budesonide twice daily on O₃-induced neutrophilia in normal volunteers. In our study, although there was a significant effect of O₃ alone on percent PMNs, a substantial number of persons examined failed to have an absolute neutrophil response (using neutrophils per milligram sputum as a measure) after placebo treatment. Thus, it is possible that Nightingale et al.’s (2000) results were influenced by a study population that included a high proportion of O₃ “non-responders.” In contrast, Vagaggini et al. (2001) examined the effect of 4 weeks of pretreatment with 400 µg inhaled budesonide twice daily on O₃-induced neutrophilia in asthmatics, and reported a significant decrease in airway neutrophils present 6 hr after 0.27 ppm O₃ challenge compared with placebo pretreatment; most volunteers in the Vagaggini et al. (2001) study appeared to be O₃ responsive. Furthermore, the objective of the present study was not to test the efficacy of FP on O₃-induced changes in monocyteic cell populations, as there is evidence that O₃ exposure results in CD16, CD64, and antigen presentation in subjects with preexisting airway disease.

In addition to its effects on airway neutrophilia, we have recently reported that O₃ challenge (0.4 ppm, 2 hr) causes an increase in expression of cell-surface phenotypes CD11b, mCD14, CD16, CD86, and HLA-DR on sputum monocytes recovered from normal volunteers (Alexis et al. 2004a). We also reported an increase in the numbers of sputum monocytes (in addition to neutrophils), suggesting that O₃ exposure resulted in an influx of activated monocytes. These data are supported by a recent animal study showing that O₃ enhanced the expression of interstitial lung cell-surface molecules associated with antigen presentation and increased the number of antigen-presenting cells in the lung (Koike and Kobayashi 2004). In the present study, we found that 2 mg inhaled FP decreased the expression of CD11b, mCD14, CD16, CD64, CD86, and HLA-DR on sputum monocytes after O₃ challenge compared with placebo. The 0.5 mg dose of FP decreased the expression of CD86 and HLA-DR on sputum monocytes after O₃ challenge compared with placebo. Given that these surface molecules are involved with mediating innate immune responses (CD11b and mCD14), acquired immune responses (CD16, CD64), and antigen presentation (CD86, HLA-DR), we speculate that O₃ exposure may play a role in modifying how airway cells respond to a number of pathologic agents in the airborne environment. It is unclear whether the effect of FP on O₃-induced changes in monocyteic cell populations is due to effects on monocytes present in the airway when exposure began, or on the subsequent influx of monocytes that are activated from the circulation. Decreased monocyteic cell influx could be mediated by an effect of FP on production of monocyte-associated chemotactic factors or decreased adhesion molecule expression on endothelial cells lining the postcapillary venules.

The use of CCP16 as a systemic marker for injury of the epithelium has been examined by several investigators (Blomberg et al. 2003; Helleday et al. 2006). O₃ exposure is associated with increased serum levels of CCP16 (Blomberg et al. 2003). We likewise observed that O₃ exposure caused an increase in serum CCP16 and further showed that pretreatment with either 0.5 mg or 2 mg inhaled FP prevented the O₃-induced increase in CCP16. We compared CCP16 with SP-D, an innate immune molecule produced by type II alveolar epithelial cells and Clara cells. We previously showed that SP-D plays a protective role in O₃-induced injury of the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Expression (MFI; mean ± SE) of cell-surface phenotypes on sputum monocyteic cells and DCs after O₃ exposure with 0.5 mg FP, 2 mg FP, or placebo pretreatment. (A) CD11b/CR3. (B) mCD14. (C) CD64/FcγRII. (D) CD16/FcγRII. (E) HLA-DR. (F) CD86. Only results in which at least one dose of FP resulted in a change in surface marker expression compared with placebo are shown.

* *p < 0.05 for CD11b, mCD14, CD64, CD16, and CD86 compared with 2 mg FP and for HLA-DR and CD86 with compared with 0.5 mg FP.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** CCP16 levels (mean ± SE) in serum pre-O₃ and 8 hr post-O₃ for placebo and 0.5 and 2 mg FP.

* *p < 0.05 compared with placebo. * *p < 0.05 for post-O₃ compared with pre-O₃.
lung (Kierstein et al. 2006), but whether release of this protein into the circulation could parallel the inflammatory airway changes was unclear. Our study showed that CCP16 is a superior serum marker for injury of the airway epithelium and is a more sensitive biomarker for the effect of inhaled FP on airway inflammation compared with SP-D or, indeed, compared with the wide range of cytokines, chemokines, and inflammatory mediators we investigated.

The apparent discrepancy we observed between serum SP-D and CCP16 was likely influenced by many factors, including changes in lung concentrations. We previously showed that intracellular SP-D mRNA and protein expression are very sensitive to corticosteroids, cAMP, and cytokine levels (Cao et al. 2004) and that SP-D in bronchoalveolar lavage fluid is subject to rapid breakdown after O3 exposure of mice (Kierstein et al. 2006). Although no formal comparisons have been made between local (pulmonary) expression of SP-D and CCP16, we speculate that the CCP16 molecule is more resistant to O3-induced breakdown than is SP-D. This is supported by the fact that CCP16 serum levels in a number of animal and human studies accurately reflected the extent of increases in capillary permeability after acute exposure to lipopolysaccharide, chlorine, or O3 (Lakind et al. 2007; Michel et al. 2005). Thus, the discrepancy we observed between serum levels of SP-D and CCP16 after O3 exposure may be due to different structure, regulation of expression, and sensitivity to O3-induced molecular changes. This discrepancy highlights the specific importance of CCP16 as a biomarker for lung injury and treatment effectiveness.

Overall, our observations are consistent with the hypothesis that FP will inhibit acute airway inflammation due to O3 exposure in a dose-dependent fashion that includes the therapeutic dose of 0.5 mg FP. Apart from percent PMNs, however, several of our findings with 0.5 mg FP did not reach statistical significance. This was likely due to an insufficient number of subjects examined at this dose. Subsequent studies using the 0.5 mg of FP and extended the findings by demonstrating decreased neutrophilic inflammation with the 0.5 mg FP treatment, as well. We also observed that both doses of FP inhibited the up-regulatory effect of O3 on airway monocytic cell surface phenotypes and that 2 mg FP inhibited serum levels of CCP16. Taken together, these observations suggest that brief treatments with inhaled corticosteroids by persons in anticipation of exposure to air pollution may offer protection against the inflammatory effects of ambient air O3, particularly for those individuals with preexisting airway disease. However, it is important to note that inhaled corticosteroids had no protective effect on the spirometric decrements induced by O3, suggesting this component of airway function, particularly in individuals with preexisting airway disease, remains susceptible to the modifying effects of O3 exposure. A second conclusion is that O3 challenge with subsequent analysis of airway sputum and serum CCP16 is a good acute disease model for phase I screening of novel anti-inflammatory agents intended for use in asthma and COPD.

**Conclusion**

We confirmed original observations that O3-induced airway neutrophilic inflammation was inhibited by a single administration of 2 mg FP and extended the findings by demonstrating decreased neutrophilic inflammation with the 0.5 mg FP treatment, as well. We also observed that both doses of FP inhibited the up-regulatory effect of O3 on airway monocytic cell surface phenotypes and that 2 mg FP inhibited serum levels of CCP16. Taken together, these observations suggest that brief treatments with inhaled corticosteroids by persons in anticipation of exposure to air pollution may offer protection against the inflammatory effects of ambient air O3, particularly for those individuals with preexisting airway disease. However, it is important to note that inhaled corticosteroids had no protective effect on the spirometric decrements induced by O3, suggesting this component of airway function, particularly in individuals with preexisting airway disease, remains susceptible to the modifying effects of O3 exposure. A second conclusion is that O3 challenge with subsequent analysis of airway sputum and serum CCP16 is a good acute disease model for phase I screening of novel anti-inflammatory agents intended for use in asthma and COPD.

**References**

Alexis NE, Becker S, Bromberg PA, Devlin R, Peden DB. 2004a. Circulating CD11b expression correlates with the neutrophil response and airway inflammation following ozone exposure in humans. Clin Immunol 111(1):126–131.

Alexis NE, Eldridge MW, Peden DB. 2003. Effect of inhaled endotoxin on airway and circulating inflammatory cell phagocytosis and CD11b expression in atopic asthmatic subjects. J Allergy Clin Immunol 112(2):393–396.

Alexis NE, Eldridge MW, Peden DB. 2004b. Effect of inhaled endotoxin on airway and circulating inflammatory cell phagocytosis and CD11b expression in atopic asthmatic subjects. J Allergy Clin Immunol 123:393–396.

Alexis NE, Lay JC, Zeman K, Bennett WE, Peden DB, Szpak JM, et al. 2006. Biological material on inhaled coarse fraction particulate matter activates airway phagocytes in vivo in healthy volunteers. J Allergy Clin Immunol 117:1396–1403.

Alexis NE, Peden DB. 2001. Blunting airway eosinophilic inflammation results in a decreased airway neutrophil response to inhaled LPS in patients with atopic asthma: a role for CD11b. J Allergy Clin Immunol 108:577–580.

Alexis N, Sopuck J, Dho A, Becker S. 2000a. Sputum phagocytes from healthy individuals are functional and activated: a flow cytometric comparison with cells in bronchoalveolar lavage and peripheral blood. Clin Immunol 97:21–22.

Alexis N, Urch B, Tarlo S, Corey P, Penglasy D, O’Byrne P, et al. 2000b. Cyclooxygenase metabolites play a different role in ozone-induced pulmonary function decline in asthmatics compared to normals. Inhal Toxicol 12(12):1205–1224.

American Thoracic Society and European Respiratory Society. 2005. Recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide. Am J Respir Crit Care Med 171(12):912–930.

Bernsten JA, Alexis N, Barnes C, Bernstein IL, Ne A, Peden D, et al. 2004. Health effects of air pollution. J Allergy Clin Immunol 114(1):119–123.

Blomberg A, Mudway IS, Nordenhall C, Hedenstrom H, Kelly FJ, Frew AJ, et al. 1999b. Ozone-induced lung function decrements do not correlate with early airway inflammatory or antioxidant responses. Eur Respir J 13(6):1418–1428.

Blomberg A. Mudway I, Svensson M, Hagenbjork-Gustafsson A, Thomasson L, Hellday R, et al. 2003. Clara cell protein as a biomarker for ozone-induced lung injury in humans. Eur Respir J 22:893–898.

Cao Y, Tao JG, Bates SR, Beers MF, Haczku A. 2004. IL-4 induces production of the lung collectin surfactant protein-D. J Allergy Clin Immunol 114(1):439–444.

Enright PL. 2003. How to make sure your spirometry tests are of good quality. Respir Care 48:772–778.

Gong H, Wong R, Sarma RJ, Linn WS, Sullivan ED, Shamaa DA, et al. Cardiovascular effects of air pollution on human volunteers. 1998. Am J Respir Crit Care Med 167(2):538–546.

Haczku A. 2006. Role and regulation of lung collectins in allergic airway sensitization. Pharmacol Ther 110(1):14–36.

Hauzucha MJ, Madden M, Pape G, Becker S, Devlin R, Koren HS, et al. 1996. Effects of cyclo-oxgenase inhibition on ozone-induced respiratory inflammation and lung function changes. Eur J Appl Physiol Occup Physiol 73(1–2):17–27.

Hellday R, Segerstedt B, Forsberg B, Mudway I, Nordberg G, Bernard A, et al. 2006. Exploring the time dependence of serum Clara cell protein as a biomarker of pulmonary injury in humans. Chest 130(5):1391–1397.

Hill AT, Campbell EJ, Bayley DL, Hill SL, Stockley RA. 1999. Evidence for excessive bronchial inflammation during an acute exacerbation of chronic obstructive pulmonary disease in patients with α1-antitrypsin deficiency. J Respir Crit Care Med 160:1968–1975.

Holz D, Jorres FA, Timm P, Mucke M, Richter K, Koschyk S, et al. 1999. Ozone-induced airway inflammatory changes differ between individuals and are reproducible. Am J Respir Crit Care Med 159:776–784.

Holz D, Tal-Singer R, Kannies F, Simpson KJ, Gibson A, Vermeulen R, et al. 2005. Validation of the human ozone challenge model as a tool for assessing anti-inflammatory drugs in early development. J Clin Pharmacol 45:498–503.

Kierstein S, Poulain FR, Cao Y, Grous M, Mathias R, Kierstein G, et al. 2006. Susceptibility to ozone-induced airway inflammation is associated with decreased levels of surfactant

**Correction**

In the original manuscript published online, Brad Harris was not included as an author. His name has been added here.
Fluticasone propionate protects against airway inflammation.

Koike E, Kobayashi T. 2004. Ozone exposure enhances antigen-presenting activity of interstitial lung cells in rats. Toxicology 196:217–227.

Lakind JS, Holgate ST, Ownby DR, Mansur AH, Helms PJ, Pyatt D, et al. 2007. A critical review of the use of Clara cell secretory protein (CC16) as a biomarker of acute or chronic pulmonary effects. Biomarkers 12(5):445–467.

Lay JC, Alexis NE, Kleeberger SR, Roubey RAS, Harris BD, Bromberg PA, et al. 2007. Ozone exposure enhances expression of surface markers of innate immunity and antigen presentation on airway monocytes in healthy individuals. J Allergy Clin Immunol 120(3):719–722.

Louis R, Lau LCK, Bron AJ, Roldaan AC, Radermecker M, Djukanovi R, 2000. The relationship between airways inflammation and asthma severity. Am J Respir Crit Care Med 161:9–16.

Maneechotesuwan K, Essii-lie Quaye S, Khairtanov SA, Adcock IM, Barnes PJ. 2007. Loss of control of asthma following inhaled corticosteroid withdrawal is associated with increased sputum interleukin-8 and neutrophils. Chest 132(1):98–105.

McDonnell WF, Stewart PW, Andreoni S, Seal E Jr, Kehrl HR, Horstman DL, et al. 1997. Prediction of ozone-induced FEV, changes. Effects of concentration, duration, and ventilation. Am J Respir Crit Care Med 156:715–722.

Michel O, Murdoch R, Bernard A. 2005. Inhaled LPS induces blood release of Clara cell specific protein (CC16) in human beings. J Allergy Clin Immunol 115:1143–1147.

Nightingale JA, Rogers DF, Fan CK, Barnes PJ. 2000. No effect of inhaled budesonide on the response to inhaled ozone in normal subjects. Am J Respir Crit Care Med 161:479–486.

Pauwels RA. 2004. Similarities and differences in asthma and chronic obstructive pulmonary disease exacerbations. Proc Am Thorac Soc 1(2):73–76.

Wenzel SE. 2003. Severe/fatal asthma. Chest 123:405S–410S.

Singh R, Tal-Singer R, Falferman I, Lasenby S, Henderson A, Wessels D, et al. 2006. Plethysmography and impulse oscillometry assessment of tiotropium and ipratropium bromide; a randomised, double blind, placebo controlled, crossover study in healthy subjects. Br J Clin Pharm 61(4):398–404.