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Fluticasone Propionate Protects against Ozone-Induced Airway Inflammation and Modified Immune Cell Activation Markers in Healthy Volunteers

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BACKGROUND: Ozone exposure induces airway neutrophilia and modifies innate immune monocytic cell-surface phenotypes in healthy individuals. High-dose inhaled corticosteroids can reduce O₃-induced airway inflammation, but their effect on innate immune activation is unknown.

OBJECTIVES: We used a human O₃ inhalation challenge model to examine the effectiveness of clinically relevant doses of inhaled corticosteroids on airway inflammation and markers of innate immune activation in healthy volunteers.

METHODS: Seventeen O₃-responsive subjects [$> 10\%$ increase in the percentage of polymorphonuclear leukocytes (PMNs) in sputum, PMNs per milligram vs. baseline sputum] received placebo, or either a single therapeutic dose (0.5 mg) or a high dose (2 mg) of inhaled fluticasone propionate (FP) 1 hr before a 3-hr O₃ challenge (0.25 ppm) on three separate occasions at least 2 weeks apart. Lung function, exhaled nitric oxide, sputum, and systemic biomarkers were assessed 1–5 hr after the O₃ challenge. To determine the effect of FP on cellular function, we assessed sputum cells from seven subjects by flow cytometry for cell-surface marker activation.

RESULTS: FP had no effect on O₃-induced lung function decline. Compared with placebo, 0.5 mg and 2 mg FP reduced O₃-induced sputum neutrophilia by 18% and 35%, respectively. A similar effect was observed on the airway-specific serum biomarker Clara cell protein 16 (CCP16). Furthermore, FP pretreatment significantly reduced O₃-induced modification of CD11b, mCD14, CD64, CD16, HLA-DR, and CD86 on sputum monocytes in a dose-dependent manner.

CONCLUSIONS: This study confirmed and extended data demonstrating the protective effect of FP against O₃-induced airway inflammation and immune cell activation.

KEY WORDS: inhaled corticosteroids, innate immune markers, ozone, sputum neutrophils *Environ Health Perspect* 116:799–805 (2008). doi:10.1289/ehp.10981 available via <http://dx.doi.org/> [Online 28 February 2008]

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 (Manechotesuwan 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/FcγRIII, 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 (Kierstein 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.

Materials and Methods

Subjects. Seventeen (nine male and eight female) nonsmoking healthy volunteers (10 from the Center for Environmental Medicine, Asthma and Lung Biology; 7 from Rancho Los Amigos National Rehabilitation Center) between 18 and 50 years of age (age, 26.4 ± 7.4 years, mean ± SD; body mass index, 20–30 kg/m²) were recruited for this

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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 ($\geq 1 \times 10^6$ total cells, $\geq 50\%$ cell viability, $\leq 20\%$ squamous epithelial cells) as measured on their first baseline visit (sputum with no O₃ exposure), and all were responsive to O₃ (defined as $\geq 10\%$ 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., postexposure). 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 \times 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, Hoechst, 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 (F_{res}), reactance at 5 Hz, and low-frequency reactance area (area of reactance integrated from 5 Hz up to F_{res}) were recorded at the 5-, 10-, 15-, and 20-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 expectoration 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

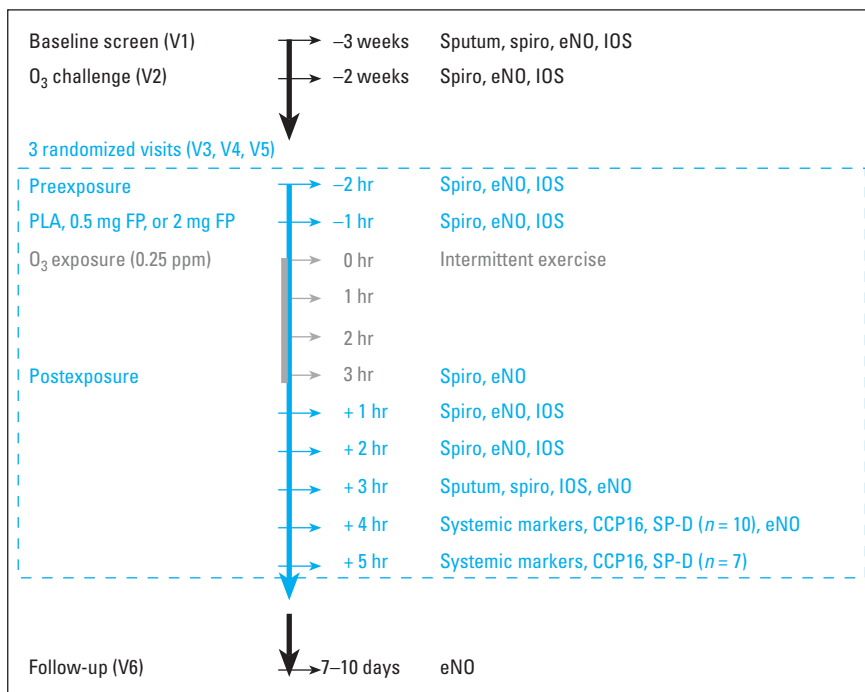


Figure 1. Schematic of the study design. Abbreviations: PLA, placebo; Spiro, spirometry; V, visit. The study was a double-blinded, randomized, cross-over design with a 2-week washout period between visits. Except for the first visit (screen) and last visit (follow-up), all visits included an O₃ exposure (0.25 ppm, 3 hr).

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-1Ra, IL-17, eotaxin, and IL-12P40 using fluorometric custom-designed validated Multiplex kits (Pathway Diagnostics, Malibu, CA). CCP16 and SP-D were assayed using commercially available ELISA kits (Biovendor, Candler, NC) according to the manufacturer's instructions.

Flow cytometry and immunofluorescent staining. All flow cytometry acquisitions and analyses (surface markers, phagocytosis) were performed as previously described (Alexis et al. 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. 2003, 2006). In brief, cells (100 µL, 1 × 10⁶/mL) were incubated with 10 µL fluorochrome-labeled monoclonal antibodies, washed in Dulbecco's phosphate-buffered saline (DPBS), fixed with 0.5% paraformaldehyde in DPBS, and analyzed by flow cytometry within 48 hr of fixation. Viable macrophages, monocytes, neutrophils, lymphocytes, and DCs in sputum were initially identified and gated on the basis of light-scatter properties and positive expression for CD45 (pan-leukocyte marker). Cell populations were then confirmed by positive staining with CD16 (neutrophils), mCD14 (monocytes), HLA-DR (macrophages), HLA-DR/CD86 (DCs), and CD3 (lymphocytes). The acquired data were analyzed using CellQuest Pro v5.3 software, and results were expressed as a rightward shift from control in mean fluorescence intensity (MFI) on histogram analysis. Control cells were incubated with appropriately labeled isotypic control antibodies. Surface markers analyzed included markers of innate (CD11b/CR3, mCD14/LPS receptor, CD16/FcγRIII, CD64/FcγRI) and adaptive (HLA-DR/MHC class II, and CD86/B7.2 co-receptor)

immune function. All monoclonal antibodies were purchased from Beckman Coulter Corporation (Miami, FL).

Phagocytosis. We analyzed phagocytosis using fluorescein 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 (Aerocrine 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 FEV₁ during all

exposures. Decrements in FVC and FEV₁ were evident immediately after O₃ exposure during placebo, 0.5 mg, and 2 mg FP treatments but were subsiding by 1 hr postexposure for each treatment condition (Table 2). Decrements in FVC and FEV₁ 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 × 10⁴ cells/mL (34.78–125.41 cells/mL), 56.87 × 10⁴ cells/mL (30.15–107.27 cells/mL), and 37.49 × 10⁴ 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 (95% CI), 66.05 × 10⁴ 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	
Female	9
Male	8
Race	
Caucasian	10
African American	3
American Hispanic	2
Asian	1
Other	1
Height (cm)	170 ± 2.6
Weight (kg)	78 ± 3.9

62.20×10^4 cells/mL [−10.17 to 312.97 cells/mL], respectively]. Other than percent macrophages, FP exerted no statistically significant effect on total leukocytes per milliliter or total and percent eosinophils, lymphocytes, and bronchial epithelial cells. Relative to placebo, we observed a 24% and 48% increase in percent macrophages with 0.5 mg and 2 mg FP, respectively.

We observed no statistically significant treatment effect of 0.5 mg or 2 mg FP on MPO or total protein levels in sputum. There was, however, borderline evidence of a difference in levels of the MPO/total protein ratio relative to placebo for 2 mg FP. We observed, on average, reductions of 18% and 43% in the MPO/total protein ratio for 0.5 mg and 2 mg FP, respectively, suggesting a dose–response relationship.

Effects of FP on 0.25 ppm O₃-induced changes in surface marker expression and phagocytosis on sputum monocytes, macrophages, DCs, and neutrophils. Figure 3 shows the effect of 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 macrophages, 16 ± 3 DCs; for mCD14, 65 ± 16 macrophages, 59 ± 14 monocytes, 61 ± 11 DCs; for CD64, 5 ± 1 monocytes; for CD16, 238 ± 44 , macrophages, 195 ± 28 DCs; for HLA-DR: 31 ± 5 monocytes; and for CD86, 22 ± 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 monocytic cell-surface markers. We also observed a significant decrease in CD16/FcγRIII expression on neutrophils after 2.0 mg FP compared with placebo (MFI, 406 ± 64 vs. 515 ± 72 ; $p < 0.05$). We observed no significant drug effect of 0.5 mg or 2 mg FP versus placebo on sputum cells as measured by 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 ± 5.4 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).

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-12P40, IL-15, IL-17, IL-1β, IL-1Ra, INF-γ, TNF-α), mediators (MPO, eotaxin), or eNO (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.

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,

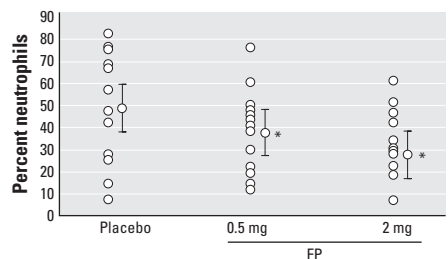


Figure 2. The percent sputum neutrophils after O₃ exposure for each pretreatment dose of FP (0.5 mg or 2 mg) or placebo. * $p < 0.05$ compared with placebo.

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

	FEV ₁ (L)	FVC (L)	eNO (ppb)	IOS		
				R5	X5	F _{res} (Hz)
Baseline						
Pretreatment	3.76 ± 0.18	4.69 ± 0.21	12.54 ± 1.55	0.376 ± 0.008	−0.112 ± 0.003	12.27 ± 0.30
Placebo						
Preexposure (0 hr)	3.84 ± 0.01	4.70 ± 0.02	11.49 ± 1.26	0.409 ± 0.007	−0.161 ± 0.002	12.03 ± 0.28
Immediately after exposure	3.52 ± 0.07	4.39 ± 0.08	13.51 ± 1.39	0.379 ± 0.008	−0.099 ± 0.002	11.85 ± 0.27
1 hr postexposure	3.71 ± 0.05	4.60 ± 0.06	14.24 ± 1.49	0.404 ± 0.04	−0.102 ± 0.01	11.87 ± 1.14
2 hr postexposure			8.94 ± 1.15	0.414 ± 0.04	−0.221 ± 0.11	12.60 ± 1.38
3 hr postexposure						
0.5 mg FP						
Preexposure (0 hr)	3.84 ± 0.02	4.75 ± 0.03	11.65 ± 1.91	0.383 ± 0.007	−0.112 ± 0.002	12.10 ± 0.24
Immediately after exposure	3.51 ± 0.05	4.43 ± 0.05	14.80 ± 1.68	0.356 ± 0.007	−0.106 ± 0.002	12.01 ± 0.25
1 hr postexposure	3.69 ± 0.04	4.60 ± 0.05	15.53 ± 1.70	0.382 ± 0.05	−0.101 ± 0.01	12.26 ± 1.43
2 hr postexposure			15.85 ± 1.60	0.393 ± 0.05	−0.196 ± 0.08	12.99 ± 1.76
3 hr postexposure			12.16 ± 1.2			
2.0 mg FP						
Preexposure (0 hr)	3.73 ± 0.02	4.61 ± 0.02	10.81 ± 1.75	0.382 ± 0.008	−0.113 ± 0.003	12.23 ± 0.25
Immediately after exposure	3.51 ± 0.07	4.35 ± 0.08	13.84 ± 1.32	0.367 ± 0.007	−0.109 ± 0.003	11.70 ± 0.24
1 hr postexposure	3.60 ± 0.04	4.41 ± 0.05	14.87 ± 1.57	0.365 ± 0.04	−0.099 ± 0.01	11.47 ± 1.07
2 hr postexposure			13.65 ± 1.18	0.373 ± 0.04	−0.188 ± 0.08	11.45 ± 1.07
3 hr postexposure			11.88 ± 1.45			

Abbreviations: R5, total respiratory resistance (cm H₂O/L/sec); X5, reactance (cm H₂O/L/sec).

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 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 the O₃ model, but rather to determine whether clinically relevant doses of FP could be assessed to support subsequent larger studies 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 monocyte cells after O₃ challenge compared with placebo treatment. 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 monocyte 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 monocyte 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

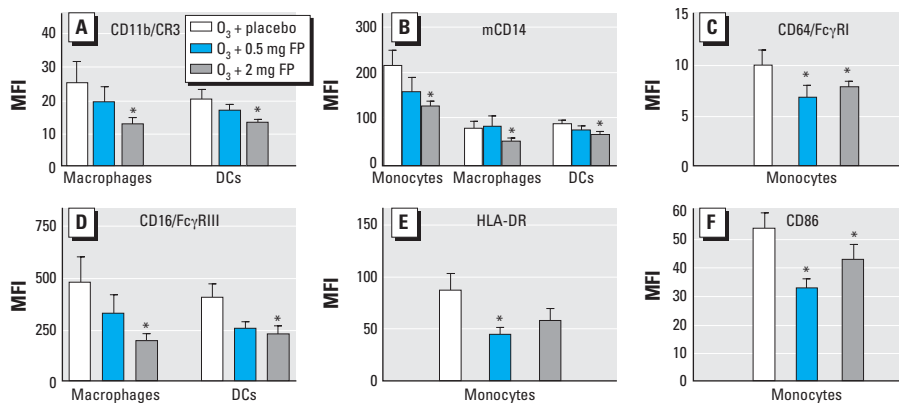


Figure 3. Expression (MFI; mean ± SE) of cell-surface phenotypes on sputum monocyte cells and DCs after O₃ exposure with 0.5 mg FP, 2 mg FP, or placebo pretreatment. (A) CD11b/CR3. (B) mCD14. (C) CD64/FcγRI. (D) CD16/FcγRIII. (E) HLA-DR. (F) CD86. Only results in which at least one dose of FP resulted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

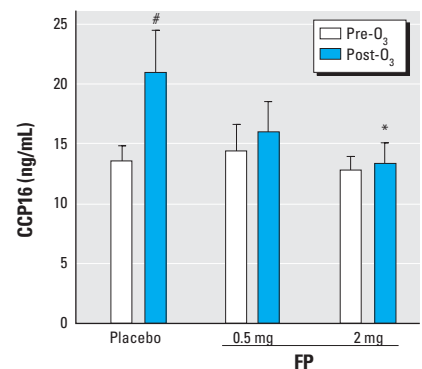


Figure 4. CCP16 levels (mean ± SE) in serum pre-O₃ and 8 hr post-O₃ for placebo and 0.5 and 2 mg FP. **p* < 0.05 compared with placebo. #*p* < 0.05 for post-O₃ compared with pre-O₃.

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 dose of FP will require a larger sample size. It is also possible that a more prolonged pretreatment with FP before O₃ challenge would have resulted in a more pronounced effect of 0.5 mg FP on airway inflammation, but our single-use administration of FP provided adequate drug exposure over the challenge time. We also note that this study was conducted in normal healthy volunteers. We chose healthy volunteers to avoid the potentially high variation in baseline inflammation associated with subjects with preexisting airway disease. Lower variability in healthy subjects would reduce the need to examine a large cohort of

subjects in this study and allow us to attain an initial proof of pharmacology for new anti-inflammatory chemical entities. One cannot rule out, however, that because asthmatics have been reported to have an increased pulmonary sensitivity to O₃ exposure (Alexis et al. 2000b; Scannell et al. 1996), although we did not observe a statistically significant effect using a single administration of 0.5 mg FP on airway inflammation, this might have been observed in a cohort of asthmatics. As noted above, Vagaggini et al. (2001) reported a significant inhibition of O₃-induced inflammation in asthmatics with treatment of 400 µg budesonide administered twice daily. Using endotoxin as an inflammatory stimulus, we previously observed that 440 µg FP for 2 weeks delivered via a metered dose inhaler twice daily inhibited the effect of endotoxin on neutrophilic airway inflammation in allergic asthmatics (Alexis and Peden 2001). Thus, implementation of a longer treatment period in healthy individuals may have resulted in demonstration of anti-inflammatory efficacy of a single 500 µg dose of inhaled FP.

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.

CORRECTION

In the original manuscript published online, Brad Harris was not included as an author. His name has been added here.

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