PULMONARY FUNCTION, DIFFUSING CAPACITY AND INFLAMMATION IN HEALTHY AND ASTHMATIC SUBJECTS EXPOSED TO ULTRAFINE PARTICLES

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ABSTRACT

Particulate air pollution is associated with asthma exacerbations and increased morbidity and mortality from respiratory causes. Ultrafine particles (particles less than 0.1 μm in diameter) may contribute to these adverse effects because they have a higher predicted pulmonary deposition, greater potential to induce pulmonary inflammation, larger surface area, and enhanced oxidant capacity when compared with larger particles on a mass basis.

We hypothesized that ultrafine particle exposure would induce airway inflammation in susceptible humans. This hypothesis was tested in a series of randomized, double-blind studies by exposing healthy subjects and mild asthmatic subjects to carbon ultrafine particles vs. filtered air. Both exposures were delivered via a mouthpiece delivery system during rest and moderate exercise. Healthy subjects were exposed to particle concentrations of 10, 25 and 50 $\mu g/m^3$, while asthmatics were exposed to 10 $\mu g/m^3$. Lung function and airway inflammation were assessed by symptom scores, pulmonary function tests, and airway nitric oxide parameters. Airway inflammatory cells were measured via induced sputum analysis in several of the protocols.

There were no differences in any of these measurements in normal or asthmatic subjects when exposed to ultrafine particle at concentrations of 10 or 25 $\mu g/m^3$. However, exposing 16 normal subjects to the higher concentration of 50 $\mu g/m^3$ caused a reduction in maximal mid-expiratory flow rate (- 4.34 ± 1.78% [ultrafine particles] vs. + 1.08 ± 1.86% [air], p = 0.042) and carbon monoxide diffusing capacity (- 1.76 ± 0.66 ml/min/mmHg [ultrafine particles] vs. - 0.18 ± 0.41 ml/min/mmHg [air], p = 0.040) 21

hours after exposure. There were no consistent differences in symptoms, induced sputum, or exhaled nitric oxide parameters in any of these studies.

These results suggest that exposure to carbon ultrafine particles results in mild small airways dysfunction together with impaired alveolar gas exchange in normal subjects. These effects do not appear related to airway inflammation. Additional studies are required to confirm these findings in normal subjects, compare them with additional susceptible patient populations, and determine their pathophysiologic mechanisms.

INTRODUCTION

Epidemiological studies have shown a direct relationship between ambient particle concentrations and mortality (Samet, *et al*. 2002). A 50 μg/m^3 increase in <10 μm diameter particulate matter (PM_{10}) concentration is associated with a 3 - 8% higher risk of death, particularly cardiopulmonary death in the elderly (EPA, 1996). Pope and colleagues (2002) found an even more potent long-term effect by analyzing data from 1.2 million adults in the US from 1982 to 1998, linking 10 μg/m^3 elevations in PM10 with a 6% increase in death from cardiopulmonary disease. Pulmonary morbidity from ambient particulate matter is evidenced by associations between PM concentrations and asthma exacerbations, increased respiratory symptoms, decreased lung function, and increased hospital admissions (EPA, 1996).

While the epidemiologic data cited above links PM to adverse health consequences, it is not certain which particles in the atmosphere are directly responsible for these effects. Ultrafine particles (UFP), or particles smaller than 0.1 μm, are candidates for this toxicity because: 1) UFP induce inflammatory changes at lower mass concentrations than larger-sized particles (Oberdoster, *et al*., 1995, Li, *et al*., 1999); 2) UFP have a much higher number concentration and surface area when compared with larger particles at the same mass concentration (Oberdoster, *et al*., 1995); 3) inhaled UFP have a very high predicted deposition efficiency in the pulmonary region (ICRP publication No. 66, 1994); 4) UFP have higher oxidant capacity compared to larger particles on an equal mass basis (Brown, *et al*., 2001, Li, *et al*., 2003); 5) UFP are more likely to penetrate epithelium and reach the interstitium (Stearns, *et al*., 1994). Moreover, the biological mechanisms involved in the toxicity of PM are unclear, and elucidation of

these mechanisms has been identified as a high priority research goal (EPA, 1998, National Research Council, 1998). Human studies are essential for accurate determination of these mechanisms in healthy and susceptible populations (Utell, *et al*., 1998). Pulmonary inflammation may be an operative mechanism, and the aforementioned properties of UFP may uniquely enhance their ability to generate an inflammatory response. These same characteristics could also enhance the capacity of UFP to produce pulmonary endothelial dysfunction as a complementary yet separate mechanism. Recent studies suggest that pulmonary (Batalha, J.R., *et al*., 2002) and systemic (Brook, R.D., *et al*., 2002) vasoconstriction may occur in response to inhalation of PM. To our knowledge, similar studies utilizing UFP have not yet been performed.

Asthmatics appear to be a subgroup with enhanced susceptibility to the adverse health consequences of ambient particles. Ambient PM exposure is associated with increased emergency room visits (Tolbert, PE, *et al*., 2000, Lipsett, M., *et al*., 1997, Atkinson, R.W., *et al*., 2001), increased dyspnea, and increased bronchodilator use among asthmatics (Hiltermann, *et al*., 1998). This may be due to the airway inflammation that is present at baseline in many asthmatics (Ohkawara, Y., *et al*., 1995, Montefort, S, *et al*., 1994, Lee, Y.C, *et al*., 1997), which could enhance particle toxicity. Peters, *et al*. (1997) recently showed that peak flow measurements in asthmatics correlate more closely with UFP particle number than fine particle mass concentration, suggesting that UFPs contribute to airway effects in asthmatics.

We hypothesized that UFP generate reactive oxygen species, inducing pulmonary inflammation, endothelial dysfunction, and pulmonary vasoconstriction. These effects should be detectable by measuring pulmonary function, airway inflammation and

pulmonary diffusing capacity. The latter is dependent on pulmonary capillary blood volume so it should reflect a vasoconstrictive response. We further hypothesized that subjects with underlying pulmonary dysfunction would demonstrate greater alterations in these measurements, since they should be more susceptible to the toxic effects of UFP. We conducted a series of clinical studies in which healthy subjects and mild asthmatics were exposed to either filtered air or varying concentrations of carbon UFP. Symptoms, pulmonary function, airway inflammation, and diffusing capacity were measured before and after exposure.

METHODS

Subjects

The studies were approved by the Institutional Research Subjects Review Board. Healthy subjects and mild asthmatic subjects were recruited and paid a stipend. All participants were non-smokers. Healthy subjects were required to have normal spirometry (FEV_1 and FVC ≥80% predicted, FEF_{25-75} ≥60% predicted), a normal 12-lead ECG, and no history of chronic respiratory disease. Subjects were considered to have asthma if they had a history of repetitive symptoms characteristic of intermittent bronchoconstriction (wheezing, shortness of breath) and one of the following: 1) ≥ 12% improvement in FEV_1 after inhaling albuterol, if the baseline FEV_1, or FEV_1/FVC ratio were low compared to predicted values (Morris, J. *et al*., 1971), *or* 2) bronchial hyperresponsiveness with methacholine challenge testing (American Thoracic Society, 2000), in subjects with normal baseline spirometry. The severity of asthma in the subjects to be recruited for these studies was consistent with mild intermittent, mild persistent, or moderate persistent asthma, according to National Institutes of Health guidelines (NIH, 1997). Subjects with FEV_1 <70% of predicted at baseline screening, or with >20% reduction in FEV_1 following the screening exercise, were excluded. Other exclusion criteria for both normal and asthmatic subjects were: regular marijuana use within the past five years, pregnancy, ischemic heart disease, active psychiatric disorder, occupation involving regular, heavy dust or particle exposure, inability to complete the required exercise, inability to produce sputum with sputum induction (see below), or current drug or alcohol abuse. Subjects were required to avoid the following medications before each study day for the indicated interval: systemic steroids (1 month), short-acting

bronchodilators (6 hours), long-acting bronchodilators and inhaled steroids (12 hours), oral bronchodilators and antihistamines (1 week), nonsteroidal anti-inflammatory drugs including aspirin (1 week), and vitamins C and E (1 week). Subjects with atopy or allergic rhinitis were eligible as long as they did not require regular treatment with antihistamines or systemic steroids. Subjects were not studied within six weeks of a respiratory infection.

Study Design

A series of four randomized, double-blinded, controlled studies were performed using a crossover design in which each subject was exposed to filtered air and UFP. Exposure orders were randomized, and the randomization was blocked by order of presentation and gender. Exposures were blinded to both subjects and investigators. The first study, UPREST, involved 12 subjects exposed at rest to 10 $\mu g/m^3$ UFP or filtered air for 2 hours. Exposures were separated by at least 2 weeks. The second, UPDOSE, involved 12 subjects with 3 exposures for each subject, with exposures separated by at least 2 weeks: 10 $\mu g/m^3$ UFP, 25 $\mu g/m^3$ UFP, and filtered air. For safety reasons, the order of exposure was randomized in a restricted fashion, so that each subject received the 10 $\mu g/m^3$ exposure before the 25 $\mu g/m^3$. To simulate outdoor activities subjects exercised on a bicycle ergometer for 15 minutes every half-hour, with the intensity adjusted to achieve minute ventilation of approximately 20 $L/min/m^2$ BSA. The third protocol, UPASTHMA, involved 16 subjects with asthma exposed to 10 $\mu g/m^3$ UFP vs. air for 2 hours with intermittent exercise, with exposures separated by at least 3 weeks. The fourth protocol, UP50, was specifically modified to investigate whether UFP inhalation

alters vascular function. To that end, we included measurement of the carbon monoxide diffusing capacity (DL_{CO}), in 16 normal subjects exposed to 50 $\mu g/m^3$ UFP vs. air for 2 hours with intermittent exercise, with exposures separated by at least 3 weeks.

Each study required 5 to 7 visits for each subject. In all studies, visit 1 was a screening day. Informed consent was obtained, and subjects completed a standardized questionnaire for assessment of respiratory symptoms, medical history, and smoking history. A physical examination was performed, followed by routine spirometry. Subjects exercised on the bicycle ergometer for 15 minutes to determine the intensity necessary to achieve a minute ventilation ($\dot{V}_E$) of 20 $L/min/m^2$. For females, pregnancy testing was performed. Finally, in all studies except UP50, subjects underwent sputum induction by inhaling nebulized saline (see below).

On Visit 2, at least 1 week after the screening day, subjects arrived at 7:15 AM for the following: symptom questionnaire, phlebotomy, exhaled nitric oxide (NO) including NO diffusing capacity (DL_{NO}), and spirometry. In UP50, DL_{CO} was also measured. These procedures required about 2 hours. Subjects were then exposed by mouthpiece for 2 hours to either filtered air or UFP, with intermittent exercise. Subjects breathed room air on the mouthpiece for 5 minutes before the exposure was actually started. A 10-minute break off the mouthpiece was taken after 1 hour of exposure. During the break subjects were asked about dyspnea; if they answered affirmatively, spirometry was repeated. The exposure was terminated if FEV_1 had fallen by more than 20% from the pre-exposure baseline value. Immediately ("0 hours") and 3.5 hours after exposure, the pre-exposure measurements were repeated. However, in UP50 time constraints did not permit measurement of exhaled NO or DL_{CO} at these time points. Subjects returned the next

morning at 8:00 AM (visit 3). The series of measurements were again performed. Sputum induction was performed during visit 3 in all protocols except UP50 where it was replaced by DL_{CO} measurement. In UPASTHMA and UP50, subjects returned for a final series of measurements 45 hours after exposure (visit 4). DL_{CO} was again measured in UP50. All subjects then underwent the alternative exposure at least 2 weeks (UPREST and UPDOSE) or 3 weeks (UPASTHMA and UP50) later, using an identical protocol.

Symptoms were assessed by questionnaire at regular intervals following exposure; subjects ranked the severity of each symptom on a scale from 0 ("not present") to 5 ("incapacitating").

Exposure System

The experiments utilized a mouthpiece exposure system within an Environmental Chamber in the General Clinical Research Center at the University of Rochester Medical Center. Details of this mouthpiece system and method of particle generation have been published elsewhere (Chalupa, *et al.*, 2002). Briefly, ultrafine carbon particles were continuously generated from pure graphite electrodes by spark discharge in anhydrous argon, using a commercial generator (Palas Co., Germany). For example, the generator was set to 25% of full output and mixed with the appropriate amount of filtered air to produce a mass concentration of 10 $\mu g/m^3$. This resulted in nominal particle size parameters with a count median diameter (CMD) of 0.025 μm and a diameter of average mass of 0.035 μm on the basis of a log normal distribution with an average GSD of 1.6. The measured values for all protocols are listed in Table 2. CMD was measured immediately prior to inhalation, confirming that subjects were exposed to ultrafine

particles rather than agglomerated larger particles. Particles were passed through a charge neutralizer after generation, in order to achieve Boltzman's equilibrium.

The particles were diluted with filtered air in a reservoir; an overflow line exhausted the excess aerosol. The subject inhaled from a mouthpiece, and wore a nose clip. One-way valves (Hans Rudolph Inc., Kansas City, MO) prevented rebreathing of UFP. Condensation particle counters (TSI, Inc., St. Paul MN) and an electrical detection mobility analyzer (TSI, Inc., St. Paul MN) determined particulate number, surface area, and volume concentrations of the inspired and expired aerosols. The target exposure mass concentrations were 10, 25 and 50 $\mu g/m^3$. The mass concentrations were determined by the use of a tapered element oscillating microbalance (TEOM, Rupprecht and Patachnick, Albany, NY). Electronic integration (HPChem Integrating Software, Hewlett Packard, MD) of a pneumotachographic airflow transducer (E for M Co., White Plains, NY) on the expiratory limb provided continuous measurements of V_T, respiratory rate, and minute ventilation.

Pulmonary Function

Spirometric measurements of forced vital capacity and FEV_1 were performed with a pneumotachograph interfaced with a microcomputer (Model CPF-S, Medical Graphics, St. Paul, MN). Lung volumes (by plethysmography) and DL_{CO} were measured in the clinical pulmonary function laboratory using equipment from Morgan Scientific Inc., Haverhill, MA.

Airway Nitric Oxide

Measurement of airway nitric oxide (NO) production provides a non-invasive method for assessing airway inflammation (ATS, 1999). We have developed methods for separately measuring NO production in the conducting (or upper) airways ($\dot{V}_{UNO}$), and NO production in the alveolar (or lower) airways ($\dot{V}_{LNO}$). Methodologic details have been previously described (Hyde, *et al*., 1997, Pietropaoli, *et al*., 1999, Perillo, *et al*., 2001). In brief, the technique involves determination of the single-breath diffusing capacity for NO ($D_{L_{NO}}$) and measurement of the partial pressure of exhaled NO (P_E) at different constant expiratory flow rates. Nitric oxide concentrations in the exhaled breath were measured with a rapidly responding chemiluminescence NO analyzer (model 270B, Sievers, Boulder, CO). $D_{L_{NO}}$ was measured by inhaling 10 ppm NO from a bag-in-box apparatus. After 2 seconds of breath-holding, the subject exhaled at a constant flow rate of 0.5 L/sec into the recording spirometer. The rate of change of NO concentration at increments of exhaled volume provided the data to calculate $D_{L_{NO}}$ (Perillo, *et al*., 2001).

For determination of $\dot{V}_{UNO}$ and $\dot{V}_{LNO}$, subjects inhaled NO-free air to total lung capacity from the bag-in-box, breath-held for 10 sec, and then exhaled at each of six different constant expiratory flow rates. In the initial studies, (UPREST and UPDOSE), the partial pressure of NO in the alveoli (P_A) and $\dot{V}_{UNO}$ were determined by plotting the inverse of the more rapid flow rates on the x-axis vs. the corresponding exhaled NO measurements on the y-axis. P_A was determined as the y-intercept (infinite flow rate) of this plot, and $\dot{V}_{UNO}$ was calculated from its slope. In the later protocols (UPASTHMA and UP50), these variables were calculated simultaneously with a curve-fitting method that utilizes a greater range of expiratory flow rates. This method offers the advantage of simultaneous

calculation the NO diffusing capacity of the upper, conducting airways (DU_{NO}), and thus provides a complete assessment of NO exchange in this lung region (Pietropaoli, et. al., 1999). $\dot{V}_{LNO}$ was determined from the following equation:

$$\dot{V}_{LNO} = D_{LNO}(P_A)$$

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Sputum Induction

The cells obtained in induced sputum are representative of the lower airways, and provide a non-invasive measure of airway inflammation. Sputum induction was performed as part of baseline determinations on the screening day. Subjects unable to produce an adequate sample (>0.7 x 106 cells with ≥ 70% non-epithelial cells) were excluded from the study. Sputum was induced 21 hours after each exposure. Only one sputum induction was performed after each exposure, because sputum induction itself induces a transient airway inflammatory response that influences repeated measurements (Nightingale, *et al*., 1998, Holtz, O, *et al*., 1997). Sputum induction and the "plug selection" technique for sputum processing were based on the methods of Pizzichini *et al*. (1996). Cytospin slides were prepared for microscopic differential counts (~500 cells counted). The cell-free supernatant was aliquoted and stored at –80°C for subsequent analysis of interleukins-6 (IL-6) and –8 (IL-8) using ELISA.

Data Handling and Statistical Methods

UPREST, UPASTHMA, and UP50 utilized a standard, two-period cross-over design in which each subject received both particles and air. Equal numbers of males and

females were included. The order of presentation was randomized separately for each gender, with half of each group of subjects receiving each of the two possible orders. A wash-out period was included between the two exposures, of sufficient duration that carry-over effects from the first period to the second were expected to be minimal or nonexistent. UPDOSE utilized a three-period cross-over design in which each subject received both low (10 $\mu g/m^3$) and high (25 $\mu g/m^3$) concentrations of particles, and air. There were then three possible exposure sequences, depending on where in the sequence the air exposure was placed. Equal numbers of subjects were randomly assigned to each sequence.

The standard analysis for continuous endpoints is a repeated measures analysis of variance (ANOVA). In this analysis, order of presentation is a between subjects factor, while treatment period and time (when there are repeated measurements after each exposure) are within subject factors. The analysis included tests for period and carry-over effects, although the latter were expected to be minimal because of the nature of the exposures and the length of the wash-out period. In cases where carry-over effects were highly significant, first period data were examined separately (Jones, B., *et al*., 1989). For some endpoints repeated measurements were made at uniform intervals after each exposure. In this case the ANOVA included tests for an effect of time as well as interactions with other effects in the model. The ANOVA model and its interpretation have been discussed by Wallenstein and Fisher (Wallenstein, *et al*., 1977). Each ANOVA included an examination of residuals as a check on the required assumptions of normally distributed errors with constant variance. If these assumptions were not satisfied, data transformations (for example, square-root transformation for cell counts)

were considered. In the special case that only one measurement is made in each period, the ANOVA simplifies to three different t tests, for treatment, period and carry-over effects (71). In this case nonparametric tests such as the Wilcoxon rank sum test can be used if the observations are not normally distributed. Paired t-tests were also performed comparing UFP vs. filtered air exposure for each time point. A level of 5% was required for statistical significance. Data are shown as means ± SE, unless otherwise indicated.

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RESULTS

Subject and UFP Characteristics

A total of 56 subjects were studied, 28 male and 28 female. Sixteen subjects were enrolled with mild asthma. All subjects provided written, informed consent. Table 1 shows the subject characteristics and pulmonary function at screening for each of the four clinical studies. Table 2 shows characteristics of the inhaled UFP in each of the protocols.

Respiratory Symptoms

UFP exposure had no effects on respiratory symptoms in any of the study protocols. Total symptom scores and individual sums of scores for respiratory symptoms were similar with air and UFP exposures. Subjects were not able to identify the exposure atmosphere more often than expected by chance.

Pulmonary Function

In normal and asthmatic subjects, there were no significant or meaningful effects of UFP exposure on pulmonary function when dosages were less than 50 $\mu g/m^3$. Figure 1 shows the changes from baseline FEV_1 in UPDOSE and UPASTHMA. In contrast, the FEF_{25-75} decreased 21 hours after exposure to 50 $\mu g/m^3$ UFP, and was lower than the corresponding value after air exposure (change in FEF25-75 after UFP = -4.34 ± 1.78% vs. air = 1.08 ± 1.86%, p = 0.04, Figure 2). This difference disappeared when re-measured 45 hours after exposure.

We observed a significant change in DL_{CO} 21 hours after exposure to 50 $\mu g/m^3$ UFP when compared with air (change in DL_{CO} after UFP = -1.76 ± 0.66 ml/min/mmHg vs. air = -0.18 ± 0.41 ml/min/mmHg, p = 0.04, Figure 3). This difference also disappeared when DL_{CO} was re-measured at 45 hours (change in DL_{CO} after UFP = -0.67 ± 0.0.65 ml/min/mmHg vs. air = -0.54 ± 0.37 ml/min/mmHg, p = 0.90). An earlier effect on DL_{CO} cannot be determined from this study, because measurements were limited to 21 and 45 hours after exposure. There were no other differences in pulmonary function between air and UFP exposure in the UP50 protocol.

Minute ventilation was measured during exposure in each of the protocols, and it was slightly lower during resting exposure to 10 $\mu g/m^3$ UFP in UPASTHMA (UFP = 12.75 ± 0.46 vs. air = 13.78 ± 0.58, p=0.007). In UP50, the tidal volume during exercise was slightly lower during UFP exposure (UFP = 1774.2 ± 146.3 ml vs. air = 1903.4 ± 152.6 ml, p = 0.035), although minute ventilation and respiratory rate did not change significantly.

Measures of Airway Inflammation

There were no consistent UFP-induced changes in alveolar or conducting airway nitric oxide variables in any of the protocols. Flow rates sufficient for calculation of all conducting and alveolar NO parameters were available in 11 subjects from UPASTHMA and 14 subjects from UP50. There were no differences in conducting airway NO parameters. Figure 4 shows the results for alveolar airway NO exchange parameters in the UP50 protocol. There was a trend toward a reduction in DL_{NO} after exposure to 50 $\mu g/m^3$ UFP, but this change did not achieve statistical significance. In UP50, changes

in $\dot{V}_{LNO}$ and P_A were significantly different after UFP exposure vs. after filtered air by ANOVA (UFP exposure x time, p = 0.030 and p = 0.039 respectively) but not by individual t-test comparisons at the different time points after exposure. .

There were no convincing changes in airway cell differential counts or sputum cytokine concentrations in response to carbon UFP exposure in any of the three studies in which these endpoints were examined. Figure 5 shows the sputum cell differential counts. UFP exposure was associated with a small but statistically significant increase in the percentage of alveolar macrophages in patients with asthma (UFP = 62.6 ± 3.0 vs. air = 51.8 ± 5.7%, p=0.019 [ANOVA]), without significant changes in other cell types, or in total cell recovery. Concentrations of inflammatory cytokines interleukin-6 and -8 in the sputum were unaffected by UFP exposure in any of the studies.

DISCUSSION

This report describes the first human studies examining clinical responses to ultrafine particle inhalation. These studies demonstrate that there are only minimal changes in pulmonary function after carbon ultrafine particle inhalation in healthy and asthmatic subjects. The most notable findings were a reduction in FEF_{25-75} and DL_{CO} after 50 $\mu g/m^3$ UFP exposure in normal subjects. The first effect appeared dose-dependent, since it was only observed during inhalation of the highest particle concentration. Both findings were transient and reversible, since they resolved when measurements were repeated 45 hours after exposure.

The reduction in FEF_{25-75} suggests mild airways obstruction. The small magnitude of the change and the lack of a significant concomitant change in FEV_1 indicates that this effect has no clinical significance in healthy subjects. However, in patients with underlying lung disease and tenuous respiratory status, even small increases in airways obstruction could be of major clinical significance. In this context, it is relevant to note that many exacerbations of asthma or chronic obstructive pulmonary disease occur without clinically identifiable precipitants.

The effect on FEF_{25-75} could be due to increased airway secretions, or a reduction in airway luminal diameter. The former is less likely, since there were no convincing changes in sputum cells or cytokines after inhalation of lower particle concentrations. However, an effect on airway secretions cannot be entirely ruled out since sputum induction was not performed with the UP50 protocol. It is more likely that UFP exposure caused a reduction in airway caliber. Such an effect could be due to UFP-mediated

airway inflammation, stimulation or inhibition of endogenous bronchoconstrictors or bronchodilators, or direct stimulation of airway smooth muscle. We assessed airway inflammation by measuring airway nitric oxide dynamics and induced sputum parameters. The lack of significant change in conducting airway nitric oxide production, coupled with the lack of recruitment of inflammatory cells to the airways, provides evidence that these concentrations of carbon UFP do not induce significant airway inflammation. We could not assess endogenous bronchodilators or bronchoconstrictors, or direct effects of UFP on airway smooth muscle. Thus, the specific mechanism responsible for this increase in airway resistance remains unknown. It is conceivable that the significance value for this finding by paired t-test was observed by chance alone, since the ANOVA did not yield a significant result. Additional studies will be required to confirm our observation.

The DL_{CO} declined in healthy subjects twenty-one hours after inhalation of 50 $\mu g/m^3$ UFP, but returned to the baseline value when re-measured after 45 hours. In considering the potential etiology of this transient yet robust finding, it is useful to consider the components of the diffusing capacity measurement as originally defined by Roughton and Forster (Roughton, et al., 1957). Their model states that gas uptake in the lungs depends on its diffusion across the alveolar-capillary membrane and through the plasma (extra-erythrocytic component), and its subsequent reaction with hemoglobin in the pulmonary capillary blood (intra-erythrocytic component). The latter, in turn, depends on the reaction rate between the gas and hemoglobin (Θ) and the volume of pulmonary capillary blood (V_C). It is evident, therefore, that the reduction in DL_{CO} after carbon UFP inhalation could result from factors which slow gas diffusion or reduce its

reaction rate with hemoglobin. The former include pulmonary edema or an acute pulmonary inflammatory response. The latter include anemia or pulmonary vasoconstriction (both causing a decline in V_C), or poisoning of the hemoglobin molecule itself (reducing Θ). Transient pulmonary edema or inflammation of the lower respiratory tract might be expected to induce a restrictive ventilatory defect, which was not observed -- the forced vital capacity did not change after UFP exposure. Moreover, an inflammatory response sufficient to cause a reduction in gas diffusion is unlikely since alveolar airway nitric oxide production ($\dot{V}_{LNO}$) did not rise. Anemia did not occur. Of the remaining possible etiologies, UFP-induced pulmonary vasoconstriction seems more likely than direct UFP-induced hemoglobin poisoning. We are unaware of any evidence supporting direct effects of PM on hemoglobin function. Conversely, there is experimental data supporting particle-induced vasoconstriction. This link is suggested by data showing that inhaled particulate matter can induce endothelial dysfunction and systemic vasoconstriction as assessed by brachial artery ultrasound examinations (Brook, 2002). Moreover, a reduced lumen/wall area ratio of small pulmonary arteries was recently shown in rats exposed to concentrated ambient particles (Batalha, 2002), suggesting that inhaled PM can induce pulmonary vasoconstriction.

The precise mechanisms underlying reduced gas diffusion 21 hours after UFP exposure cannot be confidently determined from these clinical studies. Again, the significant p-value obtained by t-test was less striking when the data was subjected to ANOVA ($p = 0.062$, UPF, exposure x time). Additional studies are necessary to confirm the significance of this preliminary finding, and investigate whether vasoconstriction is an important mechanism accounting for adverse health effects of UFP. This will require

studies correlating measures of pulmonary gas diffusion with levels of endogenous vasodilators, vasoconstrictors, and markers of endothelial dysfunction in the blood. Changes in blood pressure and heart rate were not observed in any of these studies. If there is a systemic vasoconstrictive effect, it is probably more subtle than can be detected by these gross measurements. Alternative techniques, such as forearm occlusion plethysmography and brachial artery ultrasound, may be more useful and should be considered in future studies. Finally, since the DL_{CO} effect appears acute and reversible, additional measurements between exposure and 21 hours will be helpful in determining its time course.

The reduction in minute ventilation during UFP exposure is small and of uncertain etiology and significance. It is unlikely to be a voluntary effect since the subjects and investigators were blinded to exposure. Instead, mild airway irritant effects may explain these observations. This speculation is supported by the concomitant reduction in FEF_{25-75} during UP50, and because only asthmatics, with their baseline abnormalities in airway function, were susceptible to this effect at lower UFP concentrations.

Our choices of particle and exposure system were based several considerations. First, a laboratory exposure setting permitted careful and reproducible control of exposure conditions and particle concentrations. Second, elemental carbon is a universal component of ambient combustion particles, and carbon content is often considered a signature of combustion-related particles. Finally, because these were the first health studies of UFP exposure, we chose a relatively inert particle composition for safety reasons. We must stress that the findings observed with carbon UFP may not be

applicable to ambient ultrafine particles. Indeed, since these manufactured particles are probably less toxic than ambient particles, it is conceivable that the observed effects would be magnified after ambient ultrafine particle exposure.

Our maximum exposure concentration of 50 $\mu g/m^3$ is within the range of measured ambient UFP levels. For example, background urban levels of UFP have been reported as approximately 40-50,000 particles/cm^3, equivalent to an estimated mass concentrations of 3-4 $\mu g/m^3$ (Peters, et al. 1997). However, episodic increases have been documented up to 300,000 particles/cm^3, equal to ~50 $\mu g/m^3$ as an hourly average after subtraction of the background larger particles (Brand, et al. 1991; Brand, et al. 1992). More recently, Kittelson and colleagues found up to 10^7 particles/cm^3 inside a vehicle travelling on a major highway (Kittelson, et al. 2001), similar to the number concentration used in UP50. In Southern California UFP near major highways may periodically account for up to 1/3 of $PM_{2.5}$, with UFP mass concentrations exceeding 50 $\mu g/m^3$ (Zhu, et al. 2002). Thus, peak ambient exposure concentrations, whether mass or number, may exceed the concentrations used in these studies. By choosing a 50 $\mu g/m^3$ maximum exposure concentration, we strove to detect biological effects while minimizing subject risk.

There is sparse evidence from other human studies indicating that exposure to concentrated ambient particles (not ultrafine) can induce lung inflammation or significant changes in pulmonary function. Human clinical studies of exposure to diesel exhaust (Salvi, et al., 1999) showed evidence of distal airway inflammation although no acute effects on lung function were observed. Ghio, et al. (2000) found modest increases in polymorphonuclear leukocytes recovered in bronchoalveolar lavage fluid 24 hours

following 2-hour exposures to concentrated ambient particles (CAP), at concentrations up to 311 $\mu g/m^3$. These investigators also did not observe any effects on symptoms or pulmonary function. Gong, et al. (2003) studied healthy and asthmatic subjects exposed to CAP at a concentration of 174 $\mu g/m^3$, with intermittent exercise. There were no effects on lung function and no evidence for airway inflammation as assessed by induced sputum analysis.

It is difficult to compare the findings from these studies with our own, largely because of differences in the exposure atmospheres. The ambient particle concentrators used in all of the above studies are only able to concentrate ambient fine particles, so their exposure systems could not specifically assess the effects of UFP. In addition, ambient particulate matter represents a complex mixture of chemical species, including organic compounds and metals, which may be key mediators of PM effects (Nel A.E., et al., 2001, Ghio, A.J., et al., 1999, Huang, Y., et al., 2003). In our own studies, mass concentrations were approximately an order of magnitude lower than in the CAP studies, but particle number and surface area were certainly higher. Our particles consisted of elemental carbon, without significant content of organic species, metals, oxides, or sulfates. However, even elemental carbon ultrafine particles, by virtue of their large surface area, may carry an increased burden of reactive oxygen species in comparison with larger particles on a mass basis (Li, N., et al. 2003). Ambient ultrafine particles have been shown to induce heme-oxygenase *in vitro* (Li, N., et al., 2000). It is possible that these reactive oxygen species cause bronchial smooth muscle contraction and pulmonary vasoconstriction (perhaps via endothelial dysfunction), while causing minimal or even negligible cellular inflammatory responses. This could explain our observations

of relative reductions in FEF_{25-75} and DL_{CO} in the absence of detectable airway inflammation.

It is also possible that our methods for assessing airway inflammation (exhaled nitric oxide and induced sputum) lacked sufficient sensitivity to detect the inflammatory response generated by UFP. Both methods, however, are accepted as sensitive techniques for assessing lung inflammation (Pizzichini, 1996, ATS, 1999). Moreover, newer methods for analyzing exhaled NO which permit separate measurements of NO exchange in the conducting and alveolar airways (Tsoukias, et al., 1998, Pietropaoli, et al., 1999, Jorres, et al., 2000), as utilized in UPASTHMA and UP50, have been validated in other settings (Silkoff, 2000, Shin, 2002, Girgis, 2002). These methods provide more specific data about the origin of exhaled NO than currently recommended methods allow (Kharitonov, 1997, ATS, 1999). Our findings of statistically significance changes in $\dot{V}_{LNO}$ and P_A with ANOVA are difficult to interpret. Visual inspection of figure 4 demonstrates inconsistent variation in these parameters after air exposure. Therefore, we are unable to attribute the ANOVA results to any significant clinical or pathopthysiologic process associated with UFP exposure. Additional studies should help to clarify this issue. Importantly, there is no evidence that $\dot{V}_{LNO}$ increases after UFP, a finding we would expect if carbon UFP induced alveolar inflammation. We consider it unlikely that a significant inflammatory reaction in either the alveolar or conducting airways was missed.

Available animal studies also show only minimal and inconsistent pulmonary inflammatory effects resulting from UFP exposure. Oberdörster and colleagues (2000)

studied mice and rats of varying ages with either normal or abnormal underlying pulmonary function. When normal animals were exposed to carbon or platinum UFP at concentration of approximately 110 $\mu g/m^3$ for 6 hours, pulmonary inflammation was not observed, as assessed by biochemical and cellular analyses of bronchoalveolar lavage fluid and tissue extracts, as well as histological assessment. When aged animals with underlying emphysema were studied there were small inflammatory changes evident, including very slight increases in lavage neutrophils and peribronchial lymphocytes. Only additional pretreatment with endotoxin or co-treatment with ozone during carbon UFP inhalation revealed a slightly augmented percentage of neutrophils in lavage fluid (Elder, et al. 2000). More obvious pulmonary inflammation was evident when titanium UFP were intratracheally instilled at higher doses, and when UFP generated by heating with polytetraflouroethylene were co-inhaled with their gas phase constituents, but the relevance of these findings to routine human UFP exposure is unknown. These data indicate that UFP do not cause significant lung inflammation in isolation. Furthermore, UFP require either a susceptible host, toxic co-factors, or both to induce even subtle inflammatory pulmonary responses in animals.

In summary, these studies of carbon UFP exposure demonstrate subtle effects on pulmonary function in normal human subjects. The main findings were small increases in airways resistance and reductions in carbon monoxide diffusing capacity, occurring in the absence of a detectable pulmonary inflammatory response. These effects were dose related and reversible. Based on these data, we speculate that UFP may induce bronchoconstriction (from alterations in endogenous mediators of airway tone or direct effects on airway smooth muscle), and pulmonary vasoconstriction. Concentrations of

carbon UFP below 25 $\mu g/m^3$ do not have appreciable effects on pulmonary function in normal or asthmatic subjects. Variations in particulate matter composition may alter these pulmonary function effects, and the threshold dose for their occurrence. Further clinical studies are needed to confirm these findings, determine their relationship to particulate matter composition, and investigate their mechanisms.

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FIGURE LEGENDS

Figure 1. Per cent change in FEV_1 before and after exposure to filtered air vs. UFP -- UPDOSE and UPASTHMA. There were no significant differences between filtered air and UFP in either study. Data represent means ± S.E.

Figure 2. Per cent change in FEF_{25-75} before and after exposure to filtered air vs. UFP -- UP50. FEF_{25-75} decreased progressively after exposure to 50 $\mu g/m^3$ UFP. After 21 hours, FEF_{25-75} increased back toward the baseline value in the UFP-exposed subjects. *p = 0.04 UFP vs. filtered air at 21 hours, paired t-test. ANOVA was not significant.

Figure 3. Change in DL_{CO} before and after exposure to filtered air vs. UFP -- UP50. There was a significant decline in DL_{CO} 21 hours after exposure to 50 $\mu g/m^3$ UFP. This difference resolved when the measurement was repeated 45 hours after exposure. ♣p = 0.06, ANOVA, UFP exposure x time; *p = 0.04 UFP vs. filtered air at 21 hours, paired t-test.

Figure 4. Change in alveolar airway NO parameters after exposure to filtered air vs. UFP -- UP50. DL_{NO} declined in normal subjects after exposure to 50 $\mu g/m^3$ UFP, but the difference was not statistically significant. Consistent patterns of change in $\dot{V}_{LNO}$ and P_A were not detected, although the differences between exposures were statistically significant by ANOVA. Significant differences were not observed when the values measured at individual time points were compared with paired t-tests. ♣p = 0.03, ANOVA, UFP exposure x time; *P = 0.04, ANOVA, UFP exposure x time.

Figure 5. Percentage of cells in induced sputum after exposure to filtered air vs. UFP -- UPREST, UPDOSE, and UPASTHMA. The only significant change was a slight increase in the percentage of macrophages in asthmatics after exposure to 10 $\mu g/m^3$ UFP.

Table 1. Subject characteristics and baseline pulmonary function*

	UPREST	UPDOSE	UPASTHMA	UP50
Age (yrs)	30.1±8.9	26.9±5.8	23.0±2.7	26.9±6.5
M/F	6/6	6/6	8/8	8/8
FEV_1 (% predicted)	103.8±8.0	106.3±16.6	97.6±5.0	102.8±9.5
FVC (% predicted)	99.9±8.3	103.5±15.6	106.2±14.5	105.3±11.0
FEV_1/FVC (%)	87.9±4.2	86.8±5.2	77.8±6.9	82.6±5.2
FEF_{25-75} (% predicted)	113.9±24.2	106.3±22.0	77.6±29.7	92.8±16.9
D_LCO (% predicted)	90.9±11.5	89.8±18.2	99.7±12.5	92.7±3.8

*Data are means±SD.

Table 2. UFP Parameters for all study protocols*

	Uprest (n=12)	**Updose(n=12) (Low dose)**	**Updose(n=12) (Higher dose)**	**Upasthma (n=16)**	**Up50 (n=16)**
Target Mass Conc. ($\mu g/m^3$)	10	10	25	10	50
Measured Mass Conc.($\mu g/m^3$)	10.00 ± 2.14	13.87 ± 4.02	28.46 ± 5.13	11.08 ± 3.11	49.97 ± 3.88
Number Conc. ($x10^6 p/cm^3$)	1.88 ± 0.09	2.04 ± 0.07	6.96 ± 0.10	2.20 ± 0.10	10.79 ± 1.66
CMD (nm)	27.30 ± 2.50	25.22 ± 1.71	26.46 ± 1.54	23.12 ± 1.56	27.92 ± 2.17
Dia. of Avg. Vol.(Mass) (nm)**	38.64 ± 3.63	35.30 ± 2.41	36.92 ± 2.30	33.38 ± 2.23	40.90 ± 3.39
GSD	1.62 ± 0.02	1.60 ± 0.02	1.60 ± 0.02	1.64 ± 0.01	1.65 ± 0.02

*Mean ± SD. All numbers were calculated in one-hour intervals.
**Assumes a log normal distribution given the stated GSD
Abbreviations: CMD – Count Median Diameter, GSD – Geometric Standard Deviation.

Figure 1.

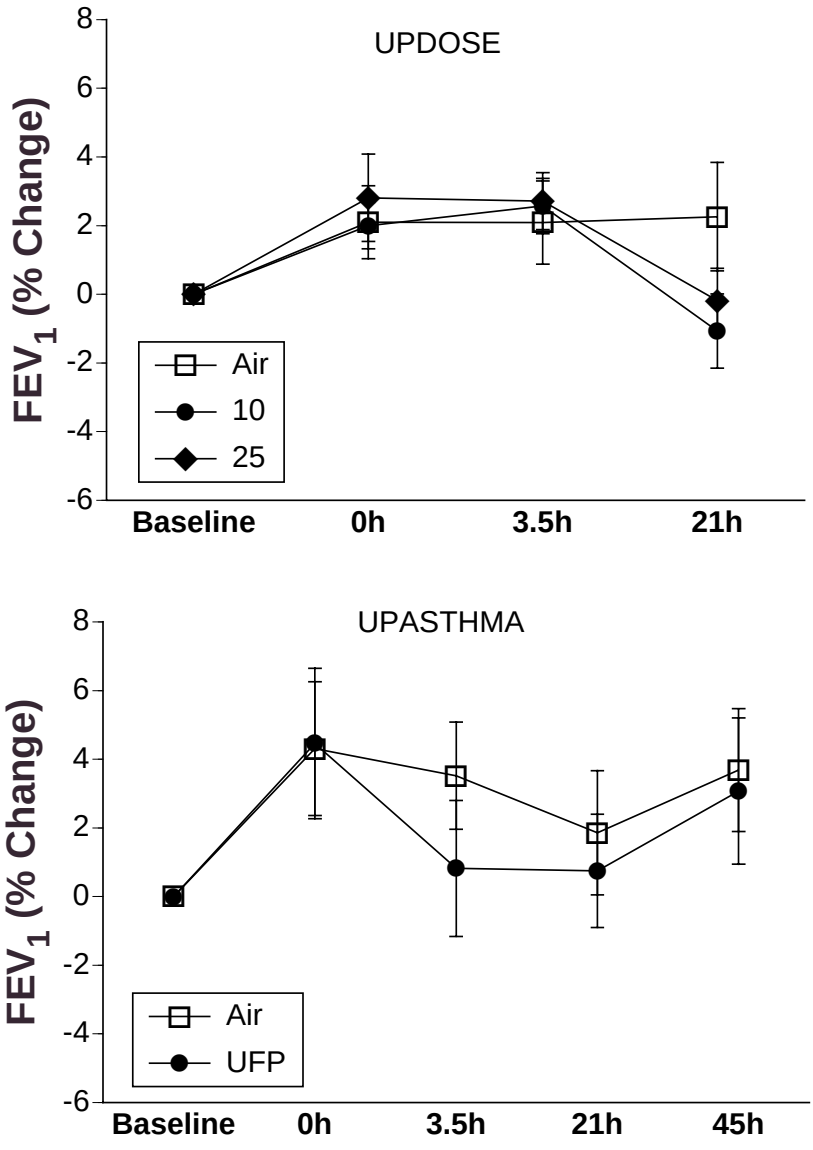

UPDOSE
FEV1 (% Change)
8
6
4
2
0
-2
-4
-6
Air
10
25
Baseline
0h
3.5h
21h
UPASTHMA
FEV1 (% Change)
8
6
4
2
0
-2
-4
-6
Air
UFP
Baseline
0h
3.5h
21h
45h

Figure 2.

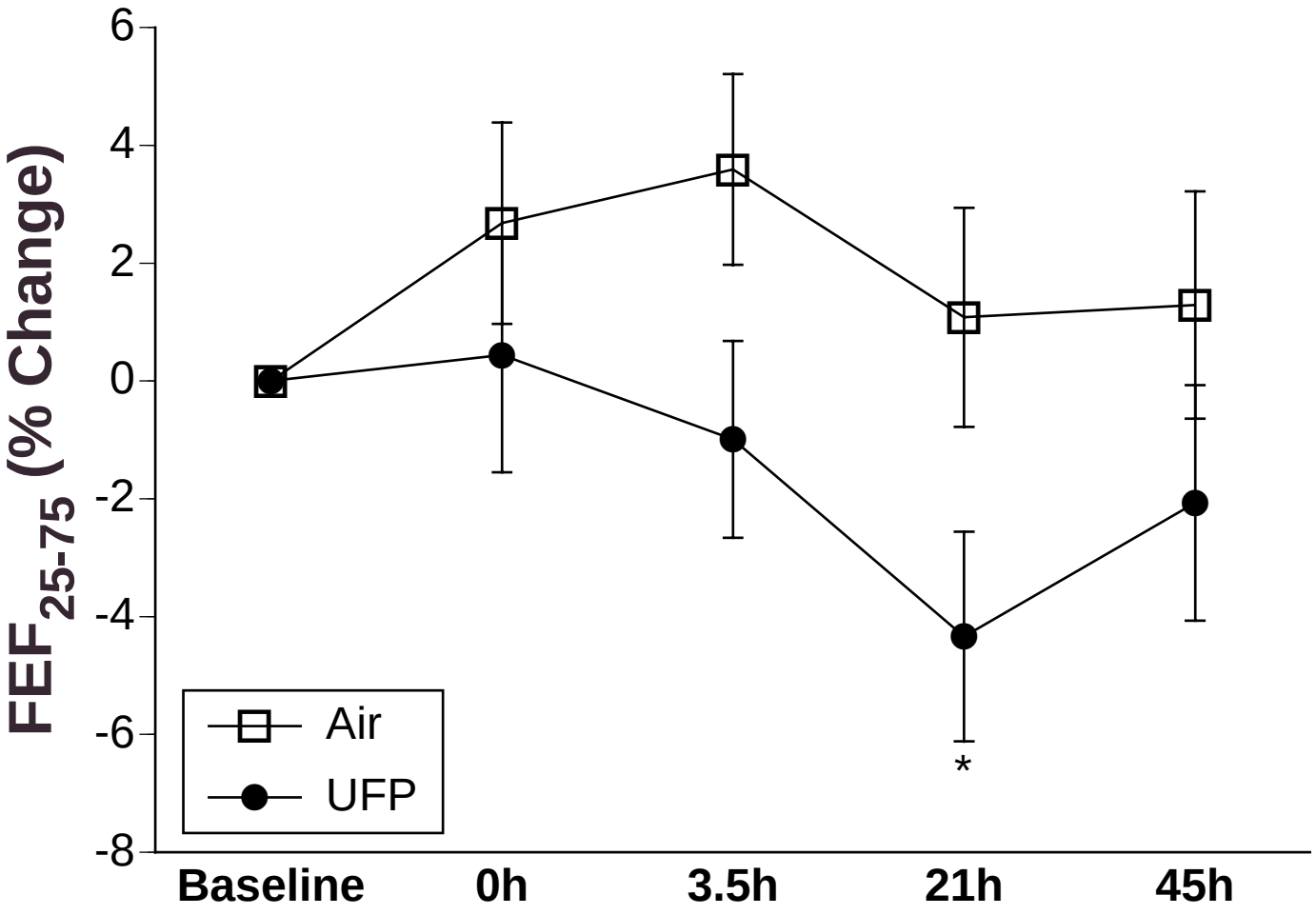


Figure 3.

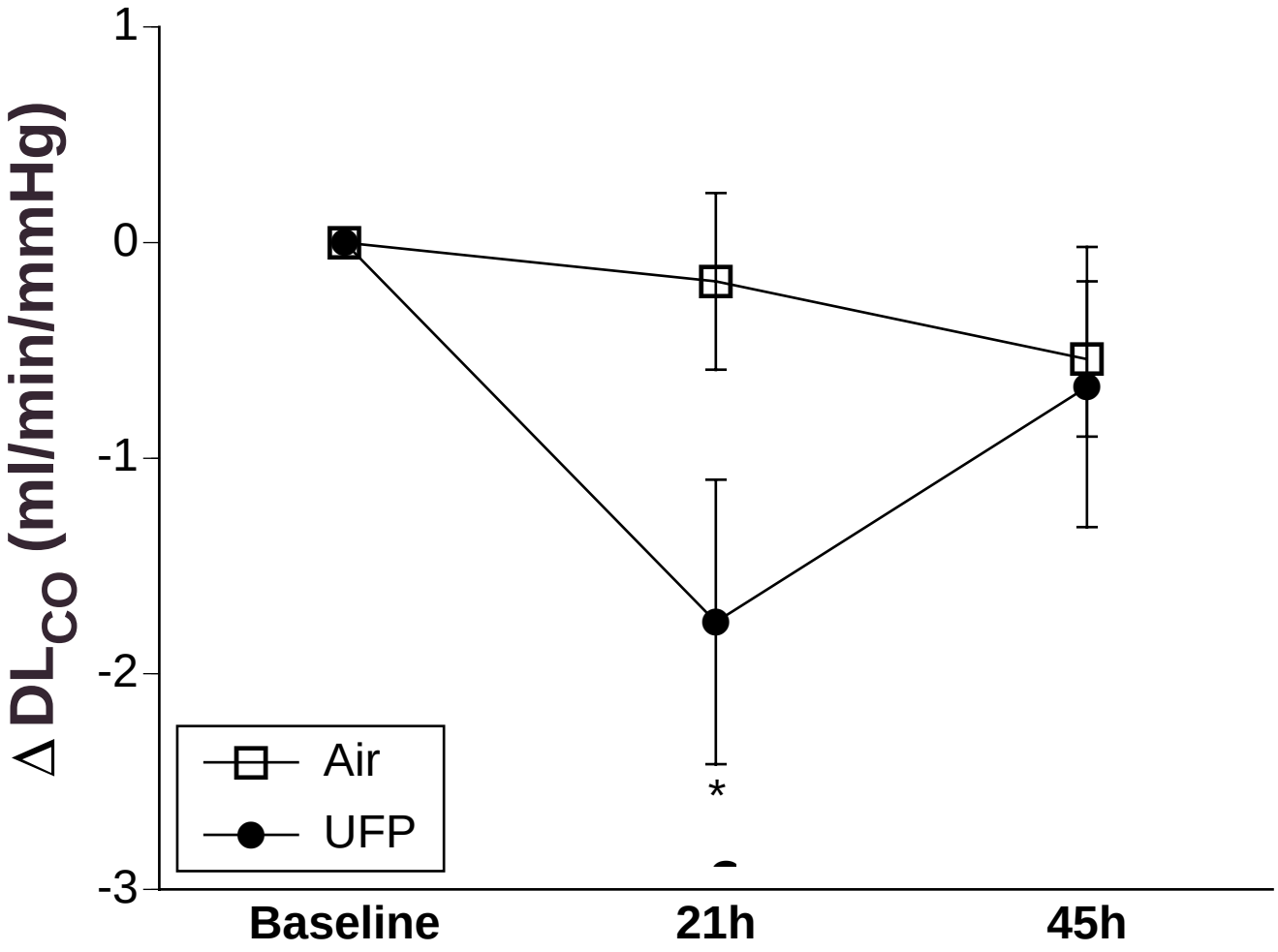


Figure 4.

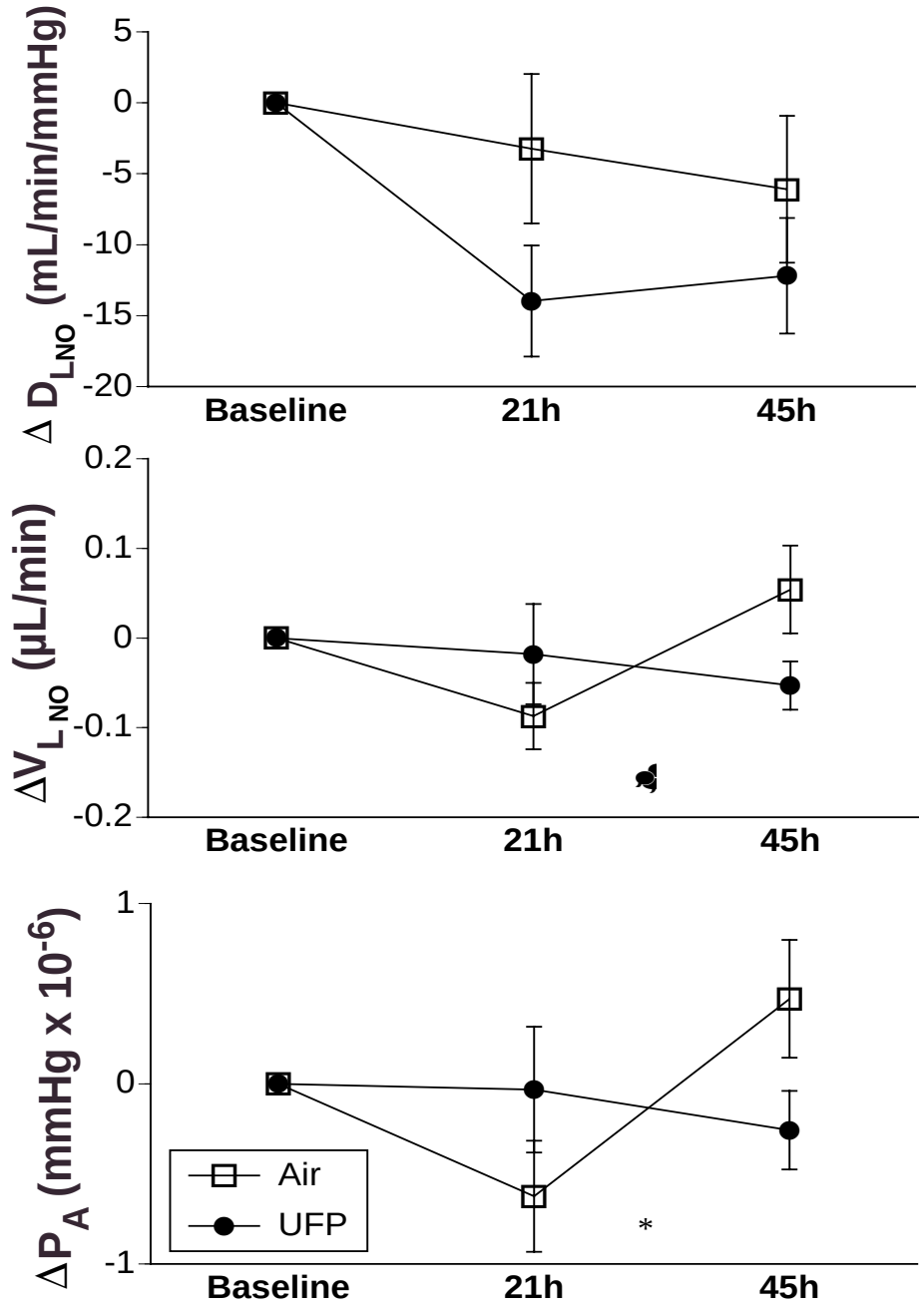
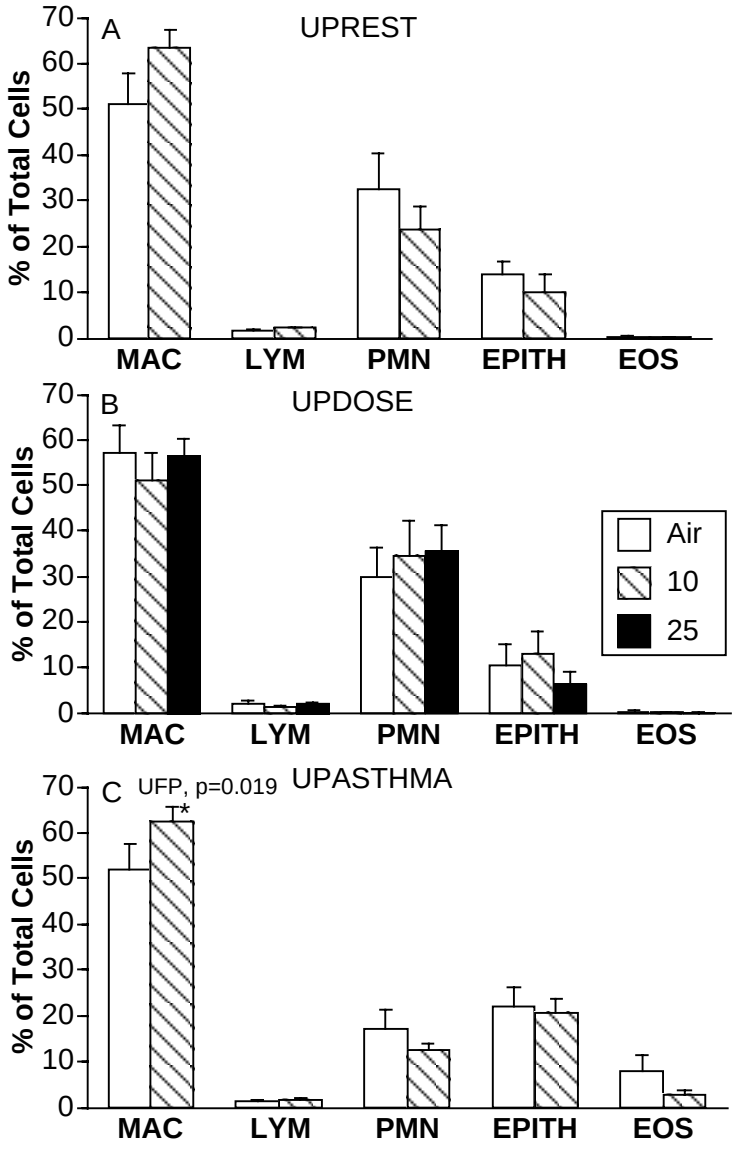

Δ D_LNO (mL/min/mmHg)
5
0
-5
-10
-15
-20
Baseline
21h
45h
ΔV_L NO (μL/min)
0.2
0.1
0
-0.1
-0.2
Baseline
21h
45h
ΔP_A (mmHg x 10^-6)
1
0
-1
Air
UFP
*
Baseline
21h
45h

Figure 5.


A
UPREST
% of Total Cells
70
60
50
40
30
20
10
0
MAC
LYM
PMN
EPITH
EOS
B
UPDOSE
Air
10
25
C
UFP, p=0.019
UPASTHMA
*