FINAL REPORT

Mechanisms of Particulate Toxicity: Health Effects in Susceptible Humans

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Abstract

Particulate air pollution is an issue of global health importance, being a major component of air pollution, and having been associated with increased human cardiorespiratory morbidity and mortality. Individuals with asthma could be particularly susceptible to the respiratory health effects of particulate air pollution, due to the airway inflammation and hyper-reactivity components of the disease. This project was designed to test the hypothesis that airway inflammation would be increased, and spirometric pulmonary function (SPF) and heart rate variability (HRV), would be decreased as a function of a single particle exposure, and to a larger degree, as a function of a combined particle and O_3 exposure and a serial particle exposure, compared to filter air exposure. This project consisted of two controlled human exposure experiments. Experiment One: (N = 15; subjects with asthma); Exposure conditions were separate single (4 hr) exposures to each of Filtered-Air (FA); carbon particles and ammonium particles [300 $\mu g/m^3$] (P); P and O_3 [0.2 ppm] (PO). Experiment Two: (N = 10; subjects with asthma); separate single exposures to FA; and P; and three serial-day exposures to P (P3). Bronchoscopy was conducted 18 hr post-exposure (final exposure in P3), SPF was measured immediately pre- and post-exposure and 18 hr post-exposure, and HRV was measured immediately pre-exposure and during the final 25 min of the exposure. Experiment One: In PO, compared to FA and/or P, in the airway lavages there were significantly higher inflammation-related cell numbers (neutrophils, lymphocytes, eosinophils), protein concentrations (total protein, granulocyte-monocyte colony stimulating factor, C-reactive protein), and cytokine gene expression (interluekin-8 and –10); significantly lower SPF (forced vital capacity, forced expired volume in 1 s; FEV_1); and significantly lower heart rate variability (time domain, and low and high frequency domain). In both PO and P, compared to FA, there was significantly lower SPF (forced expired volume between 25-75% of FVC; FEF_{25-75}). For P, compared to FA, there was significantly lower HRV (low frequency domain). Experiment Two: In P3 compared to both FA and P, in the airway lavages there was a significantly lower cell number (lymphocytes), and in P compared to FA, there was a significantly higher cell number (epithelial cells). With-in P3 at 18 hr post-exposure three, compared to post-exposure two and pre- and post-exposure three, there was significantly lower SPF (FEV_1). With-in the P3 condition at 18 hr post-exposure three, compared to both pre- and post-exposure two, there was significantly lower SPF (FEF_{25-75}). The results of this project indicate that in individuals with asthma, both single and serial exposures to carbon and ammonium nitrate particles can result in significant decreases in SPF, a low number of changes in airway cell distribution and HRV, and no changes in cytokine protein or gene expression. However, combined exposure to particles and O_3, in addition to decreases in SPF, produces increases in several inflammatory associated cells, increases in protein and gene expression, and multiple changes in HRV. It is expected that for the combined exposure these changes are due, at least primarily, to the O_3 component of the exposure environment. Further studies are required to investigate the health effects and control mechanisms of these changes in both healthy and other susceptible individuals.

Executive Summary

Background

Particulate air pollution is an issue of local, regional, and global importance, and is a major component of air pollution in both outdoor and indoor environments. Specifically, particulate air pollution has been associated with negative health effects pertaining to respiratory tract disease, including asthma. Additionally, particulate air pollution has been associated with adverse changes in a specific component of cardiac function, heart rate variability (HRV). As air pollution can be a combination of gases, liquids, and particles, these components could have interactive effects on the respiratory and cardiac systems. Potentially susceptible individuals, including individuals with asthma, could be at increased risk of negative health effects from exposure to both particulate and gaseous air pollution. Currently the mechanisms controlling particle-induced airway inflammation and changes in HRV in asthma are incompletely understood.

It was hypothesized that airway inflammation (as indicated by cell distribution, protein concentration; gene expression) would be increased, and spirometric pulmonary function (SPF) and HRV would be decreased, as a function of single particle exposure, and to a larger degree, as a function of combined particle and O_3 exposure and serial particle exposure, compared to filtered air exposure.

Materials and Methods

This project consisted of two controlled human exposure experiments. All subjects for both experiments were individuals with mild to moderate asthma. Experiment One: (15 subjects); exposure conditions were separate single exposures to each of Filtered-Air (FA); carbon and ammonium-nitrate particles [300 $\mu g/m^3$] (P); P and O_3 [0.2 ppm] (PO): Experiment Two (10 subjects); exposure conditions were separate single exposures to FA; and P; and three serial-day exposures to P (P3). The duration of all the exposures was 4 hr, during which subjects alternated exercise and rest at 30 min intervals. Bronchoscopy for bronchoalveolar lavage, epithelial brushing, and bronchial biopsies, was conducted 18 hr post-exposure (final exposure in P3), SPF was measured immediately pre- and post-exposure and 18 hr post-exposure, and HRV was measured immediately pre-exposure and during the final 25 min of the exposure.

Results

Experiment One:

In PO, compared to FA and/or P, in the airway lavages there were significantly higher inflammation-related cell numbers (neutrophils, lymphocytes, eosinophils), protein concentrations (total protein, granulocyte-monocyte colony stimulating factor, C-reactive protein), and cytokine gene expression (interluekin-8 and –10); significantly lower SPF (forced vital capacity, forced expired volume in 1 s; FEV_1); and significantly lower heart rate variability (time domain, and low and high frequency domain). In both PO and P, compared to FA, there was significantly lower SPF (forced expired volume between 25-

75% of FVC; FEF_{25-75}). For P, compared to FA, there was significantly lower HRV (low frequency domain).

Experiment Two:

In P3 compared to both FA and P, in the airway lavages there was a significantly lower cell number (lymphocytes), and in P compared to FA, there was a significantly higher cell number (epithelial cells). With-in P3 at 18 hr post-exposure three, compared to post-exposure two and pre- and post-exposure three, there was significantly lower SPF (FEV_1). With-in the P3 condition at 18 hr post-exposure three, compared to both pre- and post-exposure two, there was significantly lower SPF (FEF_{25-75}).

Discussion

The results of this project indicate that both single and serial exposures to carbon and ammonium nitrate particles result in low-level changes in airway cell distribution, decreases in SPF, and a decrease in HRV in individuals with asthma. Further, combined exposure to particles and O_3 produced increases in inflammatory associated cells, and protein and mRNA levels, decreases in SPF, physiologically relevant decreases in HRV. It is expected that the changes in the combined particle and O_3 exposure condition were due, at least primarily, to the O_3 component of the exposure environment. The main novel finding in the combined exposure was the decrease in multiple indices of HRV, which potentially has direct implications for O_3-induced adverse cardiac health effects. Further studies are required to investigate the health effects and control mechanisms of these changes in both healthy and other susceptible individuals.

Introduction

Particulate air pollution is an issue of local, regional, and global importance (Akimoto, 2003), as it is a major component of air pollution in both outdoor and indoor environments (Dockery *et al.,* 1993; Pope *et al.,* 1991; Schwartz, 1993; Stern *et al.,* 1989). Airborne particulate pollution has been associated with increased human morbidity and mortality (Reviewed: UNEP and WHO 1994; US EPA, 1995). Specifically, particulate air pollution has been associated with negative health effects pertaining to respiratory diseases, including asthma (Reviewed: ATS, 1996). Additionally, particulate air pollution has been associated with negative changes in a specific component of cardiac function, heart rate variability (HRV) (Gold *et al.* 2000; Pope *et al.* 1999), which have been associated with increased cardiac morbidity and mortality (ESC and NASPE, 1996). As environmental air pollution can be a combination of gases, liquids, and particles, these components could have interactive effects on the respiratory and cardiac systems. Potentially susceptible individuals, including individuals with asthma, have been shown to be at increased risk of negative health effects from exposure to both particulate and gaseous air pollution (Reviewed: Peden, 2002). Currently, the biological mechanisms controlling particle-induced airway inflammation and changes in HRV in asthma are incompletely understood.

Respiratory Effects of Particulate Air Pollution:

Epidemiological Studies:

Epidemiological research has indicated that there are associations between increased levels of ambient particulate matter and increased adverse respiratory health effects (Dockery *et al.,* 1993; Greenburg *et al.,* 1967; Knight *et al.,* 1989; Martin, 1964; Pope and Kranner, 1993; Pope *et al.,* 1991; Samet *et al.,* 1981; Schenker, 1993; Schwartz, 1993; Stern *et al.,* 1989; Whittemore and Korn, 1980). For airborne particle matter with a mass median aerodynamic diameter (MMAD) of less than 10 μm in diameter (PM_{10}), there are positive associations between particulate levels and decreased pulmonary function (Pope *et al.,* 1991; Stern *et al.,* 1989; Pope and Kranner, 1993), increased number of asthma attacks (Whittemore and Korn, 1980; Schenker, 1993), increased asthma medication usage (Pope *et al.,* 1991), increased emergency room visits for respiratory illness, and increased hospital admissions (Greenburg *et al.,* 1967; Knight *et al.,* 1989; Martin, 1964; Samet *et al.,* 1981), and increased daily mortality (Dockery *et al.,* 1993, Schwartz, 1993). The positive associations between PM_{10} exposure and health effects have been found at particle concentration levels below the USA national standard of 150 $\mu g/m^3$ (24 hr average) (Reviewed: US EPA, 1995).

Controlled Human Exposure Experiments:

In healthy subjects, controlled exposure to concentrated ambient particles (CAPS) results in an increase in neutrophils in bronchoalveolar lavage fluid (BAL) (Ghio *et al.* 2000), and a decrease in columnar cells in induced-sputum (Gong *et al*. 2003). However, in healthy subjects CAPS exposure does not result in any changes in

inflammatory cytokines [interleukin (IL)-6, IL-8] in BAL (Ghio *et al.* 2000), indices of inflammation in bronchial tissue (Holgate *et al.* 2003), or spirometric pulmonary function (SPF) (Ghio *et al.* 2000; Gong *et al*. 2003).

In subjects with asthma, CAPS exposure also results in a decrease in columnar cells in induced-sputum, but no change in SPF (Gong *et al*. 2003). Controlled exposure of subjects with asthma to generated ammonium bisulfate particles at a concentration of 450 $\mu g/m^3$ resulted in a decrease in specific airway conductance (Utell *et al.*, 1983). Other controlled exposures of subjects with asthma to generated ammonium nitrate particles (Kleinman *et al.*, 1980), carbon particles (Anderson *et al.*, 1992), and sodium bisulfate, and ammonium sulfate particles (Utell *et al.*, 1983), did not result in any changes in SPF.

Indices of Airway Inflammation:

Changes in differential leukocyte cell counts (specifically increased neutrophils and macrophages), in BAL are used as the primary measure of airway inflammation following air pollutant exposures (Balmes *et al.*, 1996; Solomon *et al.*, 2000). Specific proteins measured in the fluid phase of BAL are indicative of airway inflammation and/or injury including, total protein which is an indicator of epithelial permeability, measuring protein extravagation into the airway, C-reactive protein which is an non-specific indicator of inflammation, IL-8 which is a neutrophil chemoattractant, and granulocyte and macrophage colony stimulating factor (GMCSF) which is an indicator of leukocyte recruitment.

To add to the information obtained from protein levels, and when protein levels can not be determined, measurement of mRNA expression in cells from BAL, and bronchial epithelial cells, for inflammation-associated genes can be used. The mediators for which measurement of mRNA is appropriate include, IL-1β,?an early-phase pro-inflammatory cytokine, IL-6, a pro-inflammatory and pyrogenic cytokine, IL-8, a neutrophil chemoattractant, IL-10, an anti-inflammatory cytokine, and high in normals-1 (HIN-1), an anti-proliferation cytokine involved in epithelial cell proliferation.

The timing of the measurement of these indices of airway inflammation is important so that a potential change in a variable is not missed. Inhaled toxin-induced changes in cells and cytokines in BAL have been measured at; 6 h (Salvi *et al.* 2000), and 18 h (Rudell *et al.* 1999), post-exposure to diesel exhaust; 18 h post-exposure to concentrated ambient particles (Giho *et al.* 2000); at 1 h, 6 h, and 24 h post-exposure to ozone (O_3) in healthy individuals (Schelege *et al.* 1991); and 18 h post-exposure to O_3 in individuals with asthma (Balmes *et al.* 1996). As the exposure conditions in this project consisted of both particulate and O_3 components, toxins for which airway inflammatory changes (cells and cytokines) have been measured at 18 hr post-exposure (Balmes *et al.* 1996, Giho *et al.* 2000), this time interval was utilized for this project.

Heart Rate Variability and Particulate Air Pollution:

Heart Rate Variability:

Heart rate variability HRV is an indicator of cardiac autonomic function, reflecting the balance between sympathetic and parasympathetic nervous system input to the heart (Akselrod *et al.*, 1981; ESC and NASPE, 1996). Decreased HRV is correlated with increased risk of cardiovascular morbidity and mortality (Bigger *et al.*, 1992; ESC and NASPE, 1996, Kleiger *et al.*, 1987; Truji *et al.*, 1994; Truji *et al.*, 1996). This correlation may be explained by that loss of autonomic nervous system balance being the leading event in cardiovascular death.

Epidemiological Studies:

Epidemiological studies have indicated positive associations between the level of ambient particulate matter and changes in HRV (Gold *et al*, 2000; Pope *et al,* 1999). Increases in ambient air pollution particles have been associated with decreases in the standard deviation of all normal R-R intervals (SDNN) (Pope *et al,* 1999); and both SDNN and the root mean square of successive differences (r-MSSD) (in elderly individuals) (Gold *et al*, 2000).

Controlled Human Exposure Experiments:

Controlled human exposure to CAPS results in a decrease in the high frequency (HF) component of HRV, in elderly subjects, but not in younger subjects (Devlin *et al,* 2003). However, another controlled human exposure to CAPS found an increase in HF exposure in healthy and asthmatic subjects (Gong *et al,* 2003). In a semi-controlled (the exposure concentration was measured but not controlled) exposure study on environmental tobacco smoke, an increase in particle concentration was associated with a decrease in SDNN (Pope *et al*, 2001).

Mechanisms of Particle-Induced Effects in Cardiac Function:

It is possible that particle-associated mortality is explained, at least in part, by alterations in cardiac autonomic nervous system balance. Heart rate, cardiac contractility and myocardial conduction velocity are affected by autonomic balance. Thus, both heart rate variability and dysrhythmias are intimately connected with the autonomic nervous system and with each other. Multiple studies have demonstrated an association between cardiac autonomic balance and all-cause mortality (Truji *et al.*, 1994), sudden cardiac death (Algra *et al.*, 1993), and death due to congestive heart failure (Szabo *et al.*, 1997). Specifically, increased cardiac parasympathetic activity is likely to be protective against fibrillation, while both enhanced sympathetic activity and parasympathetic impairment are associated with a decrease in the arrhythmia threshold and an increased risk of malignant ventricular arrhythmias and death (Algra *et al.*, 1993; Klingenheben *et al.*, 1993; Szabo *et al.*, 1993). The physiological mechanisms underlying particle-induced changes in HRV are unknown, but may involve elevation of

systemic inflammatory mediators (Schwartz, 2001) or activation of pulmonary irritant receptors that mediate stimulation of parasympathetic pathways (Watkinson *et al*, 2001).

Indices of Heart Rate Variability:

Both time domain variables and frequency domain variables are used for the measurement of HRV.

Time-Domain Analysis:
In time-domain analysis, the principal measure is the standard deviation of the normal sinus R-R intervals (NN intervals) (termed the heart period) over time. Four time-domain variables that have been used to determine the effect of particle exposure on HRV in humans are: 1) the SDNN, a measure of overall HRV; 1) the standard deviation of means of all NN intervals (SDANN), a measure of the long-term components of HRV; 3) the standard deviation of all NN intervals index (SDNNi), a measure of the short-term components of HRV; and 4) the root mean square of successive differences (r-MSSD), a measure of the short-term component of HRV.

Frequency-Domain Analysis:
Frequency domain variables can be correlated with physiologic responses (ESC and NASPE, 1996). The low frequency (LF) is a marker for both sympathetic and parasympathetic modulation. The HF is a marker of parasympathetic influence on heart rate (vagal tone). The LF and HF can be normalized using the very low frequency (VLF) and total power, and these values better represent these physiologic relationships. The LF/HF ratio is an indicator of sympathovagal balance (Pagani *et al*, 1986). The physiologic significance, if any, of VLF is not known.

<u>Asthma Status and Particle Exposure</u>:

Under ambient conditions, both healthy individuals, and potentially susceptible individuals, including those with asthma, can be non-differentially exposed to particulate, and other, air pollution.

Asthma and Airway Inflammation:

Individuals with asthma have been shown to be at increased risk of negative respiratory health effects from exposure to both particulate and gaseous air pollution (Bosson *et al.* 2003; Reviewed: Peden, 2002; Scannell *et al.*, 1996). The increased response to air pollution in individuals with asthma, compared to healthy individuals, could be due to the underlying airway inflammation and/or non-specific airway hyperresponsiveness, which characterizes the disease.

Asthma and Heart Rate Variability:

Chronic pulmonary disease may decrease the tolerance for adverse effects of particles, resulting in a lower threshold for observable cardiorespiratory effects. Pre-existing pulmonary inflammation, as occurs in asthma, could facilitate particle-induced release of pro-inflammatory mediators, resulting in additional pulmonary inflammation, bronchoconstriction, hypoxemia, and cardiac effects including ventricular fibrillation and death (Godleski, 1996). It has been proposed that exposure to particles could provoke alveolar inflammation, causing exacerbations of existing lung disease and increased blood coagulability, leading in turn to cardiovascular deaths through direct effects on either the lungs or the heart (Seaton *et al.*, 1995).

Particle Composition:

Carbon and ammonium nitrate are major constituents of ambient air particulate matter in specific regions (Chow *et al.*, 1992; Gebhart and Malm, 1993; Hering, 1993; Solomon *et al.*, 1989). Carbon is a major component of ambient particulate matter (Akimoto, 2003), and forms the core many airborne particles, including combustion derived. Ammonium nitrate is present in ambient air in concentrations up to 120 $\mu g/m^3$, and is the most abundant particle compound in the Western United States (Hering, 1993; Solomon *et al.*, 1989). As both carbon and ammonium nitrate particles are major components of both outdoor and indoor air, they are relevant for investigating the effects of inhaled particles on airway inflammation and HRV in ambient environments. Both of these particle types may have toxic effects in the airway simply by mechanical impact or presence in the airway epithelium. However, as chemical composition determines the solubility, and oxidative and acid/base characteristics of the particle, chemical composition will have direct effect on the inflammatory capacity of different inhaled particles. This project will investigate carbon and ammonium nitrate particles only. However, due to the potential generic mechanical action of inhaled particles on or in the airway epithelium, the data obtained using these particle types could be applicable to particles of different chemical compositions.

Particle Concentration:

As particle concentration directly effects the total inhaled dose of particles, for any specific exposure time, the particle concentration is a primary factor in the potential toxicity of inhaled particles. The current USA national standard for PM_{10} is 150 $\mu g/m^3$ (as a 24 hr average). The particle mass concentration of 300 $\mu g/m^3$ used in this project was selected based on ambient environmental concentrations, the potential to find meaningful biological/physiological changes in the measured dependent variables, and for subject safety and health.

Combined Particle and Ozone Exposure:

Ambient air pollution is a combination of gases, liquids, and particles. Of the gaseous air pollutants, O_3 is one of the most predominant globally (Akimoto, 2003). Therefore, to

assess the effects of a combined particle-gas exposure on airway inflammation and HRV, the combination of O_3 and the described carbon and ammonium nitrate particles, is a relevant mixture.

Ozone and Asthma:

In individuals with asthma, controlled exposure to O_3 results in airway inflammation, including increases in neutrophils, total protein, IL-8, and granulocyte macrophage colony stimulating factor (GMCSF), BAL, and decreases in SPF including, FVC and FEV_1 (Scannell *et al.* 1996). Individuals with asthma, compared to healthy individuals, have a larger inflammatory response to inhaled O_3 (Bosson *et al.* 2003; Reviewed: Peden, 2002; Scannell *et al.*, 1996).

Ozone and Heart Rate Variability:

Epidemiological studies have indicated positive associations between increases in ambient O_3 and decreases in r-MSSD (Gold *et al*, 2000); and the HF and LF components of HRV (hypertensive individuals) (Holguin *et al*, 2003); and between increases in particles and O_3 combined, and decreases in r-MSSD (Gold *et al*, 2000). While no controlled human exposure studies have investigated the effects of O_3 or particles and O_3 combined on HRV, other cardiovascular effects have been reported; exposure to O_3 resulted in increased heart rate and rate-pressure product (health and hypertensive individuals) (Gong *et al,* 1998), and exposure to particles and O_3 combined decreased brachial artery diameter (Brook *et al,* 2002).

Objective and Specific Aims:

Currently the mechanisms controlling particle-induced airway inflammation and changes in HRV are incompletely understood. Specifically, the effects of single exposure to carbon and ammonium nitrate particles, combined particle and O_3 exposure, and serial particle exposures, on airway inflammation and HRV in asthma are unknown. The overall objective of this project was to investigate the effect of inhaled particles (single and serial exposures, and in combination with O_3) on airway inflammation and HRV in individuals with asthma.

Specific Aim One:

To determine the effect of a single exposure to carbon and ammonium nitrate particles on airway inflammation and HRV in individuals with asthma.

Specific Aim Two:

To determine the effect of exposure to the combination of carbon and ammonium nitrate particles and O_3 on airway inflammation and HRV in individuals with asthma.

Specific Aim Three:

To determine the effect of serial-day exposure to carbon and ammonium nitrate particles on airway inflammation and HRV in individuals with asthma.

Hypotheses

The hypotheses of this project were:
1) Airway inflammation, as indicated by cell distribution (macrophages, neutrophils, lymphocytes, eosinophils, epithelial cells), and protein (total protein, IL-8, GMCSF, CRP), and mRNA (IL-1β, IL-6, IL-8, IL-10, HIN-1) expression in BAL would be increased, and SPF (FVC, FEV_1, FEF_{25-75}) would be decreased, as a function of single particle exposure, and to a larger degree, as a function of combined particle and O_3 exposure and serial particle exposure, compared to filter air exposure.
2) Heart rate variability, as indicated by both time and frequency domain indices, would be decreased as a function of single particle exposure, and to a larger degree, as a function of combined particle and O_3 exposure, compared to filter air exposure.

Materials and Methods

Design:

This project consisted of two controlled human exposure experiments. Experiment One utilized 15 subjects and Experiment Two utilized 10 subjects. All subjects for both experiments were individuals with mild to moderate asthma. The exposure conditions were: Experiment One: separate single exposures to each of Filtered-Air (FA); carbon particles at a target concentration of 150 $\mu g/m^3$ and ammonium-nitrate particles at a target concentration of 150 $\mu g/m^3$, for a target total concentration of 300 $\mu g/m^3$ (P); P and O_3 at a concentration of 0.2 ppm (PO): (Figure 1.): Experiment Two: separate single exposures to FA; and P; and three serial-day exposures to P (P3): (Figure 2.). The duration of all the exposures was 4 hr, during which subjects completed 4 x 30 min exercise periods, separated by 4 x 30 min rest periods. Bronchoscopy was conducted 18 hr following each of the single exposures and, 18 hr following exposure three in P3 (Figure 1.; Figure 2.). The HRV measurements were collected immediately pre-exposure and during the final 25 min of the exposure.

For both experiments, each subject attended the laboratory for one characterization session, and subsequently for three or four exposure and bronchoscopy sessions. The characterization session was used to collect physical and pulmonary characteristics, and to familiarize the subject with the procedures of the experiment. Each of the experiments utilized a single-blind (subjects being unaware of the exposure conditions), repeated measures design, each subject completing each condition within the experiment. The order of the experimental conditions was counter-balanced/randomized within each experiment.

Controls:

For both experiments, a control exposure condition of FA was used. To allow recovery from preceding sessions, a minimum of three weeks separated each of the exposure conditions within each experiment.

Independent Variables:

The independent variables were the exposure conditions.

Experiment One:

1) FA; single exposure.
2) P; at 300 $\mu g/m^3$; single exposure.
3) PO; at 300 $\mu g/m^3$ and 0.2 ppm; single exposure.

Experiment Two:

1) FA; single exposure.
2) P; at 300 $\mu g/m^3$; single exposure.
3) P3; at 300 $\mu g/m^3$; three serial-day exposures.

Dependent Variables:

Experiment One:

1) Cell distribution: (Bfx and BAL) total and differential cell counts (macrophages, neutrophils, lymphocytes, eosinophils, epithelial cells, squamous cells).
2) Protein: (Bfx and BAL) total protein, IL-8, GMCSF, CRP.
3) mRNA: (Bfx, BAL, and epithelial cells) IL-1β, IL-6, IL-8, IL-10, HIN-1.
4) Spirometric pulmonary function: FVC, FEV_1, FEF_{25-75}.
5) Airway inflammation grading (visual).
6) Symptoms (general and respiratory).
7) Heart-rate variability.

Experiment Two:

1) Cell distribution: (Bfx and BAL) total and differential cell counts (macrophages, neutrophils, lymphocytes, eosinophils, epithelial cells, squamous cells).
2) Protein: (Bfx and BAL) total protein, IL-8, GMCSF, CRP.
3) Spirometric pulmonary function: FVC, FEV_1, FEF_{25-75}.
4) Airway inflammation grading (visual).
5) Symptoms (general and respiratory).

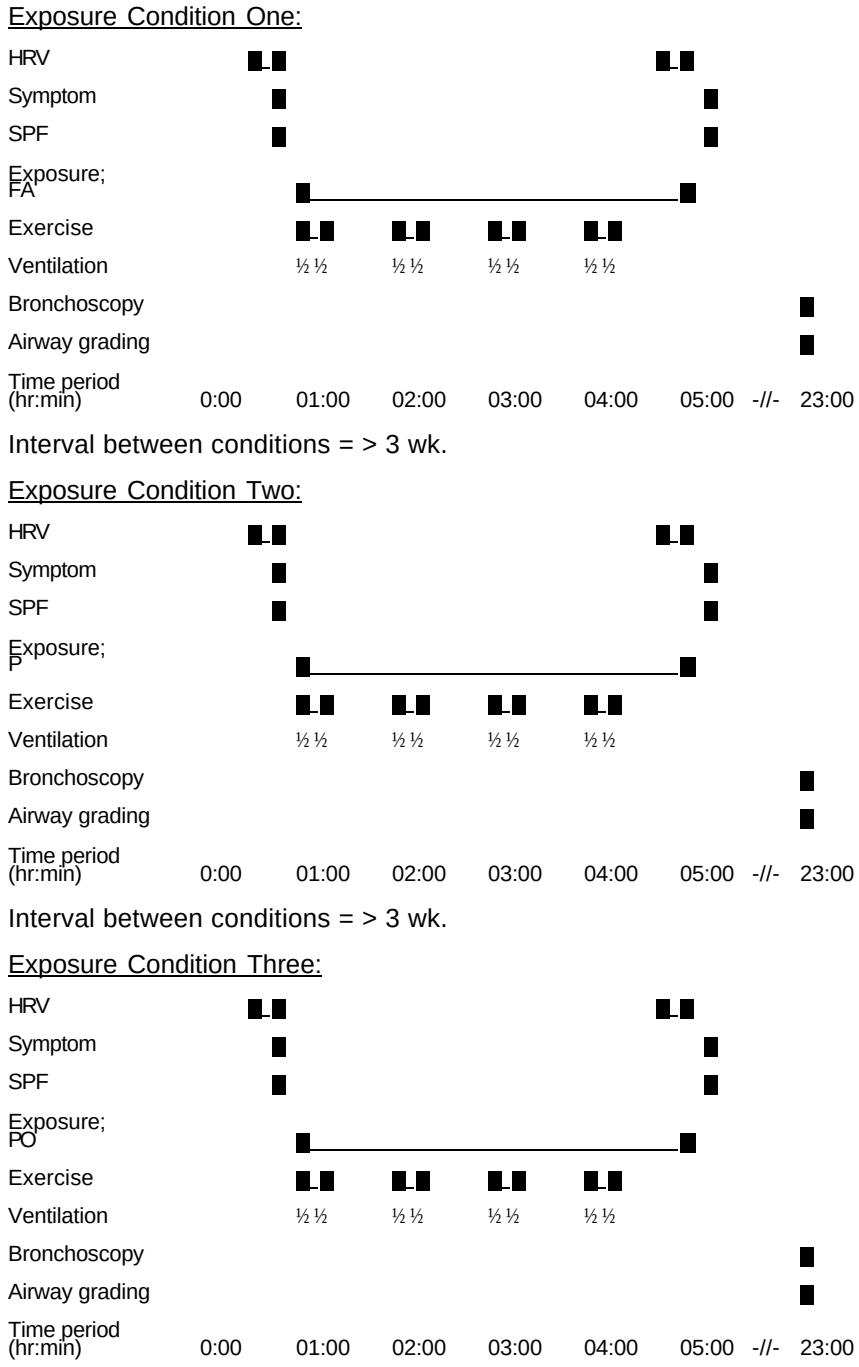

Figure 1. Experiment One design. Abbreviations: HRV = heart rate variability; SPF = spirometric pulmonary function; FA = filtered air; P = carbon and ammonium nitrate particles; PO = P + ozone.

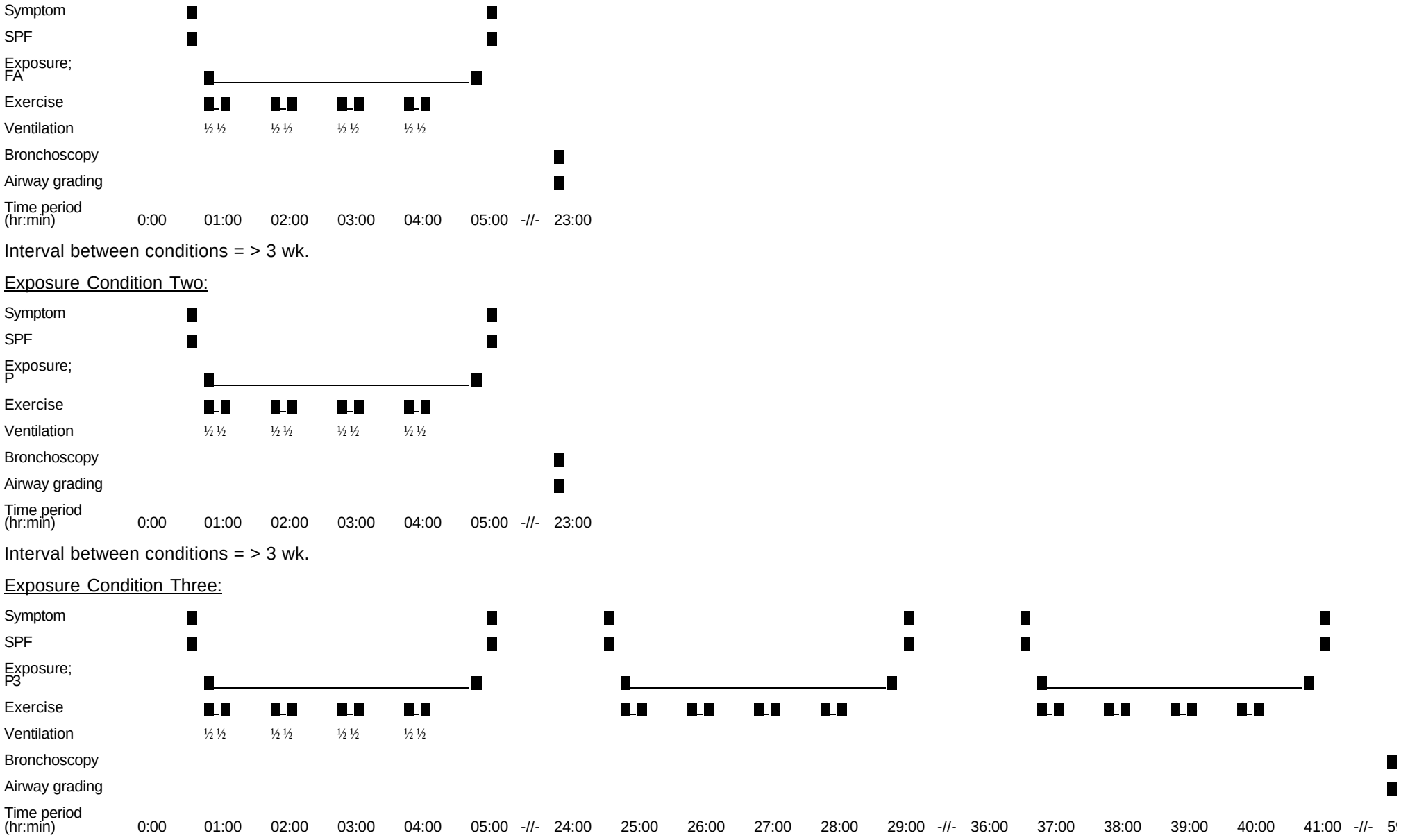

Figure 2. Experiment Two design. Abbreviations: SPF = spirometric pulmonary function; FA = filtered air; P = carbon and ammonium nitrate particles; P3 = P x 3 serial-day exposures; Exp-1 = Exposure-1; Exp-2 = Exposure-2; Exp-3 = Exposure-3.

Subjects:

All subjects were informed of the risks of the experiment and provided informed consent prior to participation. The procedures for the experiments were approved by the University of California, San Francisco, Institutional Review Board, the Committee on Human Research. All subjects completed a medical history questionnaire, were current non-smokers, and had no history of regular smoking, and had no serious health problems. Female subjects were not pregnant throughout the project. Subjects had no respiratory-tract illness in the three weeks preceding, or during, each session. Subjects were characterized by physical characteristics, SPF, non-specific airway reactivity, and allergy skin test. Asthma status was determined using the guidelines of the National Asthma Education Program (National Asthma Education Program Expert Panel, 1997). All subjects had non-specific airway reactivity of < 10 mg/ml methacholine.

Experiment One:

The subject group consisted of 15 volunteers with mild to moderate asthma who were otherwise healthy. Subjects were characterized by physical, pulmonary, allergy, and medication characteristics (Table 1.).

Experiment Two:

The subject group consisted of 10 volunteers from Experiment One who had mild to moderate asthma who were otherwise healthy. Subjects were characterized by physical, pulmonary, allergy, and medication characteristics (Table 2.).

Controls and Medications:

Subjects abstained from caffeine for 8 h prior to each session, and were instructed not to take any medication with known or potential anti-inflammatory or broncho-active properties for appropriate periods both before or during the exposure and bronchoscopy periods. Subjects abstained from all inhaled steroids for a minimum of two weeks prior to all testing sessions.

Equipment and Procedures:

Laboratory:

All sessions, excluding bronchoscopy (Refer to Bronchoscopy section), were conducted in the Human Exposure Laboratory at the Lung Biology Center, San Francisco General Hospital Campus, University of California San Francisco.

Spirometric Pulmonary Function:

Spirometry for the determination of indices of pulmonary function; FVC, FEV_1, FEF_{25-75}, was conducted using a dry, rolling-seal spirometers (Anderson Instruments; Spirotech

Division, Model No. S400; or Collins, Survey Plus), using standardized procedures (Crapo *et al.*, 1995). Spirometry was conducted for subject characterization, non-specific airway reactivity, immediately pre-and post-exposure, and pre-bronchoscopy.

Non-Specific Airway Reactivity:

Non-specific airway reactivity was determined by the FEV_1 response to inhalation of nebulized (Devilbiss, Model No. 646) phosphate-buffered saline (PBS) and doubling concentrations of methacholine in PBS (0.313, 0.625, 1.25, 2.5, 5.0, 10.0 mg ml) delivered via a dosimeter (Rosenthal) at the rate of 0.01 ml breath (Kranner *et al.*, 1994). Non-specific airway reactivity was determined for subject characterization.

Allergy Skin Test:

Epicutaneous skin-prick testing with nine local aeroallergens (DP plus *aspergillus fumigatus*, birch mix, chinese elm, cat, dog, mountain cedar, mugwort sage, olive tree, perennial rye) and controls of saline (0.9%) and histamine were performed on the forearm. Sensitivity was defined as a >2 x 2 mm skin wheal response. Allergy skin testing was determined for subject characterization, and to determine the allergen to which each individual subject had the primary response, this specific allergen being utilized for *in-vitro allergen* exposure of bronchial tissue at UCD (refer to corresponding UCD report).

Exposure Chamber:

The exposure sessions were conducted in a custom-built steel and glass exposure chamber (Nor-Lake Inc., Model No. W00327-3R), which is 2.5 m x 2.5 m x 2.4 m in size, and has an average airflow rate of $100/m^3$ min. The chamber air supply is sourced from ambient air, which is filtered by passing through purifying (Purafil Model No. 6239), and high efficiency particle (Aeropac Model No.53 HEPA 95) filters. The filtered air is dehumidified by passing through a drier (Cargocaire Engineering Corp.). HC-575), and the air temperature is decreased with a chilled-water coil. Subsequently, both the temperature and humidity are increased with steam (Nortec Model No. NHMC-050), to obtain the pre-set temperature (20 ^{O}C) and relative humidity (50%) conditions in the chamber. The temperature and relative humidity inside the chamber are monitored (LabView, v 6.1) and controlled throughout the exposures (Johnson Controls, Model No. DSC 8500).

Particle Generation and Measurement:

The carbon and ammonium nitrate particles were generated using a unique purpose-built system, which was constructed and installed as part of the Sub-Contract with UCI.

A solution of 2% carbon (Monarch Corp. Cabot 100) and 2% ammonium nitrate (Fisher Scientific, Cat. No. A676-500) in water was made immediately before each particle exposure session. For the initial four particle exposures, the solution was loaded into

two nebulizers (Babington) which were located in the center of a sealed plexi-glass box (30 cm^3). The air supply to the nebulizers, and a separate inflow to the box were sourced from a common supply of compressed medical grade air at 20 psi, which could be adjusted to control the proportion of flow to the nebulizers and the box. The particle-air out-flow from the box was piped to the inlet duct of the chamber through 10 mm stainless steel tubing, being connected to a dedicated inlet for this system positioned at ~10 ft from the chamber inlet. Subsequently, the system was re-designed to allow less frequent loading of the solution and for easier operation. For all subsequent particle exposures, the solution was loaded into a set of five nebulizers (Acorn), connected in series by 15 mm plastic T-pieces. The air supply for the nebulizers was compressed medical grade air at 20 psi. The nebulizer set was connected directly into the dedicated inlet in the in-flow duct of the exposure chamber.

For the initial characterization of the particle exposure system, the particle size distribution and concentration and were determined using a scanning mobility particle analyzer (SMPS, Model No. 3936; TSI Inc.). The system produced particles with a size distribution that had an average diameter of: mean = 0.61 μm; geometric mean = 0.53 μm, geometric SD = 1.8; at a mean concentration of 220 $\mu g/m^3$.

During the exposure sessions, the total particle concentration in the chamber was measured at the subjects breathing zone using a filter holder and filter (Pallflex; 0.22 μm), and vacuum pump sampling at 12.67 l min. The filter mass was determined pre- and post-sampling (Micro-systems), and the particle concentration calculated from the mass delta and flow-rate. Particle concentration samples were collected throughout the 4 hr exposure sessions (Table 4., Table 5.).

Quantification of the relative carbon and ammonium nitrate levels on the filters are currently being conducted through the Sub-Contract with UCI (data to be included). Preliminary data from a total of 24 samples indicate that carbon constitutes 91.4% of the total mass of the particles collected, and ammonium nitrate constitutes 8.6% of the total mass of the particles collected.

Ozone Generation and Measurement:

The O_3 was produced using compressed O_2 (balance argon) and a corona-discharge O_3 generator (Model T 408; Polymetrics, San Jose, CA). The O_3 concentration was measured at the subjects breathing zone using an ultraviolet-light photometer (Model 1008 PC; Dasibi). The O_3 concentration was maintained at the target concentration of 0.2 ppm by adjusting the voltage of the generator. For Experiment One, in the PO exposures the O_3 concentration was: mean $\pm$ SD; 0.204 ± 0.007 ppm.

Exercise and Pulmonary Ventilation:

During each exposure, exercise was utilized to induce mouth breathing and to increase minute ventilation. The exercise consisted of 4 x 30 min periods of either walking/running on a treadmill (Cybex, Model No. T400) or pedaling a cycle-ergometer

(Monark, Model No. 90818e). The exercise intensity was adjusted for each subject to a target expired minute ventilation of 25 l/min/m^2 body surface area. During exercise, expired ventilation was calculated from tidal volume and breathing frequency measured using a pneumotachograph (Fleisch, Model No. 3) at the 10- and 20-min intervals of each 30 min exercise period. Following each 30 min exercise period, subjects were seated at rest for 4 x 30 min periods.

Bronchoscopy:

Due to the subjects in both experiments having mild to moderate asthma, each subject under-went a standard inhaled nebulized albuterol procedure, 30 min before the bronchoscopy to minimize the chance of bronchoconstriction during the bronchoscopy.

The bronchoscopies were conducted in a dedicated room at San Francisco General Hospital. Vital signs were measured pre- and post-bronchoscopy. Throughout the procedure, intravenous access was maintained, and arterial hemoglobin:oxygen percent saturation, the electrocardiograph, and blood pressure were monitored. Atropine, to decrease airway secretions, and if required, midazolam and fentanyl to maintain subject comfort, were administered intravenously. The posterior pharynx was anesthetized using a 1% lidocaine spray, and 4% lidocaine-soaked cotton-tipped plegets applied to the mucosa over the ninth cranial nerve. Supplemental oxygen was delivered via a nasal cannula at 2 l/min. The bronchoscope (Pentax, Model No. FB 18x), tipped with lidocaine jelly, was introduced through the mouth, and the larynx and airways were anesthetized using 1% lidocaine solution as required. The bronchoscope was directed and wedged into the right middle lobe orifice (2 x 50 ml lavage), and subsequently into the lingula (1 x 50 ml lavage). The lavages were conducted using 0.9% saline heated to 37 ^{O}C. The first 15 ml of lavage fluid returned was designated as the bronchial fraction (Bfx). The lavage fluids were immediately centrifuged at 200 g for 15 minutes (Girofuge model No. 1805), and the supernatant separated and recentrifuged at 1800 g for 15 minutes to remove any cellular debris. The supernatant was then frozen and stored at -80 ^{O}C for biochemical analysis.

Following the lavages, five epithelial brushings were conducted by passing a specialized cytology brush (Mill-Rose Laboratories, Inc., Model No. 149; working diameter: 1.6 mm), against the airway wall. This technique collects samples that are > 95 % epithelial cells. Following the brushing, 6-8 endobronchial biopsies were obtained from multiple sites of the airway bifurcations within the right middle lobe and carina using spiked forceps (Pentax Precision Instrument Corporation). All biopsy samples were collected directly into MEM (Joklik modified Minimal Essential Medium and 1 mM HEPES) on ice, packaged in wet ice and transported overnight to collaborators at UCD for the *in-vitro* allergen exposure and RT RT-PCR analysis (refer to corresponding UCD report). The bronchoscopy was conducted 18 hr (range 17 to 19 hr) post-exposure.

Cell Counts:

Total cells were counted in unspun aliquots of Bfx and BAL using a hemacytometer (Fisher Scientific, Cat. No. 0267110). Differential cell counts were conducted on slides prepared using a cytocentrifuge at 200 g for 5 min (Shandon Southern Products Ltd., Model No. Cytospin 2) and stained in Diff-Quik (Diff-Quik, Baxter, Cat. No. B4132-1). All differential leukocytes cell counts were expressed as a percent of total leukocytes (macrophages, lymphocytes, neutrophils, eosinophils), and differential epithelial cell counts were expressed as a percent of total leucocytes + epithelial cells. Two readers blinded to the exposure condition and sample type, each performed the cell counts in duplicate.

Protein Assays:

Protein levels in both the Bfx and BAL supernatant were determined for, total protein (Pierce BCA), IL-8 and GMCSF (ELISA; R&D Systems), and CRP (ELISA; Diagnostics Automation Inc.).

Gene Expression:

Total RNA was extracted from the Bfx and BAL cell pellets, and the epithelial cell pellet (RNeasy, Qiagen). The RNA purity and quality was checked for all samples (Agilent RNA 6000 Nano assay). This procedure allows isolation of 20-30 μg of total RNA per sample. The RNA was used for determining the mRNA level for specific genes, IL-6, IL-8, IL-10, HIN-1, using quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (R-T RT-PCR) (Applied Biosystems, TaqMan).

For R-T RT-PCR 100 ng of total RNA was used as a template for single-strand complimentary DNA (cDNA) synthesis using gene-specific reverse transcription (RT) primers and Reverse Transcriptase (Superscript II). The cDNA was pre-amplified by PCR using Advantage Klentaq DNA polymerase and a mixture of gene-specific primers (TaqMan primers) that are located internal to the RT primers (2 minutes at 94 °C, followed by 15-25 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 70 °C for 45 seconds). Samples were processed in parallel without RT to evaluate DNA content and assess RNA quality.

Equal volumes of pre-amplified cDNA were mixed with forward and reverse TaqMan primers (TM primers) along with a gene-specific fluorescence-labeled TaqMan probe. Samples were analyzed using 40 cycles of PCR with real-time fluorescence measurement (Applied Biosystems 2700 Sequence Detector). The mean number of cycles to threshold (C_T) of fluorescence detection was calculated for each sample and the results normalized to the mean C_T of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for each sample tested.

Heart Rate Variability:

The HRV data was collected using five standardized electrode sites on the subjects chest wall that allow for recording of two channels, and a ground lead. The Holter monitor (Forest Medical: Trillium Model 3000) leads were attached according to the manufacturers instructions. The subject then performed the following maneuvers during the 25-minute Holter monitoring session (Gold *et al.*, 2000):
1) Resting in the supine position (breathing frequency and two blood pressure measurements were conducted); 5 min.
2) Resting in the standing position; 5 min.
3) Exercise, walking in place; 5 min.
4) Resting in the supine position (breathing frequency and two blood pressure measurements were conducted); 5 min.
5) Twenty complete full inhalations and exhalations.

The HRV data was collected immediately pre-exposure, and during the final 25-min of each of the exposure sessions. Following storage, all data were scanned and edited for mislabeled beats. The number of episodes, if present, of ventricular tachycardia, ventricular fibrillation and episodes of bradyarrhythmia/asystole was calculated for each monitoring session. The HRV analysis was performed using the manufacturers computer software (Forest Medical: Trillium 3000 Holter monitor). The two principle types of HRV parameters, time-domain variables and frequency-domain variables, were analyzed. In the HRV analysis, only sinus R-R intervals were used. Artifacts, ectopy (both supraventricular and ventricular), and uninterpretable complexes were not considered for analysis. Also, intervals in duration of is <80% or >120% of that of the running R-R average were excluded to eliminate intervals related to premature supraventricular complexes and ventricular arrests (defined as R-R intervals >2 s). Prolonged intervals after a short interval were excluded as partial compensatory pauses.

Time-Domain Analysis:
In time-domain analysis, the principal measure used was the standard deviation of the sinus R-R intervals (termed the heart period) over time. This analysis used the following variables:
1) The standard deviation of all NN intervals (SDNN) is the mean of the standard deviations of heart periods derived from successive 5 min periods. The SDNN is sensitive to heart period variations over short (5 min) periods.
2) The standard deviation of means of all NN intervals (SDANN) is the standard deviation of the means of heart periods derived from successive 5-min periods. The R-R intervals of normal sinus rhythm beats are averaged within each 5 min period, and the standard deviation of the 5-min averages is used. The SDANN is relatively resistant to beat misclassification, which can occur with artifact or ectopy. It is also the HRV variable best able to demonstrate the circadian variations in HRV.
3) The standard deviation of all NN intervals index (SDNNi) is the mean of the standard deviations of all NN intervals for all 5 min periods of the entire recording period. The SDNNi is a descriptor of the short-term components of HRV.

4) The root mean square of successive differences (r-MSSD) is the square root of the mean of the summed squared differences between successive normal R-R intervals. This measure assesses beat-to-beat variability rather than 5 min variability (SDNN) or longer-term variability (SDANN). Therefore, the r-MSSD is particularly sensitive to the misclassification of beats. As the vagus nerve is responsible for very short-term variations in heart period, the r-MSSD reflects vagal tone.

Frequency-Domain Analysis:
The frequency domain component of HRV analysis, also referred to as power spectral analysis, separates the heart rate signal into the frequency components, then quantifies those components in terms of their relative intensity, or power. By separating high-frequency components from low-frequency components, power spectrum analysis delineates parasympathetic and sympathetic effects. The high-frequency components (0.15-0.40 Hz) related to respiratory sinus arrhythmia represent parasympathetic neural activity. Low-frequency components (0.04-0.15 Hz) represent predominately sympathetic neural activity, but could also reflect some parasympathetic activity.

Airway Grading:

The level of inflammation in the airways was graded visually by the bronchoscopist on a scale of: 0 = Normal, 1 = Mildly inflamed, 2 = Moderately inflamed, 3 = Severely inflamed; during each bronchoscopy.

Symptoms:

Subjects current self-graded symptoms; anxiety, chest discomfort or chest tightness, chest pain on deep inspiration, cough, eye irritation, headache, nasal irritation, nausea, phlegm or sputum production, shortness of breath, throat irritation, wheezing; were graded on a scale of; 0 = None, 1 = Minimal (symptom is barely noticeable), 2 = Mild (symptom is present but not annoying), 3 = Moderate (symptom is somewhat annoying), 4 = Severe (symptom is very annoying and/or limits performance. Symptoms were graded immediately pre- and post-exposure.

Statistical Analysis:

Current data are for 13 subjects in Experiment One and 9 subjects in Experiment Two. Comparisons of the exposure particle concentrations were made using un-paired t-Tests, as the data are used for exposure description. The data for the majority of dependent variables measured in this project were not normally distributed, therefore, non-parametric methods were utilized. For the paired comparisons both with-in and between, the exposure conditions, the Wilcoxon Signed-Rank test was used. For the R-T RT-PCR data, the n of 4-6 precluded the use of ranked tests, therefore, for the paired comparisons between the exposure conditions, the paired t-Test was used. All comparisons were made using only complete individual sets of repeated-measured data. Differences were assigned as statistically significant at an alpha of <0.05.

Table 1. Experiment One: Individual subjects physical, spirometric pulmonary function, airway responsiveness, allergen, and medication characteristics.

Subj.	Gender	Age (yr)	Height (cm)	Mass (kg)	FVC (l)	FEV_1 (l)	FEV_1/FVC (%)	NSAR PC_{20} (mg ml)	Allergen	Medication
1	M	45	183	82	5.07	3.95	78	10	Birch	AH
2	F	26	157	59	3.77	3.49	93	10	Mite	AH
3	M	32	175	73	4.56	3.53	77	1	Dog	SB
4	F	21	168	64	3.90	3.03	80	1	Olive	SB
5	M	44	173	68	3.88	2.21	57	0.25	Cedar	SB
6	M	34	170	80	4.09	3.29	80	1	Mite	SB
7	F	41	168	61	3.27	2.64	81	8	Cat	IS
8	F	29	160	88	3.19	2.38	75	2	Mite	SB, LB
9	F	36	165	132	2.52	2.04	81	2	Rye	SB, LB, IS LA, NS, AH
10	M	54	165	77	3.87	2.52	65	0.25	Olive	Nil
11	F	41	163	80	2.21	1.83	83	0.25	Olive	SB, LB, IS, NS, AH
12	F	51	165	88	2.27	1.82	80	0.25	Dog	Nil
13	F	41	168	127	3.18	2.66	84	4	Mite	SB, NS
14	F	32	165	75	3.40	2.73	80	1	Olive	SB
15	M	25	178	90	5.35	3.61	67	4	Mite	SB
Mean	(F=9)	36.8	168.2	82.9	3.64	2.78	77.4	3.0		
± SD	(M=6)	9.6	6.8	21.2	0.93	0.68	8.7	3.5		

Abbreviations: FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; NSAR PC_{20} = non-specific airway reactivity, methacholine provocative concentration at which FEV_1 decreased 20%, maximum dose = 10 mg ml; Allergen = allergen to which the subject had the largest response at skin test; SB: short term bronchodilator; LB: long term bronchodilator, IS: inhaled steroid; LA: leukotriene antagonist; NS: nasal steroid; AH: antihistamines; DE: decongestant/expectorant.

Table 2. Experiment Two: Individual subjects physical, spirometric pulmonary function, airway responsiveness, allergen, and medication characteristics.

Subj.	Gender	Age (yr)	Height (cm)	Mass (kg)	FVC (l)	FEV_1 (l)	FEV_1/FVC (%)	NSAR PC_{20} (mg ml)	Allergen	Medication
1	M	45	183	82	5.07	3.95	78	10	Birch	AH
2	F	21	168	64	3.90	3.03	80	1	Olive	SB
3	M	44	173	68	3.88	2.21	57	0.25	Cedar	SB
4	M	34	170	80	4.09	3.29	80	1	Mite	SB
5	F	29	160	88	3.19	2.38	75	2	Mite	SB, LB
6	F	36	165	132	2.52	2.04	81	2	Rye	SB, LB, IS LA, NS, AH
7	M	54	165	77	3.87	2.52	65	0.25	Olive	Nil
8	F	41	163	80	2.21	1.83	83	0.25	Olive	SB, LB, IS, NS, AH
9	F	51	165	88	2.27	1.82	80	0.25	Dog	Nil
10	F	32	165	75	3.40	2.73	80	1	Olive	SB
Mean	(F=6)	38.7	167.7	83.4	3.44	2.58	75.9	1.9		
± SD	(M=4)	10.2	6.5	18.7	0.91	0.68	8.3	3.1		

Abbreviations: FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; NSAR PC_{20} = non-specific airway reactivity, methacholine provocative concentration at which FEV_1 decreased 20%, maximum dose = 10 mg ml; Allergen = allergen to which the subject had the largest response at skin test; SB: short term bronchodilator; LB: long term bronchodilator, IS: inhaled steroid; LA: leukotriene antagonist; NS: nasal steroid; AH: antihistamines; DE: decongestant/expectorant.

Table 3. Experiment One: Exposure Particle Concentrations.

	Exposure Condition		
	FA	P	PO
[Particle] ($\mu g/m^3$)			
Mean	66	283	237
± SD	21	44	49

Values are mean ± SD. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles; PO = P + ozone [0.2 ppm].

Table 4. Experiment Two: Exposure Particle Concentrations.

	Exposure Condition				
	FA	P	P3 Exp-1	Exp-2	Exp-3
[Particle] ($\mu g/m^3$)					
Mean	66	288	271	274	255
± SD	30	44	42	67	52

Values are mean ± SD. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles; P3 = P x 3 serial-day exposures; Exp-1 = Exposure-1; Exp-2 = Exposure-2; Exp-3 = Exposure-3.

Results

Experiment One:

Cell Distribution:

Bronchial Fraction:
For the Bfx there was no difference between the FA, P, and PO exposure conditions in the total leukocyte concentration (Table 6.). In PO compared to both FA and P, there was a significantly higher percent and concentration of both neutrophils and lymphocytes, and concentration of eosinophils (Table 5; Table 6.). Corresponding to the increases in differential percentages, in PO compared to both FA and P, there was a significantly lower percent of macrophages (Table 5). There were no other significant differences between the FA, P, and PO exposure conditions in any other cell type percents or concentrations (Table 5; Table 6.).

Bronchoalveolar Lavage:
For the BAL, in PO compared to FA, there was a significantly higher percent and concentration of eosinophils, and significantly lower percent of macrophages (Table 5). In PO compared to P, there was a significantly lower concentration of epithelial cells (Table 6). There were no other significant differences between the FA, P, and PO exposure conditions in any other cell type percents or concentrations (Table 5; Table 6.).

Protein:

Bronchial Fraction:
For the Bfx, in PO compared to both FA and P, there was a significantly higher total protein concentration (Table 7.). In PO compared to P, there was a significantly higher GMCSF concentration, and for PO compared to FA, there was a significantly higher CRP concentration (Table 7.). There was no significant difference between the FA, P, and PO exposure conditions in the IL-8 concentration (Table 7.).

Bronchoalveolar Lavage:
For the BAL, in PO compared to FA, there was a significantly higher total protein concentration (Table 7.). There were no significant differences between the FA, P, and PO exposure conditions in the IL-8 or GMCSF concentrations (Table 7.). In the BAL all CRP concentrations were below the level of detection of the assay; 0.00035 μg ml.

mRNA:

Bronchial Fraction:
For the Bfx, in PO compared to both FA and P, there were significantly higher IL-8 and IL-10 mRNA levels (lower Ct) (Table 8.). There were no significant differences between the FA, P, and PO exposure conditions in the IL-1β, IL-6, or HIN-1 mRNA levels (Table 8.).

Bronchoalveolar Lavage:
For the BAL, there were no significant differences between the FA, P, and PO exposure conditions in the IL-1β, IL-6, Il-8, IL-10, or HIN-1mRNA levels (Table 8.).

Bronchial Epithelial Cells:
For the bronchial epithelial cells, there were no significant differences between the FA, P, and PO exposure conditions in the IL-1β, IL-6, Il-8, IL-10, or HIN-1mRNA levels (Table 8.).

Spirometric Pulmonary Function:

In PO at post-exposure, compared to FA post-exposure, P post-exposure, and PO 18 hr post-exposure, there was a significantly lower FVC (Table 9.). In PO at 18 hr post-exposure, compared to FA 18 hr post-exposure, P 18 hr post-exposure, and PO at pre-exposure, there was a significantly lower FVC (Table 9.). In PO at post-exposure, compared to both FA post-exposure and PO pre-exposure, there was a significantly lower FEV_1 (Table 9.). In PO at 18 hr post-exposure, compared to both FA 18 hr post-exposure and P 18 hr post-exposure, there was a significantly lower FEV_1 (Table 9.). In both PO at post-exposure and P post-exposure, compared to FA post-exposure, there was a significantly lower FEF_{25-75} (Table 9.). There were no other significant differences either with-in or between the FA, P, and PO exposure conditions in any other SPF values (Table 9.).

Airway Grading:

There were no significant differences either with-in or between the FA, P, and PO exposure conditions in the airway grading score (Data not presented).

Symptoms:

There were no significant differences either with-in or between the FA, P, and PO exposure conditions in the symptom scores (Data not presented).

Heart-Rate Variability:

Time Domain:
For the time domain variables, in PO at post-exposure, compared to FA post-exposure, and PO pre-exposure, there was a significantly lower SDNN, and in PO the delta, compared to the FA delta, was a significantly larger (Table 10.). In PO at post-exposure, compared to FA post-exposure, there was a significantly lower SDNNi, and in PO the delta, compared to the FA delta, was significantly larger (Table 10.). In PO at post-exposure, compared to FA post-exposure, there was a significantly lower r-MSSD (Table 10.). There were no significant differences with-in or between, the FA, P, and PO exposure conditions in the SDANN, or in any other of the time domain variables (Table 10.).

Frequency Domain:
The analyses for the frequency domain variables were conducted independently using the separate 5 min periods during which the subject was positioned in the; 1) supine position; and 2) standing position.

For the supine position, there were no differences in the total power between the FA, P, and PO exposure conditions (Table 11.). In FA at pre-exposure, compared to P pre-exposure, there was a significantly higher LF-norm, lower HF-norm, and higher LF/HF ration (Table 11), therefore, only the delta values for these variables were considered for between condition comparisons for these two conditions. In both P post-exposure and PO post-exposure, compared to FA post-exposure, and in PO pre-exposure, compared to PO pre-exposure, there was a significantly lower LF (Table 11.). In PO at post-exposure, compared to P post-exposure, there was a significantly higher LF-norm, and in PO the delta, compared to the P delta, was significantly larger (Table 11.). In PO at post-exposure, compared to FA post-exposure, P post-exposure and PO pre-exposure, there was a significantly lower HF, and in PO the delta, compared to the P delta, was significantly larger (Table 11.). In PO at post-exposure, compared to P post-exposure, there was a significantly lower HF-norm, and significantly higher LF/HF ratio (Table 11.).

For the standing position, in PO at post-exposure, compared to FA post-exposure, there was significantly lower total power, and in PO the delta, compared to the P delta, was significantly larger (Table 12.). In P at pre-exposure, compared to PO pre-exposure, there was a significantly higher VLF (Table 12), therefore, only the delta values for this variable was considered for between condition comparisons for these two conditions. In PO the delta, compared to the P delta for VLF was significantly larger (Table 12.). In PO at post-exposure, compared to both FA post-exposure and PO pre-exposure, there was a significantly HF (Table 12.).

There were no other significant differences for the supine or standing positions, either with-in or between, the FA, P, and PO exposure conditions in the frequency domain variables (Table 12.).

<u>Experiment Two</u>:

Cell Distribution:

Bronchial Fraction:
For the Bfx there was no difference in the total leukocyte concentration between the FA, P, and P3 exposure conditions (Table 14.). In P3 compared to both FA and P, there was a significantly lower percent of lymphocytes (Table 13). There were no other significant differences between the FA, P, and P3 exposure conditions in any other cell type percents or concentrations (Table 13.; Table 14.).

Bronchoalveolar Lavage:
For the BAL, in P compared to both FA, there was a significantly higher concentration of epithelial cells (Table 14).. There were no other significant differences between the FA, P, and P3 exposure conditions in any cell type percents or concentrations (Table 13.; Table 14).

Protein:

Bronchial Fraction:
For the Bfx, there were no significant differences between the FA, P, and P3 exposure conditions in the total protein, IL-8, GMCSF, or CRP concentrations (Table 15.).

Bronchoalveolar Lavage:
For the BAL, there were no significant differences between the FA, P, and P3 exposure conditions in the total protein, IL-8, or GMCSF concentrations (Table 15.). In the BAL all CRP concentrations were below the level of detection of the assay; 0.00035 μg ml.

Spirometric Pulmonary Function:

There were no significant differences either with-in the FA or P conditions, or between the FA, P and P3 conditions in any SPF variables (Table 16.; Table 16 A). With-in the P3 condition at 18 hr post-exposure three, compared to post-exposure two, and both pre- and post-exposure three, there was a significantly lower FEV_1 (Table 16 A.). With-in the P3 condition at 18 hr post-exposure three, compared to both pre- and post-exposure two, there was a significantly lower FEF_{25-75} (Table 16 A.). There were no other significant differences with-in the P3 condition in the SPF values (Table 16 A.).

Airway Grading:

There were no significant differences either with-in or between, the FA, P, and P3 exposure conditions in the airway grading score (Data not presented).

Symptoms:

There were no significant differences, either with-in or between the FA, P, and P3 exposure conditions in the symptom scores (Data not presented).

<u>Multiple Comparisons:</u>
It must be noted that due to the multiple paired and un-paired comparisons made for the statistical analyses of the data in this project, it is possible that a difference could be assigned as statistically significant due to the number of comparisons conducted.

Table 5. Experiment One: Leukocyte and epithelial cell differential percent in bronchial fraction, and bronchoalveolar lavage fluid, post-exposure to filtered air, particles, and particles + ozone.

Cell	Exposure Condition					
	FA		P		PO	
	Bfx	BAL	Bfx	BAL	Bfx	BAL
Macrophages (%)						
Median	92.5 [A]	92.4 [B]	91.6 [C]	86.1	72.8 [A C]	85.3 [B]
25-75% range	90.4 - 95.5	89.2 - 96.0	85.9 - 96.4	84.0 - 95.8	66.6 - 75.8	77.4 - 95.0
Neutrophils (%)						
Median	4.5 [A]	3.7	3.9 [B]	2.9	17.2 [A B]	5.4
25-75% range	2.5 - 5.4	1.5 - 5.3	2.4 - 5.6	1.2 - 5.2	10.7 - 23.8	1.9 - 9.4
Lymphocytes (%)						
Median	2.7 [A]	2.8	1.9 [B]	4.1	7.7 [A B]	4.5
25-75% range	0.0 - 3.6	0.3 - 5.5	0.0 - 5.9	0.0 - 8.6	0.3 - 8.9	0.3 - 8.4
Eosinophils (%)						
Median	0.3	0.0 [A]	0.6	0.6	1.7	1.3 [A]
25-75% range	0.0 - 0.3	0.0 - 0.3	0.3 - 2.4	0.3 - 1.9	0.6 - 3.8	0.3 - 2.3
Epithelial Cells (%)						
Median	16.8	4.8	10.5	4.8	10.3	5.0
25-75% range	9.3 - 26.8	1.8 - 9.0	9.0 - 22.9	2.5 - 12.8	7.0 - 17.5	2.0 - 5.5

Values are median and 25-75% range. Epithelial cells = percent of total non-squamous cells; all other cell types = percent of leukocytes. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; BFx = bronchial fraction; BAL = bronchoalveolar lavage. [A B C] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 6. Experiment One: Leukocyte and epithelial cell concentration in bronchial fraction, and bronchoalveolar lavage fluid, post-exposure to filtered air, particles, and particles + ozone.

Cell	Exposure Condition					
	FA		P		PO	
	Bfx	BAL	Bfx	BAL	Bfx	BAL
Total Leukocytes ($x10^4$ ml)						
Median	10.0	17.8	11.3	16.5	14.8	15.5
25-75% range	6.8 - 18.5	15.0 - 21.5	6.3 - 13.3	14.3 - 18.3	11.3 - 18.0	12.3 - 20.3
Macrophages ($x10^4$ ml)						
Median	9.2	15.5	9.8	15.3	8.7	11.6
25-75% range	6.0 - 16.7	12.8 - 21.1	5.4 - 12.1	12.9 - 16.4	7.6 - 13.6	10.1 - 18.1
Neutrophils ($x10^4$ ml)						
Median	0.6 [A]	0.5	0.3 [B]	0.6	2.2 [A B]	0.9
25-75% range	0.3 - 0.7	0.2 - 0.9	0.3 - 0.6	0.2 - 0.9	1.6 - 2.8	0.2 - 1.1
Lymphocytes ($x10^4$ ml)						
Median	0.3 [A]	0.6	0.1 [B]	0.4	1.2 [A B]	0.6
25-75% range	0.0 - 0.8	0.1 - 0.8	0.0 - 0.3	0.0 - 1.9	0.1 - 1.7	0.0 - 1.4
Eosinophils ($x10^4$ ml)						
Median	0.0 [A]	0.0 [B]	0.0 [C]	0.1	0.2 [A C]	0.2 [B]
25-75% range	0.0 - 0.0	0.0 - 0.1	0.1 - 0.1	0.0 - 0.3	0.1 - 0.4	0.1 - 0.4
Epithelial Cells ($x10^4$ ml)						
Median	2.0	1.0	2.5	1.8 [A]	2.3	0.8 [A]
25-75% range	1.5 - 3.3	0.5 - 1.8	0.8 - 3.8	0.8 - 2.5	0.5 - 3.5	0.5 - 1.5

Values are median and 25-75% range. Epithelial cells = percent of total non-squamous cells; all other cell types = percent of leukocytes. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; BFx = bronchial fraction; BAL = bronchoalveolar lavage. [A B C] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 7. Experiment One: Protein concentrations in bronchial fraction, and bronchoalveolar lavage fluid, post-exposure to filtered air, particles, and particles + ozone.

Protein	Exposure Condition					
	FA		P		PO	
	Bfx	BAL	Bfx	BAL	Bfx	BAL
Total Protein (μg ml)						
Median	85 [A]	158 [B]	93 [C]	174	216 [A C]	179 [B]
25-75% range	46 - 160	125 - 169	39 - 188	115 - 174	114 - 319	138 - 240
IL-8 (pg ml)						
Median	24.2	8.1	35.4	13.7	24.3	18.9
25-75% range	8.4 - 44.8	5.3 - 16.8	22.2 - 89.3	10.1 - 27.7	16.0 - 47.2	5.5 - 30.3
GMCSF (pg ml)						
Median	0.7	4.1	0.6 [A]	3.3	1.6 [A]	3.7
25-75% range	0.4 - 1.1	3.7 - 4.5	0.2 - 0.9	2.9 - 4.1	1.5 - 1.8	1.3 - 6.9
CRP (μg ml)						
Median	0.002 [A]	<0.00035	0.002	<0.00035	0.005 [A]	<0.00035
25-75% range	0.001 - 0.003		0.001 - 0.003		0.004 - 0.006	

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; BFx = bronchial fraction; BAL = bronchoalveolar lavage; IL-8 = interleukin-8; GMCSF = granulocyte macrophage colony stimulating factor; CRP = C-reactive protein. [A] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 8. Experiment One: Normalized mRNA levels (RT-PCR cycles) in bronchial fraction, bronchoalveolar lavage, and bronchial epithelial cells, post-exposure to filtered air, particles, and particles + ozone.

Gene	Exposure Condition								
	FA			P			PO		
	Bfx	BAL	Epi	Bfx	BAL	Epi	Bfx	BAL	Epi
IL-1β									
Mean	7.1	5.7		7.2	5.7		6.9	6.0	
± SE	0.3	0.8		0.7	0.7		0.7	1.0	
IL-6									
Mean	12.6	10.4		11.2	10.8		11.9	10.5	
± SE	0.3	0.6		0.8	0.1		0.5	0.5	
IL-8									
Mean	8.9 [A]	9.8	9.3	9.6 [B]	7.7	8.5	7.5 [A B]	8.3	8.6
± SE	0.3	1.5	0.3	0.5	0.7	1.0	0.5	0.8	1.1
IL-10									
Mean	15.8 [A]	15.5	15.3	16.0 [B]	14.1	14.5	14.4 [A B]	14.0	15.1
± SE	0.4	1.4	0.5	0.4	0.2	0.5	0.4	1.0	0.2
HIN-1									
Mean	17.7	10.9	5.4	16.4	8.3	5.0	18.1	10.5	5.9
± SE	2.0	2.3	2.8	1.2	0.6	2.5	2.5	1.3	2.7

Data are number of reverse transcription polymerase chain reaction cycles; Note: decreased value equals increased RNA level. Values are mean ± standard error. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; BFx = bronchial fraction; BAL = bronchoalveolar lavage; Epi = epithelial cell; IL-1β = interleukin-1beta; IL-6 = interleukin-6; IL-8 = interleukin-8; IL-10 = interleukin-10; HIN-1 = high in normals-1. [A B] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 9. Experiment One: Spirometric pulmonary function, immediately pre- and post-exposure, and 18 hr post-exposure, to filtered air, particles, and particles + ozone.

	Exposure Condition								
	FA			P			PO		
	Pre-Exp	Post-Exp	Post-18	Pre-Exp	Post-Exp	Post-18	Pre-Exp	Post-Exp	Post-18
FVC (l)									
Median	3.50	3.46 [A]	3.56 [B]	3.54	3.39 [C]	3.50 [D]	3.61 [E]	3.23 [A C F]	3.23 [B D E F]
25-75% range	2.98 - 3.98	2.99 - 3.91	2.94 - 3.96	2.93 - 3.92	2.99 - 4.01	2.90 - 3.84	3.02 - 3.88	2.77 - 3.76	2.85 - 3.78
FEV_1 (l)									
Median	2.60	2.63 [A]	2.67 [B]	2.64	2.58	2.57 [C]	2.56 [D]	2.31 [A D]	2.57 [B C]
25-75% range	2.20 - 3.15	2.07 - 3.16	2.10 - 3.12	2.05 - 3.14	2.11 - 3.12	2.02 - 3.12	2.03 - 3.10	1.90 - 2.95	1.91 - 3.03
FEF_{25-75} (l s)									
Median	2.23	2.32 [A B]	2.23	2.04	1.93 [A]	1.97	2.28	1.61 [B]	1.87
25-75% range	1.55 - 2.97	1.48 - 3.23	1.57 - 3.05	1.57 - 3.10	1.54 - 2.99	1.61 - 2.94	1.52 - 2.87	1.45 - 2.92	1.36 - 2.75

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; BFx = bronchial fraction; Pre-Exp = pre-exposure; Post-Exp = post-exposure; Post-18 = 18 hr post-exposure; FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; FEF_{25-75} = forced expired flow-rate at 25-75% FVC. [A B C D E F] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 10. Experiment One: Heart-rate variability, time-domain indices, pre-exposure and end-exposure to filtered air, particles, and particles + ozone.

Indices	Exposure Condition								
	FA			P			PO		
	Pre-Exp	End-Exp	Delta	Pre-Exp	End-Exp	Delta	Pre-Exp	End-Exp	Delta
SDNN (ms)									
Median	104	100 [A]	3 [B]	99	80	-19	147 [C]	77 [A C]	-31 [B]
25-75% range	90 - 156	94 - 159	-4 - 4	94 - 149	74 - 156	-19 - 7	76 - 168	66 - 92	-55 - -25
SDANN (ms)									
Median	71	79	8	65	51	-10	95	53	-25
25-75% range	67 - 88	50 - 79	-9 - 17	63 - 93	42 - 83	-21 - -8	41 - 112	41 - 66	-59 - -21
SDNNi (ms)									
Median	57	73 [A]	3 [B]	59	60	-4	91	52 [A]	-28 [B]
25-75% range	53 - 127	57 - 96	-3 - 6	55 - 93	51 - 88	-5 - 1	51 - 94	36 - 63	-32 - -11
r-MSSD (ms)									
Median	46	41 [A]	-2	42	37	-5	43	27 [A]	-16
25-75% range	27 - 95	32 - 60	-5 - 5	32 - 49	27 - 39	-7 - 0	24 - 45	17 - 35	-24 - -7

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 μg/m^3]; PO = P + ozone [0.2 ppm]; Pre-Exp = pre-exposure; End-Exp = final 25 min. of exposure; Delta = End-Exp minus Pre-Exp; SDNN = standard deviation; SDANN = mean of the standard deviations; SDNNi = mean standard deviation for all 5-minute intervals ; r-MSSD = root mean square successive differences. [A B C] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 11. Experiment One: Heart-rate variability, frequency-domain indices, supine position, pre-exposure and end-exposure to filtered air, particles, and particles + ozone.

Indices	Exposure Condition								
	FA			P			PO		
	Pre-Exp	End-Exp	Delta	Pre-Exp	End-Exp	Delta	Pre-Exp	End-Exp	Delta
TOTAL (ms^2)									
Median	1302	1432	-36	1188	998	-115	3083	226	-406
25-75% range	1166 - 3446	1206 - 3410	-384 - 40	1141 - 1488	909 - 1176	-143 - -12	576 - 3365	170 - 1530	-1553 - -246
VLF (ms^2)									
Median	587	873	305	600	550	-35	1497	124	-221
25-75% range	568 - 780	849 - 1500	-227 - 354	488 - 787	453 - 606	-181 - 66	294 - 1963	80 - 1069	-894 - -141
LF (ms^2)									
Median	449	463 $^{A B}$	123	336	295 ^{A}	32	743 ^{C}	97 $^{B C}$	-137
25-75% range	297 - 683	280 - 839	28 - 156	285 - 758	199 - 342	-137 - 57	187 - 825	50 - 420	-405 - -71
LF-norm									
Median	64 ^{A}	71	9	66 ^{A}	59 ^{B}	-7 ^{C}	59	76 ^{B}	-17 ^{C}
25-75% range	35 - 70	46 - 79	-3 - 20	63 - 81	47 - 75	-13 - 7	57 - 66	63 - 91	-6 - 19
HF (ms^2)									
Median	391	120 ^{A}	-15	200	257 ^{B}	-19 ^{D}	294 ^{C}	41 $^{A B C}$	-215 ^{D}
25-75% range	124 - 876	98 - 1070	-293 - -4	111 - 392	50 - 331	-66 - 57	95 - 518	12 - 47	-253 - -56
HF-norm									
Median	36 ^{A}	29	-9	34 ^{A}	41 ^{B}	7	41	23 ^{B}	-17
25-75% range	29 - 65	21 - 54	-20 - 3	20 - 37	25 - 53	-7 - 13	34 - 43	9 - 38	-20 - -6
LF/HF									
Median	1.8 ^{A}	2.4	0.5	1.9 ^{A}	1.4 ^{B}	-0.4	1.4	3.3 ^{B}	2.0
25-75% range	0.5 - 2.4	0.9 - 3.9	-0.9 - 1.5	1.7 - 4.1	0.9 - 3.0	-0.5 - 0.3	1.3 - 2.0	1.7 - 10.2	0.4 - 7.4

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; Pre-Exp = pre-exposure; End-Exp = final 25 min. of exposure; Delta = End-Exp minus Pre-Exp; TOTAL = total power; VLF = very low frequency; LF = low frequency; LF-norm = LF normalized to VLF and TOTAL; HF = high frequency; HF-norm = HF normalized to VLF and TOTAL; LF/HF = ratio of LF to HF.
$^{A B C D}$ = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 12. Experiment One: Heart-rate variability, frequency-domain indices, standing position, pre-exposure and end-exposure to filtered air, particles, and particles + ozone.

Indices	Exposure Condition								
	FA			P			PO		
	Pre-Exp	End-Exp	Delta	Pre-Exp	End-Exp	Delta	Pre-Exp	End-Exp	Delta
TOTAL (ms^2)									
Median	2654	2294 [A]	579	2904	2442	283 [B]	4196	1417 [A]	-1262 [B]
25-75% range	967 - 5991	1546 - 3550	-3825 - 1519	1345 - 3145	2148 - 3842	-462 - 697	1076 - 5267	1025 - 2008	-3850 - -653
VLF (ms^2)									
Median	1280	1273	142	1682 [A]	1436	457 [B]	1964 [A]	817	-482 [B]
25-75% range	596 - 4773	738 - 1743	-3828 - 1282	723 - 1700	1187 - 3144	-246 - 1444	786 - 5014	741 - 1082	-3932 - -277
LF (ms^2)									
Median	819	680 [A]	190	1111	575	-179	347	262 [A]	-149
25-75% range	376 - 1242	624 - 1789	84 - 377	531 - 1326	555 - 820	-751 - -165	246 - 2056	198 - 917	-947 - -135
LF-norm									
Median	82	84	3	87	82	-9	84	78	-3
25-75% range	74 - 86	84 - 91	1 - 8	86 - 91	75 - 85	-11 - -2	80 - 85	77 - 89	-7 - 8
HF (ms^2)									
Median	123	127 [A]	27	112	123	4	105 [B]	86 [A B]	-35
25-75% range	69 - 399	91 - 167	-187 - 44	91 - 119	63 - 140	-52 - 5	88 - 175	72 - 139	-36 - -33
HF-norm									
Median	19	16	-3	13	18	9	16	22	3
25-75% range	15 - 26	9 - 17	-8 - -1	9 - 14	15 - 25	2 - 10	15 - 20	11 - 23	-8 - 7
LF/HF									
Median	4.4	5.4	1.0	6.9	4.7	-2.8	5.1	3.6	-1.6
25-75% range	2.8 - 5.9	5.1 - 9.8	0.6 - 1.8	6.3 - 9.9	3.0 - 5.9	-3.3 - -1.0	3.9 - 5.6	3.4 - 8.0	-1.8 - 2.2

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; Pre-Exp = pre-exposure; End-Exp = final 25 min. of exposure; Delta = End-Exp minus Pre-Exp; TOTAL = total power; VLF = very low frequency; LF = low frequency; LF-norm = LF normalized to VLF and TOTAL; HF = high frequency; HF-norm = HF normalized to VLF and TOTAL; LF/HF = ratio of LF to HF. [A B] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 13. Experiment Two: Leukocyte and epithelial cell differential percent in bronchial fraction, and bronchoalveolar lavage fluid, post-exposure to filtered air-one exposure, particles-one exposure, and particles-three serial exposures.

Cell	Exposure Condition					
	FA		P		P3	
	Bfx	BAL	Bfx	BAL	Bfx	BAL
Macrophages (%)						
Median	95.2	92.6	91.6	89.0	96.3	93.0
25-75% range	92.5 - 96.1	89.2 - 97.5	86.4 - 96.4	79.4 - 95.8	90.7 - 97.1	88.9 - 97.8
Neutrophils (%)						
Median	2.6	3.6	3.9	2.6	2.7	2.4
25-75% range	2.2 - 4.5	1.5 - 4.5	2.4 - 5.3	1.2 - 5.2	1.0 - 5.8	0.8 - 4.7
Lymphocytes (%)						
Median	1.1 [A]	2.8	1.9 [B]	4.1	0.0 [A B]	0.3
25-75% range	0.0 - 3.6	0.3 - 5.5	0.3 - 5.9	0.0 - 8.6	0.0 - 0.4	0.0 - 0.8
Eosinophils (%)						
Median	0.0	0.0	0.6	0.6	1.0	1.1
25-75% range	0.0 - 0.3	0.0 - 0.3	0.0 - 1.0	0.4 - 1.3	0.0 - 2.1	0.8 - 1.4
Epithelial Cells (%)						
Median	9.8	3.8	17.3	5.5	12.3	7.5
25-75% range	8.5 - 22.3	1.8 - 5.3	9.8 - 27.5	4.3 - 15.3	10.3 - 27.5	3.8 - 8.8

Values are median and 25-75% range. Epithelial cells = percent of total non-squamous cells; all other cell types = percent of leukocytes. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; P3 = P x 3 serial-day exposures; BFx = bronchial fraction; BAL = bronchoalveolar lavage. [A B] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 14. Experiment Two: Leukocyte and epithelial cell concentration in bronchial fraction, and bronchoalveolar lavage fluid, post-exposure to filtered air-one exposure, particles-one exposure, and particles-three serial exposures.

Cell	Exposure Condition					
	FA		P		P3	
	Bfx	BAL	Bfx	BAL	Bfx	BAL
Total Leukocytes ($x10^4$ ml)						
Median	12.5	21.0	12.3	16.0	11.3	19.8
25-75% range	6.3 - 18.8	15.0 - 22.8	6.3 - 13.3	13.0 - 19.5	8.8 - 15.8	14.0 - 23.5
Macrophages ($x10^4$ ml)						
Median	11.0	18.9	9.8	14.1	8.8	18.5
25-75% range	6.0 - 18.4	12.8 - 21.3	5.8 - 12.1	10.1 - 19.3	8.2 - 13.4	13.2 - 20.5
Neutrophils ($x10^4$ ml)						
Median	0.3	0.5	0.3	0.6	0.2	0.2
25-75% range	0.3 - 0.7	0.2 - 0.7	0.3 - 0.6	0.2 - 0.9	0.2 - 0.6	0.1 - 1.7
Lymphocytes ($x10^4$ ml)						
Median	0.1	0.7	0.1	0.3	0.0	0.1
25-75% range	0.0 - 0.8	0.1 - 1.0	0.0 - 0.3	0.0 - 2.2	0.0 - 0.1	0.0 - 0.1
Eosinophils ($x10^4$ ml)						
Median	0.0	0.0	0.0	0.1	0.1	0.2
25-75% range	0.0 - 0.0	0.0 - 0.1	0.0 - 0.1	0.0 - 0.3	0.0 - 0.3	0.1 - 0.3
Epithelial Cells ($x10^4$ ml)						
Median	2.0	1.0^{A}	3.3	2.5^{A}	3.3	1.5
25-75% range	1.5 - 3.3	0.5 - 1.3	2.3 - 4.3	1.5 - 3.5	1.3 - 5.0	1.0 - 2.0

Values are median and 25-75% range. Epithelial cells = percent of total non-squamous cells; all other cell types = percent of leukocytes. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; P3 = P x 3 serial-day exposures; BFx = bronchial fraction; BAL = bronchoalveolar lavage. ^{A} = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Table 15. Experiment Two: Protein concentrations in bronchial fraction, and bronchoalveolar lavage fluid, post-exposure to filtered air-one exposure, particles-one exposure, and particles-three serial exposures.

Protein	Exposure Condition					
	FA		P		P3	
	Bfx	BAL	Bfx	BAL	Bfx	BAL
Total Protein (μg ml)						
Median	106	158	113	174	111	155
25-75% range	61 - 160	125 - 169	39 - 188	115 - 231	63 - 133	133 - 177
IL-8 (pg ml)						
Median	24.8	6.6	35.4	20.8	12.5	9.0
25-75% range	8.4 - 44.8	5.2 - 23.9	22.2 - 89.3	9.0 - 28.4	6.9 - 32.3	6.5 - 12.3
GMCSF (pg ml)						
Median	0.9	4.2	0.7	3.5	0.6	3.2
25-75% range	0.5 - 1.1	3.8 - 4.5	0.6 - 0.9	3.2 - 4.1	0.4 - 2.4	3.0 - 3.9
CRP (μg ml)						
Median	0.002	<0.00035	0.002	<0.00035	0.002	<0.00035
25-75% range	0.001 - 0.004		0.001 - 0.003		0.001 - 0.006	

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; P3 = P x 3 serial-day exposures; BFx = bronchial fraction; BAL = bronchoalveolar lavage; IL-8 = interleukin-8; GMCSF = granulocyte macrophage colony stimulating factor; CRP = C-reactive protein.

Table 16. Experiment Two: Spirometric pulmonary function, immediately pre- and post-exposure, and 18 hr post-exposure, to filtered air-one exposure, and particles-one exposure.

	Exposure Condition					
	FA			P		
	Pre-Exp	Post-Exp	Post-18	Pre-Exp	Post-Exp	Post-18
FVC (l)						
Median	3.59	3.70	3.75	3.84	3.82	3.64
25-75% range	3.20 - 4.05	3.21 - 3.97	3.32 - 4.08	3.11 - 3.99	3.24 - 4.04	3.04 - 3.89
FEV_1 (l)						
Median	2.65	2.68	2.71	2.70	2.71	2.69
25-75% range	2.25 - 3.02	2.10 - 2.98	2.10 - 2.90	2.06 - 3.04	2.16 - 3.06	2.03 - 3.06
FEF_{25-75} (l s)						
Median	2.31	2.19	2.23	1.72	1.76	1.77
25-75% range	1.47 - 2.94	1.60 - 3.07	1.51 - 3.03	1.59 - 2.95	1.55 - 2.89	1.70 - 2.85

Values are median and 25-75% range. Abbreviations: FA = filtered air; P = carbon and ammonium nitrate particles [total 300 $\mu g/m^3$]; PO = P + ozone [0.2 ppm]; Pre-Exp = pre-exposure; Post-Exp = post-exposure; Post-18 = 18 hr post-exposure; FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; FEF_{25-75} = forced expired flow-rate at 25-75% FVC.

Table 16 A. Experiment Two: Spirometric pulmonary function, immediately pre- and post-exposure, and 18 hr post-exposure, to particles-three serial exposures.

	Exposure Condition						
	P3 Exp-1		P3 Exp-2		P3 Exp-3		
	Pre-Exp	Post-Exp	Pre-Exp	Post-Exp	Pre-Exp	Post-Exp	Post-18
FVC (l)							
Median	3.78	3.84	3.82	3.81	3.86	3.79	3.74
25-75% range	3.06 - 3.86	3.03 - 3.86	3.11 - 4.05	3.08 - 4.03	3.23 - 3.96	3.16 - 3.95	2.84 - 4.03
FEV_1 (l)							
Median	2.63	2.73	2.67	2.99 [A]	2.73 [B]	2.61 [C]	2.57 [A B C]
25-75% range	2.19 - 2.97	2.09 - 2.94	2.20 - 3.29	2.16 - 3.21	2.23 - 3.02	2.28 - 2.97	2.11 - 2.88
FEF_{25-75} (l s)							
Median	1.77	1.67	2.39 [A]	1.91 [B]	1.87	1.74	1.63 [A B]
25-75% range	1.58 - 2.98	1.48 - 2.77	1.96 - 2.94	1.68 - 2.98	1.55 - 2.65	1.68 - 2.46	1.48 - 2.38

Values are median and 25-75% range. Abbreviations: P3 = P x 3 serial-day exposures; Exp-1 = exposure-1; Exp-2 = exposure-2; Exp-3 = exposure-3; Pre-Exp = pre-exposure; Post-Exp = post-exposure; Post-18 = 18 hr post-exposure; FVC = forced vital capacity; FEV_1 = forced expired volume in 1 s; FEF_{25-75} = forced expired flow-rate at 25-75% FVC. [A B C] = values with same subscript letter with-in each variable are significantly different ($P < 0.05$).

Discussion

This project was designed to test the hypothesis that airway inflammation would be increased, and SPF and HRV would be decreased, as a function of single particle exposure, and to a larger degree, as a function of combined particle and O_3 exposure, and serial particle exposure, compared to filter air exposure. The results of the two experiments support, in part, this hypothesis, there being low-level particle-induced changes in cell distribution, decreases in SPF, and a decrease in HRV. Further, the particle and O_3 combination exposure resulted in larger and differential changes in cell distribution, SPF, and HRV.

The absence of any particle-induced changes in macrophages and neutrophils in the Bfx or BAL in Experiment One differs from the increase in monocytes and neutrophils in the Bfx, and total cells and neutrophils in BAL, resulting from CAPs exposure in healthy subjects (Ghio *et al.* 2000). As the particles used in the current project were pure carbon and ammonium nitrate only, there would be a large number of both soluble and insoluble components of CAPs, including metals and acids, which were not present in/on the currently used particles, which could be responsible for the CAPs-induced inflammatory response. With-in the soluble components of CAPs, the sulfate/iron/selenium component was associated with the CAPs-induced increase in BAL neutrophils (Huang *et al.* 2003). In the CAPs exposure experiment (Ghio *et al.* 2000), for the group which had the increase in neutrophils, the average exposure particle concentration was 206.7 $\mu g/m^3$ the particle MMAD was 0.65 μm, and the exposure duration was 2 hr, and in the current experiment, the particle concentration was 283 $\mu g/m^3$, the mean particle size was 0.61 μm, and the exposure duration was 4 hr, providing approximately double the total exposure/dose. As there was no indication of a macrophage or neutrophil mediated airway inflammatory response in the current experiment, comparison of these experiments could indicate that for particle of this size, at these concentrations, there is no general mechanical (non-chemical), effect of inhaled particles on airway inflammation, and that it is the chemical components of the particles that are responsible for the induction of inflammation.

As epithelial cells are the initial cells to contact inhaled particles, these cells would be expected to be involved in the initiation of an inflammatory response and the uptake and clearance of particles. In subjects with asthma, CAPS exposure also results in a decrease in columnar cells in induced-sputum (Gong *et al*. 2003). In Experiment Two of this project, there was a particle-induced increase in epithelial cells in the BAL. It is not possible to directly compare cell counts from induced-sputum and Bfx or BAL, as these techniques sample different region of the respiratory tract. However, the difference in these results requires further investigation.

As there were no changes in neutrophils, lymphocytes, and eosinophils, and a decrease in lymphocytes in the particle only exposure condition, and there is a well characterized airway inflammatory response attributable to O_3 exposure in individuals with asthma (Reviewed: Peden, 2003) it could be expected that the combined particle and O_3

exposure-induced increases in neutrophils, lymphocytes, and eosinophils, were due to the O_3 component of the exposure environment.

Particle exposure did not result in any inflammatory related changes in protein levels in Bfx or BAL. This finding is in accord with CAPs exposure experiment (Ghio *et al.* 2000). The absence of any particle-only induced changes in total protein, GMCSF and CRP, would indicate that the increases in these proteins in the combined particle and O_3 exposure were due, at least primarily, to the O_3 component of the exposure environment. Similarly, compared to both the filtered air and particle-only conditions, the combined particle and O_3 exposure induced increases in IL-8 and IL-10 mRNA levels, indicates that these pro- and anti-inflammatory responses were due to the O_3 component of the exposure environment. These counteractive changes in IL-8 and IL-10 are an indication of the dynamic balance between pro- and anti-inflammatory processes in response to inhaled pollutants.

Particle exposure resulted in decreases in spirometric pulmonary function. In the single particle exposure there was a decrease in FEF_{25-75}, and in the serial particle exposure there were decreases in both FEV_1 and FEF_{25-75}. These changes are in contrast to the finding of no change in SPF in other controlled human particle exposure experiments in subjects with asthma, which have used generated ammonium nitrate particles (Kleinman *et al.*, 1980), carbon particles (Anderson *et al.*, 1992), and sodium bisulfate particles, and ammonium sulfate particles (Utell *et al.*, 1983), and CAPs (Gong *et al.,* 2003). It is possible that differences in exposure particle concentration, duration, and exercise could have contributed to the differing results of these studies. In the current project, the changes in SPF occurred in the absence of any macrophage or neutrophil related inflammation. Therefore, it is probable that pulmonary function and airway inflammation is not linked under these conditions. Assessment of the correlation between acute O_3-induced changes in FVC and FEV_1 and subsequent airway inflammation have shown no correlation or a negative correlation between pulmonary and airway inflammation indices (Balmes *et al.,* 1996; Schelegle *et al.,* 1991). The particle-induced changes in SPF in this project require further investigation.

This study is the first controlled human exposure study to investigate the combined effects of exposure to particles and O_3 on HRV. In previous studies, ambient air particles, CAPS, and ambient O_3 have been associated with decreased HRV (Gold *et al*, 2000; Gong *et al,* 2003; Pope *et al,* 1999). The physiological mechanism underlying this effect is unknown, but may involve elevation of systemic inflammatory mediators (Schwartz, 2001) or activation of pulmonary irritant receptors that mediate stimulation of parasympathetic pathways (Watkinson *et al*, 2001). Across the combined particle and O_3 exposure there was a decrease in the time domain variable SDNN, and delta values for this exposure, compared to the delta values for the filter air exposure were decreased for both the SDNN and the SDNNi. For the frequency domain variables, comparison of pre-exposure supine and standing values followed the expected pattern, validating the physiologic relevance of the results. The normalized LF and the LF/HF ratio values were increased by standing, evidence of increased sympathetic tone (Pagani *et al*, 1986). Supine and standing measurements were then analyzed

separately to investigate the effect of exposure on frequency domain variables. In the supine position, the LF-norm, and HF decreased, and in the standing position, the total power and VLF decreased, from particle-only to combined particle and O_3 exposure. Controlled human exposure to CAPs resulted in a 35.7% decrease in HF in elderly subjects, but a similar effect was not seen in younger subjects (Devlin *et al,* 2003). However, another human exposure study found an increase in HF with CAPS exposure in subjects with asthma (Gong *et al,* 2003).

The magnitude of the HRV effect found with PO exposure in this study could be clinically relevant. In a prospective study of HRV and mortality in congestive heart failure patients reported a relative risk for mortality of 1.62 (95% CI, 1.16 to 2.44) with a decrease in SDNN of 41.2ms, and annual mortality rates for three separate HRV-defined subgroups: 5.5% for SDNN>100 ms, 12.7% for SDNN 50-100 ms, and 51.4% for SDNN<50ms (Nolan *et al,* 1998). In a study of recent myocardial infarction patients who had an SDNN <50 ms, the relative risk for cardiac mortality was 2.94 (Lanza *et al,* 1999). The risk for sudden death, mortality from coronary artery disease, and all-cause mortality in a Dutch cohort of men with SDNN measurements of <20 ms, compared with men with SDNN between 20ms and 39ms was 2.1 (95% CI, 1.4 to 3.0) for middle-aged men and 1.4 (95% CI, 0.9 to 2.2) for elderly men (Dekker *et al,* 1997).

For HRV, the primary limitation of this study was the lack of respiratory monitoring or control during ECG monitoring. Respiration is known to affect heart rate through vagally-mediated, parasympathetic pathways, with controlled respiration at rest inducing an increase in HF and a decrease in LF, with a corresponding decrease in the LF/HF ratio (Pagani *et al,* 1986). Acute O_3 inhalation has been shown to cause a significant increase in respiration rate with a concurrent decrease in tidal volume (Weinmann *et al,* 1995). The interaction between O_3-induced respiration changes and respiration-induced HRV changes could have effected the finding of this project. However, in a study designed to investigate the impact of metronome-paced breathing on particle-related effects on HRV, the association between $PM_{2.5}$ exposure and reduced r-MSSD did not diminish after controlling for respiratory rate through metronome-paced breathing, or after adjusting for the observed respiratory rate (Gold *et al,* 2000). This suggests that it is unlikely that the PM effect on HRV is explained solely by alterations in respiration.

The HRV results of this study suggest that exposure to generated carbon and ammonium nitrate particles alone had little effect on cardiac autonomic function, while exposure to generated particles plus O_3 had the expected effect of decreasing HRV in asthmatic adults. A decrease in HRV was seen with O_3 exposure, which causes marked airway inflammation and stimulation of pulmonary irritant receptors, than with carbon and ammonium nitrate particle exposure, which caused essentially no airway inflammation. These preliminary HRV results suggest that airway inflammation and modulation of parasympathetic pathways may be the physiologic mechanisms by which particles and O_3 affect HRV. Future studies are needed to explore the physiologic link between O_3 inhalation and HRV changes, possibly through measurement of systemic inflammation mediators, endothelins, or renin-angiotensin system proteins.

The particles used in this project consisted of pure carbon and ammonium nitrate, and were of one size distribution. Although unknown, it is possible that particles consisting of more biologically active compounds (metal, acids), and of different size distributions, could be expected to induce larger changes in airway inflammation, SPF, and HRV, for the same concentration and exposure duration. The direct effect of various particle chemical compositions and size distributions requires further investigation.

In this project, all subjects were required to withhold all prescribed asthma and allergy medications, and any other medications with potential anti-inflammatory properties, for appropriate periods prior to each testing session. It is possible that if the subjects had continued to use their medications during the project, that the airway inflammation and SPF changes, and subsequently the HRV changes resulting from the current exposures would have been diminished due to the anti-inflammatory and bronchodilator effects of these medications. The design and results of this project do not allow quantification of this issue.

Summary and Conclusions

The results of this project indicate that both single and serial exposures to carbon and ammonium nitrate particles result in low-level changes in airway cell distribution, decreases in SPF, and a decrease in HRV in individuals with asthma. Further, combined exposure to particles and O_3 produced increases in inflammatory associated cells, and protein and mRNA levels, decreases in SPF, physiologically relevant decreases in HRV. It is expected that the changes in the combined particle and O_3 exposure condition were due, at least primarily, to the O_3 component of the exposure environment.

Recommendations

The results of this project need to be further investigated in healthy individuals, elderly individuals, and individuals with respiratory and/or cardiovascular disease. Further investigations need to include determination of the effect of particle composition, and size distribution. Additional investigations need to be conducted to determine the effects of O_3-only exposures on HRV in all subject groups.

Table 17. Table of abbreviations

Exposure conditions:
FA = filtered air; single exposure
P = carbon and ammonium nitrate particles at 300 $\mu g/m^3$; single exposure
PO = carbon and ammonium nitrate particles at 300 $\mu g/m^3$ and O_3 at 0.2 ppm; single exposure
P3 = carbon and ammonium nitrate particles at 300 $\mu g/m^3$; serial (3-day) exposures

Bronchoscopy:
Bfx = bronchial fraction (first 15 ml of bronchoalveolar lavage)
BAL = bronchoalveolar lavage

Protein:
CRP = C-reactive protein
IL-8 = interleukin-8
GMCSF = granulocyte and macrophage colony stimulating factor

mRNA:
IL-1β = interleukin-1 beta
IL-6 = interleukin-6
IL-8 = interleukin-8
IL-10 = interleukin-10
HIN-1 = high in normals-1

Spirometric pulmonary function:
SPF = spirometric pulmonary function
FVC = forced vital capacity
FEV_1 = forced expired volume in 1 s
FEF_{25-75} = forced expired flow-rate at 25-75% FVC

Heart Rate Variability:
Time Domain:
SDNN = standard deviation of all NN intervals
SDANN = standard deviation of means of all NN intervals
SDNNi = standard deviation of all NN intervals index
r-MSSD = root mean square of successive differences

Frequency Domain:
Total = total power
VLF = very low frequency
LF = low frequency
LF-norm = normalized low frequency
HF = high frequency
HF-norm = normalized high frequency

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