Final Report

Mechanisms of Particulate Toxicology: Systemic Effects in Sensitive Animal Models and Susceptible Humans

Principal Investigator: M.T. Kleinman, Ph.D.
Co-Investigators: D. Meacher, Ph.D.; A. Hamade, M.S.
University of California, Irvine
Collaborators: K. Pinkerton, Ph.D[1]; C. Solomon, Ph.D.[2]
[1]University of California, Davis
[2]University of California, San Francisco

Contract # 99-316
Project Period: 3/01/00 - 5/23/04

AIR POLLUTION HEALTH EFFECTS LABORATORY
Department of Community and Environmental Medicine
University of California, Irvine
Irvine, CA 92697-1825

Submitted to:
THE CALIFORNIA AIR RESOURCES BOARD

May, 2004

Disclaimer

The statements and conclusions in this report are those of the University and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein is not to be constructed as actual or implied endorsement of such products.

Acknowledgements

Aged rats for this study were obtained with partial support from the National Institute on Aging, one of the National Institutes of Health (NIH). We are particularly indebted to Drs. Christine Nadjieko and Terry Gordon for the loan of several telemetry modules to supplement the number we were able to purchase. We also thank Charles Bufalino, and Gary Devillez for their technical assistance in conducting this project, Dr. Robert F. Phalen for his help in coordinating the facilities of the Air Pollution Health Effects Laboratory and Ms. Susan Aquivan for her assistance in preparing and submitting this report. This report was submitted in fulfillment of ARB Contract 99-316, " Mechanisms of Particulate Toxicology: Systemic Effects in Sensitive Animal Models and Susceptible Humans," by the University of California, Irvine under the support of the California Air Resources Board. Work was completed March, 2004.

Abstract

Particle-induced lung injury and heart responses were measured in young adult and geriatric (senescent) rats exposed to laboratory generated particles composed of elemental carbon and ammonium nitrate. The particle compositions and concentrations used were consistent with ambient aerosols collected and characterized in California. This study was part of a coordinated multicampus program. The biological responses studied focused on cardiophysiologic factors (changes in blood pressure, variability in heart rate and abnormal heart rhythms) in the animal studies and macrophage superoxide production in the human studies.

Acute (4 hour) single day exposures to particles alone were compared with the effects of a mixture of particles and ozone at approximately the same particle concentrations. Both exposures produced decreases in blood pressure and heart rate. Blood pressure changes (differences between pre-exposure and post-exposure levels) were significantly different from controls. Heart rate was decreased by pollutant exposure but the response was not statistically significant. The changes induced by single day exposure to particles alone were not significantly different from those induced by the single day ozone + particle mixture. Thus, the effects observed may be those of the particles and not due to the presence of ozone in the mixture.

Repeated 3-day (4 hour per day) exposures to particles caused changes in heart rate and blood pressure that were statistically significant immediately post-exposure, but there were no changes in baseline levels measured 20 hours after exposure. Heart rate variability was not significantly changed after a single exposure to particles, but showed progressive reduction after 2 and three days of consecutive exposure, compared to clean air exposures.

Table of Contents

List of Figures

List of Tables

Executive Summary

Background

This study analyzed the effects of acute, short-term exposures to particulate matter (PM) on the cardiopulmonary system. Cardiopulmonary effects were examined because epidemiological studies established associations of increased risks of respiratory illness and mortality in people exposed to elevated levels of ambient PM. This study used laboratory-generated particles containing two components of ambient PM, ammonium nitrate (AMN) and elemental carbon (EC). AMN and EC are major constituents of fine particle ambient PM (i.e. PM2.5) in California. The concentrations of nitrate in polluted cities of the Northeastern US are much lower than in California, and very few studies had examined the potential health effects of nitrate-containing particles.

The primary objective of this project are: (1) to examine the cardiopulmonary health effects of atmospheric mixtures that realistically model sizes and compositions of particles in California air and (2) to examine mechanisms that mediate systemic changes and other adverse effects of inhaled particles. This project is a component of a larger multicampus, interdisciplinary research program. The studies performed at UCI are designed to complement both the human clinical studies of air pollution effects at UCSF on healthy volunteers and people with asthma. The studies at UCI focus on fine particles and their role in inducing cardiopulmonary responses in a sensitive animal model, the senescent or 'geriatric' rat. The senescent rat model was chosen because it is an accepted model for certain characteristics of the aging human population. In addition, samples of lung cells collected following human exposures at UCSF and animal exposures at UCD were analyzed for oxidative stress-related mediators. Since this study was part of a coordinated, multicampus program, particle formulation, chemical composition and exposure parameters were intended to be similar at each campus.

Approach

The first specific aim of this study was to determine the cardiopulmonary responses to combination ozone-particle (PO) inhalation by human volunteers and sensitive laboratory animal models and compare those to the effects of particles alone (PM). To accomplish this aim, senescent rats were exposed nose-only to aerosols of ammonium nitrate (AMN; 150 $\mu g/m^3$) and elemental carbon (EC; 100 $\mu g/m^3$) with and without ozone (O_3; 0.2 ppm). The exposures were 4 hours per day for a single day. These studies involved three atmospheres, (1) filtered air (FA); (2) particles alone (PM); and (3) particles and ozone (PO). The particle mass median aerodynamic diameter (MMAD) and total particle mass concentrations used were 0.7 μm and 250 $\mu g/m^3$, respectively.

The rats were implanted with telemetry devices that monitored cardiac function (blood pressure, heart rate and electrocardiographic data) in unrestrained animals. A major difficulty in this study was that the blood pressure sensor did not function well in the geriatric rats and we monitored the blood pressure using a tail cuff sphygmomanometer designed for rodents. We used ANOVA followed by a Tukey multiple comparison to test the hypothesis that biological responses to PM+O_3 mixtures were worse than responses to PM alone for macrophage free radical production, heart rate, blood pressure and the heart rate x blood pressure product (double product – an index of cardiac output).

Specific Aim 2 was to compare the effects of single day PM exposure with those of a multiple-day exposure to PM. The overall approach was described above for Aim 1. The three-day exposures were on consecutive days and involved two atmospheres, (1) FA; and (2) PM. We used ANOVA followed by a Tukey multiple comparison to test the hypothesis that biological responses to 3-day PM exposures were worse than responses to PM alone for macrophage free radical production, heart rate, blood pressure and the heart rate x blood pressure product (double product – an index of cardiac output) and heart rate variability. The data for each biological endpoint were analyzed using ANOVA to specifically address the hypothesis that the responses following multiple day exposures were significantly greater than those after a single day of exposure.

Discussion and Conclusions

Acute (4 hour) single day exposures to particles alone were compared with the effects of a mixture of particles and ozone at approximately the same particle concentrations. Both exposures produced decreases in blood pressure and heart rate. Blood pressure changes (differences between pre-exposure and post-exposure levels) were significantly different from controls. Heart rate was decreased by pollutant exposure but the response was not statistically significant. The changes induced by single day exposure to particles alone were not significantly different from those induced by the single day ozone + particle mixture, although th PO difference from control was significant and the PM difference from control was not.

Superoxide production by macrophages obtained by BAL from human subjects after 1 day of PM or PO exposure was elevated relative to controls, however only the PO exposure-induced effect was significantly different from that measured after FA exposure.

Repeated 3-day (4 hour per day) exposures to particles caused changes in blood pressure that were statistically significant immediately post-exposure, but there were no changes in baseline levels measured 20 hours after exposure. Heart rate variability was not significantly changed after a single exposure to particles, but showed progressive reduction after 2 and three days of consecutive exposure, compared to clean air exposures.

We conclude that our findings support the associations of cardiophysiological changes observed in epidemiological studies and ambient PM exposures. We observed changes in blood pressure and heart rate variability that are consistent with an adverse effect of PM on the heart. At the levels of exposure used in this study, which are comparable to those now being studied in experiments that use particle concentrators, we found that significant changes could be seen in a susceptible animal model the senescent, or geriatric, rat. We also showed increased activation of macrophages from humans exposed under conditions that were nearly identical with those used in the UCI rodent studies. This study has provided an opportunity to compare animal responses and human responses to similar exposures and similar conditions. This should be a great aid in developing strategies for extrapolating from animals to humans. This study suggests that more information is needed to assess the longer term effects of biological responses to particle exposure, especially for improving our understanding of how long term, low-level exposures contribute to the development of human lung and heart disease, and that the senescent rat is an appropriate model for such future studies.

Introduction

Background

Human exposures to particulate matter (PM) have been associated with acute illness and death, especially in people older than 65 years of age or people with pre-existing lung or heart disease. Current federal and California ambient air quality standards were established for particles smaller than 10 μm (PM_{10}) in mass median aerodynamic diameter (MMAD), because particles in this size range are inhaled and penetrate deeply into the respiratory tract, and they are associated with adverse effects on human health. More recently both the federal EPA and the State of California have imposed standards on particles smaller than 2.5 μm in MMAD. The California and federal $PM_{2.5}$ standards were promulgated to (a) prevent excess deaths from short-term exposures and from exacerbation of symptoms in sensitive individuals with respiratory disease; and (b) prevent excess seasonal declines in pulmonary function, especially in children. The current National Ambient Air Quality Standard (NAAQS) for $PM_{2.5}$ is 15 $\mu g/m^3$ (annual arithmetic mean). The State of California has set more stringent $PM_{2.5}$ standards of 12 $\mu g/m^3$ (annual mean). The federal government is now reviewing a NAAQS for the coarse fraction of PM ($PM_{2.5-10}$)

$PM_{2.5}$ concentrations are strongly influenced by emissions from combustion sources and atmospheric chemical reactions, whereas larger particles (>2.5 μm) are primarily generated by mechanical processes such as abrasion and resuspension.[1,2] The chemical constituents of the larger sized particles are predominantly those characteristic of the earth's crust (e.g. iron, silica, aluminum). The $PM_{2.5}$ particles are more enriched in heavy metals, elemental carbon, organic carbon, and products of atmospheric chemical and photochemical processes including sulfate and nitrate salts.[3,4]

Exposures to urban PM_{10} at levels near the State's (50 $\mu g/m^3$, 24 hr average) and often below the PM10 national (150 $\mu g/m^3$, 24 hr average) ambient air quality standard (NAAQS) are associated with increased hospital admissions and emergency room visits for respiratory illnesses,[5,6,7,8] increased incidences of asthma attacks,[9,10] increased asthma medication use,[11] reduced pulmonary function,[11, 12,13] and increased daily mortality.[14,15,16,17] People whose deaths have been attributed to $PM_{2.5}$ and PM_{10} exposures tend to be older than 65 years of age, although health effects have also been reported for children and people with asthma. The study

reported by Pope et al. [18] showed increased mortality for individuals 35 to 64 years of age associated with PM 2.5 exposures at or below the promulgated PM $_{2.5}$ NAAQS. Individuals with pre-existing cardiovascular or chronic pulmonary diseases are at even greater risk of PM-related mortality.[19]

Other pollutants, such as ozone (O_3), sulfur dioxide (SO_2), nitrogen dioxide (NO_2) and carbon monoxide (CO) have been examined in conjunction with PM health effects in some studies, as have meteorological variables such as temperature, relative humidity and season. Interactions of O_3 or CO with PM-induced health effects have been reported and there is some contention that in some studies it is not possible to disentangle the effects of the co-pollutants from those of PM. However, in most of the reported epidemiological studies, PM has been shown to have an independent association with mortality which remains significant even when other copollutants are introduced into the statistical model, whereas the copollutants often do not. Most laboratory studies of particle-induced health effects have focused on the pulmonary effects. However, heart disease is the leading cause of death and hospitalization among adults 65 years of age or older,[19] which makes the identification of preventable causes of heart disease-related morbidity and mortality a major research need. Numerous epidemiologic time series studies have shown positive associations of outdoor ambient particulate matter (PM) air pollution with cardiovascular hospital admissions and mortality. However, the causal components driving the relationship of cardiovascular morbidity and mortality with PM remain to be identified. Some data suggest that associations between mortality and fine particle or acidic particle concentrations are stronger than those between mortality and coarse particle concentrations.[20] For example, exposures to respirable fine (≤ 2.5 μm MMAD) particles and sulfates have been associated with increased total annual mortality rates.[21,22] Other data suggest that ultrafine particles ($d_p \leq 0.1$ μm) may be an especially toxic subfraction of PM. However, there are some studies that show stronger associations between health effects and exposure to coarse particles[23,24,25] than between health effects and fine particle exposures.

There is relatively little laboratory animal data regarding the basic science of how particulate matter causes development of and triggering of cardiovascular disease. Exposure of animals to particulate matter and residual oil fly ash have been associated with arrhythmias and altered ECGs. Monocrotaline treated rats exposed to residual oil fly ash demonstrated variable

R-R interval, premature atrial and ventricular contractions, multiple premature ventricular contractions, bundle branch block, and skipped beats on their electrocardiograms.[26]

Dogs exposed to very high concentrations of PMs demonstrated ST elevation.[27] ECG changes, including changes in PQ and ST, were observed with exposure to ambient air particles in normal dogs and worsened in dogs exposed to a five minute coronary artery occlusion.[28, 29] Exposures to an average concentration of 290 $\mu g/m^3$ concentrated ambient particles (CAPs) enhanced occlusion-induced ST-segment elevation in dogs, which was significantly correlated with silicon and other crustal elements. [30] Rats with left ventricular myocardial infarctions (MIs) induced by thermocoagulation, exhibited increased premature ventricular complexes (PVC's) when exposed by inhalation to residual oil fly ash (ROFA). [31] There was no difference in arrhythmia frequency in sham-exposed rats with MIs. Muggenberg [32] reviewed other reports of small numbers of animals that demonstrated pulmonary hypertension and spontaneous hypertension upon exposure to air pollutants.

Project Objectives and Specific Aims

A. Objectives

The primary objective of this project are: (1) to examine the cardiopulmonary health effects of atmospheric mixtures that realistically model sizes and compositions of particles in California air and (2) to examine mechanisms that mediate systemic changes and other adverse effects of inhaled particles. This project is a component of a larger multicampus, interdisciplinary research program. The studies performed at UCI are designed to complement both the human clinical studies of air pollution effects at UCSF on healthy volunteers and people with asthma as well as the laboratory animal studies at UCD designed to examine mechanisms of asthma in juvenile rats and other animal models. The studies at UCI focus on fine particles and their role in inducing cardiopulmonary responses in a sensitive animal model, the senescent or 'geriatric' rat. In addition, samples of lung cells collected following human exposures at UCSF and animal exposures at UCD were analyzed for oxidative stress-related mediators. Since this study was part of a coordinated, multicampus program, particle formulation, chemical composition and exposure parameters were intended to be similar at each campus. It was intended that these similarities would facilitate cross study comparisons between findings with laboratory animals and human subjects and improve our ability to draw conclusions that would

be more generalizable than they would be if data were restricted to any single, isolated experiment. Also, a broader spectrum of endpoints was possible since some of the techniques used at each campus could be applied to samples collected at other campuses.

This inhalation study examined biological endpoints that were associated with PM-induced toxicity using aged rats. Aged rats were used as laboratory models of older people in the human population. People older than 65 years of age, or people with pre-existing lung or heart disease have been reported to be especially susceptible to PM-induced toxic effects. However, several epidemiological studies have established an association of PM exposures with increased risk of cardiovascular diseases, including myocardial infarctions (heart attacks), hypertension and decreases in heart rate variability. These effects have not been well studied under controlled conditions and we therefore initially focused our efforts on measures of heart function (blood pressure and heart rate) as endpoints in our study. Technological improvements in cardiac monitoring capability also enabled us to add measures of heart rate variability to this study.

The PM components that were studied were elemental carbon (EC) and ammonium nitrate (AMN), both of which are important constituents of PM in California. In addition to the animal studies at UCI, a cohort of human subjects with asthma was exposed to PM at UCSF under conditions that were mirrored by those at UCI for the aged rat studies. The concentrations and particle sizes used in the studies at UCI and UCSF were similar.

B. Specific Aims.

The original proposal had three specific aims. As the project developed, it we modified those aims so that cross-project integration could be enhanced. The original specific aims were as follows -

Specific Aim One:

Determine the cardiopulmonary responses to combination ozone-particle inhalation by human volunteers (UCSF) and sensitive laboratory animal models (UCI and UCD).

Specific Aim Two:

Determine the effect of the size of inhaled particles on airway inflammation, cellular function, and cardiopulmonary function.

Specific Aim Three:

Determine the effect of the total dose of inhaled particles on airway inflammation, cellular function, and cardiovascular responses.

As noted in Specific Aim 1, UCI was integrated into the UCSF human clinical exposure studies by helping to install the particle generation system, assisting in atmosphere characterization and performing assays on cells obtained from bronchoalveolar lavage after the exposures. The UCSF exposure design that was carried out exposed subjects to particle mixtures (PM) containing elemental carbon (EC) and ammonium nitrate (AMN) under four conditions: (1) single day exposure to PM; (2) single day exposure to PM + 0.2 ppm O_3 (PO); (3) three-day exposures to PM; and filtered air (FA) controls. In the interest of tighter integration, we focused on the size of particles used at UCD and UCSF, added heart rate variability time domain assessment as a new endpoint and incorporated Aims 2 and 3 into modified Aim 2.

Modified Specific Aim 2:

Compare the effects of single day PM exposure with those of multiple-day exposures to PM.

Experimental Design

This study built upon the results of a previous study, performed in our laboratory, that demonstrated statistically significant and mechanistically relevant PM-induced pulmonary effects in senescent and young, healthy rats exposed to mixtures of laboratory-generated particles and ozone. That previous study examined effects following exposure to particles in three size ranges. An inertial separator was used to fractionate a high concentration primary aerosol into the three size ranges. By use of this system, the mass concentration and chemical composition of the particles was held constant for all particle sizes. Thus, observed differences in biological response in exposed groups were most likely attributable to some characteristic related to particle size. The study demonstrated greater effects on cardiophysiology (blood pressure and heart rate) in rats exposed to $PM_{0.2}$ particles (near-ultrafine) and rats exposed to $PM_{1.0\text{-}3.0}$ particles ('coarse') than in rats exposed to $PM_{0.2\text{-}1.0}$ particles ('fine'). However, when the amount of material deposited in the gas exchange region of the lung of the rats was estimated using a computer model of airway deposition the results were found to be a function of deposited dose. The results also demonstrated that senescent rats were more sensitive to these cardiophysiologic effects than were young adult rats. The present program addressed in this report utilized these findings and

human studies and laboratory animal studies were designed to use similar exposure conditions so that results could be intercompared. Exposure experiments were conducted over the three-year period of this contract to provide the data with which to test the key hypotheses.

The first specific aim of this study was to determine the cardiopulmonary responses to combination ozone-particle (PO) inhalation by human volunteers and sensitive laboratory animal models and compare those to the effects of particles alone (PM). To accomplish this aim, human volunteers were exposed at UCSF and senescent rats were exposed at UCI to aerosols of ammonium nitrate (AMN; 150 $\mu g/m^3$) and elemental carbon (EC; 100 $\mu g/m^3$) for single day with and without ozone (O_3; 0.2 ppm). The exposures were 4 hours per day. Humans were exposed in a whole body chamber at UCSF and rats were exposed nose-only at UCI. The single day studies involved three atmospheres, (1) filtered air (FA); (2) particles alone (PM); and (3) particles and ozone (PO). The particle size and total particle mass concentrations used were similar for the human and rat exposures (MMAD = 0.7 μm; 250 $\mu g/m^3$, respectively). Only a single particle size was used in this study because the previous study demonstrated that deposited dose was the key factor and that particle size (chemical composition being held constant) was less important than the amount that deposited in the gas exchange region of the lung, with respect to cardiac function.

One can estimate that the fraction of particles that would deposit as a function of particle size using a computer model. As shown in Figure 1, approximately 5% of inhaled particles with an MMAD of 0.7 μm would deposit in the gas exchange region of the rat's lung while approximately 8% would deposit in the comparable region of the human lung. If one estimates that the approximately 70 kg body weight humans in the UCSF study were lightly exercising (minute ventilation averaging about 20 $Lmin^{-1}$) in an exposure atmosphere containing 0.7 μm particles at a concentration of 250 $\mu g/m^3$, the human deposited dose in a 4 hour period would be about:

Human Gas Exchange Region Deposition = 0.02m^3/min x 240 min x 250 $\mu g/m^3$ x 0.08 μg Deposited/μg Inhaled x 1/70 kg body weight = 1.4 μg/kg body weight.
Similarly, if one estimates that the 0.5 kg body weight rats in the UCI study were exposed at rest with an average minute ventilation of 0.2 $Lmin^{-1}$, in an exposure atmosphere

containing 0.7 µm particles at a concentration of 250 µg/m^3, the rat's deposited dose in a 4 hour period would be about:

Rat Gas Exchange Region Deposition = 0.0002m^3/min x 240 min x 250 µg/m^3 x 0.05 µg Deposited/µg Inhaled x 1/0.5 kg body weight = 1.2 µg/kg body weight.

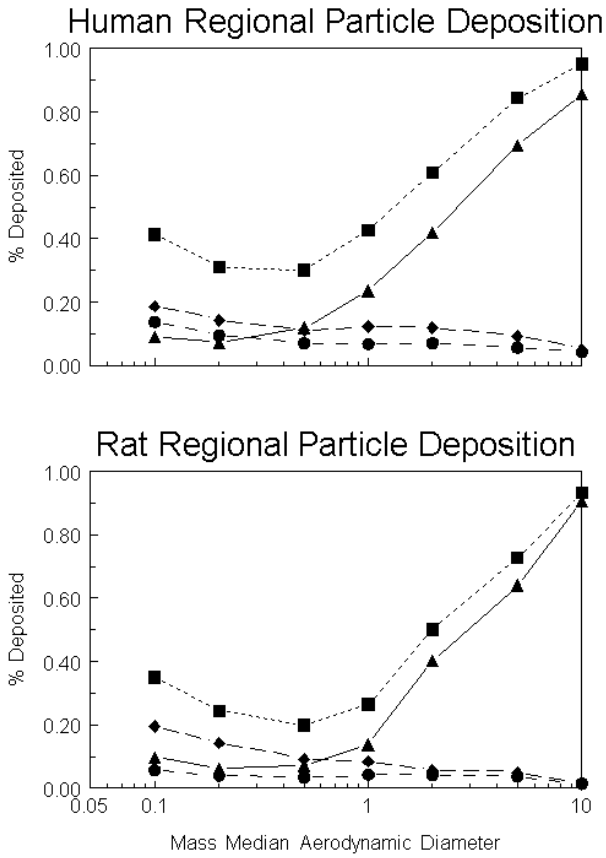

Figure 1. Estimated deposition in rat and human lungs as a function of Mass Median Aerodynamic Diameter (■=Total Deposition, ▲= Upper Respiratory Tract Deposition [non-thoracic], ●= Tracheobroncheal Deposition,♦=Gas Exchange Region Deposition [Alveolar]).

Thus, the selection of a single particle size us to achieve reasonable comparability between the human and rat studies.

The population groups represented different susceptible populations, in that the humans were individuals with asthma (which coordinated the UCSF studies with laboratory animal studies conducted at UCD) while the senescent rats used in the UCI studies were chosen to model the aging human population. **Following human exposures, cardiopulmonary measurements (pulmonary function, heart rate, blood pressure) were made. The human volunteers were examined bronchoscopically and lavaged 24 hr after each of their exposures. Cells isolated from bronchoalveolar lavage fluids were centrifuged and sent to UCI for analysis of macrophage superoxide production as an indicator of inflammatory cell activation.** Other assays had been intended as well but the numbers of cells obtained were too few to allow other assays. Cells from BAL of a rodent model of airway allergy exposed to PM at UCD were also to be sent to UCI; however insufficient sample was available after assays were performed at UCD to permit measurements at UCI. The cardiopulmonary endpoints for humans will be presented in a separate report[33] and the rodent allergy model data will be presented in a second separate report[34]. The rats exposed at UCI were implanted with telemetry devices that monitored cardiac function (blood pressure, heart rate and electrocardiographic data) in unrestrained animals. A major difficulty in this study was that the blood pressure sensor did not function well in the geriatric rats and we monitored the blood pressure using a tail cuff sphygmomanometer designed for rodents. We used ANOVA followed by a Tukey multiple comparison to test the hypothesis that biological responses to PM+O_3 mixtures were worse than responses to PM alone for macrophage free radical production, heart rate, blood pressure and the heart rate x blood pressure product (double product – an index of cardiac output).

Specific Aim 2 was to compare the effects of single day PM exposure with those of a multiple-day exposure to PM. The overall approach was described above for Aim 1. The three-day exposures were on consecutive days and involved two atmospheres, (1) FA; and (2) PM. We used ANOVA followed by a Tukey multiple comparison to test the hypothesis that biological responses to 3-day PM exposures were worse than responses to PM alone for macrophage free radical production, heart rate, blood pressure and the heart rate x blood pressure product (double product – an index of cardiac output) and heart rate variability. The data for each biological endpoint were analyzed using ANOVA to specifically address the hypothesis that the

responses following multiple day exposures were significantly greater than those after a single day of exposure.

Materials and Methods

Exposures and Atmospheres

Elemental carbon (EC), organic carbon and nitrates are found in both fine and coarse fractions of PM, although they represent larger fractions of $PM_{2.5}$ than they do of PM_{10}. Atmospheric speciation data suggests that most of the nitrates are present as ammonium nitrate (AMN). Given this and the fact that EC and AMN represent a significant fraction of PM, it was reasonable to study mixtures that contain both EC and AMN.

Ozone (O_3), another pollutant whose concentration frequently exceeds Federal and State standards in Southern California, is often present at high concentrations in ambient air in cities with high concentrations of ambient PM, albeit the times of peak exposures are not necessarily the same. Never the less, previous studies of inhaled pollutant mixtures showed that particle plus ozone mixtures produced significant changes in lung morphology and immunological endpoints. Therefore, this study examined combinations of PM components with ozone in the single day inhalation exposures. This was consistent with the human studies at UCSF. The multiple day UCSF human exposures did not include O_3. Therefore, the multiple day rat exposures were to PM but not to PO atmospheres.

The sizes of particles in the exposure atmospheres tested were selected based upon estimates of peak exposures in the South Coast Air Basin. Both EC and nitrate (NO_3^{-1}) particles are found in the 0.3-0.6 μm size range. The concentrations of particles in the exposure atmospheres tested were similar to those used in our previous PM study that examined effects of particle size. In that study we selected target concentrations of 100 $\mu g/m^3$ for EC concentrations and target concentrations of 150 $\mu g/m^3$ for AMN.

Inhalation Exposures

Senescent Fischer 344N rats (22-24 months old) were shipped to the laboratory in filter-topped boxes and maintained under barrier conditions during the experiment. The rats were separated into groups (n=10). Exposures of rats were nose-only to a continuous stream of the

test atmosphere. Nose-only exposures were performed in order to prevent artifacts due to contamination of chamber air by dander, ammonia and dried excreta. Between exposures, rats lived in purified-air barrier housing and they were given purified water and fed NIH-31 Teklad Premier Laboratory Diet (Teklad, Bartonville, IL) *ad lib*. Animals were housed in wire cages over beds of rock salt that dried feces and urine and suppressed dust and ammonia production. Personnel wore gowns, hair bonnets, masks, shoe covers and surgical gloves when handling the animals to prevent the spread of pathogens to the rats. Exposure tubes were inspected daily and thoroughly machine-washed in hot soapy water after each use. Animal cages were washed and sterilized twice weekly, and quarters were cleaned daily.

Pollutant generation

Ozone was generated by passing medical grade oxygen through two corona-discharge ozonizers (Sander Ozonizer, Type III, Osterberg, Federal Republic of Germany). The ammonium nitrate + carbon aerosol was generated by preparing a suspension of Monarch 120 carbon black particles (Cabot Corp., Boston, MA) in a dilute solution of ammonium nitrate. Monarch 120 was chosen because it consists of primary particles 75 nm in diameter. This material was more amenable to suspension in aqueous media without the need to use surfactants to facilitate suspension. Monarch 120 contains less than 1% total extractable organic compounds and PAH concentrations of about 0.01 to 0.02%. Thus, for a concentration of 100 $\mu g/m^3$ in the exposure atmosphere, total PAH concentrations would be on the order of 10 to 20 ng/m^3, about the same as measured in ambient air in cities with high levels of ambient PM. The suspension was nebulized using a high output ultrasonic nebulizer (Devilbis) Particles were dried by rapid dilution, discharged to Boltzmann equilibrium using ^{85}Kr sources and then introduced into a nose-only exposure chamber. This produced an aerosol with a mass median aerodynamic diameter (MMAD) of approximately 0.7 μm and a geometric standard deviation (GSD) of about 2.2. This aerosol was similar in size to that used in the UCSF studies.

Pollutant Characterization

Aerosol particles were collected on pre-weighed and equilibrated (50% relative humidity [R.H.]) Pallflex T60A20 fluorocarbon-coated glass fiber filters (PALL Corp., Cincinnati, OH) **These filters were chosen for three reasons. First, because the glass fibers used in their manufacture are fluorocarbon coated which reduces possible interactions with trace gas**

phase impurities. Second, because the filters are lighter in weight which reduced the uncertainty in our gravimetric analyses and provide less pressure drop per unit flow, which reduces the potential loss of semivolatile nitrates from the deposited filtered material. Third, because in our hands these filters collect 0.7 µm particles with better than 99% efficiency as measured using a light scattering particle counter (Climet 208). The filters were weighed after collection to determine the total collected mass. Pallflex filters were extracted with dilute carbonate/bicarbonate buffer and the extract analyzed for NO_3^- by ion chromatography. Samples for particle size analysis were collected using an 8-stage cascade impactor (Andersen Model 208) and pre-cut and pre-weighed Pallflex substrates. The substrates and backup filter were weighed after collection to determine the total collected mass. Pallflex substrates and the backup filter were extracted with dilute carbonate/bicarbonate buffer and the extracts analyzed for NO_3^- by ion chromatography. Cumulative mass percentages were plotted versus impactor stage cutoff diameters on log-probability paper and the mass median aerodynamic diameters and geometric standard deviations were estimated.

Samples for carbon analysis were collected on acid-treated quartz fiber filters (Microquartz; Gelman, Ann Arbor, MI). Carbon was determined by combustion in oxygen in a flow-through furnace. The evolved CO_2 was measured using a non-dispersive infrared absorption analyzer (Dasibi Model 3003, modified with a CO_2 absorption cell). The analyzer was calibrated against known amounts of CO_2 generated by the decomposition of oxalic acid.

Surgical Preparation of Rats for Cardiophysiology Monitoring

The rats were surgically implanted with small transmitting devices (C50-PXT devices) used to acquire research data as part of a telemetry system (PhysioTel Telemetry system, Data Sciences International, St. Paul, MN). C50-PXT devices are designed to measure physiologic pressure, biopotential, temperature, and physical activity in rats and other animals of similar body size. The implantable device consists of the following major components: (1) device body, (2) pressure catheter, and (3) biopotential leads. The C50-PXT device is soaked in sterile saline for 15 min prior to being placed in the peritoneal cavity. This placement has excellent biocompatibility, the ability to measure core temperature, and is relatively insensitive to changes in the ambient temperature. Aseptic technique is used throughout the implantation procedure. Sterile gloves are used for all procedures. Gloves are replaced between animals. The surgeon

wears a clean lab coat while performing surgery. A surgical mask and a cap is worn to reduce the risk of gross contamination of the surgical site. All instruments and supplies that come in contact with the surgical site are sterile.

Isofluorene is administered via inhalation to anesthetize the rats. Dr. Kleinman is experienced in administering anesthesia to rats using isofluorene via inhalation. After being anesthetized, the animal's eyes are lubricated with a sterile ophthalmic ointment to prevent corneal drying. The body hair of the rat is shaved using electric clippers (#40 blade) from the ventral abdomen from the subxiphoid area to the pelvis, and the sites of subcutaneous electrode placement. The shaved area is cleaned and disinfected with alcohol and an iodine-based disinfectant. While under general anesthesia, the animals are provided a heat source during surgery from lamp and be covered with a blanket.

A sterile drape impermeable to moisture is adhered tightly to the skin, and a 4-6 cm midline abdominal incision is made. The contents of the abdomen are exposed using a retractor. The intestines are held back using saline moistened gauze sponges, and the aorta is delicately dissected from the surrounding fat and connecting tissue using sterile cotton applicators. All excess tissue from the aorta is cleared to allow good hemostasis following catheterization. Fine, smooth tipped, forceps are carefully used to separate the aorta from the vena cava just caudal to the point where the left dorsal muscular branch crosses over the aorta. An occlusion ligature is carefully inserted between the aorta and the vena cava just caudal to the left dorsal muscular branch. A loop is formed underneath the aorta that will allow occlusion of aorta blood flow for no longer than 3-4 minutes. Blood flow is restricted using the proximal ligature to elevate the vessel in preparation for catheterization. The aorta is punctured just cranial to the bifurcation using a 21-gauge needle bent 90° at the beveled end. The tip of the catheter is slid under the needle and the catheter is passed cranial until the entire thin-walled section is within the vessel. The puncture site and surrounding tissue are dried thoroughly, one drop of tissue adhesive is applied to the puncture site and a small cellulose patch is immediately placed on the glue over the puncture site. The catheter is lifted gently to allow the glue to flow under the catheter to bond to the vessel wall, and the glue is allowed to dry for 10-15 seconds. The tension on the suture is carefully released and leakage is looked for. If bleeding occurs, tension is reapplied and the area is dried before another drop of glue is placed at the area of leakage. Once hemostasis has been assured, the catheter is tested for proper placement and readjusted, if necessary. Any gauze

sponges are removed, the abdominal cavity is flushed with sterile saline, and hemostasis is verified. The intestines are replaced into their original position, and the device is placed on top of the intestines parallel to the long axis of the body with the catheter directed caudally.

EKG Lead Placement

A skin incision is made at the site of negative lead placement. Using a trocar, a tunnel is made subcutaneously from the abdominal incision to approximately 1 cm beyond the lead incision. A plastic sleeve is slid over the end of the trocar, and the trocar is used to guide the sleeve into the prepared tunnel. The trocar is removed leaving the sleeve in place. A 14-gauge needle is passed through the abdominal wall lateral to the cranial aspect of the incision going from the outside into the abdominal cavity. One of the leads is passed through the needle and out of the abdomen. The needle is withdrawn leaving the lead externalized. The lead is passed through the waiting plastic sleeve to the desired site, and the sleeve is removed. The silicone tubing is cut around on the tip of the lead using a sharp sterile blade and 1.5 cm of the silicone is removed to expose the stainless steel wire. A tip cover is screwed counter clockwise onto the exposed wire,leaving at least 1 cm of the wire exposed. The muscle fibers are bluntly dissected at the desired site to provide a shallow area in which to place the electrode. The lead is placed with the muscle tissue and secured by suturing the muscle tissue up over the lead using 4-0 nonabsorbable suture. These steps are repeated for the positive lead. The device body is secured in place by closing the abdominal incision and incorporating the suture rib on the device into the closure using nonabsorbable sutures (3-0 or 4-0) in a simple interrupted pattern. The skin incision is closed using skin staples.

Recovery: The animal is placed into a warm environment and the breathing air is supplemented with additional oxygen. The animal's recovery is monitored until it is fully awake. Analgesia, buprenorphine (.01 - .05 mg/kg subcutaneously every 12 hours for three days) is provided to all animals post surgery. Enrofloxacin (Baytril) 3 mg/kg BW is administered by subcutaneous injection twice per day for 7 days. **The sutures were removed 7 to 10 days post-surgery. Animals were allowed to recover for a minimum of 3 weeks after cessation of medications before beginning exposures. This allowed sufficient time for any confounding effects of the analgesics or antibiotics on the physiological endpoints to be abrogated.**

Acquisition of Cardiophysiology Data

Blood pressure and electrocardiographic data were acquired using a PhysioTel Telemetry system (Data Sciences International, St. Paul, MN). The C50-PXT devices that had been implanted in the rats were interrogated using a telemetry receiver linked to a computerized data acquisition and analysis system (Dataquest A.R.T. 2.3). Electrocardiographs were obtained over a 15 min acquisition period from each rat before and after each exposure. As mentioned elsewhere, while the ECG telemetry worked extremely well for heart rate and HRV. Electrocardiograph (ECG) data were collected continuously for 22 minutes before and after each exposure. The data were analyzed using the DataQuest 2.3 software, which determined estimates of the node to node interval between heart beats. Changes in beat to beat interval can be quantified as the standard deviation of the node to node interval (SDNN), which is an index of overall heart rate variability (HRV). The quantity provided by the DataQuest software is called the interbeat interval (IBI). To examine HRV we computed average IBI for each animal for every 2 minutes during the 22 minute monitoring period, providing approximately 11 values for each animal. Group mean 2 minute averages and standard deviations were computed for each time point.

The pressure transducers were unreliable in the senescent rats and time did not permit us to improve the system. Fortunately we were able to use an alternative method to acquire systolic blood pressure data. Blood pressure and heart rate were measured in unanaesthetized rats immediately before and after each exposure using a tail-cuff sphygmomanometer. Rats were placed into a warming chamber with their tails exposed. A blood pressure cuff and pressure transducer, designed for use on a rodent's tail, were placed on the tail and monitored using a calibrated recorder/integrator system (Gould, Inc.) until a reproducible heart pulse was obtained. The systolic blood pressure (SBP) was determined by automatically inflating the pressure cuff to a pressure sufficient to occlude the pulse. The cuff was slowly deflated and the resulting pressure/heart rate pattern recorded. The pressure at which the pulse reappeared was taken as the systolic blood pressure. Each measurement was repeated up to 5 times and averaged or until 3 valid pressure pulse curves were recorded with SBP that agreed to within 10%.

Sacrifice of rats and timing of endpoints

Animals were euthanized 24 hr measured from the midpoint of their final exposure. Tissue and biological fluids were collected and stored for endpoint analyses. The 24 hr. timepoint was selected because this timing was suitable for detecting inflammatory processes, such as influx of inflammatory cells, release of inflammatory cytokines and observation of functional changes in macrophages.

Bronchoalveolar Lavage

The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The abdominal aorta was severed and a polyethylene catheter was placed and tied in the trachea. An incision was made in the diaphragm to allow lung expansion during the lavage. Lungs were lavaged by introduction of 28 mL/kg body weight HEPES buffered Hanks Balanced Salt Solution (HBSS) without Ca^{+2} or Mg^{+2} through the tracheal catheter followed by withdrawal of the fluid. The introduction and withdrawal of this fluid was repeated three times and the first 5 mL of lavage fluid was recovered and transferred to a 15 ml polypropylene centrifuge tube. The process of introduction and withdrawal of lavage fluid was repeated two additional times with fresh HBSS. The fluid from the last two lavages was pooled in a separate centrifuge tube. The tubes containing lavage fluid from each animal were centrifuged at 300 g for 10 min. The cell-free supernatant from the first lavage was analyzed for total protein and serum albumin. The cells from the two tubes were pooled for each animal.

Inflammatory Cell Infiltration (PMNs)

Cells from lavage fluid were deposited on slides using a cytocentrifuge and stained with Diff Quik (Baxter Healthcare Corp., McGaw, IL) for differential cell counts. Cells were scored as being macrophages or monocytes, lymphocytes, PMNs or "other" cells (the other category included epithelial cells, multinuclear macrophage-derived cells or cells which were not otherwise distinguishable).

Respiratory Burst Activity in Human Macrophages

One hallmark of inflammation is the activation of inflammatory cells, presumably to respond to threats such as invasion by pathogenic organisms. Activated macrophages produce increased levels of toxic mediators such as superoxide which can be measured in cells recovered

after bronchoalveolar lavage **when the cells are stimulated. Stimulation simulates the macrophage's activity when it encounters a 'pathogenic' organism. Activated macrophages may, or may not exhibit increased activity in the unstimulated state.** Macrophages from human volunteers exposed at UCSF were sent to UCI for study. Integrated superoxide production during respiratory burst activity after stimulation with phorbol myrastyrate acetate (PMA; Sigma Chemical, St. Louis, MO) was measured by a chemiluminescence method. **PMA was selected because PMA-activation is independent of other macrophage functions such as phagocytosis.** Lung lavage cells (2×10^5) were added to luminometer cuvettes (LKB Pharmacia) in 1 ml HHBS. Samples were incubated at 37°C in 5% CO_2 for 90 min and non-adherent cells were removed by gentle washing. The medium was replaced with RPMI 1640 supplemented with 10 mM glucose. Superoxide production was determined by lucigenin-amplified chemiluminescence using an LKB-Pharmacia Model 1251 Luminometer. Chemiluminescence measurements were made beginning immediately after the addition of 200 mM bis N Methylacridinium Nitrate (lucigenin, Sigma), with or without stimulating agent (50 ng/mL PMA). Measurements were continued until readings returned to near baseline levels (typically 30 min). Duplicate cuvettes with SOD-treated samples were used to correct readings to yield SOD-inhibitable chemiluminescence readings.

Statistical Analyses

Data were analyzed using one-way or two way analyses of variance (ANOVA) in SYSTAT. Tukey multiple comparison tests were then used to assess significant differences between exposure groups.[35] This test, although conservative, allows one to compare all group means while preserving the α=0.05 Type I error. Some data were not normally distributed, however the conclusions were not changed when non-parametric methods were applied. Only the parametric analyses are shown in this report. Regression analyses in SYSTAT were used to determine whether observed trends were statistically significant. Two-tailed tests with $\alpha \leq 0.05$ were used to establish statistical significance for all analyses.

Results

Initial Considerations

Integrity of Air Samples

Preliminary studies were performed to determine whether AMN might volatilize during collection to form ammonia and nitric acid vapor and thus yield erroneous estimates of AMN concentrations in the exposure atmosphere. This phenomenon had been noted in ambient air measurements of nitrates and was especially problematical during periods of high temperature. AMN was generated under conditions identical to those under which rat exposures were planned. The sample collection system was also identical, with the following difference. A nylon (Nylasorb®) filter was place in tandem behind the PallFlex filter. Nylon filters have a high affinity for nitric acid vapor and if AMN did volatilize during the collection, nitrate should be detected on the nylon filter. We ran several samples. The amounts of nitrate on the nylon backup filter were at or below the detection limit of the analysis method (3 $\mu g/m^3$). Thus, we concluded that under the conditions of this study, there was no significant error due to estimating nitrate concentrations from the nitrate measured on the PallFlex filter.

The method used to generate ozone (corona discharge) has been reported to produce ultrafine particles. To minimize particle formation we generated ozone using oxygen rather than air to eliminate nitrogen and reduce the flow of gas needed to supply ozone for the experiments. We measured the numbers of ultrafine particles in a chamber with 0.2 ppm ozone to which no particles were added. There was a small addition of particles (~34 per liter) to the chamber air when ozone was introduced, however this is unlikely to influence our study since we deliberately added more than 10^6 particles per liter during the exposures.

Specific Aim 1: Comparison of the Effects of Aerosols Containing Particles or Particle+O_3 Mixtures

The first specific aim was to determine the cardiopulmonary responses to combination ozone-particle inhalation (PO) by human volunteers and sensitive laboratory animal models and to compare those responses with those of exposure to particulate matter alone (PM). To accomplish this aim, human volunteers were exposed at UCSF and senescent rats were exposed at UCI to aerosols of ammonium nitrate (AMN) and elemental carbon (EC) for single day and

three-day periods. The exposures were 4 hours per day. Humans were exposed in a whole body chamber at UCSF and rats were exposed nose-only at UCI.

PM vs. PO in Senescent Rats

The experimental conditions for rat exposures to PM and PO are summarized in Table 1. AMN and EC concentrations agreed between the exposure conditions, within experimental error and were close (± ~20%) to the preselected target values.

Table 1 Atmospheres and Endpoints Tested in Aim 1 for Senescent Rat Exposures

Group	Atmosphere	Target Size and Conc.	Actual Concentration	Exposure	Endpoints
1[a]	Purified Air	NA	NA	4 hour	Blood Pressure Heart Rate Heart Rate Variability
2	PO[b] Carbon + AMN + Ozone	$0.5 \leq d_p \leq 1.0$ 100 μg/m^3 150 μg/m^3 + 0.2 ppm	0.69 ±0.05 MMD[d] 2.43±0.09 GSD[e] 91.35 ± 12.15 176 ± 38 0.194 ± 0.004	"	Blood Pressure Heart Rate
3[a]	PM[c] Carbon + AMN	$0.5 \leq d_p \leq 1.0$ 100 μg/m^3 150 μg/m^3	0.74±0.07 MMD 2.13±0.09 GSD 83 ± 23 198 ± 26	"	Blood Pressure Heart Rate Heart Rate Variability

Notes: [a] Rats in groups 1were instrumented for ECG data acquisition. These rats were used in a repeated measures design and received both both FA and PM exposures. The Group 2 rats were not instrumented and were euthanized after the single day PO[b] exposure; [b]Particle +O_3 mixture ;[c]Particles alone; [d]MMD = mass median diameter; [e]GSD = geometric standard deviation.

Observed biological responses

Inflammatory Response

The presence of inflammatory cells (macrophages, polymorphonuclear leucocytes and lymphocytes) in the lungs of exposed rats was examined as an indicator of inflammatory response. Cells recovered from rats by bronchoalveolar lavage had, on the average, viabilities greater than 94%. There was a small reduction in the total numbers of viable cells recovered from the group exposed to the particle mixture + O_3. The percentages of macrophages and lymphocytes recovered in BAL averaged 97 ± 1 and 2.2 ± 0.5, respectively, and were not significantly different among the exposure groups (Table 2).

Table 2 Inflammatory Cells in BAL of Rats Exposed to Ammonium Nitrate and Carbon Particles (PM) in the Presence or Absence of Ozone (O3), N = 10 per group.

(Number of Cells; Mean ± SE)				
Atmosphere	Total Cell Number ($\times 10^{-6}$)	Macrophages ($\times 10^{-6}$)	PMNs	Lymphocytes
FA	3.8 ± 0.3	3.7 ± 0.3	26600 ± 2400	95000 ± 19000
PM	3.7 ± 0.2	3.6 ± 0.2	29600 ± 3500	67000 ± 23000
PO	3.4 ± 0.3	3.3 ± 0.3	70400 ± 5400*	37400 ± 5400

Note: *p≤0.05

The percent of PMN's and lymphocytes were analyzed using a 1-way ANOVA to test the null hypothesis of no significant effect of exposure. There was a significant increase in the percent of polymorphonuclear leucocytes (PMNs) recovered from the bronchoalveolar lavage (p≤0.05) in PO exposed rats compared to controls. There were no significant differences between total number, macrophages or lymphocytes between FA or exposure groups. There were greater numbers of PMN's in lavage fluid from rats exposed to ozone-containing atmospheres.

Physiological Endpoints

Systolic blood pressure and heart rates were measured before and after a single 4-hr exposure to FA, PO or PM atmospheres. The blood pressure x heart rate product was also determined. The data were summarized in Table 3. Blood pressure was slightly elevated (p=0.13) after PM exposure but not PO exposure, compared to FA exposures. Heart rate was slightly lower after PM exposure compared to FA but the difference was not statistically significant. Double product, which is the product of diastolic blood pressure and heart rate, and is a surrogate measure for cardiac work capability, was not significantly changed by these exposures.

Table 3 Cardiovascular Outcomes Following Single Day Exposure to Particle-Containing Atmospheres

Exposure	SBP[a]	HR[b]	DP[c]
FA[d] (n=6)	99±4	422±17	42000±1800
PO[e] (n=6)	103±4	420±17	43000±2300
PM[f] (n=10)	110±4	402±7	45000±1900

Notes: [a]Systolic Blood Pressure; [b]Heart Rate; [c]Double Product; [d]Filtered Air; [e]Particles + O3; [f]Particles alone.

PM vs. PO in Human Subjects With Asthma

Macrophage Superoxide Production

Human subjects were exposed to FA, PO and PM atmospheres under controlled conditions at UCSF. Following exposure bronchoalveolar lavage was performed and cells were shipped to UCI for analyses. Exposure conditions (particle sizes and chemical composition of the aerosol) were, by design, similar to those used in the senescent rat exposure discussed above.

Cells were counted by hemocytometry and the number of viable cells was determined by Trypan Blue Exclusion. Macrophages were isolated from this sample by adherence to treated plastic cuvettes. The numbers of cells obtained were generally small, but adequate for the determination of superoxide production by a chemiluminescence method. Superoxide

production and release by unstimulated macrophages and by macrophages stimulated by incubation with PMA were determined and the data are summarized in Table 4. **Although samples from about 10 individuals were provided for these analyses, not every subject completed the entire experimental design.**

Exposure to the PO atmosphere increased macrophage superoxide production slightly, but not significantly in unstimulated macrophages, but nearly tripled the superoxide production after PMA stimulation, which approached significance in a one-way ANOVA ($p=0.07$). Rats exposed to PO following similar one day exposures in a previously reported study also showed increased macrophage superoxide production compared to rats exposed to FA. These data were analyzed in two ways. The design of the human study used repeated measures to improve the ability to detect small changes that might not be significant if one were comparing independent groups. Not all subjects completed the entire experimental design. Complete data were available for 4 subjects and one additional subject had complete data except for FA. Repeated-measures ANOVA does not allow missing data. We therefore inserted the group average FA value so that repeated measures analysis could be performed with five subjects. Following this analysis the Tukey multiple comparison test was applied and demonstrated that superoxide production by PMA-stimulated macrophages obtained from BAL after single day PO exposures was significantly ($p \leq 0.05$) elevated compared to macrophages obtained from the same subjects after FA exposure. **When analyzed as independent groups (standard one way ANOVA), the particle exposure effects approached statistical significance ($p \leq 0.1$), but the group mean values for either the PO or the PM exposures were not significantly different from FA.**

Table 4 Chemiluminscent Measurement of Superoxide Production in Unstimulated and PMA[a]-Stimulated Macrophages from Human Subjects with Asthma after Particle Exposures (Relative Units)

Exposure	Unstimulated	PMA-Stimulated
Fa[b] (n=7)	1.4±0.15	32±7
PO[c] (n=10)	1.6±0.0.06	87±18[e]
PM[d] (n=10)	1.5±0.0.07	53±9

Notes: [a]Phorbol Myristyrate Acetate; [b]Filtered Air; [c]Particles + O_3; [d]Particles alone; [e]Significantly different from FA, $p \leq 0.05$.

Specific Aim 2: Compare the effects of single day PM exposure with those of multiple-day exposures to PM.

Single day PM exposure vs. Multiple-Day PM exposure in Senescent Rats

Senescent rats were implanted with telemetry devices to permit acquisition of blood pressure, heart rate and electrocardiographic (ECG) data from unrestrained animals. The experiment followed a repeated measures design, with each animal receiving each treatment. Although ideally the rats would have been exposed to the atmospheres randomly, that was precluded by practical considerations.

The exposure concentrations and conditions are summarized in Table 5. Rats were exposed to FA and PM for 3 consecutive days. Blood pressure, heart rate and ECG's were assessed before and after each daily exposure. The concentrations of carbon averaged about 19% below the target level and the AMN concentrations averaged about 32% above the target level. The standard deviations of both component concentrations were 25 $\mu g/m^3$ Most of the variation was due to a generator problem on the first day of the study which was corrected by increasing the ratio of carbon to AMN in the nebulizer suspension.

Table 5 Particle Size and Chemical Composition of PM Aerosol Used in Single and Multiple Day Exposures of Senescent Rats.

Group	Atmosphere	Target Size and Conc.	Actual Concentration	Exposure	Endpoints
1	Purified Air (1,2 and 3 Days)	NA	NA	4 hour	Blood Pressure Heart Rate Heart Rate Variability
2	3-Day Average PM Carbon + AMN	$0.5 \leq d_p \leq 1.0$ 100 $\mu g/m^3$ 150 $\mu g/m^3$	0.78±0.02 MMD 2.1±0.1 GSD 81±25 $\mu g/m^3$ 198±25 $\mu g/m^3$	"	Blood Pressure Heart Rate Heart Rate Variability

Rats were exposed to PM atmospheres on three consecutive days for 4 hr per day. One week following the exposures the rats received three consecutive exposures to FA. Systolic blood pressure, heart rate and ECGs were measured before and after exposure. The rats were euthanized 24 hours after the last exposure and lung tissue, heart tissue and cells from bronchoalveolar lavage were obtained.

Inflammatory Cell Infiltration

The numbers of total cells, macrophages, lymphocytes and polymorphonuclear cells (PMNs) recovered from lavage fluid in rats exposed for 1 day and 3 days to PM and 1 day and 3 days to FA are summarized in Table 6. Macrophages accounted for more than 95% of the recovered cells and no exposure-related differences were seen between PM-exposed or FA-exposed rats after either 1-day or 3-day exposures. PMN cell counts were increased after 1-day and 3-day PM exposures, compared to FA exposures. These changes were significant after the 1-day exposure. Increased numbers of lymphocytes were noted after the 3-day exposure to PM

as compared to FA, but there were large animal to animal variations and the differences were not statistically significant.

Table 6 Total and Inflammatory Cells Recovered from Bronchoalveolar Lavage after 1-Day and 3-Day Exposures to FA and PM (mean ± se)

	FA N=10	Single Day PM Exposure N=10	Multiple Day FA Exposure N=5	Multiple Day PM Exposure N=5
Total Cells	$(3.8 \pm 0.3) \times 10^6$	$(3.7 \pm 0.2) \times 10^6$	$(3.2 \pm 1.7) \times 10^6$	$(3.4 \pm 1.5) \times 10^6$
PMN	23900 ± 1900	33700 ± 1800**	57600 ± 17200	88400 ± 49300
Macrophages	$(3.7 \pm 0.3) \times 10^6$	$(3.6 \pm 0.2) \times 10^6$	$(3.0 \pm 0.1) \times 10^6$	$(3.0 \pm 0.2) \times 10^6$
Lymphocytes	98800 ± 8000	65000 ± 3500	131200±51300	272000±153000

**$p \leq 0.01$

Systolic Blood Pressure

The pre-exposure and post exposure systolic blood pressure (SBP) data are summarized in Figure 2. The upper panel of Figure 2 shows the data obtained during FA exposures and the lower panel shows the data obtained during the PM exposures. The chart shows pre-exposure and post-exposure SBP results and the approximate time from the start of the first exposure. There are small decreases in SBP after each FA exposures, but these are not statistically significant. However, there is a small SBP increase with days of exposure that approaches statistical significance (p=0.054). The PM post-exposure SBP values are all significantly lower than the pre-exposure values (p=0.013). The Post-Pre differences were computed and the values were analyzed using two-way repeated measures ANOVA to test for a main effect of atmosphere (FA vs. PM), a main effect of day and the atmosphere*day interaction. The results of that analysis showed that there was a significant difference between FA and PM exposures, but that there was no significant day effect or atmosphere*day interaction. Tukey multiple comparison tests were performed. Pre to post-exposure changes in SBP after PM exposures were significant ($p \leq 0.05$) for Days 1 and 3. These are noted in Figure 2 with '*'

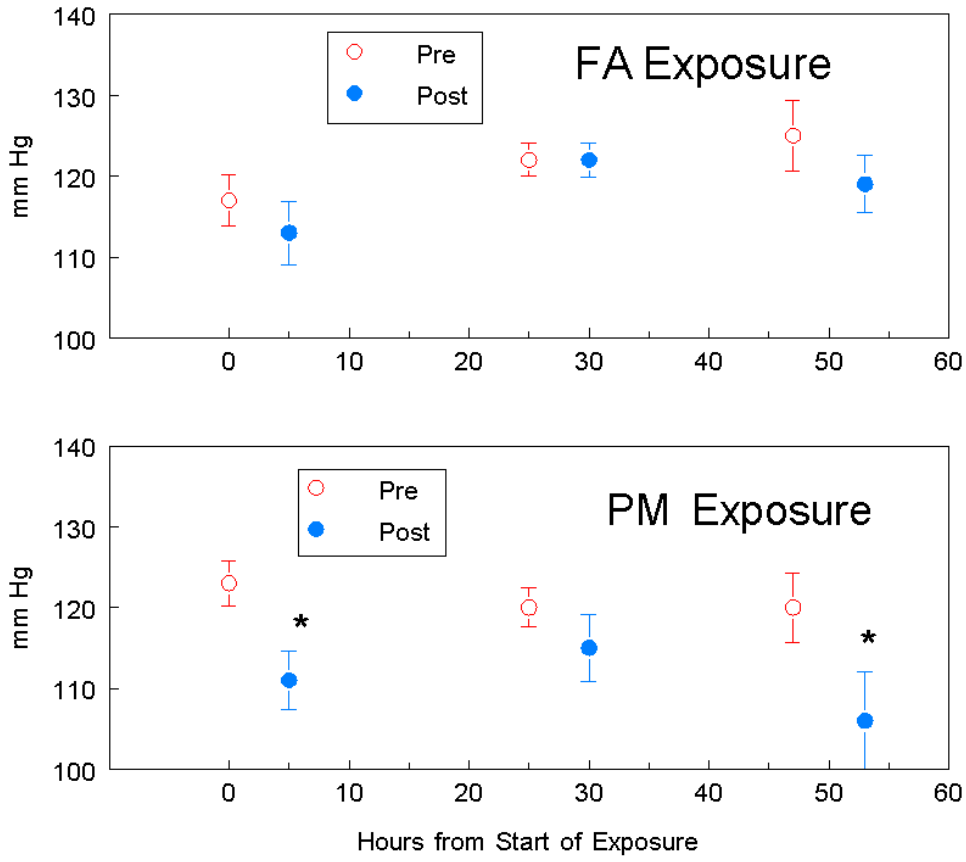

Figure 2 Systolic Blood Pressure Measured in Senescent Rats after FA and PM Exposures

Heart Rate

The pre-exposure and post exposure heart rate (HR) data are summarized in Figure 3. The upper panel of Figure 3 shows the data obtained during FA exposures and the lower panel shows the data obtained during the PM exposures. As mentioned above, the chart shows, in addition to pre-exposure and post-exposure HR results, the approximate time from the start of the first exposure. As can be seen in Figure 3, HR was increased on the last 2 days of the FA exposures. This is reflected in the ANOVA as a main effect of day that approaches significance ($p=0.11$). There were no significant main effects, interactive effects or pre to post-exposure changes in heart rate following the PM exposure.

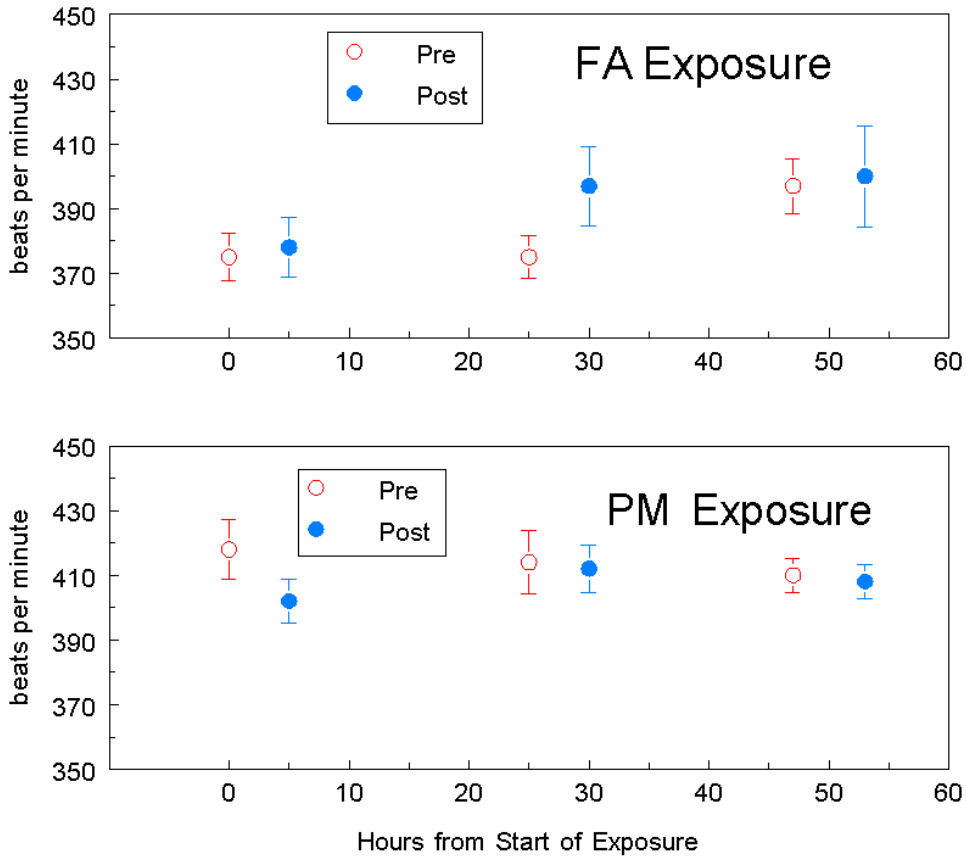

Figure 3 Heart Rate Measured in Senescent Rats after FA and PM Exposures

Double Product

The pre-exposure and post exposure double product (DP = HR*SBP) data are summarized in Figure 4. The upper panel of Figure 4 shows the data obtained during FA exposures and the lower panel shows the data obtained during the PM exposures. As mentioned above, the chart shows, in addition to pre-exposure and post-exposure HR results, the approximate time from the start of the first exposure. Repeated measures analysis of variance was performed and showed no significant differences in the pre-exposure to post exposure differences in DP after FA exposures, but the pre-exposure to post exposure differences in DP after PM exposures were significant (p=0.052). The Tukey multiple comparison test indicated that the pre to post difference for PM exposure day 1 was statistically significant, but that the other differences were not.

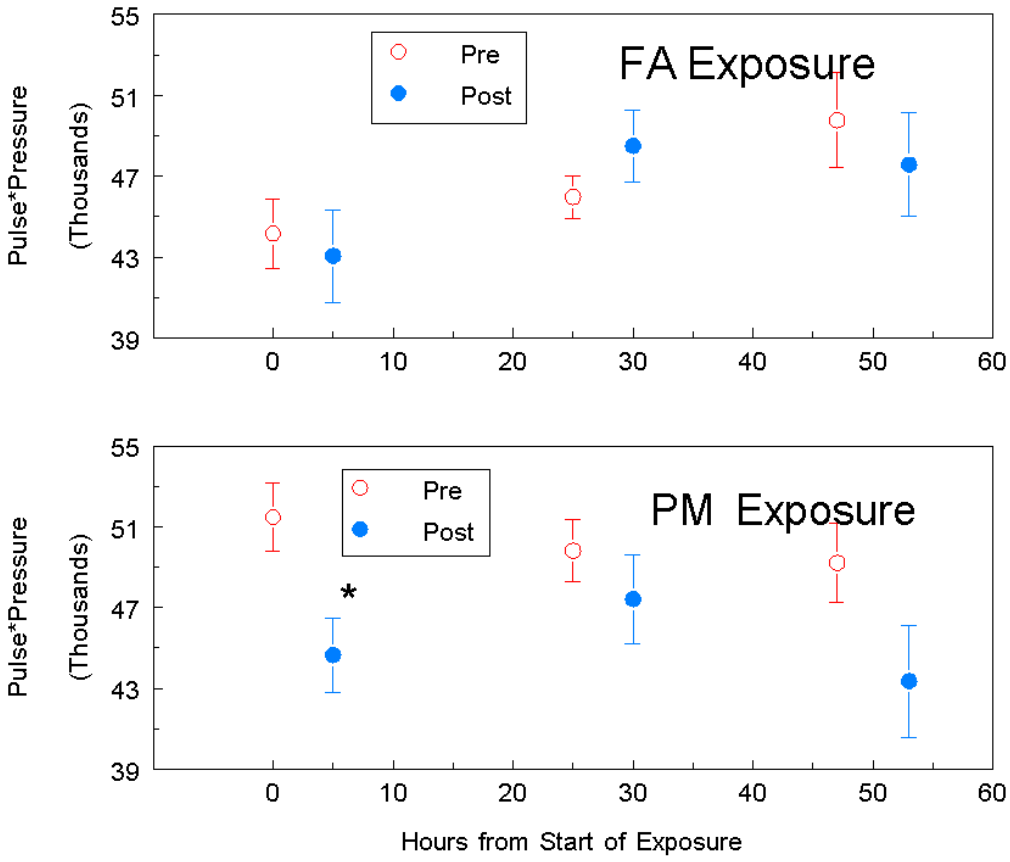

Figure 4 Double Product Measured in Senescent Rats after FA and PM Exposures

Significant changes in heart rate and blood pressure can be strongly correlated with exposure-associated decreases in body core temperature (hypothermia). We examined the possibility that hypothermic physiological responses could have influenced our observed changes in blood pressure and HRV. As shown in Figure 5, there were significant body temperature changes in the rats between the pre-exposure measurement (7AM to 10AM) and the post-exposure measurement (3PM to 6PM). This was true for both FA and PM exposures, which were not significantly different. It may be that there is some stress associated with nose only exposures. However in our case it appears that if there is, it affects the control and exposed animals equally and should not be a source of bias, i.e. the exposure-related differences in HR and BP are unlikely to be due to hypothermia.

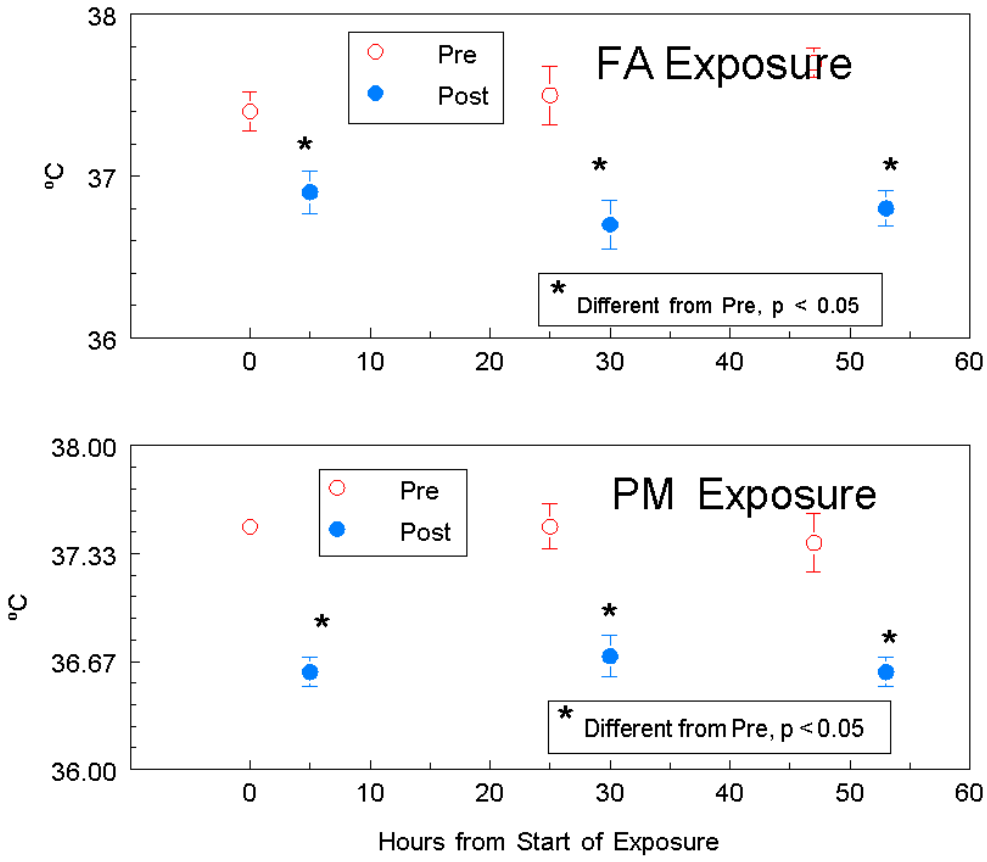

Figure 5. Body core temperature changes during FA and PM exposures (mean ± se)

Heart Rate Variability

Average interbeat interval data (IBI) were acquired for each animal for every 2 minutes during the 22 minute monitoring period, providing approximately 11 values for each animal. Group mean averages and standard deviations were computed for each 2 minute time point and these were plotted in Figure 6. Each cluster of points in the figure represents 8 to 10 group means and standard deviations of IBI (equivalent to the SDNN). This method of presentation was selected because it provides a visualization incorporating both long term and short term variations in the same presentation. The SDNN is one of several methods used for assessing overall HRV. The SDNNs after 1, 2 and 3 days of PM exposure were compared to those measured in the same rats after 1,2 and 3 days of FA exposure. The error bars in Figure 6 provide a visually interpretable appreciation of how PM exposures influence HRV. Figure 6

shows that there is no difference between the HRV measured on the first day of PM exposure and the HRV on the first day of FA exposure. However HRV gets progressively smaller over the second and third days of PM exposure, while no changes are seen over the second and third days of FA exposure. Thus, Figure 6 shows that there is a progressive reduction in HRV over the 3 day PM exposure period. The difference in variance between PM and FA for equivalent time points on the second and third days was highly significant ($p \leq 0.01$).

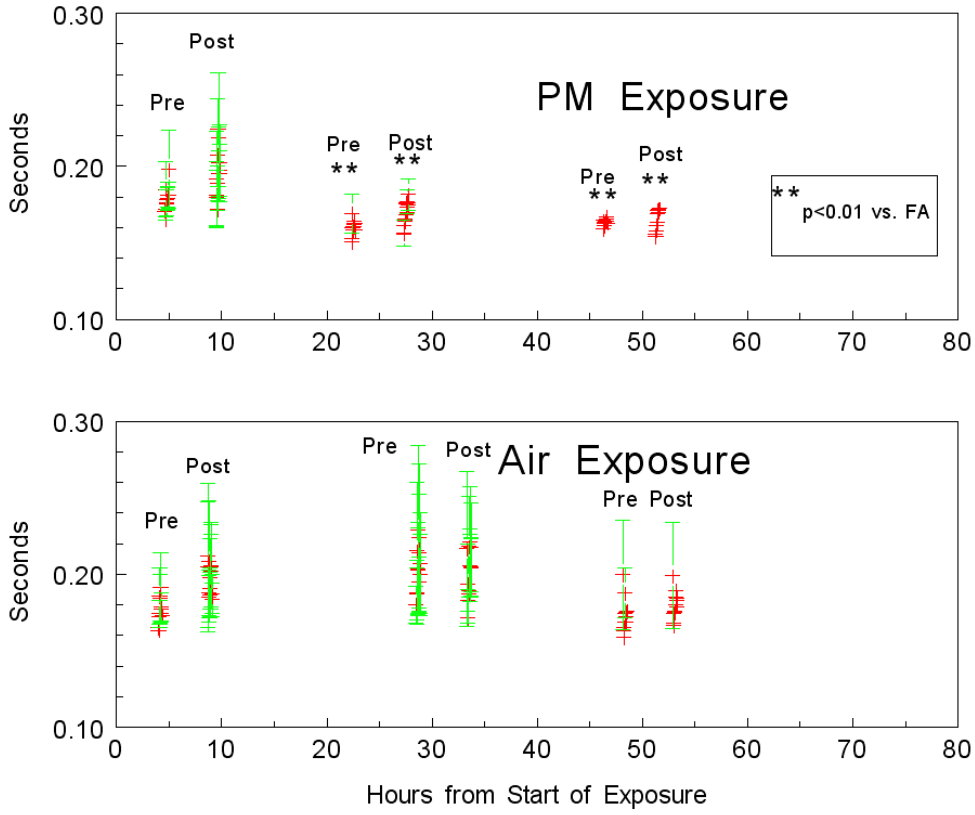

Figure 6. Heart Rate Variability (SDNN) measured in Senescent Rats after Exposure to FA or PM for 3 Consecutive Days

Single day PM exposure vs. Multiple-Day PM exposure in Human Subjects

Human subjects were exposed to FA for one day, PM for one day and PM atmospheres for 3 consecutive days under controlled conditions at UCSF. Following the single day FA and

PM exposures and after the 3rd day of the multiple day PM exposure, bronchoalveolar lavage was performed. The cells were shipped to UCI for analyses. Exposure conditions (particle sizes and chemical composition of the aerosol) were, by design, similar to those used in the senescent rat exposure discussed above.

Cells were counted by hemocytometry and the number of viable cells was determined by Trypan Blue Exclusion. Macrophages were isolated from this sample by adherence to treated plastic cuvettes. The number of cells obtained were generally small, but adequate for the determination of superoxide production by a chemiluminescence method. Superoxide production and release by unstimulated macrophages and by macrophages stimulated by incubation with PMA were determined and the data are summarized in Table 7.

Table 7 Chemiluminescent Measurement of Superoxide Production in Unstimulated and PMA-Stimulated Macrophages from Human Subjects with Asthma after FA, Single Day Particle Exposures or Multiple Day Particle Exposures (Relative Units)

	FA	Single Day PM Exposure	Multiple Day PM Exposure
Unstimulated	1.36±0.15	1.48±0.09	1.50±0.16
PMA-Stimulated	32.3±7.5	52.2±9.1	55.2±18.3

ANOVA was used to test the null hypothesis that macrophage superoxide production after PM exposure was not significantly different from that measured after FA exposures. The analysis showed that PM exposure increased superoxide production and that the difference approached statistical significance ($p=0.076$). However none of the treatment means reached significance in the Tukey multiple comparison test. There is a trend in these data. If one estimates the subjects breathed an average of 20 LPM over the 4 hour exposure period, and if we assume the lung deposition efficiency to be about 0.25, we can estimate that the subjects received a daily dose of about 300 μg PM per day. A regression analysis assuming FA = 0, 1-day PM = 300 μg deposited and 3-day PM = 900 μg of particle mass deposited was performed. The trend was approached but did not achieve significance.

Discussion

The relationship between environmental PM exposure and human health effects is of continuing concern. The present study analyzed the effects of two PM components, ammonium nitrate (AMN) and elemental carbon (EC), which were selected because they represent important fractions of ambient PM in California. The initially selected concentrations of each component were representative of estimated peak concentrations, based on extrapolations from ambient air data, and the sizes of the particles used were chosen based upon reported sizes of inorganic aerosols in ambient air.[36]

The specific aims of this study were: (1) to contrast the effects of a particle + O_3 mixture with those of the particle mixture alone; and (2) to determine whether the effects of a single day PM exposure were significantly different from those of a multiple day PM exposure. To accomplish this we used two different models: human subjects exposed in a chamber at UCSF and senescent rats exposed nose-only at UCI.

One important feature of this study was that the experimental conditions used in the animal and human studies were comparable with respect to chemical composition, particle size and exposure duration. Another important feature was that to some extent parallels could be drawn between the some of the human and rat endpoints. Thus, it is thought that the manner by which inhaled particles cause or exacerbate lung and heart diseases is through mechanisms involving the production of free radicals in excess of the organ's ability to defend against injurious effects of these reactive oxygen and nitrogen chemicals. These free radicals are an intrinsic part of the bodies innate immune system, but when pesent in excess they cause oxidative stress and tissue injury. Activated macrophages are an important source of these radicals in the lung and heart. Along with production of free radicals, macrophages release cytokines that stimulate the systemic production of acute phase proteins. These proteins alter sympathetic and parasympathetic balances that mediate changes in cardiac function and blood pressure. Our findings that PM exposures can alter heart rate variability, which is a biomarker for changes in the balance of sympathetic and parasympathetic pathways, in both rats and humans is of significance since that suggests that animal models are useful for further exploring the mechanisms by which particle exposures may adversely affect health, especially under conditions in which

human clinical studies might be much more difficult. An example might be the study of chronic effects of long term, low level exposures.

There were significant difficulties in conducting this study. A major issue was that the implantation of telemetry devices into the senescent rat turned out to be extremely difficult b ecause of the animal's fragility and sensitivity to anesthetics. After successfully implanting sufficient rats to perform the study we found that, contrary to our experience with younger rats, the blood pressure module did not function properly. Fortunately, we were able to use a tail cuff sphygmomanometer to obtain blood pressure readings.

Particle-induced changes in blood pressure, heart rate and heart rate variability have been assessed in several animal models but here have been few inhalation studies. Watkinson et al.[37] summarized the cardiovascular and systemic responses reported in a series of several studies addressing the effects of inhaled particles and ozone in rats. Most of those studies used residual oil fly ash (ROFA) or metal salts as atmospheric surrogates. The doses administered to the rats were estimated to be in the 100 to 300 μg range. Significant changes in heart rate occurred, but they were strongly correlated with exposure-associated decreases in body core temperature (hypothermia). Sham exposed rats showed a diurnal pattern, but this pattern was exaggerated in the particle-exposed animals. This is a normal physiologic response of rodents to stress. A similar effect was seen in rats exposed to ROFA. Since rodents are prominently used in air pollution exposure studies, these author point out the necessity to consider the impact of these physiological changes in the interpretation of cardiovascular data.

We examined the possibility that these physiological responses could have influenced our observed changes in blood pressure and HRV. As shown in Figure 4, there were significant body temperature changes in the rats between the pre-exposure measurement (7AM to 10AM) and the post-exposure measurement (3PM to 6PM). This was true for both FA and PM exposures, which were not significantly different. It may be that there is some stress associated with nose only exposures. However in our case it appears that if there is, it affects the control and exposed animals equally and should not be a source of bias. Second, our exposures are much lower than those described for the ROFA or metal exposures discussed above. Our estimated doses for the rats were about 3 μg for the 1-day exposed animals and 9 for the 3-day exposed animals. Thus, it appears that the effects that we have observed are not due to the anomalous physiology of rodents.

This experiment and the overall project provide an opportunity to examine the ability of these data to be extrapolated to humans, since for the first time very comparable studies have been performed.

This study has demonstrated that HRV, which is becoming more important as a sensitive indicator of adverse effects of PM, did not change appreciably after a single exposure but showed progressive reduction (a change in the adverse direction) with successive days of exposure. We don't know what would happen to HRV in rats exposed at lower concentrations for longer periods of time. Future studies are needed to address that issue.

The significance of decreased HRV in the rat may be open to question. However in humans decreased HRV is associated with future cardiac morbidity and mortality.[38] Decreased HRV is associated with the risk of developing coronary heart disease[39-41] and has been reported in patients with congestive heart failure.[42-44] PM exposure clearly affected HRV in the rat after multiple exposures suggesting potential consequences for elderly humans after episodes of high ambient PM exposure. These findings are certainly consistent with the epidemiologic literature linking ambient PM exposure to decreased HRV in people.

Summary

This study analyzed the effects of two PM components, ammonium nitrate (AMN) and elemental carbon (EC) on the cardiopulmonary system. AMN and EC were selected because they represent important fractions of ambient PM in California. The concentrations of each component used in this study were representative of estimated peak concentrations, based on extrapolations from ambient air data. The sizes of the particles used were chosen based upon reported sizes of inorganic aerosols in ambient air.

The findings of this study are summarized symbolically in Table 8. The direction of changes are shown and those trends that are significant are marked with '*'.

Table 8 Summary of Observed Effects - Trends Compared to FA Exposures and Statistical Significance

Endpoint	Single Day PM	Single Day PO	Multiple Day PM
Inflammatory Cells	↑	↑*	≈
Systolic Blood	↓	↓*	↓*

Pressure			
Heart Rate	↓	≈	≈
Double Product	↓	↓	↓*
Heart Rate Variability	≈	No Data	↓**
Macrophage Superoxide Production (UCSF Humans)	↑	↑*	↑

Notes: ↑ Increase compared to control; ↓Decrease compared to control; ≈ No difference; *Significant trend ($p \leq 0.05$); **Significant trend ($p \leq 0.01$).

Acute (4 hour) single day exposures to particles alone were compared with the effects of a mixture of particles and ozone at approximately the same particle concentrations. Both exposures produced decreases in blood pressure and heart rate. Blood pressure changes (differences between pre-exposure and post-exposure levels) were significantly different from controls. Heart rate was decreased by pollutant exposure but the response was not statistically significant. The changes induced by single day exposure to particles alone were not significantly different from those induced by the single day ozone + particle mixture, although the PO difference from control was significant and the PM difference from control was not.

Superoxide production by macrophages obtained by BAL from human subjects after 1 day of PM or PO exposure was elevated relative to controls, however only the PO exposure-induced effect was significantly different from that measured after FA exposure.

Repeated 3-day (4 hour per day) exposures to particles caused changes in blood pressure that were statistically significant immediately post-exposure, but there were no changes in baseline levels measured 20 hours after exposure. Heart rate variability was not significantly changed after a single exposure to particles, but showed progressive reduction after 2 and three days of consecutive exposure, compared to clean air exposures.

Conclusions

We conclude that our findings support the associations of cardiophysiological changes observed in epidemiological studies and ambient PM exposures. We observed changes in blood

pressure and heart rate variability that are consistent with an adverse effect of PM on the heart. At the levels of exposure used in this study, which are comparable to those now being studied in experiments that use particle concentrators, we found that significant changes could be seen in a susceptible animal model the senescent, or geriatric, rat. We also showed increased activation of macrophages from humans exposed under conditions that were nearly identical with those used in the UCI rodent studies. This study has provided an opportunity to compare animal responses and human responses to similar exposures and similar conditions. This should be a great aid in developing strategies for extrapolating from animals to humans. This study suggests that more information is needed to assess the longer term effects of biological responses to particle exposure. It is extremely difficult to do such studies in humans under sufficiently controlled conditions. We conclude that the senescent rat is an appropriate model for such future studies. [Evidence for comparability of response and function between rat and human?

Glossary

AMN	Ammonium Nitrate
Arrhythmia	Abnormal heart rhythm.
C	Coarse Particles (2000 ≤particle diameter ≤ 4000 nm)
EC	Elemental Carbon
F	Fine Particles (500 ≤ particle diameter ≤ 1000 nm).
Hypotension	Low blood pressure.
O_3	Ozone, a photochemical oxidant formed by atmospheric chemical reactions between sunlight, organic vapors and nitrogen oxides.
PM2.5	Particulate matter less than 2.5 μm mass median aerodynamic diameter.
PM10	Particulate matter less than 10 μm mass median aerodynamic diameter.
PMN	Polymorphonuclear leucocyte, a white blood cell with a multilobular nucleus.
Senescent rat	Rat older than 22 months.
Young, adult rat	Rat aged 8 to 10 weeks.
UCD	University of California, Davis
UCI	University of California, Irvine
UF	'Ultrafine' particles (particle diameter ≤ 200 nm).

References

1. Nicholson, K.W. Wind tunnel experiments on the resuspension of particulate material. Atmos. Environ., 27A(2):181-188, 1993.

2. Sehmel, G.A. Particle resuspension: A review. Environ. Int., 4:107-127, 1980.

3. Lyons, J.M., Venkataraman, C., Main, H.H., and Friedlander, S.K. Size distributions of trace metals in the Los Angeles atmosphere. Atmos. Environ., 27B 2:237-249, 1993.

4. Wolff, G.T., Ruthkosky, M.S., Stroup, D.P., and Korsog, P.E. A characterization of the principal PM10 species in Claremont (Summer) and Long Beach (Fall) during SCAQS. Atmos. Environ., 25A(10):2173-2186, 1991.

5. Martin, A.E. Mortality and morbidity statistics and air pollution. Proc. R. Soc. Med. 57:969-975, 1964.

6. Greenburg, L, Field, F., Erhardt, C., Glasser, M., and Reed, J.. Air pollution, influenza and mortality in New York City. Arch. Environ. Health 15:430-438, 1967.

7. Samet, J.M., Bishop, Y., Speizer, F.E., Spengler, J.D., and Ferris, B.G., Jr. The relationship between air pollution and emergency room visits in an industrial community. J. Air Pollut. Control. Assoc. 31:236-240, 1981.

8. Knight, K., Leroux, B., Millar, J., and Petkau, A.J. Air pollution and human health: A study based on data from Prince George, British Columbia. Tech Report #85, Department of Statistics, University of British Columbia, 1989.

9. Whittermore, A., and Korn, E. Asthma and air pollution in the Los Angeles area. Am. J. Public Health 70:687-696, 1980.

10. Schenker, M. Air pollution and mortality (editorial). N. Engl. J. Med. 329:1807-1808, 1993.

11. Pope, C.A. 3d, Dockery, D.W., Spengler, J.D., and Raizenne, M.E. Respiratory health and PM10 pollution. A daily time series analysis. Am. Rev. of Respir. Dis. 144:668-674, 1991.
12. Stern, B., Jones, L., Raizenne, M., Burnett, R., Meranger, J.C., and Franklin, C.A. 1989. Respiratory health effects associated with ambient sulfates and ozone in two rural Canadian communities. Environ. Res.:20-39.
13. Pope, C.A., and Kanner, R.E.. Acute effects of PM10 pollution on pulmonary function of smokers with mild to moderate chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. 147:1336-1340,1993.
14. Schwartz, J.. Particulate air pollution and daily mortality: A synthesis. Public Health Rev. 19:39-60, 1991.
15. Schwartz, J., and Dockery, D.W. Increased mortality in Philadelphia associated with daily air pollution concentrations. Am. Rev. Respir. Dis. 145:600-604, 1992.
16. Schwartz J. Particulate air pollution and chronic respiratory disease. Environ. Res. 62:7-13, 1993.
17. Dockery, D.W., Pope, C.A. 3rd, Xiping, X, Spengler, J.D., Ware, J.H., Fay, M.E., Ferris, B.G. Jr., and Speizer, F.E. An association between air pollution and mortality in six U.S. cities. N. Engl. J. Med. 329:1753-1759, 1993.
18. Pope CA 3rd. Adverse health effects of air pollutants in a nonsmoking population. Toxicol. 111:149-155, 1996.
19. Utell, M.J. Clinical perspectives on respiratory disease and the role of airborne particles. Proceedings of the Colloquium on Particulate Air Pollution and Human Mortality and Morbidity, R.F. Phalen ed., Irvine, CA, Jan.1994.
20. Thurston, G.D., Ito, K., Hayes, C.G., Bates, D.V., and Lippmann, M. Respiratory hospital admissions and summertime haze air pollution in Toronto, Ontario: consideration of the role of acid aerosols. Environ. Res.65:271-90, 1994.
21. Ozkaynak, H., and Thurston, G.D.. Associations between 1980 U.S. mortality rates and alternative measures of airborne particle concentration. Risk Analysis: 449-461, 1987.
22. Ozkaynak, H., and Spengler, J.D. Analysis of health effects resulting from population exposures to acid precipitation precursors. Environ. Health Perspect. 63:45-55, 1985.

23. Hefflin, B.J., Jalaludin, B., McClure, E., Cobb, N., Johnson, C.A., Jecha, L., and Etzel, R.A. Surveillance for dust storms and respiratory diseases in Washington State. Arch. Environ. Health 49:170-4, 1994.
24. Pope, C.A. 3d, Schwartz, J., and Ransom, M.R. Daily mortality and PM10 pollution in Utah Valley. Arch. Environ. Health 47:211-7, 1992.
25. Schwartz, J. Particulate air pollution and chronic respiratory disease. Environ. Res. 62:7-13, 1993.
26. Godleski JJ, Verrier RL, Koutrakis P, Catalano P. Mechanisms of morbidity and mortality from exposure to ambient air particles. Res Report Health Eff. Inst. 2000; 91:5-88.
27. Godleski JJ, Verrier RL, Koutrakis P, et al. Mechanisms of morbidity and mortality form exposure to ambient air particles. Res Report Health Eff Inst 2000; 91:5-88.
28. Godleski JJ, Lovett E, Sioutas C, et al. Impact of inhaled concentrated ambient air particles on canine electrocardiographic patterns. In the Thirteenth Health Effects Institute Annual Conference 1997; pp. 87-93. Boston: Health Effects Institute.
29. Godleski JJ, Sloutas C, Verrier RL, et al. Inhalation exposure of canines to concentrated ambient air particles. Am J Respir. Crit Care Med 1997; 155(4):A246.
30. Wellenius, G., Coull, B., Godleski, J., Koutrakis, P., Okabe, K., Savage, S., Lawrence, J. Krishna Murthy, G. and Veriier, R. Inhalation of concentrated ambient particles exacerbates myocardial ischemia in conscious dogs. Environ. Health Perspect. 2003; 111:402-408.
31. Wellenius, G., Saldiva, P., Batalha, J., Krishna Murthy, G., Coull, B., Verrier, R. and Godleski, J. Electrocardiographic changes during exposure to residual oil fly ash (ROFA) particles in a rat model of myocardial infarction. Toxicological Sci. 2002; 66:327-335.
32. Muggenborg BA, Green TL. Animal models of cardiac disease: potential usefulness for studying health effects of inhaled particles. Inhal Toxicol 2000; 12:901-925.
33. Solomon et al., 2004 Final Report to ARB
34. Pinkerton et al., 2004 Final report to ARB
35. SYSTAT: Statistics Version 5.2.1. Evanston, IL: SYSTAT, Inc., 724 pp, 1992.
36. John, W., Wall, SM, Ondo, JL and Winklmayr, W., Modes in the size distribution of atmospheric inorganic aerosol. Atmos. Environ. Part A – General Topics 1990 24:2349-2360.

37. Watkinson, WP, Campen, MJ, Nolan, JP and Costa, DL. Cardiovascular and Systemic Responses to Inhaled Pollutants in Rodents: Effects of Ozone and Particulate Matter. Env. Health Perspec. 2001 109: 539-546.

38. Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology . Heart Rate Variability: Standards of Measurement, Physiological Interpretation, and Clinical Use. *Circulation.* 1996;93:1043-1065.

39. Liao D, Cai J, Rosamond WD, Barnes RW, Hutchinson RG, Whitsel EA, Rautaharju P, Heiss G. Cardiac autonomic function and incident coronary heart disease: a population-based case-cohort study. The ARIC Study. Atherosclerosis Risk in Communities Study. Am J Epidemiol 1997; 145(8):696-706.

40. Tsuji H, Venditti FJ Jr, Manders ES, Evans JC, Larson MG, Feldman CL, Levy D. Reduced heart rate variability and mortality risk in an elderly cohort. The Framingham Heart Study. Circulation 1994; 90(2):878-883.

41. Tsuji H, Larson MG, Venditti FJ Jr, Manders ES, Evans JC, Feldman CL, Levy D. Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study. Circulation 1996; 94(11):2850-2855.

42. Casolo G, Balli E, Taddei T, Amuhasi J, Gori C. Decreased spontaneous heart rate variability in congestive heart failure. Am J Cardiol 1989; 64(18):1162-1167.

43. Casolo GC, Stroder P, Sulla A, Chelucci A, Freni A, Zerauschek M. Heart rate variability and functional severity of congestive heart failure secondary to coronary artery disease. Eur Heart J 1995; 16:360-367.

44. Mortara A, La Rovere MT, Signorini MG, Pantaleo P, Pinna G, Martinelli L, Ceconi C, Cerutti S, Tavazzi L. Can power spectral analysis of heart rate variability identify a high risk subgroup of congestive heart failure patients with excessive sympathetic activation? A pilot study before and after heart transplantation. Br Heart J 1994; 71(5):422-430.