

Final Technical Report

Date of Final Report: December 31, 2006

EPA Grant Number: RD827354

Center Name: University of Rochester–EPA PM Center

Center Director: Gunter Oberdorster

Title: Ultrafine Particles: Characterization, Health Effects and Pathophysiological Mechanisms

Investigators: Gunter Oberdorster, William Beckett, Glen Cass, Christopher Cox, David Ensor, Jacob N. Finkelstein, Mark W. Frampton, Joachim Heyder, John Looney, Victor J. Marder, P.E. Morrow, Michael O'Reilly, Annette Peters, Richard Phipps, Kimberly A. Prather, Edward Schwarz, Zachary Sharp, Barry Stripp, Mark J. Utell, Heinz-Erich Wichmann, C.P. Yu, Wojciech Zareba

Institutions: University of Rochester, GSF–Institute for Epidemiologie, Georgia Institute of Technology, Research Triangle Institute, University of California – Los Angeles, University of California – San Diego

EPA Project Officer: Stacey Katz/Gail Robarge

Project Period: June 1, 1999–May 31, 2005 (no-cost extension to May 31, 2006)

Period Covered by the Report: June 1, 1999–May 31, 2006

RFA: Airborne Particulate Matter (PM) Centers (1999)

Research Category: Particulate Matter

Objective(s) of the Research Project: The focus of the Rochester PM Center studies was an assessment of our ultrafine particle (UFP) hypothesis, which states that exposure of susceptible parts of the population to particles of the UF mode (<100 nm diameter) from the urban atmosphere can cause adverse health effects. We investigated this UFP hypothesis using a multidisciplinary team approach involving scientists from four different institutions. The five Research Cores of the Rochester PM Center integrated findings from ambient particle characterization (Core 1, R827354C001), epidemiological panel studies (Core 2, R827354C002), controlled clinical studies (Core 3, R827354C003), animal studies (Core 4, R827354C004) and *in vitro* studies (Core 5, R827354C005) to answer key questions regarding the involvement of ultrafine particles in the PM effects on respiratory and cardiovascular systems that have been observed in previous epidemiological studies. This report summarizes results of the Rochester PM Center research, demonstrating associations between specific ambient ultrafine particles and cardiovascular endpoints, effects on blood and vascular parameters following exposures to laboratory generated ultrafine carbon particles, controlled clinical studies in human subjects and in rodents, and cellular studies.

Summary of Findings: Results indicate that underlying mechanisms include direct (translocation of inhaled UFP from the portal-of-entry to extrapulmonary organs) as well as indirect mechanisms (systemic acute phase response). Results from rodent studies also demonstrate that the central nervous system appears to be yet another target organ for adverse effects of inhaled UFP. Investigation of factors associated with increased susceptibility to PM, such as asthma, chronic obstructive pulmonary disease (COPD), hypertension, inflammation, and advanced age was a major part of the Rochester PM Center studies, as described in the reports of the individual Research Cores. Some key findings of our studies from measurements of ambient

particles, from conducting epidemiological cohort studies, and using surrogate UFP in controlled clinical studies, toxicological animal studies, and *in vitro* studies confirmed that ambient UF/fine particles:

- have reactive oxygen species (ROS) so that, in addition to the potential for particulate constituents inducing the formation of ROS in the respiratory tract, the particles bring such materials to the tissues due to their composition (Venkatachari, et al., 2005);
- are associated with changes in vascular parameters indicative of an acute phase response, increased coagulation activation, and adhesion molecule expression in patients with coronary heart disease (Ruckerl, et al., 2005; von Klot, et al., 2005);
- are associated with onset of myocardial infarction 1 hour after exposure to traffic, according to time activity diaries (Peters, et al., 2004);
- induce changes in vascular parameters (acute phase response) and ECG (parasympathic stimulation) in rats following on-road exposure to highway PM (Elder, et al., 2004a), the latter assessed using a custom software program designed by our Cardiac Facility Core for rat ECG analyses (Couderc, et al., 2002).
- induce changes in cardiac function represented by heart rate variability (HRV), repolarization parameters (e.g., QT-time, T-wave amplitude, and T-wave complexity) and supraventricular and ventricular ectopic beats in patients with coronary heart disease (Ibald-Mulli, et al., 2005; Henneberger, et al., 2005) as well as changes in HRV in patients with COPD (preliminary results);
- can be measured with an Aerosol Time-of-Flight Mass Spectrometer (ATOFMS) down to particle sizes of 10 nm. These improvements permit much better characterization of the ambient aerosol as well as particles in controlled exposure systems (Su, et al., 2004);
- when concentrated in an UFP concentrator will show substantial increases in the organic carbon content as compared with the ambient particles (Su, et al., 2006).

and that inhaled laboratory-generated carbon UFP:

- have a high deposition efficiency in the respiratory tract, which is further increased in asthmatics and during exercise (Chalupa, et al., 2004; Daigle, et al., 2003);
- translocate to extrapulmonary organs via the blood circulation, dependent on particle chemistry (Oberdorster, et al., 2002; Kreyling, et al., 2002), and to the central nervous system, via olfactory neurons (Oberdorster, et al., 2004; Elder, et al., 2006);
- cause changes in adhesion molecule expression of peripheral blood leukocytes, indicative of systemic effects in healthy and asthmatic humans (Frampton, et al., 2006);

- alter ischemia-induced hyperemic blood flow in healthy subjects, indicative of particle effects on endothelial function (Pietropaoli, et al. 2004a);
- decrease the pulmonary diffusing capacity for carbon monoxide in healthy humans (Pietropaoli, et al., 2004b);
- induce greater oxidative stress in lungs of aged rats compared to adolescent rats and that ozone co-exposure can increase this response (Elder, et al., 2004a);
- accelerate venous thrombus formation in rats (Silva, et al., 2005);
- and that the greater sensitivity to UFP-induced oxidative stress in the aged organism is also evident in *in vitro* primary cell cultures (Finkelstein, et al., 2002).

Rochester PM Center Outlook

Our studies show that ambient PM can have significant oxidative capacity, that UFP can induce significant effects not only in the respiratory tract but more importantly affect the vascular and cardiac system and that age and underlying disease (susceptibility factors) are critical modifying factors. Furthermore, our demonstration of their translocation from deposition sites in the respiratory tract to other organs such as heart and central nervous system (CNS) provides plausible hypotheses for UFP-induced oxidative stress in those organs. This could be particularly detrimental in susceptible individuals with dysfunctional vascular endothelium as the earliest manifestation of atherosclerotic vascular disease, such as seen in type 2 diabetes. These hypotheses will be tested in our future studies. The scope of these multidisciplinary studies focuses on detailed characterization of ambient UF/fine PM from different sources and the molecular basis of injury to cells, animals, individuals, and populations.

Characterization of the Chemical Composition of Atmospheric Ultrafine Particles (RD827354C001)

Investigators: Philip K. Hopke, Kimberly A. Prather, Glen Cass, Ann Dillner

Objective(s) of the Research Project: The objectives of this Core were to provide an improved understanding of the chemical and physical nature of the ultrafine ambient aerosol. In 1999, there was relatively little data available that provides distinct information on the chemical and physical characteristic of particles in the size range <100 nm. Because of the relatively small concentrations of particle mass in this size range, sampling and chemical analysis is extremely difficult. However, such physical and chemical data provide critical information to the epidemiological and toxicological research to help guide their studies of the relationships of the ultrafine particles and adverse health effects. Initially, the focus of this Core was the development of effective methods to sample and analyze ultrafine particles. Subsequently, these methods were applied to characterize the ultrafine aerosol in a number of locations across the country to assess the variations that exist in the nature of the ultrafine particles.

Summary of Findings: In the early stage of this project, the state of knowledge of the composition of ultrafine particles was summarized by Cass, et al. (2000). The Cass/Dillner

group collected ultrafine particle samples in field experiments in a south central U.S. city (Houston, TX) and in a west coast city (Riverside, CA). A cluster analysis was applied by Dillner, et al., (2005) using data from two sites in Houston, TX; one site surrounded by refineries, chemical plants, and vehicular and commercial shipping traffic, and the other site, 25 miles inland surrounded by residences, light industrial facilities, and vehicular traffic. Twenty-four hour size-segregated ($0.056 \mu\text{m} < D_p \text{ (particle diameter)} < 1.8 \mu\text{m}$) particulate matter samples were collected during five days in August 2000. Inductively coupled plasma mass spectrometry (ICPMS) was used to quantify 32 elements with concentrations as low as a few picograms per cubic meter. Concentrations of particulate matter mass, sulfate, and organic carbon at the two sites were often not significantly different from each other and had smooth unimodal size distributions indicating the regional nature of these species. Element concentrations varied widely across events and sites and often showed sharp peaks at particle diameters between 0.1 and 0.3 μm and in the ultrafine mode ($D_p < 0.1 \mu\text{m}$) that suggested the sources of these elements were local, high temperature processes. Elements were clustered to provide normalized size distributions of all elements and to yield groups of elements with similar size distributions that were attributed to sources such as automobile catalysts, fluid catalytic cracking unit catalysts, fuel oil burning, a coal-fired power plant, and high-temperature metal working. The clustered elements were generally attributed to different sources at the two sites during each sampling day indicating the diversity of local sources that impact heavy metals concentrations in the areas around the sampling sites.

Su, et al. (2004) described the development of an improved aerosol time of flight mass spectrometry (ATOFMS) instrument to measure the chemical composition of single atmospheric particles smaller than 100 nm in particle diameter. An ultrafine particle aerosol time of flight mass spectrometry instrument has been constructed incorporating an aerodynamic lens system that allows transmission of ultrafine particles into the instrument. An effective method for detecting ultrafine particles in the systems has been developed and used in a number of ambient aerosol characterization studies including studies supported by the Center and many others. Thus, the development effort supported under this Core has led to a significant advance in ultrafine particle characterization that has broadened our understanding of their chemical composition.

To further support the field studies, Spencer and Prather (2006) used laboratory generated ultrafine particles to demonstrate the ability to quantify the amount of organic carbon (OC) on elemental carbon (EC) particles. They coated generated EC particles and developed a calibration curve that permitted the quantification of the amount of OC on EC particles. This resulting calibration curve was used to calculate the OC/EC mass fraction for particles in lab studies; field studies in Boston, San Diego, and Atlanta; and two source studies (gasoline and diesel vehicles). In addition, this calibration curve was used to show that 30% of the additional OC coating observed in particles produced by an ultrafine concentrator were being added to ultrafine particles in the concentrator (Su, et al., 2006). This change was attributed to additional gas-particle partitioning in the humidified growth region.

A study was conducted in Riverside, CA during the summer and fall of 2005. This was a large field study focused on $\text{PM}_{2.5}$ organic aerosols. In addition to standard gas, aerosol, and PM measurements, as part of this project, ultrafine particles were measured using a UF-ATOFMS for

3 weeks during both of these studies. In addition to standard ambient sampling and characterization, ambient particles were size selected using a Scanning Mobility Particle Sizer (SMPS). The aerodynamic sizes of these particles were measured in the ATOFMS. These two sizes could be used to determine the density and shape of ambient EC particles. It was determined that most of the particles in the summer had different densities on different days and times of the day. These densities were strongly dependent on the atmospheric water content. The higher the water content, the lower the particle density. This result suggested the Riverside summer aerosol was highly processed, allowing significant uptake of water (Spencer and Prather, 2007). Spencer, et al. (2006) reported the development of a procedure to make diesel lubricating oil particles and showed how similar their ATOFMS spectra were to ambient particles from diesel vehicles.

Beginning at the end of November 2001, the number concentrations of ultrafine particles have been measured at the NYS Department of Environmental Conservation (DEC) monitoring site on the central fire station in downtown Rochester, NY. Particle size distributions are being measured using an SMPS comprised of a differential mobility analyzer (DMA) and a condensation particle counter (CPC). In the diameter range of 10 to 500 nm, ambient particles are classified by a DMA (TSI 3071) and counted with a CPC (TSI 3010) every five minutes. This work was originally supported by the New York State Energy Research and Development Authority, but at the end of that support, we have continued this work with Center support. We have 1.5 years of data providing information on the number distributions of particles between 10 and 500 nm. In addition, the DEC site monitors SO₂, CO, PM_{2.5}, and meteorological variables. The results of this monitoring have been reported by Jeong, et al. (2004a; 2006). More than 70% of measured total number concentration was associated with ultrafine particles (UFP, 0.011-0.050 μm). Morning nucleation events typically peaking UFP number concentrations at around 8:00 were apparent in winter months with CO increases. These particles appear to be formed following direct emissions from motor vehicles during morning rush hour. There were also often observed increases in this smaller sized range particles in the late afternoon during the afternoon rush hour particularly in winter when the mixing heights remain lower than in summer. Strong afternoon nucleation events (> 30,000 cm⁻³) peaking at around 13:00 were more likely to occur in spring and summer months. During the prominent nucleation events, peaks of SO₂ were strongly associated with the number concentrations of UFP, whereas there were no significant correlations between these events and PM_{2.5} and CO. Increased SO₂ concentrations were observed when the wind direction was northwesterly where three SO₂ sources were located. It is hypothesized that UFP formed during the events are sulfuric acid and water from the oxidation of SO₂. There was also a more limited number of nucleation events followed by particle growth up to approximately 0.1 μm over periods of up to 18 hours. The nucleation and growth events tended to be common in spring months especially in April.

As part of the Core's effort to characterize the nature of PM_{2.5}, measurements of particle composition were made in Rochester, Philadelphia, and New York City that have been reported by Jeong, et al. (2004b,c) and Venkatachari, et al. (2006a,b). A major contribution of this Core has been the initiation of study of particle-bound reactive oxygen species (ROS). There is currently very limited information available on particle-bound ROS and thus, measurements in Rubidoux, CA (Venkatachari, et al., 2005) and New York City (Venkatachari, et al., 2007)

suggest that there can be significant concentrations of oxidant on fine particle surfaces. Studies of the effects of the particle-bound ROS are planned for the future at the University of Rochester.

Inflammatory Responses and Cardiovascular Risk Factors in Susceptible Populations (RD827354C002)

Investigators: H. Erich Wichmann, Annette Peters

Objective(s) of the Research Project: The aim of the Rochester Particle Center epidemiological studies was to assess the short-term health effects of fine and ultrafine particles on vascular and cardiac function. It was hypothesized that patients with coronary artery disease (CAD) as well as chronic obstructive pulmonary disease (COPD) would be susceptible to ambient fine and ultrafine particles. The hypothesis at the time was that the underlying systemic inflammation due to atherosclerotic disease would render CAD subjects more vulnerable. In contrast, subjects with COPD would be more vulnerable due to chronic inflammatory pulmonary disease. As the size and the composition of the particles are determined by the sources of the particles it is important for regulatory purposes to better understand the relative importance of sources in association with health effects. We had the following specific aims:

- 1) Blood markers of exacerbation of chronic inflammation and altered vascular function are elevated in association with ambient particles.
- 2) Cardiac function in patients is altered in association with ambient air pollution.
- 3) Particles from traffic and other combustion sources are associated with vascular and cardiac effects.

Summary of Findings: Two epidemiological studies were conducted to assess short-term health effects of fine and ultrafine particles in 61 patients with coronary artery disease and in 39 patients with COPD in Erfurt, Germany as part of the Rochester Particle Center. Twelve clinical visits including ECG measurements and blood withdrawals were scheduled. Ninety-eight percent of all scheduled ECG recordings and 94% of all scheduled blood withdrawals were realized.

Statistical Analyses

Continuous outcomes such as measurements of the blood coagulability, heart rate variability (HRV), and lung function measures were analyzed based on linear regression models considering repeated measurements for the subjects. The distribution of the residuals was checked carefully and additional analyses converting the continuous measurements into binary variables were conducted in case the residuals were not approximately normally distributed. These variables were analyzed using logistic regression analyses. In particular, several approaches to model the dose-response functions were applied including parametric, semi-parametric and non-parametric methods. The lag structure of the association between the air pollutants and the outcomes was analyzed to evaluate the time lags between exposure and response. Based on the experimental and clinical data collected in the other Cores, specific hypotheses were formulated before the analyses and then tested in the epidemiological data

(*specific aims 1 and 2*). The results obtained for the different sources will be compared to the results of the contributing particle fractions or gaseous pollutants (*specific aim 3*). It is unlikely that there is sufficient power to test for differences between regression coefficients for single pollutants and for specific sources. However, the biomarkers of cardiac function exhibit different response profiles when PM_{2.5}, ultrafine particles and organic or elemental carbon are considered. Additional information on source contributions will help to elucidate the role of different particle properties responsible for cardiovascular disease exacerbation via different mechanisms.

Specific Aim 1-Blood Biomarker. There was also evidence for an increase in C-reactive protein (CRP) concentrations and a shift to a more pro-coagulating state of the blood (Ruckerl, et al., 2006). For the CAD panel, an additional marker of inflammation was determined by the Immunology Core. Soluble CD40 ligand had been selected as a marker for exacerbation of chronic inflammation and altered vascular function (Phipps, 2000).

Our findings suggest an increase in sCD40L in association with ambient air particles, particularly with elevated levels of ultrafine particles and accumulation mode particles. For platelets the effects were limited to ultrafine particles showing an immediate as well as a three days delayed decrease. The regression of leukocytes showed consistently negative associations for UFP, AP, and PM_{2.5}, with lag 0 and for AP in addition with lag 3 and the 5-day average. As the effects seemed to be limited to the 24 hours prior to the blood withdrawal, we split the 24 hours up into four 6-hour periods and analyzed the results for UFP. While the effect for sCD40L was most prominent for the time period 12 to 17 hours prior to the blood withdrawal, platelets and leukocytes showed an immediate decrease in the first 5 hours and a delayed one between 18 to 24 hours (Figure 1) (Ruckerl, et al., 2007).

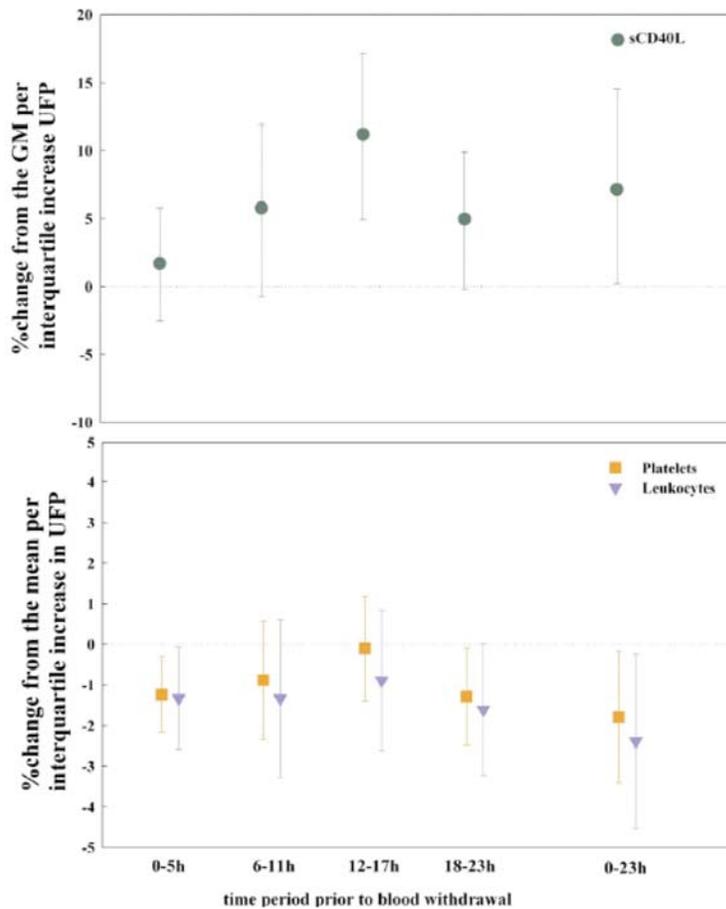


Figure 1. Effects of UFP and PM on Blood Biomarkers and Blood Cells in CAD Patients (Ruckerl, et al., 2007).

Erythrocytes and hemoglobin in contrast seemed to react more to the larger particles size fractions $PM_{2.5}$ and PM_{10} , showing a decrease in association with higher levels of air pollution. The largest negative effects for the erythrocytes were seen for PM_{10} for lag 2 and the 5-day-average exposure, a finding that is reflected to a lesser extent in the results of the hemoglobin.

For the COPD panel, differential hemograms were available. Preliminary results suggest no effect of particulate matter on all leukocytes combined. However, an increase in neutrophilic bandform granulocytes was observed in association with PM_{10} and AP immediately as well as with a 5-day average. Other leukocytic cell rows were either unaffected or showed small decreases. These results may provide evidence for a stimulation of the bone marrow by particulate matter (Socher, et al., 2005).

Specific Aim 1-Interdependence of Blood Markers and Cardiac Function. Additionally, associations within ECG recordings (time- and frequency-domain of HRV and repolarization parameters) and associations within blood markers (acute phase response, endothelial cell activation, and coagulation state markers) as well as associations between ECG recordings and

blood markers were analyzed using generalized estimating equation models adjusting for repeated measurements. Within the ECG recordings, strong significant associations were found between time- and frequency-domain parameters, and moderate but also significant associations between frequency-domain and repolarization parameters. Within blood markers, strong but significant associations existed between CRP and fibrinogen, D-dimer, E-selectin, ICAM-1, and SAA.

Between ECG recordings and blood markers, repolarization parameters and acute phase response proteins showed moderate but significant associations. HRV parameters and endothelial cell activation markers were significantly but only weakly associated. The results indicate the interplay between the autonomic nervous system and myocardial substrate as well as interactions of the acute phase response with endothelial cell activation and coagulation state. While ECG parameters and blood markers seem to vary independently, there was the suggestion for a link between systemic inflammation and repolarization as well as endothelial dysfunction and HRV (Yue, et al., 2006).

Specific Aim 2-HRV. In a study in patients with CAD, the autonomic control of the heart was altered in association with PM_{2.5} and organic (OC) and elementary carbon (EC) concentrations of PM_{2.5} (Ibald-Mulli, 2005). These findings highlight the importance of the carbonaceous component in particles. Furthermore, we were able to detect changes in the repolarization of the heart in association with PM_{2.5} and the number concentrations of accumulation mode particles (AP) (Henneberger, et al., 2005). Regarding arrhythmia, the number of supraventricular and ventricular runs showed strong effects correlated to AP and ultrafine particles as well (Berger, et al., 2006). Thereby, we found the first evidence that particles also might increase cardiac vulnerability and might modify the cardiac substrate. The effects of particulate air pollution on the autonomic nervous system as measured by heart rate (HR) and HRV in patients suffering from COPD were analyzed. Low frequency (LF) and the ratio of low to high frequency (LF/HF) increased in association with an increase in PM₁₀, OC, and EC during the 24 hours before the ECG measurement (Figure 2). Consistently, there was a significant decrease in heart rate with an increase of all particles measured 0-23 hours before the ECG recording. The analysis also showed a significant increase in root mean square successive difference (RMSSD) in response to an increase in all particle concentrations and some gases during 48-71 hours before the ECG recording. These results are contradictory to prior findings in CAD patients and our initial hypothesis. Taking both findings into account it is conceivable that the air pollution reaction depends on the disease status of the patient and that elevated concentrations of ambient particles are associated with a disturbance of the autonomic heart control manifested by an increased HRV in patients with COPD (Bero Bedada, et al., 2005).

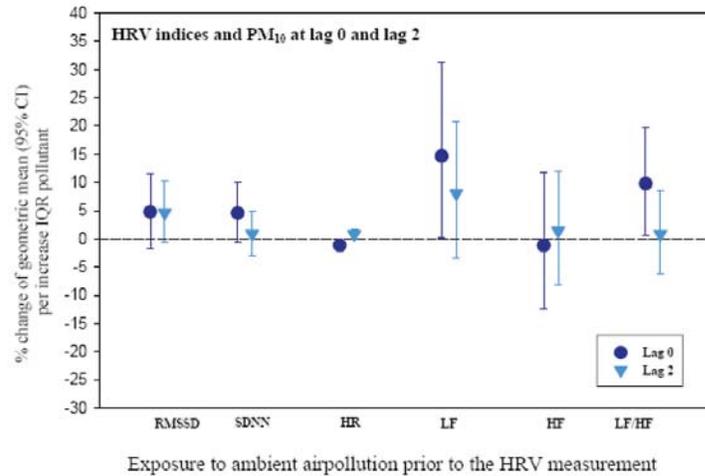


Figure 2. Effects of PM₁₀ on Heart Rate and Heart Rate Variability Parameters

Specific Aim 4-Effects of Traffic on Myocardial Infarction. A complete series of myocardial infarction survivors registered between 1999 and mid 2001 was interviewed to collect information on activities during the 4 days before MI onset. Analyses considered ambient particle concentrations as well as diary data. A total of 691 subjects were interviewed and they showed a higher prevalence of time spent in traffic 1 hour before the onset of myocardial infarctions than 24 to 72 hours earlier (Figure 3). Time spent in traffic was associated with MI onset 1 hour later (OR=2.9 (95% CI: 2.2 to 3.8) (Peters, et al., 2004). These associations were seen for times spent in cars (OR=2.6 (95% CI: 1.9 to 3.6), times spent in public transport (OR=3.1 (95% CI: 1.4 to 6.8) and on bicycles (OR=3.9 (95% CI: 2.1 to 7.2). Ambient PM_{2.5} concentrations at the urban background site also suggested an association with MI onset 2 days later (RR: 1.09 for 10 $\mu\text{g}/\text{m}^3$ PM_{2.5} (95% CI: 0.98 to 1.20) (Peters, et al., 2005).

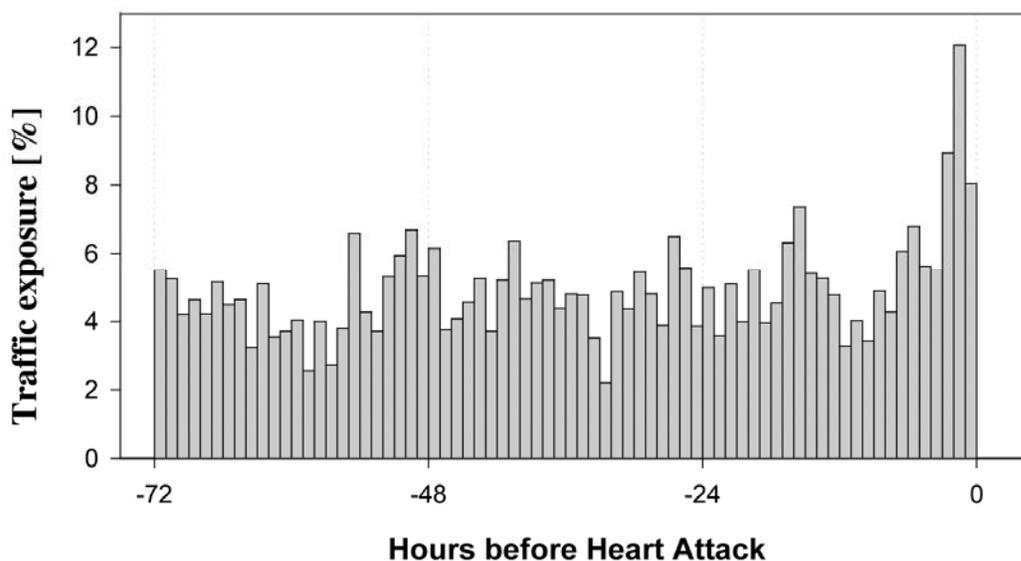


Figure 3. Prevalence of Time Spent in Traffic in 691 MI Survivors During the 72 Hours Before MI Onset

Specific Aim 3-Source Apportionment. Sources of fine and ultrafine particles were analyzed to determine the size distribution of fine and ultrafine particles. All analyses were conducted in collaboration with Core 1. The aim of this study was to use fine particle size distribution data collected between September 1997 and August 2001 in Erfurt, Germany, to investigate the sources of ambient particulate matter by positive matrix factorization (PMF). A total of 29,313 hourly averaged particle size distribution measurements covering the size range of 0.01 to 3.0 μm were included in the analysis. The particles number concentrations ($1/\text{cm}^3$) for the 9 channels in the ultrafine range, and mass concentrations (ng/m^3) for the 41 size classes of accumulation mode and fine particles were used in the PMF. The analysis was performed separately for each season. Additional analyses were performed including calculations of the correlations of factor contributions with gaseous pollutants (O_3 , NO, NO_2 , CO, and SO_2) and particle composition data (sulfate, organic carbon, and elemental carbon), estimating the contributions of each factor to the total number and mass concentration, identifying the directional locations of the sources using the conditional probability function, and examining the diurnal patterns of factor scores. These results were used to assist in the interpretation of the factors. Five factors representing particles from airborne soil, ultrafine particles from local traffic, secondary aerosols from local fuel combustion, particles from remote traffic, and secondary aerosols from multiple sources were identified in all seasons. The results can be used in epidemiological studies to investigate adverse health effects of source-specific particulate matter (Yue, et al., 2007a)

We used 56 patients' 5-minute ECG recordings for the analysis of repolarization parameters QT interval and T wave amplitude, and 57 patients' plasma samples to determine the biomarkers von Willebrand factor (vWF) and CRP. Linear and logistic regression models were used to analyze the associations between five particle source factors (airborne soil, local traffic ultrafine particles, combustion aerosols, diesel traffic particles, and secondary aerosols) and these health

parameters adjusting for trend, weekday, and meteorological variables. An increase in QT interval and a decrease in T wave amplitude were observed in association with traffic-related particles exposure during 0-23 hours before the ECG recordings. The inflammatory marker vWF increased in association with both traffic-related particles and combustion aerosols at different exposure lags. All source particles had positive associations with CRP levels above the 90th percentile (8.5 mg/l). These results suggest that traffic-related and combustion-generated particles show stronger adverse health impact with regard to cardiac effects, and that different source particles may have the potential to cause an acute phase response in these patients (Yue, et al., 2007b)

Clinical Studies of Ultrafine Particle Exposure In Susceptible Human Subjects

(RD827354C003)

Investigators: Mark Frampton; Mark J. Utell

Objective(s) of the Research Project: The project has consisted of an extensive series of controlled inhalation studies examining the effects of ultrafine carbon particles in healthy subjects, asthmatics, and diabetics. We developed a clinical inhalation facility for ultrafine carbon particles and studied particle deposition and retention, respiratory symptoms, pulmonary function, immunologic responses, endothelial function, and electrophysiologic responses to these particles using a dose-response paradigm with rest and exercise exposures. The details of the studies, findings, and implications are detailed in the following sections.

We developed a facility for experimental exposure of humans to ultrafine particles, which permits the quantitative determination of the exposure levels, respiratory intakes, and depositions of the aerosol (Chalupa, et al., 2002). Our overall objectives are to utilize controlled human exposures to examine, in healthy and potentially susceptible subjects, the role of ultrafine particles (UFP) in inducing respiratory and cardiovascular health effects. Our hypothesis was that inhalation of UFP alters pulmonary vascular function, circulating leukocyte activation, and cardiac repolarization. We speculated that these alterations reflect mechanisms involved in the observed increase in cardiovascular morbidity and mortality associated with particulate air pollution.

Summary of Findings:

(a) UFP Exposure in Healthy Subjects

For our initial studies, exposures were conducted at rest with a relatively low concentration of elemental carbon UFP ($\sim 10 \mu\text{g}/\text{m}^3$, $\sim 2 \times 10^6$ particles/ cm^3 , count median diameter 26.4 nm, GSD 2.3). The overall deposition fraction (DF) was 0.66 ± 0.12 (mean \pm SD) by number, and 0.58 ± 0.14 by mass (Daigle, et al., 2003). We found no significant differences in respiratory symptoms, blood pressure, pulse-oximetry, spirometry, exhaled NO, blood markers of coagulation and endothelial activation, leukocyte activation, or sputum cell differential counts (Pietropaoli, et al., 2004a,b). There was no convincing evidence for significant effects on heart rate variability, repolarization, or arrhythmias. We concluded that exposure to $10 \mu\text{g}/\text{m}^3$ elemental carbon UFP for 2 hours at rest does not cause significant respiratory or cardiac effects in healthy nonsmokers.

We then initiated studies to examine concentration-response effects, and to incorporate exercise. Subjects received each of three exposures (air, 10, and 25 $\mu\text{g}/\text{m}^3$ UFP). Analyses indicated that exercise further increased the relatively high resting deposition of UFP (number deposition fraction at rest: 0.63 ± 0.04 ; exercise: 0.76 ± 0.06 ; means \pm SD) (Chalupa, et al., 2004). There was evidence for a concentration-related effect of UFP exposure on the percentage of blood monocytes. In addition, monocyte expression of CD54 (ICAM-1) decreased after exposure in a concentration-response pattern ($p=0.001$), with the greatest effect occurring at 0 and 3.5 hours after exposure, and the differences resolved at 21 hours after exposure (Frampton, et al., 2006). Overall, the findings provided evidence for effects of UFP exposure, with exercise, on blood monocyte number and leukocyte expression of surface markers. In general, surface marker expression decreased in association with UFP exposure, consistent with retention of higher expressing cells within the capillary bed. ECG recording analyses showed that the response of the parasympathetic system (measured by normalized units of high-frequency [HF] components) was blunted during recovery from exercise immediately after exposure to UFP in comparison to air exposure. The analysis of QT interval duration and T wave amplitude also showed a blunted response after UFP exposure in comparison to pure air exposure. The QT and QTc shortened during exercise more substantially during UFP particle exposure than during pure air exposure, and that the QT and QTc interval remained shortened for several hours after UFP exposure but not after pure air exposure. These findings suggested that inhalation of UFP at both concentrations altered myocardial repolarization in healthy subjects.

We next initiated a study to confirm and extend these observations in a larger group of healthy men and women, using a higher, yet still environmentally relevant, concentration of UFP. Our hypothesis was that inhalation of UFP alters pulmonary vascular function, circulating leukocyte activation, and cardiac repolarization. We speculated that these alterations reflect mechanisms involved in the observed increase in cardiovascular morbidity and mortality associated with particulate air pollution.

Diffusing Capacity: In order to test our hypothesis, and to determine concentration-response relationships, we initiated exposures of healthy subjects to a higher concentration, 50 $\mu\text{g}/\text{m}^3$ UFP, using the same protocol. In these studies, we also measured the pulmonary diffusing capacity for carbon monoxide, which is affected by changes in pulmonary capillary blood volume, in 16 subjects. We also observed a significant reduction in the pulmonary diffusing capacity for carbon monoxide (DLCO), 21 hours after exposure to 50 $\mu\text{g}/\text{m}^3$ UFP when compared with air (Figure 1) (Pietropaoli, et al., 2004b). There was also a significant reduction in blood NO products throughout the post-exposure period (Figure 2). The DLCO is a function of the diffusing capacity of the pulmonary “membrane,” D_m , and the pulmonary capillary blood volume, V_c . The reduction in DLCO in these studies may be caused by mild pulmonary vasoconstriction, as a consequence of reduced NO availability, leading to a reduction in the pulmonary capillary blood volume. In addition, the results of this study confirmed our previous observations of UFP effects on leukocyte expression of adhesion molecules. Monocyte expression was significantly decreased for CD18, CD11b, and CD54.

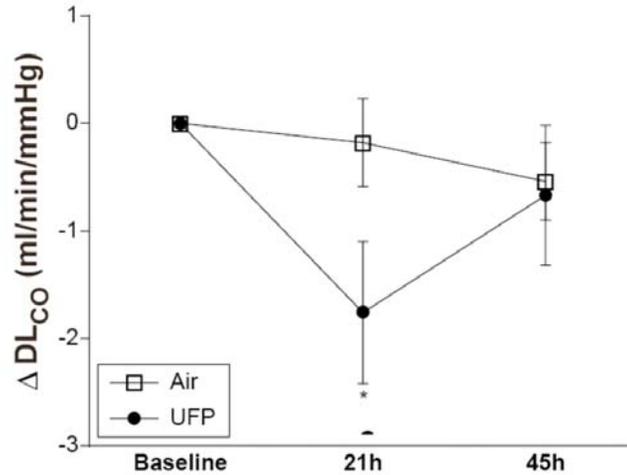


Figure 1. Change in DLCO After Exposure to Air or 50 mcg/m³ UFP

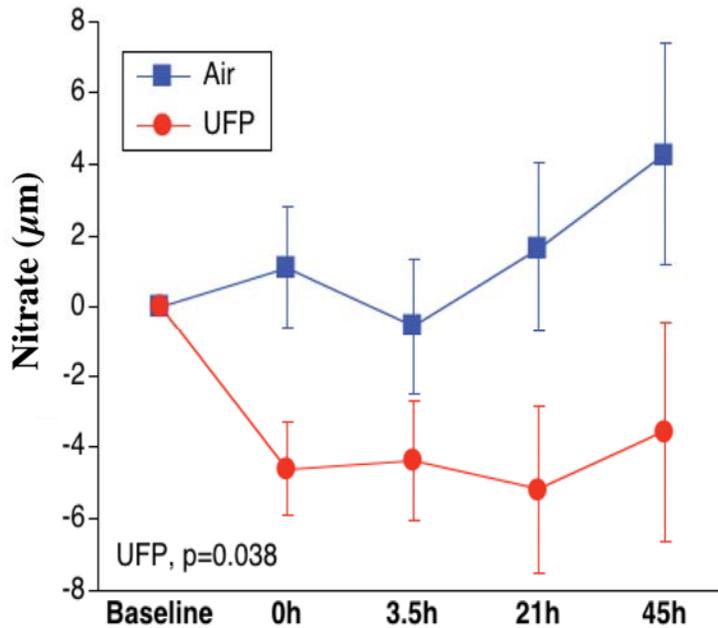


Figure 2. Change in Venous Nitrate

We subsequently tested the hypothesis that the effects on pulmonary diffusing capacity are a consequence of the high surface area of ultrafine particles, with their potential to deliver reactive oxygen species to the endothelium. In this study, 12 healthy never-smoking adults underwent three separate exposures, separated by at least 2 weeks: 1) air; 2) 50 µg/m³ UFP (count median diameter ~30 nm, particle number ~1 x 10⁷/cm³, surface area ~750 m²/g); and 3) 100 µg/m³ fine particles (FP) (count median diameter ~300 nm, particle number ~1 x 10³/cm³, surface area ~7 m²/g). The higher mass concentration used for FP relative to UFP was designed to provide an equivalent mass deposition in the lung, in view of the lower predicted deposition efficiency for FP. Exposures were by mouthpiece for 2 hours with intermittent exercise, randomized and

double-blinded. Effects on oxygen saturation, DLCO, and diffusing capacity for nitric oxide (DLNO) were assessed before and at intervals after exposure. Blood plasma was analyzed for nitric oxide metabolites and nitration products.

Our findings confirm that exposure to carbon UFP decreases the DLCO relative to air exposure. When the changes in DLCO following UFP exposure in this study were combined with data from our previous study in healthy subjects, the DLCO decreased from 30.15 ± 1.28 to 28.23 ± 1.16 ml/min/mmHg 24 hours after UFP exposure ($p=0.002$ vs. air exposure). The DLCO also decreased after exposure to fine carbon particles, but the difference was not significant. We also found an increase in the DLNO/DLCO ratio, suggesting a decrease in pulmonary capillary blood volume, with both UFP and FP exposure. However, this ratio was not significantly changed when the two studies were combined. There were no significant exposure effects on NO products, including nitrate, nitrite, S-nitrosohemoglobin, and iron-nitrosyl hemoglobin. Additional analyses are in progress. These findings provide important confirmation of effects of inhaled UFP on the pulmonary diffusing capacity for carbon monoxide. To our knowledge, no previous human clinical study has demonstrated pulmonary effects following exposures to such low mass concentrations of particulate matter. The data support the hypothesis that UFP inhalation alters endothelial function in the pulmonary vasculature of healthy nonsmokers.

Endothelial Function: In addition, we measured flow-mediated vascular dilatation of the forearm (FMD), before and at intervals up to 48 hours after exposure, using forearm plethysmography before and after ischemia, which measures the response in resistance vessels to the post ischemic increase in flow. FMD is mediated in part by endothelial NO action on vascular smooth muscle, and we hypothesized that UFP-induced reductions in vascular responsiveness would be accompanied by reduction in plasma NO reaction products. We therefore measured changes in the products of NO metabolism, nitrite and nitrate. We did not see any significant effect of UFP exposure on total forearm blood flow, either before or after ischemia. However, UFP exposure appeared to cause a blunting of the increase in peak flow in response to exercise. Figure 3 shows forearm blood flow following ischemia, measured 3.5 hours after exposure. The values represent change from the pre-exposure measurement. Peak flow (0 minutes) after air exposure increased, representing increased flow-mediated dilatation in response to exercise, which is an expected change. However, peak flow did not increase with UFP exposure. Figure 4 shows the change in peak flow at each of the time points measured. The difference in peak flows at 3.5 hours after exposure was significant by paired t-test, but not by analysis of variance. Minimal vascular resistance, which is the mean arterial pressure divided by the peak flow, was significantly increased compared with air exposure at this time point. Mean arterial pressure did not change significantly. These findings suggest that exposure to UFP reduced or delayed the exercise-induced increase in flow-mediated dilatation. Thus, inhalation of ultrafine carbon particles may have subtle vasoconstrictive effects in both the pulmonary and systemic vasculature.

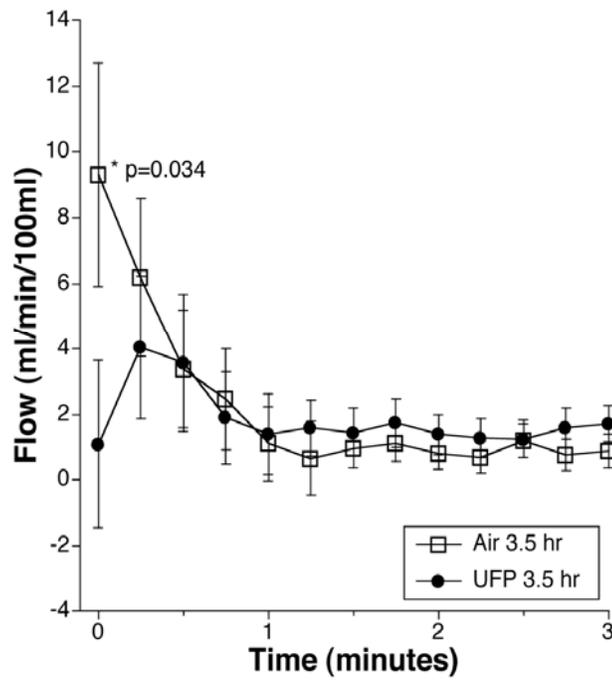


Figure 3. Forearm Blood Flow Post-Ischemia 3.5 Hours After Exposure, Difference From Pre-exposure

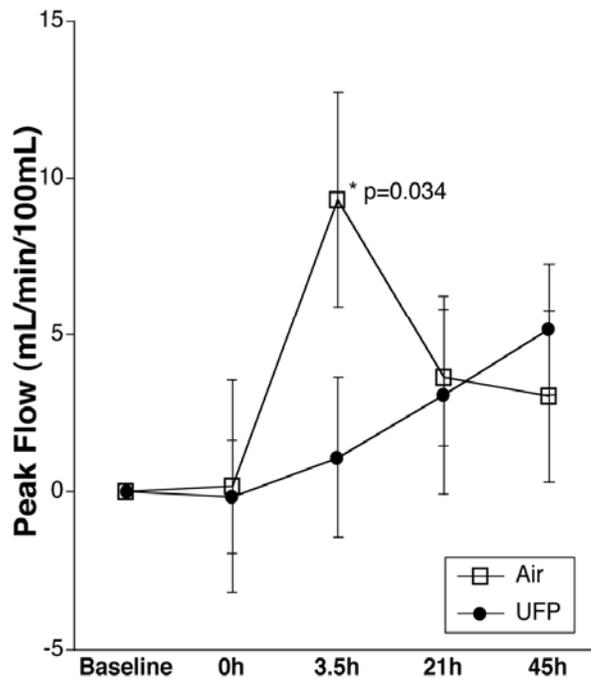


Figure 4. Peak Post-Ischemic Flow, Difference From Pre-exposure Baseline

(b) UFP Exposure in Subjects with Asthma

Subjects with asthma may represent a group with increased susceptibility to the health effects of ultrafine particles, both because of the possibility of increased airways deposition of particles, and because of underlying airway inflammation. We have completed a clinical exposure study (co-funded by the Health Effects Institute) of subjects with mild asthma. Sixteen subjects (8 male, 8 female) were exposed to air and to $10 \mu\text{g}/\text{m}^3$ carbon UFP for 2 hours with intermittent exercise. We measured effects on pulmonary function, symptoms, airway inflammation (exhaled NO and induced sputum), blood leukocyte activation, and cardiac electrophysiologic function.

In the asthmatic subjects, we found that total respiratory fractional deposition by particle number was high at rest (0.77 ± 0.05) and increased during exercise (0.86 ± 0.04) Chalupa, et al., 2004). Rest deposition was significantly increased compared with our previous study in healthy subjects at rest (0.63 ± 0.03). We conclude that ultrafine particle deposition is increased in mild asthmatic subjects compared with healthy subjects.

There were no significant changes in respiratory symptoms or pulmonary function in response to these exposures. Blood studies revealed a particle-related decrease in blood eosinophils, basophils, and CD4^+ T-lymphocytes. Blood monocytes showed a significant reduction in CD11b expression after exposure ($p=0.029$) (Frampton, et al., 2006). Expression of CD54 on polymorphonuclear neutrophil (PMN) decreased in a time-related fashion, with the greatest difference from control at 45 hours after exposure, with a significant time-exposure interaction ($p=0.031$). Expression of CD62L on PMN showed a significant exposure-gender interaction, with an increase in expression of CD62L in males only. The most significant effect on leukocyte surface molecule expression in subjects with asthma appeared to be on eosinophils. There was a small exposure-related reduction in eosinophil percentage from the blood leukocyte differential count. In addition to this early reduction in eosinophil number, there was a delayed reduction in eosinophil expression of CD32 (time-exposure interaction, $p=0.015$), and CD11b (main effect, $p=0.015$).

In summary, our study indicates that asthmatics have increased airway deposition of ultrafine particles, and that exposure to even low mass concentrations of ultrafine particles alters circulating leukocyte subsets. These data are most consistent with an alteration in leukocyte retention in the pulmonary circulation. In addition, our preliminary results suggest there are also effects on systemic endothelial function.

Conclusions

The major conclusions from our studies are as follows:

- In healthy individuals, there is a high pulmonary deposition of inhaled ultrafine particles at rest that increases with exercise.

- The reduction in diffusing capacity and changes in forearm flow mediated vascular dilatation suggest that inhalation of low mass concentrations of ultrafine carbon particles cause subtle changes in both pulmonary and systemic endothelial function.
- In addition, the findings provide evidence for effects of UFP exposure, with exercise, on blood monocyte number and leukocyte expression of surface markers. In general, surface marker expression decreased in association with UFP exposure, consistent with retention of higher expressing cells within the capillary bed.
- Asthmatics have increased airway deposition of ultrafine particles, and exposure to even low mass concentrations of ultrafine particles alters circulating leukocyte subsets.

Our findings provide support for the hypothesis that inhalation of ultrafine particles causes subtle alterations in vascular function. Our findings are consistent with the panel studies described in Core 2, which provided evidence for vascular endothelial effects of ambient particles in susceptible humans. In particular, the findings in Core 2 of increased soluble CD40L, and decreases in platelet counts, may indicate that inhalation of particulate matter causes platelet activation, which is a consequence of endothelial injury. This is consistent with our findings of changes in blood leukocyte markers, diffusing capacity, and forearm blood flow.

Animal Models: Dosimetry, and Pulmonary and Cardiovascular Events (RD827354C004)
Investigators: Gunter Oberdorster, Alison Elder

Objective(s) of the Research Project: The animal studies (Core 4) were designed to be complementary to the field and controlled clinical studies and to form a link to the mechanistic *in vitro* studies. Furthermore, they were designed to determine pulmonary and systemic responses to inhaled laboratory-generated and real-world ultrafine particles (UFP) and to develop rodent models of human disease to test our central hypothesis that UFP contribute to the increased morbidity and mortality of susceptible individuals in association with small increases in urban particles. Thus, the overall objective of the animal studies was to identify factors that are causally associated with adverse pulmonary and extrapulmonary health effects after low-level exposures to UFP. These factors were hypothesized to include particle size, dosimetry (lung deposition and disposition), host susceptibility (advanced age, cardiovascular disorders, respiratory tract inflammation), and pollutant co-exposure (e.g., ozone).

Two of the main focuses of the Core 4 research were to 1) achieve concordance for the animal studies with the other research cores in terms of endpoints measured and 2) generate and use UFP-containing atmospheres in toxicology studies that are relevant for human ambient air exposures. First, we completed studies in young and old rodents using two different respiratory tract priming agents: inhaled low-dose endotoxin, as a model for pneumonia or exacerbations of COPD, and human influenza virus, a common respiratory pathogen. Intraperitoneally-injected endotoxin was also used in some studies to prime respiratory tract cells from the blood circulation. In addition to creating compromised animal models via specific exposures, we also utilized a genetically-controlled compromised model, namely the SH rat. Several studies were completed in which ozone was used as a co-pollutant, delivered by itself or with laboratory-generated UFP. We also evaluated the clearance kinetics of inhaled UFP in rats with respect to

their translocation to extrapulmonary organs across the alveolo-capillary barrier and along sensory neurons from the upper respiratory tract. The particle types used in our studies have included laboratory-generated ultrafine carbon and ultrafine carbon/Fe particles; laboratory-generated organic UFP; concentrated fine/ultrafine particles (ambient Rochester air); and freshly-generated vehicle exhaust emission fine/ultrafine particles on highways. Aside from effects studies in the lungs, we have also focused heavily on cardiovascular and central nervous system (CNS) responses and on the uptake and tissue-specific distribution of inhaled solid carbon and metal UFP (^{13}C , mixed Mn oxides).

Summary of Findings:

Ultrafine Particle-Containing Atmosphere Generation

Laboratory-Generated Model UFP. As stated above, a main focus of these Core 4 studies was to develop model particles to be used in exposures in animals. We generated ultrafine carbon particles (~26 nm count median diameter [CMD], $15\text{-}20 \times 10^6$ particles/cm³) and similar UFPs containing ~25% Fe. In addition, ^{13}C UFP were generated for use in particle dosimetry studies. We found that the ultrafine carbon particles have a high surface area (580 m²/g) as determined via the BET method (Dr. Bice Fubini, Turin, Italy). By aging these particles for 5 minutes, they coagulated into accumulation mode particles with CMD of 280 nm. However, particle surface area remained as high as before. In addition, when rats were exposed to the larger coagulated particles at 2-fold higher concentration than uncoagulated UFP, the inflammatory response was similar to the singlet UFP, possibly due to deagglomeration in the lungs and the available high surface area. Thus, we decided that these coagulated ultrafine particles were not appropriate for use in size comparison studies.

The multigroup studies (described below) were performed with ultrafine carbon particles that were mixed with Fe (25%; i.e., generated from C/Fe graphitized rods). Having completed several of these multigroup studies, we were able to compare responses in animals inhaling ultrafine carbon vs. carbon/Fe particles; we found no evidence to support that *in vivo* inflammatory responses differ when Fe is present in the UFP. This is intriguing given the fact that the Fe in these particles is bioavailable (Dr. Ann Aust, Utah State University) and they generate more hydroxyl radicals in the presence of peroxidethan than do UFP without Fe (Dr. Vincent Castranova, NIOSH). Another interesting point regarding the laboratory-generated UFPs is that they initially contained up to 30% organic material due to a number of plastic components in the spark generator. Efforts to remove all possible sources of contamination in the spark generator were successful so that there was less than 5% of organic carbon in the laboratory-generated UFP aerosol that was used in the studies described below.

Smaller ambient UFP (<~20 nm) consist of organic compounds to a large degree, as seen by our Core 1 measurements. A significant component is used and unused motor oil. Recognizing this, studies were conducted through our Visiting Scientist Program to develop a method for generating organic UFP. To this end, a vaporization-condensation aerosol generator was assembled and characterized by Dr. John Veranth (University of Utah). Used motor oil was used in this system, which was capable of generating stable organic UFP (30-50 nm; 10^6 particles/cm³) aerosols for 6 hours. Briefly, the motor oil (in hexane and ethyl alcohol) was

nebulized such that large droplets were removed by inertial impaction on an impinger, leaving a fine mist that was then directed into a furnace with seed nuclei of NaCl. The oil droplets were completely vaporized and ultrafine aerosols formed when the vapor began to cool at the exit of the furnace. Other studies done with paraffin oil and short-chain alkanes showed that the generation system is adaptable to other low-volatility hydrocarbons.

Generation of Concentrated Ambient Ultrafine/Fine Particles. In addition to these laboratory-generated UFP, we have also performed studies using ambient PM. Through our collaboration with the Harvard PM Center (Dr. Petros Koutrakis), we performed studies using a prototypical UFP concentrator. The prototype sampled air from a moderately busy road adjacent to our laboratory in Rochester and concentrated UFP with some overlap into the fine mode (7-316 nm; CMD=35 nm, GSD=1.9). The average number concentrations during the 6-hour exposures were $\sim 2 \times 10^5$ particles/cm³. A state-of-the-art concentrator (Harvard Ultrafine Concentrated Ambient Particle System, HUCAPS) was built and sent to the University of Rochester to be assembled and permanently housed in a new research building for use in controlled animal and clinical exposures to traffic-related aerosols. This system has a 200-nm cutoff, resulting in CMDs of ~ 75 nm; the gases in the incoming aerosol are neither excluded nor concentrated. Having run the HUCAPS several times, we have found that the number concentration of the output aerosol ranges from 0.05 - 1.3×10^6 particles/cm³. Figure 1 shows an example of the variation in number concentration and the size distribution in our Rochester facility.

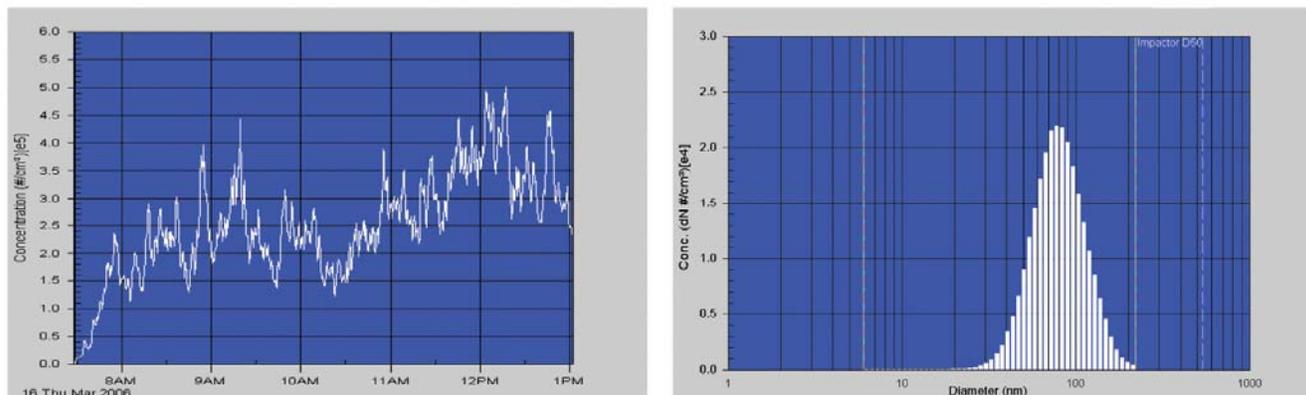


Figure 1. Variation in Particle Number Concentration over a 6-hour Exposure Period (left) and the Particle Size Distribution (right) From the Same Date

Freshly-Generated On-Road Ultrafine/Fine Particles. For toxicological studies with realistic UFP, diluted exhaust from stationary diesel engines or concentrated ambient UFP have been used, yet questions remain about how well these particle dilutions model those found in ambient air. Freshly-generated UFP are present at high concentrations on highways, and vehicle passengers are directly exposed to them when driving behind other vehicles on roads. We exposed rats to such UFP using a mobile emissions laboratory (MEL) from the University of Minnesota (D. Kittelson) for driving rats directly on highways to test the potential of highway aerosols to cause effects when the exhaust from diesel powered trucks was taken in. Since such exposures have not been done before, our objectives were to: (i) demonstrate the feasibility of

an on-road exposure study; (ii) determine if there are significant effects in aged rats; and (iii) determine if priming modulates effects in the respiratory tract. A multidisciplinary team approach was used involving PM Center investigators from all of the Research Cores, the Particle Generation and Cardiac Facility Cores, and in collaboration with University of Minnesota scientists (D. Kittelson and W. Watts). Three exposure atmospheres were generated for these studies from the incoming highway aerosol: particles + gas phase, gas phase only, or particle-filtered and gas-denuded air. The exposures in compartmentalized whole-body chambers consisted of 6 hour driving periods on I-90 between Rochester and Buffalo once or 3 days in a row. The daily average number concentration in the control (filtered air) chamber was $0.01\text{-}0.12 \times 10^5$ particles/cm³. The in-coming sampled air had a number concentration of $1.95\text{-}5.62 \times 10^5$ particles/cm³; however, losses were experienced when that air was directed into the chambers, resulting in exposure number concentrations of $0.95\text{-}3.13 \times 10^5$ particles/cm³. Details about the system itself and the aerosol characterization from our first study using it have been published (Kittelson, et al., 2001, 2004).

One limitation of these studies was the variability in the aerosol number concentration due to both the high dilution factor and long periods of low particle number concentrations. In order to achieve more continuous aerosol sampling, we performed another series of studies with MEL, this time orienting two telescoping pipes on the back of the trailer so that they would sample the engine exhaust plume from the diesel-powered MEL itself as well as—to a lesser degree—those particles and gases from surrounding vehicles. The daily average number concentration in the filtered air chamber was $<4.5 \times 10^3$ particles/cm³. The in-coming sampled air had a number concentration of $1.6\text{-}4.3 \times 10^6$ particles/cm³, but some losses were experienced when that air was directed into the chambers. Chamber number concentrations were $4.4\text{-}7.6 \times 10^5$ /cm³, 5.5 to 6.5-fold lower than in-coming air; this same phenomenon was observed in the previous study. The average geometric mean diameter was 15 nm. Despite these losses, the number concentrations in the exposure cages were about twice as high as in the previous study and the in-coming particle number concentration was an order of magnitude higher. The particle number, along with NO and CO₂ concentrations in the incoming air indicated that the MEL exhaust plume was being directly sampled at a dilution of about 400:1.

Toxicology Studies with Laboratory-Generated UFP

Studies in Young and Old Mice Exposed to Combinations of UFP and Ozone with Respiratory Tract Priming. The design for these toxicological studies, unless otherwise specified, involved 16 different groups of animals, representing all possible combinations of exposure components (UFP, ozone, priming agent) and age (young vs. old). Eight groups of young (8-10 weeks) and old (20-22 months) mice and rats were exposed ($n=5$ per group) to UFP (carbon or mixed carbon/Fe) with or without ozone for 6 hours in compartmentalized whole-body chambers with and without prior priming by endotoxin or human influenza virus. Low-dose endotoxin aerosols were used to prime the respiratory tract (~5-10% polymorphonuclear neutrophils [PMNs] in lavage fluid after 24 hours); these priming exposures lasted for ~15 minutes and were done immediately prior to UFP/ozone exposures. When human influenza virus (X-31, H3N2; 10^4 EID₅₀) was used as the priming agent, it was intratracheally instilled 48 hours prior to UFP/ozone exposures. We collected all samples for our measurements 24 hours after the start of the UFP/ozone exposure. Since there were four factors involved in these studies, all data were

analyzed via four-way analysis of variance (ANOVA) for main effects and interactions between factors. This type of ANOVA is not only the most appropriate one for the study design, but it also has high statistical power.

One “multigroup” study was performed with mixed C/Fe UFP ($\sim 100 \mu\text{g}/\text{m}^3$) and ozone (0.5 ppm) after priming with inhaled low-dose LPS. In agreement with our earlier studies in rats, all four factors (UFP, ozone, LPS, and age) had significant main effects for most of the respiratory and cardiovascular endpoints examined. The striking age effect was such that inflammatory and cell activation responses in old mice were greater than in young mice. For some endpoints (e.g., lavage PMNs, lavage AM surface ICAM-1 expression), the UFP effect was dependent upon the presence of LPS. This draws attention to the fact that there were several consistent interactions involving inhaled UFP, among them those involving LPS and age (response enhancement) and ozone (response suppression). Our findings that inhaled UFP can alter blood PMN surface ICAM-1 expression are in agreement with results from PM Center-related clinical studies. Some of the most striking effects were observed in lung and heart tissue gene expression changes. Not only were significant alterations in heart tissue gene expression observed, indicating extrapulmonary effects of inhaled UFP and ozone, but the data also suggest an imbalance in old animals between pro- and anti-inflammatory species production (Elder, et al., 2002). Examples for MIP-1 α and IL-10 are shown in Figure 2.

Another multigroup study was conducted using influenza virus to prime respiratory tract cells. The design of the study was essentially the same as that of the LPS priming study summarized in the previous section. Young and old male C57 mice (10 weeks, 21 months) were exposed to ultrafine carbon particles containing 25% Fe (CMD $\sim 26 \text{ nm}$, $\sim 140 \mu\text{g}/\text{m}^3$) and ozone (0.5 ppm) for 6 hours, alone and in combination. Lung inflammation was induced with intratracheally instilled X-31 human influenza virus 48 hours prior to UFP or ozone exposures. Parameters of inflammation in lavage fluid and blood as well as lavage cell oxidant release were measured 24 hours after exposure. RNA was also extracted from lung and heart tissue for microarray analyses. As before, a 4-way ANOVA was used to analyze the results. UFP were found to have consistent and independent effects on pulmonary inflammation and inflammatory cell activation. Ozone, influenza virus, and age had significant main effects for all endpoints examined. In addition, the interactions with UFP that were consistently significant involved influenza virus, ozone, and age. Using microarray analyses, we have also screened lung and heart tissue for changes in gene expression. There was a trend toward higher pro-inflammatory and lower anti-inflammatory gene expression in tissues from old as compared to young animals. In addition, we found evidence of significant gene changes in heart tissue (Elder, et al., 2004b). When the results from the studies using LPS and influenza virus as priming agents are considered as a whole, a consistent pattern of main effects and factor interactions emerges (see Table 1). Given the fact that there were so many endpoints analyzed in these two sets of studies, the consistency of these interactions is remarkable and strengthens the causality of associations that were found in the statistical analyses. Moreover, the results are also consistent with those of our earlier multigroup studies in young and old rats showing that effects are not species specific.

Table 1. Synopsis of Effects Tests

Multigroup Studies: Synopsis of Effects Tests <i>(both priming agents/all endpoints)</i>	
<u>Main Effects:</u>	
UFP	> 1/2 of the endpoints
Ozone	always
Priming Agent	always
Age	all but one endpoint
<u>Consistent Interactions:</u>	
UFP interacting with ...	
	Priming agent
	Ozone
	Priming agent * Ozone
	Priming agent * Age

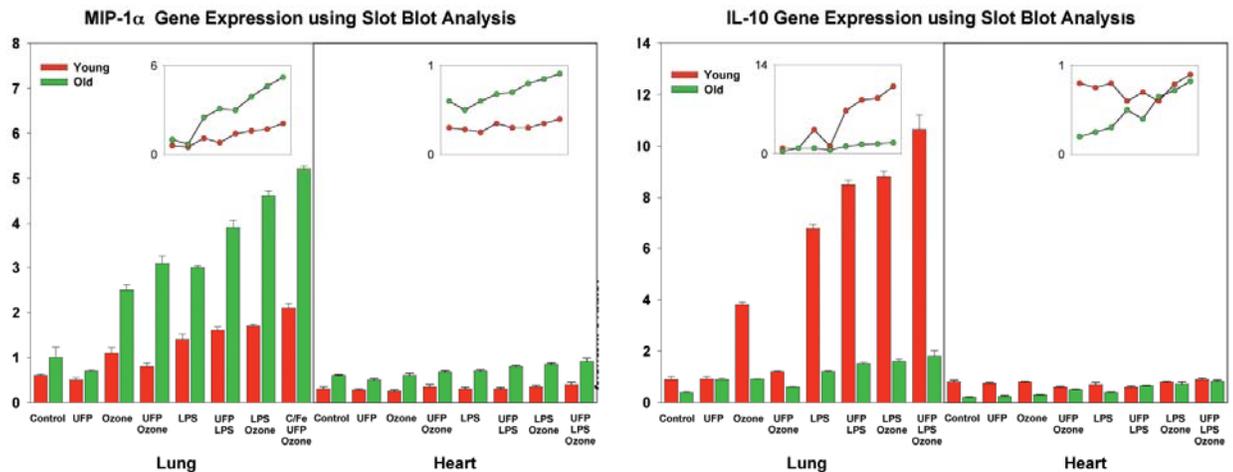


Figure 2. MIP-1 α (left) and IL-10 (right) Gene Expression in Lung and Heart Tissue After Exposure to Inhaled UFP in Combination With Ozone After LPS Priming. The insets show line plots of the group means for reference. Responses in young mice are shown in the red bars and those from old mice are shown in green.

Studies with Systemically-Delivered LPS. Other exposures with laboratory-generated ultrafine carbon particles were done after endotoxin priming via ip injection to simulate the early phase of response to an inflammatory stimulus that would prime respiratory tract and circulating cells from the systemic compartment. These studies, however, did not involve ozone. Old F-344 (23 months) and SH (15 months) rats were exposed to UFP for 6 hours, with or without ip LPS priming treatment, which immediately preceded inhalation exposures. Inflammatory lung lavage

and blood parameters were determined, including measurement of intracellular reactive oxygen species (ROS) generation by inflammatory pulmonary and blood cells (oxidation of a fluorescent dye, DCFD). Neither inhaled UFP nor ip LPS caused a significant increase in lavage PMNs or PMA-stimulated ROS release in either animal model, confirming what others have shown about the lung being somewhat protected from systemically-delivered LPS. In F-344 rats, the combination of UFP and ip LPS was suppressive in terms of lavage cell intracellular ROS activity; in SH rats this parameter was not altered by either factor. LPS significantly increased the number of circulating PMNs in both F-344 and SH rats. Interestingly, in F-344 rats, the combination of LPS with UFP led to an enhancement of response, whereas in SH rats, response was suppressed. Blood PMN intracellular DCFD oxidation was affected by exposure in both animal models; however, response was enhanced when UFP and LPS were combined in SH rats and somewhat suppressed in F-344 rats. Plasma fibrinogen was significantly increased by LPS in both animal models. Despite indications that the acute phase response (alterations in plasma fibrinogen) and blood cells were activated, blood viscosity, hematocrit, and coagulability (TAT complex increases) did not change (Elder, et al., 2002, 2004c). The results show that inhaled laboratory-generated UFP did not consistently enhance the LPS response. Although these observations are somewhat limited in scope, they do suggest that 1) the carbonaceous core of ambient PM, as modeled in these experiments, is not solely responsible for cardiovascular effects and 2) the ambient PM-induced alterations in heart rate variability (HRV) found in epidemiological studies can be independent of overt increases in coagulability or acute phase activation.

Effects of Polytetrafluoroethylene (PTFE) Fumes on Gene Expression in CNS. We used UFP of known high toxicity to determine changes in CNS gene expression as a basis against which subsequent study results with more benign UFP can be scaled. Our previous work has shown that UFP from inhaled PTFE fumes translocate rapidly to interstitial sites of the lung (Oberdorster, et al., 2000). We conducted exposures with PTFE fumes in order to determine how the translocation of highly toxic UFP affects gene expression in extrapulmonary tissues. The role of biological solubility is not yet known for this particle type. Four SH rats were exposed for 7.5 minutes to PTFE fumes (1.32×10^6 particles/cm³, 18 nm median diameter, 6.31 µg/L F⁻) and four were sham-exposed. This length of exposure resulted in a mild inflammatory response (3.74 ± 1.36 % lavage PMNs) 24 hours after exposure. The lavaged lung as well as heart, liver, olfactory bulbs, cerebrum, and cerebellum were removed for subsequent microarray analysis (J. Carter, Proctor and Gamble Co.). All of the tissues have not yet been analyzed, but results thus far show that ultrafine PTFE fume particles induce significant changes in gene expression in the cerebrum and the olfactory bulb. Genes involved in inflammation (e.g., IL-1, IL-6, TNF- α ; 6- to 14-fold changes) are markedly increased relative to sham-exposed rats in both brain regions. Interestingly, a large decrease (25- to 33-fold) in glutamate transporter gene expression was found in both regions after exposure. These changes are remarkable given the fact that they resulted from inhalation of PTFE fumes and that the response in the lung (i.e., %PMNs) was mild. However, we cannot deduce from these results that neuronal translocation of ultrafine PTFE particles *via* the olfactory nerve caused the CNS changes since a systemic inflammatory state might be responsible as well.

Toxicology Studies with Ambient UFP

Studies with Concentrated Ambient Ultrafine/Fine Particles. We conducted several exposures using the prototypical UFP concentrator from the Harvard University PM Center. We performed studies in young and old F-344 rats to measure lung inflammatory processes and lavage cell oxidant release 24 hours post-exposure. The results shown in Figure 3 (analyzed by 2-way ANOVAs per age group) show that the concentrated UFP have significant effects, but that these effects are different in the two age groups. Specifically, the response to the combination of concentrated UFP and LPS in young rats is significantly lower than to LPS alone; the responses in old rats are not significantly different from one another. In old rats, the concentrated UFP alone induced a small, but significant decrease in response as compared to sham-exposed rats; in young rats, the trend is the same, but the two groups are not different from each other. CNS tissues (cerebrum, cerebellum, olfactory bulb, trigeminus) taken from these rats were screened for gene expression changes via microarray analyses. Relative to controls (saline aerosols, sham exposures), no changes were detected in any of the brain regions in those rats (either age group) exposed to concentrated ambient UFP alone. In the olfactory bulb and cerebellum, the expression of several genes (e.g., metallothionein, *i*NOS, IL-6) increased in response to inhaled LPS; these changes were present in both shams and concentrated UFP-exposed groups. Two genes in the trigeminus increased relative to the controls (nerve growth factor, corticotropin-releasing factor). The expression of the two genes increased in olfactory tissue, but not cerebellum, as well. Gene expression appeared to be the same in the two LPS-exposed groups (i.e., no change after concentrated ambient UFP).

Results from UFP Concentrator Study in Young and Old Rats with LPS Priming

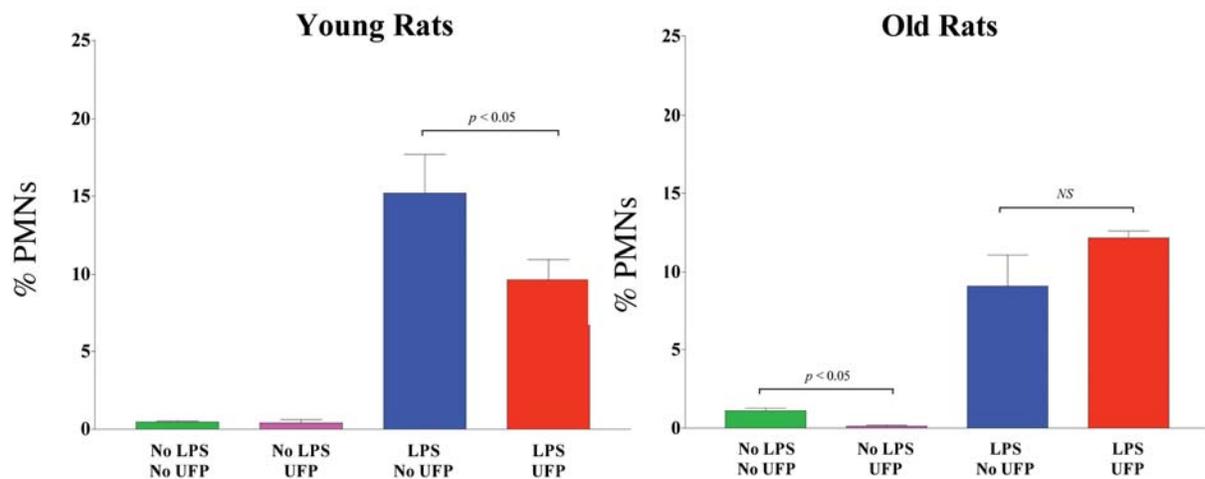


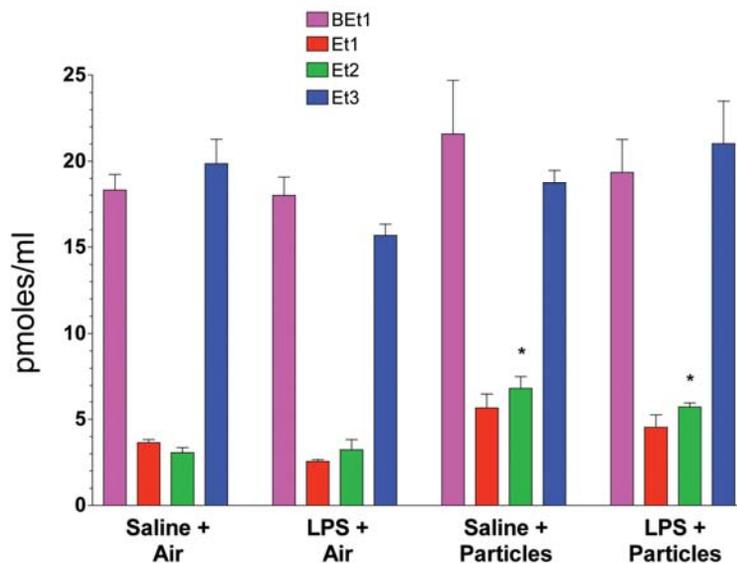
Figure 3. Lavage PMNs 24 Hours After 6-hour Exposures to Concentrated Ambient Ultrafine Particles in Combination With Inhaled LPS Priming in Young (8-10 weeks) and Old (20 months) F-344 Rats

We have now installed the new Harvard Ultrafine Concentrated Ambient Particle System for future studies using compromised animal models and for controlled clinical studies.

Studies with Freshly-Generated On-Road Ultrafine /Fine Particles: Truck Study I. For our studies with real-world UFP from diluted highway exhaust emissions, old rats were pretreated with a low dose of inhaled endotoxin or with instilled influenza virus to induce lung inflammation, as in our studies with laboratory-generated UFP. Endpoints related to lung inflammation, inflammatory cell activation, and acute phase responses were measured after exposure. In addition, two experiments were conducted in telemetered SH rats, one using inhaled and the other injected LPS priming. Heart rate, blood pressure, temperature, activity, and ECG and blood pressure waveforms were continuously recorded for 5 days after exposure (these results are in a subsequent section).

We found that the on-road highway exposures were well-tolerated by rats, as baseline values from sham-exposed animals did not differ from what has been previously published for old F-344 rats; in addition, there were no statistically significant effects of exposure on body weight. Animals were under the constant supervision of personnel in the trailer and no obvious signs of distress were noted during exposure. These results and observations suggest that on-road exposures in mobile laboratories are indeed feasible.

We observed the expected increases in response (inflammation, inflammatory cell activation) to the priming agents. Interestingly, the results suggested no differences in rats exposed to gas-phase components alone vs. the gas-phase + particle mixture. In one study, we found a significant particle-associated increase in plasma endothelin-2 (collaboration with Dr. R. Vincent, Ottawa, Canada), suggesting alterations in vascular endothelial function (Figure 4). In addition, we observed main effects of particles related to the acute-phase response and inflammatory-cell activation. Interactions between on-road particles and the priming agents were also found. The results of these studies appear in Elder, et al., 2004a.



* indicates significant effect of on-road particles, i.e., groups are significantly different from air-exposed counterparts, $p < 0.05$.

Figure 4. Endothelins in Rat Plasma as Determined by HPLC

Studies with Freshly-Generated On-Road Ultrafine /Fine Particles: Truck Study II. Another series of studies was conducted in MEL to obtain more continuous sampling of the emission aerosol. As described above, the truck was configured such that sampling of the truck's own engine exhaust plume could be achieved. As in the previous study, old rats (18-22 month F-344) were exposed directly on highways to either the gas phase/particle mixture, gas phase only, or filtered air. Some were pretreated with a low dose of inhaled endotoxin (immediately prior to on-road exposures) or with instilled human influenza virus (2 days prior to on-road exposures) to induce lung inflammation. Other groups of rats were also exposed to on-road exhaust aerosols first and then to instilled virus. Virus-exposed animals were euthanized 3 days after exposure. The on-road exposures consisted of 6-hour driving periods on I-90 between Rochester and Utica, NY, once or 3 days in a row. Endpoints related to lung inflammation, inflammatory cell activation, and acute phase responses were assessed. In addition, two experiments were conducted in telemetered SH rats. For both experiments, inhaled LPS was used to prime respiratory tract cells. One experiment consisted of a single 6-hour on-road exposure and is similar to the one done in last year's truck study; the second consisted of 3 consecutive days of exposures to the emission aerosols. Telemetry data were collected continuously for 5 days after exposure; data were also collected between each day's exposure for those rats exposed for 3 days in a row (i.e., ~12 hours recorded). The telemetry data are currently being analyzed.

For the parameters that have been analyzed thus far, no differences in response were observed in rats exposed to gas-phase components alone vs. the gas-phase/particle mixture. This was also observed in the previous year's study. We observed the expected responses to the priming agents, e.g., increased percentages of PMNs in bronchoalveolar lavage (BAL) fluid and increased ROS release from BAL inflammatory cells. The exhaust emission aerosols were found to have small but significant effects on several of the endpoints examined thus far. In one experiment, a single 6-hour exposure to on-road aerosols was found to increase the total number of cells in BAL fluid 3 days after exposure in comparison to filtered air controls. In a separate experiment, the aerosols were found to induce a decrease in the percentage of circulating PMNs relative to filtered air controls after a single 6-hour exposure regardless of pre-treatment (LPS or saline aerosol given immediately prior to on-road exposures).

The particle/gas-phase mixture caused an increase in plasma fibrinogen within 24 hours of exposure in influenza-exposed old rats. When the evaluation period was 3 days after aerosol exposure, the mixture caused decreases in fibrinogen. Regardless of priming agent, length of exposure, or length of recovery period, small increases in ICAM-1 surface expression on AMs were found due to the particle/gas-phase mixture. The data also suggest greater message expression for TNF- α and its receptor in lung, heart, and olfactory tissue from those rats exposed to the particle/gas-phase mixture. The effects of the particle/gas-phase mixture were slightly greater and more persistent than those of the gas phase alone. Compared to previous studies in MEL in which the particle number and gas concentrations were lower and less continuous (Elder, et al., 2004a; Kittelson, et al., 2004), we observed here more pronounced effects of freshly-generated vehicle exhaust aerosols, although the specific effects and the directions of the responses were consistent with the earlier study. These results show that freshly-generated

exhaust aerosols have significant effects on the pulmonary and cardiovascular systems in compromised, old rats.

Heart Rate Variability Analyses in Unrestrained, Telemetered SH Rats

Development of a Rat-Specific Algorithm. In parallel to the evaluation of the ECG recordings from the epidemiological and clinical PM Center studies, scientists in our cardiac core have developed an algorithm for analysis of recorded ECG and blood pressure signals from rats. A Windows-based algorithm was developed that was compatible with electrocardiograms and blood pressure signals acquired using the Data Sciences International system (DSI; St. Paul, MN). Based on time and frequency domain approaches, our assessment of HRV relies on a set of parameters known to provide quantitation of autonomic changes. These parameters were standard deviation of NN interval (SDNN), root mean square of successive differences in NN intervals (RMSSD), and the various frequency bands of the power spectrum computed from the tachograms and the systograms (described as low frequency [LF] and high frequency [HF] components). The validation of our algorithm for the quantitation of HRV has been implemented in a similar fashion as previous validation studies, employing pharmacological blockade to dissect the relationship between heart rate (HR), HRV, and blood pressure (BP). Signals are acquired at 1000 HZ sampling frequency with 16 bit amplitude resolution. The preliminary analyses of variability of the HRV parameters led us to conclude that at least 1,500 beats (~5 minutes) are needed to obtain reliable and reproducible estimation of HRV parameters (Couderc, et al., 2002). The first version of our software was based on ECG signals only and results were unexpectedly unstable due to profound and transient changes in QRS wave morphology occurring when the animal was moving in its cage, leading to R-peak misdetection. We thus developed another algorithm for measuring HRV from BP signals, from which we obtained higher stability and reproducibility. Greater morphological stability of the QRS waves was also achieved by placing the negative lead in the musculature surrounding the trachea and the positive lead in the musculature on top of the sternum. With this new lead placement, movement of the rats did not affect the morphology of the QRS complex and our analysis increased in stability.

Test of Analysis Software. The final analysis program has been implemented and tested using positive control data from rats exposed via ip injections to atropine or propranolol, similar to our first study. Two groups of 6 rats were randomized in a cross-over study to receive either of the two agonists or saline. Post-exposure recordings were done for 24 hours. Various time and frequency domain parameters were measured to assess autonomic control when computed from ECG and blood pressure signals. The pharmacological interventions were used to test whether our method identifies sympathetic and parasympathetic blockade. HRV parameters were computed from the tachogram and the systogram and correlations between parameters were computed in order to provide insights into the complex relationship between respiratory activity, heart rate, and blood pressure. Another aim of this study was to better understand the autonomic changes in hypertensive rats. The literature provides controversial results on the autonomic impairment in hypertensive rats. Authors have reported an impaired control of sympathetic drive in comparison to normotensive rats, whereas others revealed reduced vagal tones. Our validation study may help to investigate levels of hypertension and autonomic impairment. Results from this study are presented in a manuscript by Couderc, et al., that is in preparation. The custom

analysis algorithm (Tracking HRV in Electrocardiographic Recordings from Animals using Telemetry, THE RAT) has also been distributed to other investigators (EPA; NYU) in the toxicology field.

Analysis of HRV in Rats Exposed to On-Road Aerosols. The HRV analysis program that was developed and tested by the Cardiology Core was applied to data from telemetered SH rats exposed to on-road aerosols for 6 hours after priming with ip-injected endotoxin or saline. The results suggest that inhaled on-road aerosols activate irritant receptors in the respiratory tract, leading to increased vagal activity. These responses were of greater magnitude and were potentiated in rats pre-treated with endotoxin. Although we did not evaluate arrhythmias, it could be expected that a prolonged and unchecked imbalance favoring parasympathetic drive would lead to bradyarrhythmias. The results showed that heart rate, SDNN, and vagosympathetic balance decreased and the high-and low-frequency components of the power spectrum increased in response to on-road aerosols, suggesting a dysregulation of autonomic input (examples in Figure 5). These findings are the subject of a recently-accepted paper (Elder, et al., 2007). The results suggest that activation of CNS receptors occurs either directly or indirectly by inhaled ultrafine on-road particles and their gaseous co-pollutants. They further suggest, given evidence of parasympathetic activation and the fact that pulmonary irritant receptors are under vagal control, that the observed effects are mediated at the level of the lung as opposed to a systemically-derived response. Further studies with more continuous exposures are needed to confirm these findings.

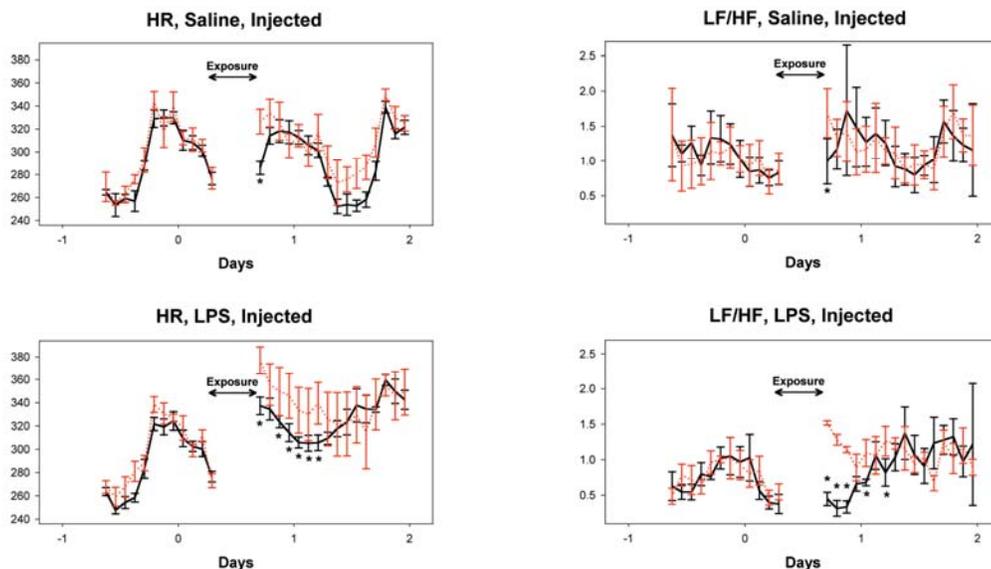


Figure 5. Autonomic Responses (HR, Left; Vagosympathetic Balance, Right) in SH Rats Exposed to Filtered Air (Red) or Freshly-Generated Exhaust Emission Aerosols (Black)

Dosimetry Studies

Translocation of ¹³C UFP to the Liver. We developed a method to generate ultrafine ¹³C particles for use in rat dosimetry studies. After several methodological improvements, we performed a study with the objective of determining whether ultrafine elemental carbon particles translocate to the liver and other extrapulmonary organs following inhalation as singlet particles by rats (Oberdorster, et al., 2002). We generated ultrafine ¹³C particles as an aerosol with CMDs of 20-29 nm (GSD 1.7) using electric spark discharge of ¹³C graphite electrodes in argon. Nine Fischer-344 rats were exposed to these particles for 6 hours in whole-body inhalation chambers at concentrations of 180 and 80 μg/m³; three animals each were killed at 0.5, 18, and 24 hours post-exposure. Six unexposed rats served as controls. Lung lobes, liver, heart, brain (including olfactory bulb), and kidney were excised, homogenized and freeze-dried for analysis of the added ¹³C by isotope ratio mass spectrometry. Organic ¹³C was not detected in the ¹³C particles. The ¹³C retained in the lung at 0.5 hours post-exposure was about 70% less than predicted by rat deposition models for UFP, and did not change significantly during the 24 hour post-exposure period. Normalized to exposure concentration, the added ¹³C per gram of lung on average in the post-exposure period was ~9 ng/g organ/μg/m³. Significant amounts of ¹³C had accumulated in the liver by 0.5 hour post-inhalation only at the high exposure concentration, whereas by 18 and 24 hours post-exposure the ¹³C concentration of the livers of all exposed rats was more than one-third the ¹³C concentration found in the lung (Figure 6). Although there was a slight increase in ¹³C in olfactory bulb, no significant increase in ¹³C was detected in the other organs which were examined. These results demonstrate effective translocation of ultrafine elemental carbon particles to the liver by one day after inhalation exposure. Potential translocation pathways include direct input into the blood compartment from ultrafine carbon particles deposited throughout the respiratory tract as well as uptake into the blood circulation of UFP particles from the GI-tract after swallowing.

Other studies performed within our PM Center's Pilot Programs used ultrafine ¹⁹²Ir particles. Iridium is the least soluble of metals in the lung and is, therefore, best suited to study its disposition after inhalation. Dr. Kreyling (GSF Munchen) found, in contrast to our results with ultrafine ¹³C particles, that after intratracheal inhalation exposure, only minimal amounts of ultrafine ¹⁹²Ir particles were translocated to extrapulmonary organs (Kreyling, et al., 2002). However, it was also found that larger (80 nm) ¹⁹²Ir particle translocation was 10-fold slower than that of smaller 15 nm ¹⁹²Ir particles. Collectively, our studies suggest that not only the particle size but also the material and surface properties, like structure and composition, may influence the efficiency of UFP translocation.

Normalized Lung and Liver Excess ^{13}C Concentration Following Ultrafine ^{13}C Particle Exposure in Rats

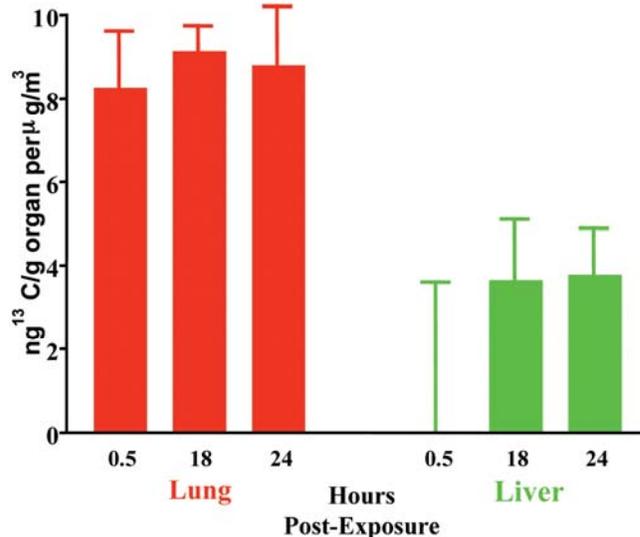
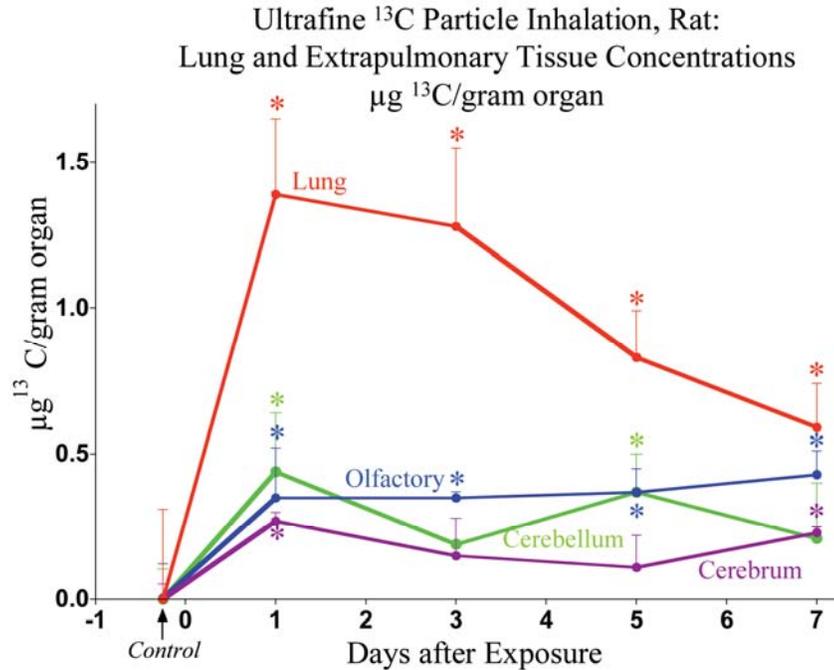


Figure 6. Lung and Liver ^{13}C Concentration in Rats at Different Times After Exposure to Ultrafine ^{13}C Particles, Normalized to the Exposure Concentration

Translocation of ^{13}C UFP to the Brain. A subsequent study with a longer post-exposure period (7 days) showed again significant amounts of added ^{13}C in the liver on day 1, but no longer on day 7. However, on day 7, significant increases in added ^{13}C in heart, brain, and olfactory bulb were found. This prompted us to conduct a follow-up study focusing on uptake of inhaled solid UFP into the CNS, expecting that UFP deposited on the olfactory mucosa of the nasal region will translocate along the olfactory nerve into the olfactory bulb, thereby resulting in high increases in that region as opposed to other areas of the CNS. We generated ultrafine elemental ^{13}C particles (CMD=36 nm; GSD=1.66) from ^{13}C graphite rods by electric spark discharge in an argon atmosphere at a concentration of $160 \mu\text{g}/\text{m}^3$. Rats were exposed for 6 hours and lungs, cerebrum, cerebellum, and olfactory bulbs were removed after 1, 3, 5, and 7 days. ^{13}C concentrations were determined by isotope ratio mass spectroscopy by Dr. Sharp, our collaborator in these studies at the University of New Mexico, and compared to background ^{13}C levels of sham-exposed controls (day 0). The background corrected pulmonary ^{13}C added as ultrafine ^{13}C particles was $1.39 \mu\text{g}$. Lung ^{13}C concentration decreased from $1.39 \mu\text{g}/\text{g}$ (day 1) to $0.59 \mu\text{g}/\text{g}$ (day 7) over the 7-day post-exposure period. There was a significant and persistent increase in added ^{13}C in the olfactory bulb of $0.35 \mu\text{g}/\text{g}$ (day 1) to $0.43 \mu\text{g}/\text{g}$ (day 7) throughout the 7-day post-exposure period with respective ^{13}C levels of 30-40 ng per organ. Day 1 ^{13}C concentrations in cerebrum and cerebellum were also significantly increased but the increase was not always significant over the following days (Figure 7). We conclude from this study that the CNS can be targeted by inhaled UFP and that a neuronal route of translocation of nasally deposited UFP via the olfactory nerve may exist. This represents a previously unrecognized pathway for clearance of solid UFP in the respiratory tract.

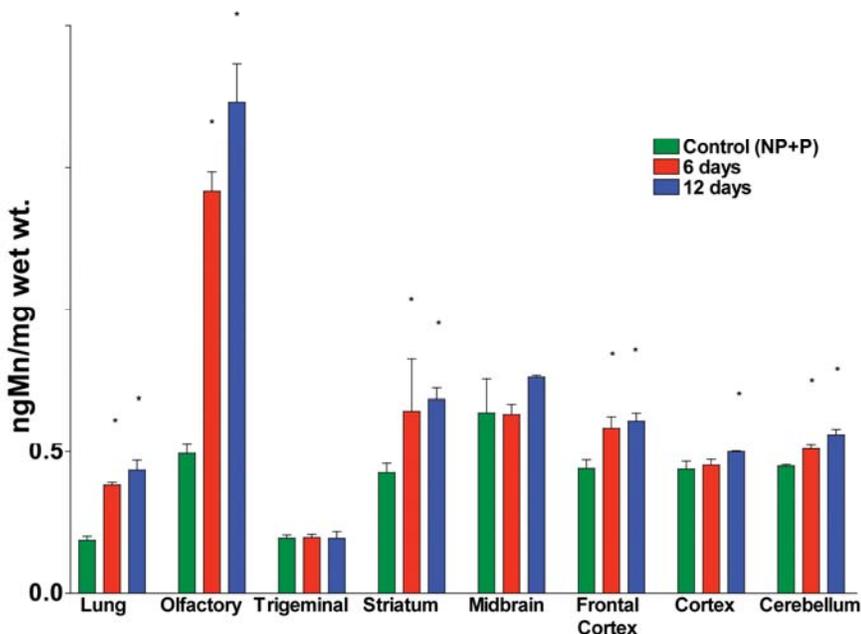


* Significantly increased ^{13}C compared to control levels ($p < 0.05$, Dunnett's test).

Figure 7. Translocation of Inhaled Ultrafine ^{13}C Particles to Tissues of the CNS Over 7 Days Post-Exposure

Translocation of Mn Oxide UFP to the Brain. We wished to confirm the olfactory translocation route for inhaled poorly soluble UFP using another material and selected ultrafine Mn oxide because Mn can be sensitively detected by AES, it is a known neurotoxicant, and is of importance for certain occupational exposures where these particles are generated as UFP (metal smelting, welding). Additionally, the EPA has recently confirmed and approved the addition of an organic manganese compound (MMT) to gasoline, thus increasing the potential for human exposures to ultrafine Mn-oxide particles from engine exhaust emissions. We had already developed a system for generating relevant metal UFP, so we made rods for use in the electric spark PALAS generator made from Mn. By introducing a small amount of O_2 into the spark chamber, we were able to generate ultrafine Mn oxide particles (CMD=31 nm; GSD=1.77). Three groups of 3 young F-344 rats were used in these studies. The data from sham-exposed rats were compared to those from rats exposed to the ultrafine Mn oxide particles for 6 or 12 days. Several tissues were harvested after exposure, including the lungs, olfactory bulbs, trigeminal ganglia, striatum, midbrain, frontal cortex, cortex, and cerebellum. Progressive large increases in the Mn content of the olfactory bulb were found (Figure 8), and smaller increases were also seen in striatum and frontal cortex, which are closer to the olfactory bulb. Interestingly, when the right naris was occluded during exposures, Mn accumulated only in the left olfactory bulb, confirming the olfactory nerve translocation pathway. Lung Mn content was slightly more than doubled, but there was no evidence of lung inflammation, as assessed by cellular and biochemical lung lavage parameters. An evaluation of the solubility of the mixed oxides

revealed that <1.5% of the Mn was solubilized per day in physiological saline at neutral pH. A comparison of the translocation to the olfactory bulb of intranasally instilled ultrafine MnO₂ and MnCl₂ was also performed and we found that olfactory bulb retention was ~10% of the amount applied regardless of the particle/solute state. In addition, more Mn was retained in the cribriform plate (representative of the olfactory mucosa) when MnCl₂ was instilled. These data support the hypothesis that the inhaled Mn oxide UFP are transported in solid particle form by the olfactory nerve. The results from these studies were recently published (Elder, et al., 2006).



* Indicates significant difference from controls (green bars).

Figure 8. Mn Concentration in Rat Lung and Brain Regions Following 6 and 12 Days of Exposure to Ultrafine MnO₂ Particles (Means ± SD).

Effects of Translocated Mn Oxide UFP. Several tissues (lung, heart, brain regions, etc.) were screened for gene and protein expression changes via microarray and proteomic analyses. Increases in glial fibrillary acidic protein and tumor necrosis factor- α (8-fold increase in message, 30-fold increase in protein), for example, were found in olfactory bulb and, to a lesser extent, in other brain regions where Mn levels were also increased as a result of exposure. These results imply that ambient UFP can potentially affect CNS function, in particular if there is continuous accumulation under even low environmental exposure conditions.

A pilot project was conducted that focused on the potential neurotoxic effects of inhaled UFP in a model of neurodegeneration (MPTP). Mice were exposed to Mn oxide UFP for 3 weeks, after which they were exposed to MPTP to cause injury to dopaminergic neurons in the nigrostriatal regions of the brain. The levels of tyrosine hydroxylase (TH, the rate limiting enzyme for the neurotransmitter dopamine) were measured in striatal tissue following the exposures. Neither

Mn nor MPTP alone significantly lowered striatal TH levels; however, when MN and MPTP were combined, significant reductions were found, suggesting impairment in the production of dopamine in this brain region. Neither exposure component had any effect on parameters of inflammation and cytotoxicity in lung lavage fluid.

Investigations of the Thrombogenic Activity of Inhaled Ultrafine Particles

We optimized a non-invasive method to study the thrombogenic potential of inhaled ultrafine particles. Initial attempts to use the surgically invasive femoral artery or vein model in rats proved to be of questionable reproducibility, mainly due to manipulation of the blood vessel, causing it to change its tension. We switched to monitoring the ear veins of rats under an inverted microscope. The principle of evaluating a thrombus inducing potential of ultrafine particles involves the local generation of singlet oxygen from i.v. Rose Bengal (RB) by illumination with a green laser beam leading to oxidative endothelial damage. Circulating platelets will adhere, forming a thrombus in the ear vein, which is visible and measurable *in vivo* under the microscope. In the presence of ultrafine particles, the illumination time needed to induce a thrombus is significantly shortened.

Intravenous administration of ultrafine (60 nm) aminated (positively charged)-polystyrene particles (0.02, 0.5, and 50 mg/kg) 30 minutes after the start of the RB infusion significantly shortened the time of thrombus formation in our RB ear model. However, carboxylated (negatively charged) PS particles of the same size failed to affect thrombus formation at any dose. These data suggest that UFP can affect coagulation directly, but that the effect will depend on particle charge (Silva, et al., 2005).

We found that the non-invasive ear vein model also works without the i.v. administration of RB; the green laser alone appears to be sufficient to activate endothelial cells. Intravenous administration of aminated particles in this system showed a significant reduction in the time of thrombus formation. Likewise, intratracheal instillation of the aminated 60 nm PS particles shortened the time to about 42% of the baseline level. These data indicate that ultrafine particles deposited in the respiratory tract can induce changes in thrombus formation without RB and that they might translocate from the lung into the blood stream to produce an effect as shown in our ear vein model.

The thrombogenic potential of more environmentally relevant particles was assessed using the ear vein model. Laboratory-generated ultrafine (30 nm) elemental carbon particles (4, 20, 100, and 500 $\mu\text{g}/\text{kg}$) were administered intravenously to rats and their effects on coagulation studied. It was shown that doses as low as 4 $\mu\text{g}/\text{kg}$ (1 μg per rat) were able to significantly (more effective) shorten the time of thrombus formation. The lower doses are significantly different from the high dose, there seems to be a trend indicating that the lower the dose of UFP in the system, the greater the effect. Similar results were obtained when these carbon particles were intratracheally instilled. In this case, even a lower dose (0.8 $\mu\text{g}/\text{kg}$ or 0.2 μg per rat) was able to produce a significant effect. These results were presented at the 2005 Society of Toxicology (SOT) meeting by Silva and colleagues. In continuation of these studies, we also observed a thrombogenic effect in rat ear veins following inhalation exposure to either 70 or 200 $\mu\text{g}/\text{m}^3$ ultrafine carbon for as little as 30 minutes duration.

Ultrafine Particle Cell Interactions: Molecular Mechanisms Leading to Altered Gene Expression (RD827354C005)

Investigator: Jacob Finkelstein

Objective(s) of the Research Project:

Hypothesis

Numerous epidemiological studies have found a correlation between exposure to respirable airborne particulate matter (PM) and increased mortality and adverse respiratory health effects, including the development of emphysema, chronic bronchitis, and asthma and acute and chronic cardiovascular effects. On the tissue and cellular level, PM-deposition insults can result in pulmonary inflammation, airway hyperreactivity, epithelial cell damage, and increased epithelial permeability. A key biological effect of inhaled PM has been the recognition of cardiac and cardiovascular effects. The mechanism of effects of inhaled PM on the cardiovascular system was more difficult to discern until recent data indicated the transport of ultrafine PM across the pulmonary epithelium into the vascular bed (Oberdorster, et al., 2002; Kreyling, 2002; Oberdorster and Utell, 2002). Thus, the possibility of direct particle cell interaction with the vascular endothelium becomes a distinct mechanistic possibility.

The formation of reactive oxygen species (ROS) and subsequent lipid peroxidation is believed to play a major role in toxicity; however, the rate of formation of ROS can depend on synergistic effects between components of PM and on the presence of relatively benign materials. The direct mechanisms by which the wide variety of airborne PM types impact target cells in the respiratory and cardiovascular system is diverse, thus severely complicating schemes to monitor the potential impact of the release of such particulates into the atmosphere.

The experiments performed within this project were designed to address specific mechanistic hypotheses regarding the interactions between inhaled ultrafine particles and specific pulmonary and cardiovascular cell populations. The proposed *in vitro* experiments were intended to provide a link between the whole animal and controlled clinical (human) exposures, described in the other programs of this PM Center, by elucidating specific mechanisms that are triggered following particle cell contact and to test the specific hypothesis that many of the subsequent physiologic effects are the consequences of cellular oxidative stress, cell activation, and apoptosis.

A key component of the studies included an expansion of the effect of PM on pulmonary cells to cells of the cardiovascular system and to assess both particle-cell and cell-cell interactions on the processes of cell activation and alteration of gene expression. Work by a number of authors has suggested that production of both inflammatory and fibrotic mediators following particle interaction is not limited to classic inflammatory cells, but that pulmonary parenchymal elements including epithelial cells (type II, Clara cells) and fibroblasts may also contribute to the milieu.

Summary of Findings: A key component of our studies was to examine particle cell interactions in individual cell populations to begin to assess the role of ultrafine particles (UFP)

in altering inflammatory gene expression by an oxidant-related mechanism. In our experiments, in collaboration with Core 4, we were able to define susceptible populations on the basis of age as well as prior or concurrent infection.

To test the hypothesis that increased susceptibility of aged animals is due to cell intrinsic differences in oxidant sensitivity, we evaluated the effect of age on the response of cells to particles. We compared macrophage production of cytokines following lipopolysaccharide (LPS) and particles from 22-27 month old rats to cells from 10-12 week old rats. Baseline (unstimulated) production of MIP-2 (and TNF) was elevated 30-50% in these cells as well as increased response to exogenous stimulus. Increased production of prostaglandin E₂ (PGE₂) by alveolar macrophages from “aged” animals, an endpoint chosen to better correlate with the animal studies (Core 4) and the human clinical studies (Core 3) was observed when cultured in the presence of LPS used as a positive particle control, and LPS plus particles confirming age effects for a number of endpoints.

An important development during this project was the ability to use laboratory generated ultrafine particles containing various metals. The choice of the specific metal was based on the data provided by our Chemical UFP characterization (Core 1) that iron is among the most abundant metal constituents. This material was produced by our particle generation Core. We compared macrophage production of cytokines following LPS and particles (with C/Fe) incubation with cells from 20-22 month old and 8-10 week old mice. Baseline MIP-2 and TNF was significantly elevated in cells from “old” mice. After stimulation, the old mice were also found to be more responsive.

When particles and LPS were combined as a stimulus an enhanced effect is observed only in the “old” cells except at the highest dose of particles. Most significant, in the context of our investigation of age effects and the ability of particles to induce effects at low dose, was the fact that in the aged animals co-administration of particles and LPS leads to synergistic effects at the lowest dose of particles. This result is somewhat similar to results obtained in the *in vivo* studies in which enhanced response to combined insult was noted in aged rats.

One marker that has proven useful in assessing cellular response to PM is the production of prostaglandins (PGs). By measuring changes in PGs, we could indirectly monitor activity of COX-2, the rate limiting enzyme and also determine the role of PGs in pulmonary and systemic inflammation. Stimulation of young and old cells with a combination of ultrafine C/Fe particles and LPS lead to an increase in PGE₂ production (Figure 1). As with MIP-2 (and TNF) this was mainly observed in the cells from the old mice. This is consistent with our other age experiments and reinforces the hypothesis that age is related to increased PM susceptibility.

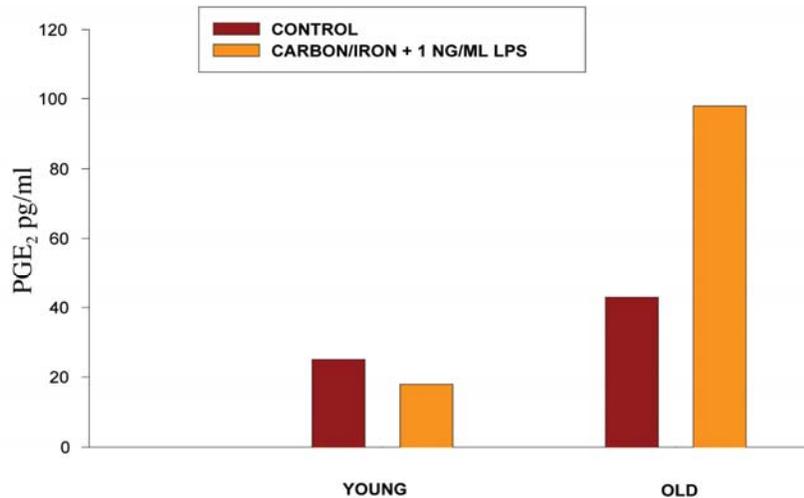


Figure 1.

We also developed reagents and approaches that would allow extension of our *in vitro* studies to human cells while also developing a test of our oxidant stress hypothesis. We developed a human lung cell line, A549, which was stably transfected with a reporter gene that in other studies has been shown to be responsive to oxidant stress. Using this transfected A549 cell line, we were able to detect changes in gene expression at particle doses below 1 $\mu\text{g}/\text{cm}^2$ (Figure 2). This clearly puts us in the realistic range of ultrafine PM mass burdens. In future studies, together with our particle generation Facility Core we will determine if this relationship will be maintained for particles of different composition or with ambient particles collected using the Harvard ultrafine particle concentrator that we have available for our use. Our initial studies comparing cytokine analysis with luciferase activity show a reasonable correlation between these two measurements.

A549 LUCIFERASE CULTURES EXPOSED TO CARBON/IRON

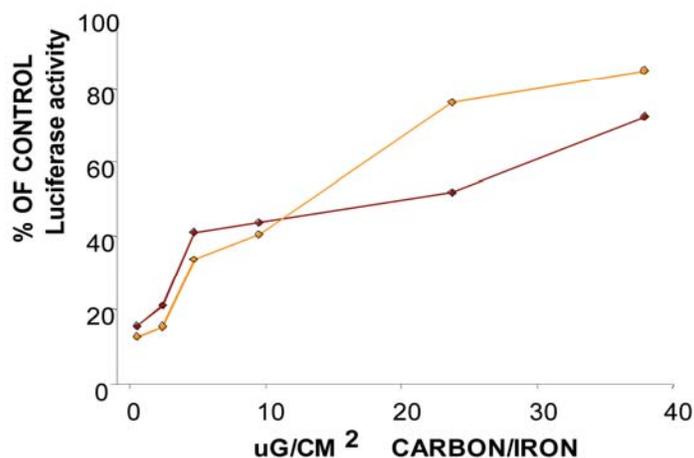


Figure 2. Luciferase Activity in Transfected A549

Particle Effects on Vascular Endothelium

Recent experiments, to better bridge the experiments that are being carried out in the Clinical Studies Core and Animal Exposure Core, have focused on vascular endothelial models that could be useful in assessing particle-induced changes in endothelial gene expression; and that may represent aspects of endothelial dysfunction. To more accurately reflect the complex nature of endothelial interactions with particles, we have used two complementary culture models.

Many of our experiments utilize a standard monolayer culture of primary vascular endothelial cells. A second model, a bilayer epithelial/endothelial co-culture system permits study of cell-cell interactions mediated by particles (Figure 3).

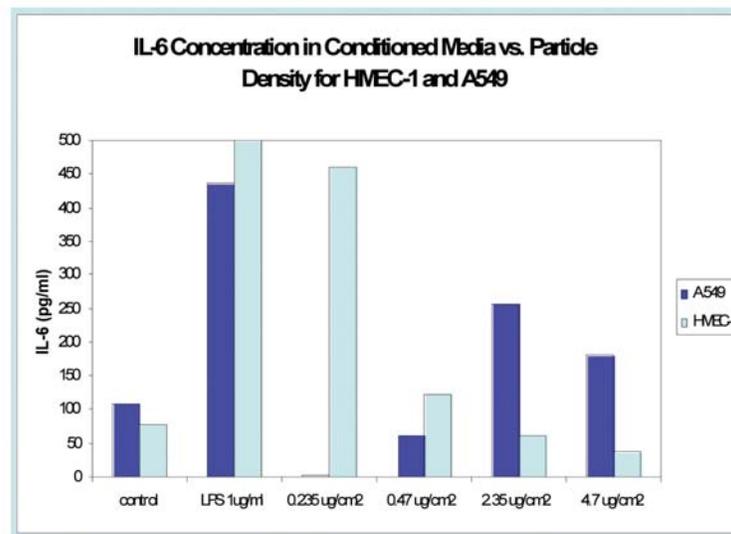


Figure 3. Comparison of IL-6 Release from Epithelial and Endothelial Cells

Monolayer cultures of human umbilical vein endothelial cells (HUVEC) were established and optimized with regard to media, serum, and other culture conditions. Production of IL-6 and PGE₂ when endothelial cells were cultured in the presence of LPS or TNF for 24 hours was used to establish the basic parameters. Our major objective with these cultures was to establish appropriate dose and time parameters of incubation with particles so that we could begin testing with UFP of various compositions. Based on our experiments with cultured epithelial cells, we began our studies using laboratory-generated particles containing 25% Fe. Particles were added to the cells at concentrations ranging from 0.47 to 19.0 $\mu\text{g}/\text{cm}^2$ and media collected at 6 and 24 hours. Particle-induced cytotoxicity was measured by LDH release. Addition of particles in the presence of a priming dose of LPS stimulated the release (production) of both IL-6 and PGE₂ at both 6 and 24 hours. The PGE₂ response appears to be more sensitive as it is observed at doses as low as 0.47 $\mu\text{g}/\text{cm}^2$ (which converts to a total mass dose of $\sim 1.5 \mu\text{g}$ of particle) (Figure 4).

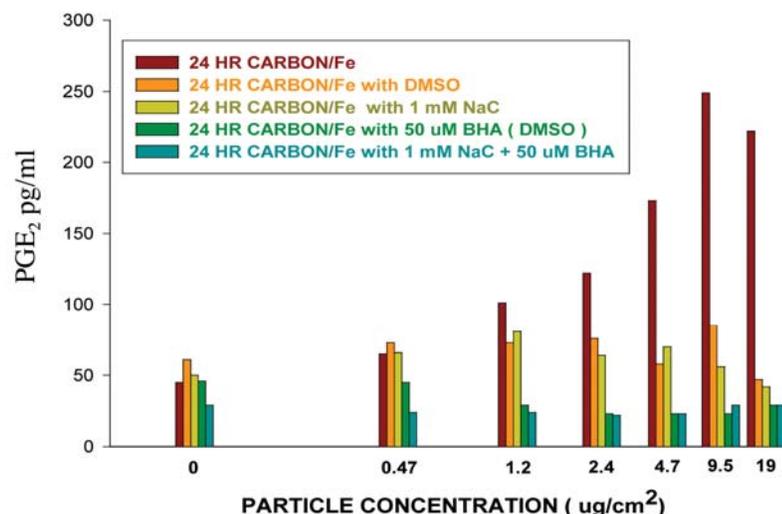


Figure 4. Response of Vascular Endothelial Cells to Particles: Effect of Antioxidants (NAC=N-acetyl cysteine; BHA=butylated hydroxyanisole)

In this system, we also assessed the response of these cells to laboratory-generated carbon, similar to the material used in the human clinical studies, TiO₂, and a laboratory-generated Mn-oxide. In contrast to our experiments with epithelial cells, the endothelial cell cultures were moderately responsive to the carbon alone. After 24 hours of incubation, PGE₂ production was increased 2-3 fold. In contrast C/Fe particles increased PGE₂ by 5-6 fold (Figure 4). Additionally, in support of the role of oxidant stress in particle effects, pretreatment with either a soluble (N-acetyl cysteine) or lipophilic (BHA) antioxidant suppressed the production of PGE₂ induced by PM.

Overall comparison of particle, dose, and time parameters suggest that PGE₂ is the most reliable marker of endothelial activation. It was also noted that particle composition was a major response factor, with TiO₂ being most active and Mn-oxide being most directly cytotoxic. We also determined if coculturing these cells with A549 pulmonary epithelial cells would alter their ability to be stimulated by LPS or by particles. Both cell types appear to be responsive to particles and LPS with apparently different concentration dependence. Using this second model, we have begun to examine cytokine production in response to various stimuli, including particle and LPS. Among the cytokines we evaluated in this model were IL-6 and PGE₂ (Figure 5).

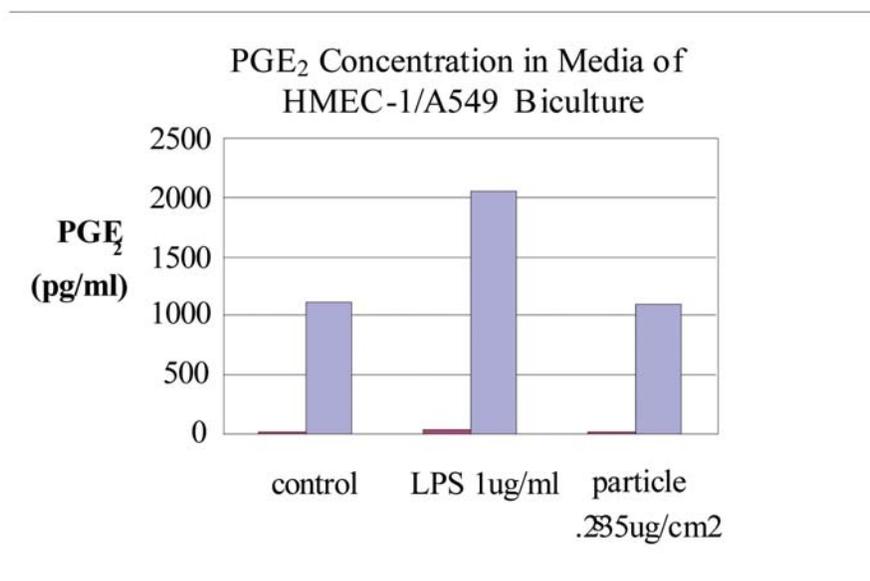


Figure 5. Endothelial Response to Particles: BiCulture Model

The endothelium appears to respond to lower particle mass burdens than does the epithelium. This may account for the enhanced sensitivity of the vascular endothelium *in vivo*. Additionally, aided by our Immunology and Vascular Core, we also measured production of prostaglandins in these culture supernatants. Since the majority of the prostaglandin was found below the membrane, this would suggest it is derived from the endothelium. This is consistent with the *in vivo* results from Core 4 showing enhanced prostaglandin production following particle exposure in a sensitive animal model.

Our experiments have focused on a number of critical issues that relate to the overall goals of the Rochester PM Center. A key point of the studies was the emphasis on real-world particles in lieu of laboratory surrogates. The delay in the characterization of the HUCAPS concentrator in Rochester has delayed this effort somewhat. However, it has enabled us to continue mechanistic studies with defined composition particles that may ultimately be important in attributing effects seen in specific cell populations to unique sources. The one source of real-world particles available for *in vitro* studies was the material collected as part of the MAPS, multi-Center multi-site particle collection effort begun at the end of the grant period.

As shown in Figure 6a and b vascular endothelial cells (HUVEC) exposed to concentrated fine and ultrafine particles respond through increased production of IL-6. This cytokine was chosen as a potential sentinel as a result of experiments from the Animal and Clinical Studies Cores that suggested a possible acute phase response following particle inhalation. Interestingly, using this marker and cell type, we revealed a differential response from particles collected from certain sites. It is hypothesized that this relates to the abundance of vehicle emissions at these sites. More detailed analyses and source calculations are planned with the help of Core 1 as the compositional data are provided.

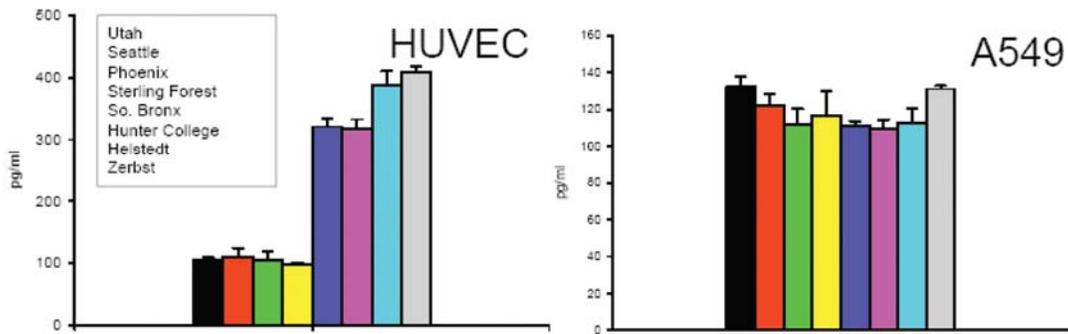


Figure 6. Comparison of IL-6 Production by Cultures Pulmonary Epithelial Cells and Vascular Endothelial Cells Exposed to UFPM Collected From Multiple Sites.

An additional important piece of data was revealed as a consequence of this study. When epithelial cells (A549 cells) were similarly exposed to these materials, no differential response based on site selection was noted. To verify this cell-specific difference and to relate this potential mechanistic difference to studies from Cores 3 and 4, we carried out a direct comparison of the response of the epithelium and vascular endothelium in a series of well characterized particles. We chose these carbon particles as they had previously been used for *in vivo* studies within the Center (Figure 7). While these studies are interesting and important, they do not necessarily address the most relevant question, that of the response of the microvascular endothelium, which is being investigated during the next project cycle.

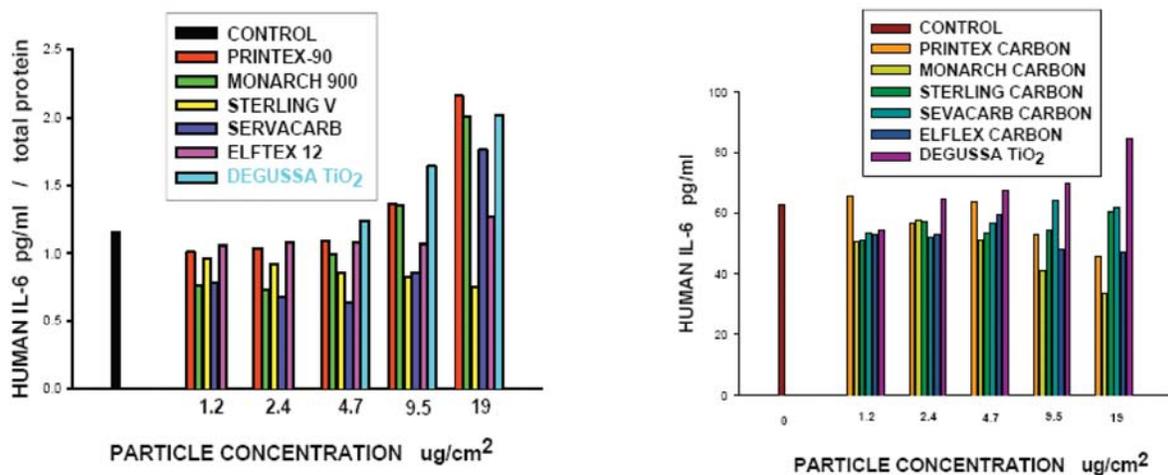


Figure 7. Comparison of IL-6 Production by Vascular Endothelial Cells(a) and Epithelial Cells (b) Exposed to Various Forms of Pure Carbon.

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Supplemental Keywords: NA

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