Rochester PM Center

Source-Specific Health Effects of Ultrafine/Fine Particles

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Exposure Assessment and PM Source Identification

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Epidemiological studies on extra pulmonary effects of fresh and aged urban aerosols from different sources

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Animal Models: Cardiovascular Disease, CNS Injury and Ultrafine Particle Biokinetics

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Ultrafine Particle Cell Interactions *In Vitro*: Molecular Mechanisms Leading to Altered Gene Expression in Relation to Particle Composition

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Cardiac Rehab Center: *Ultrafine Particles and Cardiac Responses: Evaluation in a Cardiac Rehabilitation Center*

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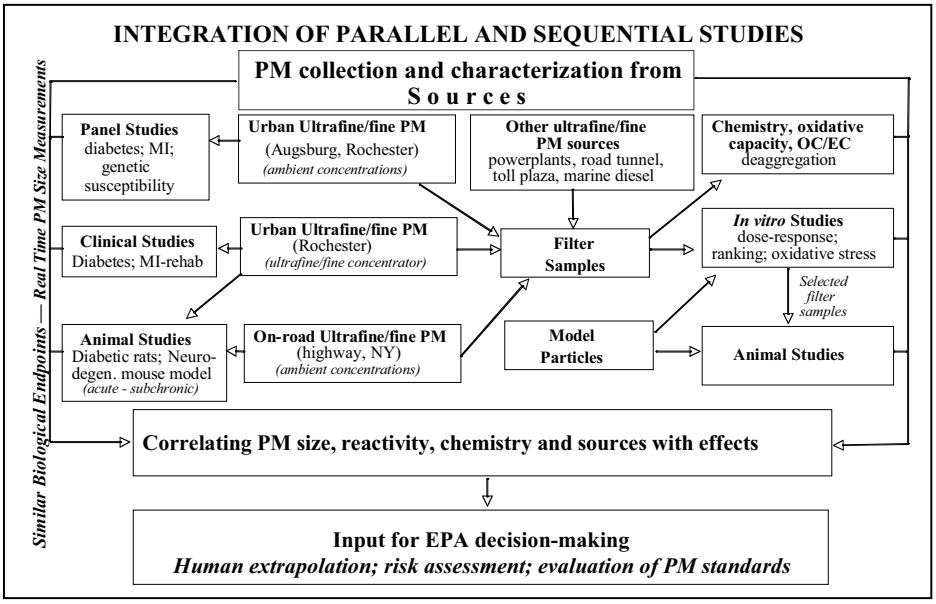
The Rochester Center on "*Source-Specific Health Effects of Ultrafine/Fine Particles"* focuses on the fine ($PM_{2.5}$) fraction, which includes the ultrafine particle (UFP) fraction. Our Center builds on results achieved in the first round of the PM Center program, where we demonstrated that ambient PM has significant oxidative capacity, that UFP can induce significant effects in both the respiratory tract and the cardiovascular system, that age and underlying disease are critical response-modifying factors, and that UFP efficiently translocate from deposition sites in the respiratory tract to other organs such as heart and brain, thus providing plausible hypotheses for UFP-induced effects in those organs.

Our focus has shifted to identifying the health hazards of source-specific physicochemical components of ambient ultrafine/fine PM (*e.g.*, UFP; organics) in epidemiological, controlled clinical, animal, and in vitro studies and the pathophysiological mechanisms by which PM triggers adverse health effects in the cardiovascular and central nervous systems. The Figure emphasizes the interactions of the five Rochester Center Research Cores with respect to (*i*) *parallel* epidemiological, clinical, animal and in vitro studies with a common focus on cardiovascular health effects and underlying mechanisms, and (*ii*) *sequential* studies starting with in vitro investigations of the biological/toxicological activity of PM from different sources followed by in vivo studies using the most potent samples. Our integrated multidisciplinary approach is designed to explore the Source-Exposure-Dose-Response paradigm with the ultimate goal of correlating the adverse pulmonary, vascular, cardiac and CNS effects of airborne UF/fine PM and the mechanisms that underlie these effects with detailed physicochemical analyses of the aerosols from different sources. This interactive and highly coordinated multidisciplinary team approach – made possible through a Center grant – has proven to be very successful and resulted in novel study designs and findings as evidenced by our publications. These achievements would have been less likely with several smaller individual grants.



Our main focus regarding health effects is on extrapulmonary organ systems, such as the vascular system, the heart and the CNS. This focus is based on epidemiological and experimental findings, including our own, of extrapulmonary PM effects and awareness of newer results related to the pathophysiology of endothelial dysfunction and thrombus formation associated with cardiac events in susceptible parts of the population. Furthermore, we are

assessing the role of gene-environment interactions by adding whole genome-wide association analyses to the epidemiological approach.

Integrated Approach: Our team of atmospheric scientists, chemists, epidemiologists, pulmonary, vascular and cardiac physicians and scientists, inhalation, neuro-, cellular, and molecular toxicologists, diabetologists, and immunologists has tackled two of the most highly responsive disease processes – atherosclerosis and diabetes – to fine/UF particles. One example is our work with diabetes, which has extended from molecules to man. Using our clinical exposure facility, we examined the effects of inhaled laboratory-generated carbon UFP in type 2 diabetics without clinical cardiovascular disease and not on "statin" medications. UFP exposure increased platelet expression of CD40 ligand (CD40L), a marker of platelet activation and a key molecule in the development of atherosclerosis. Exposure also increased platelet-associated tissue factor (TF) and increased the number of microparticles expressing TF. We concluded that inhalation of carbon UFP may transiently activate vascular endothelium and/or platelets in subjects with type 2 diabetes. This finding supports the hypothesis that exposure to ambient UFP may increase the potential for vascular thrombosis in patients with severe vascular disease or ulcerated atherosclerotic plaques.

Further support for this hypothesis came from the epidemiology core studies of patients with coronary artery disease. Evidence was found of increases in C-reactive protein in association with PM_{10}, UFP, NO_2, and CO with a lag of 2 days. A consistent decrease in Factor VII was also found for almost all pollutants for the 5-day-average exposure, indicating a cumulative effect. Fibrinogen also showed decreasing mean concentrations with an increase in ambient air pollutants. Soluble CD40L was also increased in association with elevations in UFP and accumulation mode particles.

The epidemiology core investigators are also performing Center-supported genotyping within the AIRGENE study, a multi-center European study, to analyze gene-environment interactions for inflammatory markers in response to PM. The approach involves the selection of candidate Single Nucleotide Polymorphisms and examining their impact on mean biomarker levels and their variability. Results so far indicate that PM_{10} modifies fibrinogen responses via *TLR4* and *TNF-*α genes while UFP modify IL-6 responses via the *IL-18* gene. Furthermore, 18 risk loci for type 2 diabetes and PM exposure were identified, indicating an impact on adipokine and beta cell function. Based on our epidemiological studies, we plan to examine these genes in our next set of studies involving controlled exposure of diabetic subjects to concentrated ambient UFP.

In parallel to these studies, our toxicologists have utilized JCR cp/cp rats, which are obese, hyperlipidemic, hyperinsulinemic, and have atherosclerotic and ischemic lesions, hallmark features of human type II diabetes despite not being hyperglycemic, to study the mechanisms responsible for these findings. JCR rats exposed to freshly-generated Diesel exhaust emissions in a mobile laboratory or concentrated ambient UFP-containing aerosols revealed small increases in inflammatory markers in lavage fluid and blood with a slight decrease in the number of circulating platelet microparticles. Other studies in aged rats confirmed our clinical findings of acute phase response activation (i.e. alterations in adhesion molecule expression and fibrinogen levels). Furthermore, our in vitro toxicology group has demonstrated that vascular endothelial and epithelial cells cultured in high glucose, as a model of diabetes, alters both the basal and particle-induced cytokine responses following exposures to particles collected by our atmospheric science group with high volume samplers in Rochester. This sampling scheme will allow comparison of both in vitro and in vivo responses to all three PM size fractions: $PM_{10\text{-}2.5}$, $PM_{2.5\text{-}0.1}$, and $PM_{0.1}$. The production of IL-6 is increased in response to collected UFPs and is

also potentiated in lung epithelial cells that are cultured in high glucose as compared to those grown in normal levels of glucose. We are trying to determine to what degree differences in PM composition will help explain the differences in responses at the cellular level. In summary, these are examples of how the multidisciplinary approach has allowed us to tackle the impact of UF/fine particles on vascular biology, ranging from clinical effects to cellular mechanisms.

Our future studies will also focus on the effects of fine/UFP in the central nervous system. This focus is based on our earlier data showing that the brain is a site of UFP accumulation following deposition in the upper respiratory tract and that, furthermore, inflammatory changes were found in those brain regions where the particles accumulated. Recent reports of electroencephalogram (EEG) wave alterations following controlled human clinical exposures to Diesel exhaust and decreases in cognitive function in children with increases in black carbon concentrations also lend support to the hypothesis that the CNS may be a target for adverse health outcomes following exposure to PM. To pursue this further, we are using a neurodegenerative mouse model (Huntington's disease) to measure the progression of neuropathology and associated behavioral changes following exposure to concentrated ambient UFP. The clinical and epidemiology core investigators are also pursuing strategies for the assessment of CNS effects in their studies, such as EEG measurements and review of autopsy reports for neurodegenerative changes, respectively.

Our immediate plans also take advantage of the further development of an aerosol time-of-flight mass spectrometer (ATOFMS), which is now capable of analyzing the chemical composition of UFP down to 10 nm in diameter. This highly improved instrument will be incorporated into our controlled clinical and animal studies to allow real-time aerosol characterization and avoidance of the physicochemical alterations associated with PM filter collection and storage. The development of a field model to measure PM-associated ROS in real time will enhance our ongoing study in cardiac rehab patients with severe atherosclerotic heart disease by linking changes in cardiac performance with exposure to ambient UFP measured at home, when commuting in cars, in the surrounding community, and at the rehab center.

Implications for Air Quality Science: Using real-world ultrafine and fine PM in our studies, our goal is to answer questions related to the health effects of UF/fine PM such as: What are the adverse health effects that are attributable to the PM size fractions we can study (PM_{10}, $PM_{2.5}$, $PM_{0.1}$)? What are the most appropriate dose metrics for describing these effects? How important are the chemical composition and atmospheric interactions? Which sources can be identified as emitters of toxicologically important UF/fine particles? Can specific engineering devices or process modifications be designed to lower toxicologically important components of UF/fine PM from respective sources (e.g. the new particle filter trap technology for diesel engines)? In order to answer these questions, our studies integrate detailed physicochemical analyses of ambient PM (including hourly size distributions and number concentrations for volatile and non-volatile fractions of ambient aerosols, particle mass for volatile and non-volatile fractions of ambient aerosols, particle bound polycyclic aromatic hydrocarbons, black smoke, particulate nitrate, sulphate, organic and elemental carbon, total oxidative capacity) with our epidemiological, clinical and toxicological studies when evaluating effects and mechanisms. While most of our studies are performed using ambient UF/fine PM, we also use model particles for in vitro and animal studies to address specific mechanistic questions. The great strength of our Center approach (see Figure) is the ability to coordinate epidemiological, clinical, and toxicological findings with real-world ambient UF/fine PM characterization and results from source-apportionment analyses performed by the atmospheric scientists in our team, an approach that would be much more difficult with individual R01-type projects.

ANNUAL PROGRESS REPORT, ROCHESTER PM CENTER

Title: Source Specific Health Effects of Fine/Ultrafine Particles

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Center Directors: Günter Oberdörster; Mark J. Utell

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Characterization and Source Apportionment

Grant Number: R832415C001
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Center Director: Günter Oberdörster
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Investigators: Philip K. Hopke, Kimberly A. Prather
Institution:
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Content

Objectives:

Core 1: Exposure Assessment and PM Source Identification

Introduction

A central hypothetical mechanism of how particles affect human health involves the generation of reactive oxygen species (ROS) at target sites in the lung. ROS has been defined to include families of oxygen-centered or related free radicals, ions, and molecules. The free radical family includes hydroxyl, hydroperoxyl, and organic peroxy radicals. Ions such as the superoxide, hypochlorite, and peroxynitrite ions, and molecules such as hydrogen peroxide, organic and inorganic peroxides also come under the umbrella of 'Reactive Oxygen Species'. Much of the attention has focused on the formation of ROS *in situ* after particle deposition in the respiratory tract generally through the interaction with transition metal ions (Stohs et al. 1997), organic hydrocarbons, such as polycyclic aromatic hydrocarbons and quinones (Squadrito et al. 2001), and ultrafine particle surfaces (Li et al. 2003). However, recent work has shown that ROS is present in the atmosphere on respirable particles to which we are exposed (Hung and Wang 2001, Hasson and Paulson 2003, Venkatachari et al. 2005, 2007). The hypothesis that the ROS present on particles could cause the same kind of systemic dysfunction as endogenously generated ROS has clear merit and represents a fundamental issue for further investigation. Thus, a major function of this core is the study of the concentrations of particle-bound ROS in ambient PM2.5 and studies to better understand their formation so that we can eventually estimate the amounts of particle bound ROS arising from anthropogenic and biogenic sources of the reactive hydrocarbon

precursor species.

Progress to Date:

Development of a Field-Deployable ROS Monitor

We have previously developed a laboratory version of a continuous monitor for particle-bound reactive oxygen species (ROS) (Venkatachari and Hopke, 2008a). This work demonstrated that it is possible to automate the use of dichlorofluorescin (DCFH) as a nonspecific indicator of the oxidative capacity of particle surfaces. To move this system into the field to permit routine monitoring of particle-bound ROS, there are a number of questions to be answered regarding reagent stability and system optimization.

We have conducted a series of experiments to answer these questions. From them, we have concluded that 2 μM DCFH shows a linear response only in 10-7 M H2O2 concentration range, but not in 10-6 M H2O2 concentration range. When we increase DCFH concentration to 5 μM, we extend linear range to 1 x 10-6 M H2O2. With 10 μM DCFH, this range can be extended to 1.5 x 10-6 M H2O2, with 20 μM DCFH to 2 x 10-6 M H2O2 and with 40 μM DCFH we extended that range to 2.5 x 10-6 M H2O2 concentration. We decided to use 5 μM DCFH working solution for future measurement because we do not expect higher ROS ambient concentration than 1 x 10-6 M H2O2.

HRP is catalyst in our reaction, but it can also oxidize DCFH in the absence of any other ROS species and increase fluorescence intensity measured at 535 nm. Thus, we have explored lowering the HRP concentration and make reaction more dependent on the ROS species present in solution. There were no significant differences in results with a range of concentrations so we have decided to use 0.5 units HRP/ml.

In order to test time stability of new working solution (mixture of 5 μM DCFH and 0.5 units/ml HRP), a series of 5 day measurements were conducted. It could be concluded that the solution is stable for at least 5 days (stored in refrigerator). The same working solution (5 μM DCFH) was tested for temperature stability and stored at room temperature for three days. The conclusion from there results is that the 5 μM DCFH working solution is stable for at least three days at room temperature.

The time and temperature stability of H2O2 calibration solutions (10-7M concentration range) were also tested to ensure that those standards could be used during the field measurements when it is not always possible to store them at low temperatures (refrigerator) or prepare them daily. Results for eight days measurements showed that H2O2 calibration solutions are stable for at least one week at room temperature.

Incubation at 37℃ using a water bath was one step in ROS continuous monitor system (Venkatachari and Hopke, 2008a). The residence time at this temperature was ~30 sec. Using water bath in field measurements is not always practical because of slow but continuous water evaporation from bath. Thus, we investigated the importance of the incubation step. Two sets of experiments were performed with the same DCFH working solutions: one with incubation and second without the increase in temperature to 37℃. It was found that there was little difference between the two responses. It can be concluded that incubation at increased temperature is not an important step in the ROS continuous monitor system. There are some remaining questions regarding the lower temperature. Considering the standard solution of H2O2 uses a strong oxidant such that the reaction is fast even without incubation. The question is whether this behavior holds for other less potent, but still important ROS species present on the ambient particles. Further studies are in progress.

The result of all of these experiments has led to changes in the ROS continuous system. In the new setup, we have removed the membrane reactor for HRP introduction to system because now we use a premixed solution of DCFH with HRP. The new setup is shown on Figure 1.

Further testing of the system is in progress with the expectation that it will be deployed at the NYS DEC site in Rochester some time in the early fall where it will operate for at least one year. The initial plans were to operate it for a month in each quarter, but if the improvements we have made work as they appear to, it may be possible to operate the system continuously over the entire year.

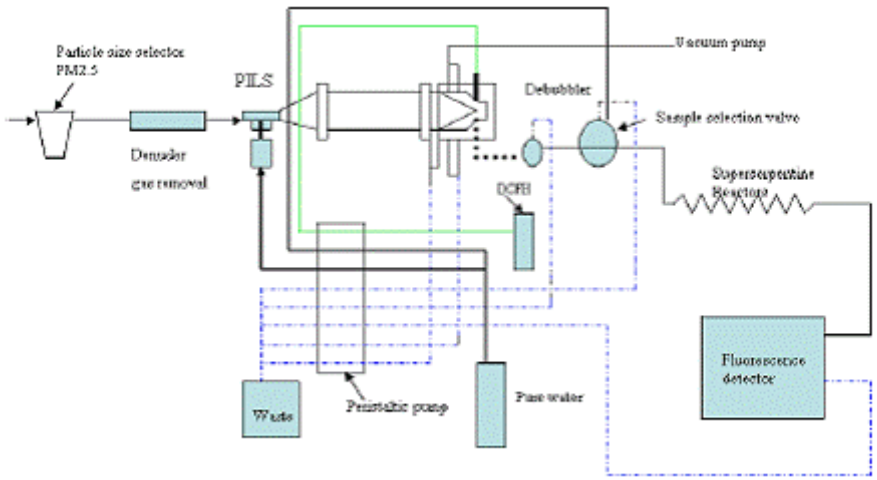

Figure 1. Scheme of the new ROS continuous monitor system

Chemical Characterization of Particle-Bound ROS

To understand the mechanisms of particle-bound ROS formation, identify its likely sources, and determine the chemical pathways that might be influenced by air quality management strategies, we have pursued the chemical characterization of the constituents in particle-bound ROS. Initially, we used the ROS particle generator described by Venkatachari and Hopke (2008b) to provide samples. These samples were collected on Teflon filters, water leached, and the resulting leachate analyzed by liquid chromatography/mass spectrometry using sequential MS analysis. Using the α-pinene/ozone reaction to produce ROS, several new peroxide species have been separated and identified (Venkatachari and Hopke, 2008c).

These species were those that were sufficiently long lived to permit their collection over extended time periods and then be stable enough for the subsequent LC/MSn analyses. However, more reactive species are not likely to be found in this manner and we have returned to our studies of spin-trap compounds that can react with radicals to stabilize them sufficiently to permit the separation and identification of the resulting adducts. Spin traps have been primarily employed to trap endogenous radical species in biological systems. We are now looking to employ them to stabilize and permit isolation and quantification of reactive radical species associated with airborne particulate matter.

One of the more common spin-trap compounds is 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) that has been widely used for hydroxyl and organic peroxyl radicals. In our studies, particulate matter samples were collected from our 2.4 m3 aerosol chamber in which the α-pinene – ozone reaction was used to produce ROS-containing particles. Samples were collected at 23 LPM during 2 hours on a precleaned, 25 mm quartz fiber filters (PALL, USA) and evenly impregnated with 0.18 mmol of DMPO solution in methanol (Sigma Aldrich, USA).

Samples collected from the aerosol reactor were analyzed first in absence of DMPO. In the MS spectra, monomer compounds with MW up to 250 and dimeric compounds with MW from 300 to 450 could be observed. The MS/MS results for parent ions of m/z 357 and 399 confirmed that the dimers originated from low molecular weight species with m/z 171 and 185. In order to better understand the formation of dimers and higher MW products, we used DMPO to capture reactive species before they can terminate reactions.

Two groups of polymeric species with base monomers m/z 256 and 314 and constant difference among the species of m/z 44 were observed in the DMPO samples, but were not observed in the absence of DMPO. The observed presence of ions with m/z 114, 130 and 146 in the spectra can be attributed to radical adducts with DMPO. An ion with m/z 114 [DMPO+H]+ corresponds to a fragment ion formed from DMPO carbon-centered radical adducts. The ions with m/z 130 [DMPO-O+H]+ and m/z 146 [DMPO-OO+H]+ correspond to fragment ions formed from DMPO

oxygen-centered radical adducts (alkoxyl and peroxyl). Other common fragmentations observed in all spectra were the loss of water molecule (-18) and CO2 (-44) group.

These results require more work to explicate the mechanisms of oligomeric compound formation and the role of the spin trap agent in the process. Further work will be done using 5-diethoxy phosphoryl-5-methyl-pyrroline-N-oxide (DEPMPO). DEPMPO is an improvement on DMPO as a spin trap. The spin adduct that it forms is more stable than DMPO, and it is detectable at lower concentrations. Thus, further studies will be made to elucidate the nature of the most reactive particle-bound ROS species.

In summary, prior work has identified the presence of particle-bound reactive oxygen species (ROS) in ambient PM. These initial measurements were made with a manual sampling and analysis system. We have now developed an automated system that would provide continuous measurements of particle-bound ROS. The laboratory system is now being engineered to be a field monitor that will be deployed in Rochester in the fall of 2008. In addition work has continued on the characterization of the important ROS species. Several new peroxide species have been identified in the PM produced by the reaction of α-pinene with ozone. Further studies of more reactive by-products will be studied using spin trapping compounds so that a better understanding of the nature of the particle-bound ROS will be obtained.

Filter samples of ambient ship emissions and wildfires PM were collected by the Prather group and sent to Rochester for in vitro studies. However it was determined that PM could not be effectively extracted from the filters. A sample recovery rate of less than 25% was encountered thus in vitro studies were not performed. Thus it was decided a better method is needed to collect samples for in vitro studies. The Prather group began exploring options and stopped collecting filter samples until this issue was resolved.

Collection of source specific samples of urban aerosol

Research on the characterization of urban ultrafine and accumulation mode particles allows composition and size analysis in real time of single particles using aerosol time-offlight mass spectrometry (ATOFMS). The objective of the research is to greatly expand the understanding of the chemical composition and impact of specific sources of ultrafine particles on human health. New sampling methods coupling ATOFMS with high volume cascade impactors are being developed such that samples will be collected that represent material primarily from specific sources. These samples will permit improved characterization of the PM from specific sources as well as providing material for in vitro and in vivo testing of their toxicity. Also, a new method for collecting size-resolved samples of ultrafine PM from

ambient air directly into an aqueous solution has been developed. This method gets around issues required with sample collection on filters, where PM species have been determined to be difficult to extract. These aqueous solutions can be used for in vitro studies and allow 100% of the ultrafine particles to be deposited on cells. Furthermore these are concentrated solutions with small volumes so low concentrations can be used in studies.

Characterization of CAPs

The ATOFMS was moved from the Hopke lab to Rochester to be used to measure the chemistry of ambient PM during exposure studies. A graduate student from the Prather group, Melanie Zauscher, traveled to Rochester to assist in getting the instrument operational.

However, there were problems encountered in measuring particles at the smallest sizes (i.e. <200 nm). A size mismatch existed between the lowest size the ATOFMS could analyze chemically and the sizes of the concentrated particles being used for exposure studies. The next step involves improving the ability of the ATOFMS at Rochester to detect particles at the smaller sizes.

To overcome both issues above, the Prather group worked with Susanne Hering to develop a method for growing small ultrafine particles to larger sizes using a condensational growth tube developed in the Hering lab. This tube is quite compact and can be placed in line with the ATOFMS to grow up size selected particles into larger size so they can be optically detected with the ATOFMS. This approach was recently implemented and tested at UCSD and works very well. It allows the detection of particles as small as 10 nm in the mass spectrometer. This will be implemented in the Clarkson ATOFMS at Rochester in the coming year.

The condensational growth tube also allows one to collect ultrafine particles with close to 100% efficiency in an impinger system. Small spots (300 microns) can also be collected and deposited directly on cells for in vitro studies. This method avoids the filter collection and extraction and allows direct deposition of 100% of the sample to cells. We tested whether the cells remained viable after being exposed to ambient air with these larger particles for different periods of time. The goal of these studies was to be sure the sampling procedure was not lysing the cells. It was shown that no detectable number of cells were destroyed by this sampling procedure. The next steps will involve collecting size resolved source samples for Rochester to use in in vitro studies and helping them set up this sampling method in their laboratories.

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Venkatachari P, Hopke PK. Characterization of Products Formed in the Reaction of Ozone with α-Pinene: Case for Organic Peroxides. *Journal of Environmental Monitoring* (in press, 2008c).

Publications:

Type	Citation	Document Sources
Presentation	Ogulei D, Hopke PK, Chalupa D, Utell M. Modeling source contributions to ultrafine particle number concentrations measured in Rochester, NY. Presented at the 2006 International Aerosol Conference, St. Paul, MN, September 10-15, 2006.	*not available*

Supplemental Keywords:

Epidemiological Studies on Extra Pulmonary Effects of Fresh and Aged Urban Aerosols from Different Sources

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Center Director: Günter Oberdörster
Title: Epidemiological Studies on Extra Pulmonary Effects of Fresh and Aged Urban Aerosols from Different Sources
Investigators: Annette Peters, Heinz-Erich Wichmann, Mark J. Utell, Petra Belcredi, Josef Cyrys, Susanne Breitner, Irene Brüske-Hohlfeld, Regina Hampel, Rolf Holle, Thomas Illig, Wolfgang Koenig, Ute Küpper, Christa Meisinger, Mike Pitz, Regina Rückerl, Alexandra Schneider
Institution:
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Report Start Date: October 1, 2006
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RFA: Particulate Matter Research Centers
NCER Research Categories: Particulate Matter

Content

Objectives:

Core 2: Epidemiological studies on extra pulmonary effects of fresh and aged urban aerosols from different sources

The objective of the epidemiological study in Augsburg, Germany, is to determine the effect of fine and ultrafine particles on an acute phase reaction in the blood and on its prothrombotic states. It is aimed to enroll 240 participants in three panels consisting of subjects with 1) type 2 diabetes mellitus (T2DM), 2) impaired glucose tolerance (IGT) and 3) potential genetic susceptibility. Particles are measured at a central measurement site in Augsburg. In addition, for a subset of 90 individuals, personal measurements of ultrafine particles using a portable condensation particle counter (CPC) as well as of temperature, humidity and noise are undertaken. Furthermore, health effects on endothelial dysfunction as a key element of coronary vulnerability as well as the health effects on cardiac function characterized by ECG measures will be assessed in this subset.

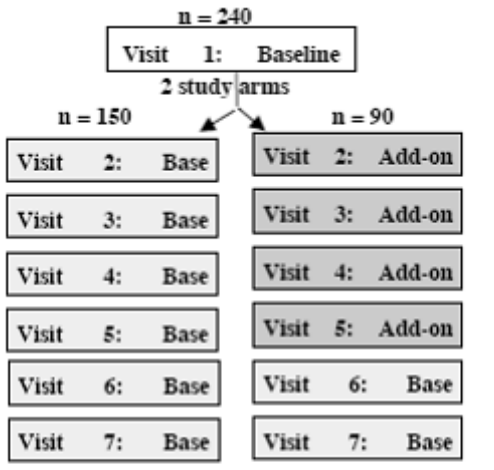
The specific aims of the study are to:

Aim #1: Determine the effect of ambient fine and ultrafine particles on an acute phase reaction in the blood of subjects with type 2 diabetes mellitus (T2DM),

impaired glucose tolerance (IGT) and potential genetic susceptibility.
Aim #2: Determine the effect of ambient fine and ultrafine particles on pro-thrombotic states of the blood in the above subject panels.
Aim #3: Determine the effect of ambient fine and ultrafine particles on endothelial dysfunction as a key element of coronary vulnerability in a subset of 90 individuals of the above subject panels.
Aim #4: Determine the effect of ultrafine particles on cardiac function as characterized by ECG measures of autonomic function and repolarization in a subset of 90 individuals of the above subject panels.

Progress to Date:

FIGURE 1 shows an overview of the study design. Subjects are invited to participate in seven repeated examinations. The visits are scheduled every 4-6 weeks on the same weekday and at the same time of the day to minimize the impact of weekly and circadian variation. For a subgroup of 30 participants in each panel the personal measurements are conducted in four examinations. A pilot phase was conducted between 19th and 23rd of February 2007 on six test patients. Newly established methods and devices were tested and feedback was given from the study nurses to the coordinating centre. No major changes in the study protocol seemed necessary; however, two special belts for an easier attachment of the 24hr Mortara ECG device were manufactured.



Baseline examination: blood withdrawal

Base program: blood withdrawal

Add-on program: blood withdrawal; personal measurements of ultrafine particles, temperature, humidity and noise; endothelial function test; 5-hour ECG

Figure 1. Study design.

The main phase started on the 19th of March 2007 and will presumably be terminated at the end of 2008. FIGURE 2 shows the recruitment and progress status of the study. The recruitment of genetically susceptible participants is already completed whereas the recruitment for subjects for the two remaining panels is still ongoing. Until 23rd of May 2008 672 subjects were contacted and 207 subjects could be invited to the study.

Exclusions and drop outs

Seven participants were excluded after the first visit (FIGURE 2), five subjects because of rheumatoid arthritis, one person because of arthropathic psoriasis and one due to Crohn's disease. One volunteer had a myocardial infarction between the fifth and sixth visit, hence only the first five visits were kept in the study.

Eight subjects dropped out of the base program, two of them already after the first visit. As at least two visits per person are required for the statistical analysis, visits of both subjects are not further considered for analysis. Seven participants of the add-on program dropped both, the base and add-on program, five participants dropped the add-on program but continued with the base program.

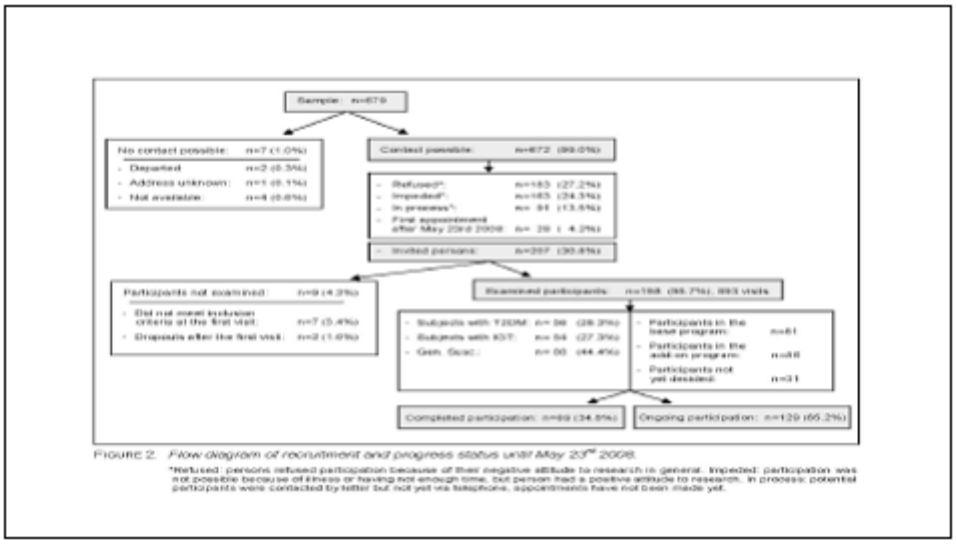


FIGURE 2. *Flow diagram of recruitment and progress status until May 23rd 2008.*

*Refused: persons refused participation because of their negative attitude to research in general. Impeded: participation was not possible because of illness or having not enough time, but person had a positive attitude to research. In process: potential participants were contacted by letter but not yet via telephone, appointments have not been made yet.

Examinations

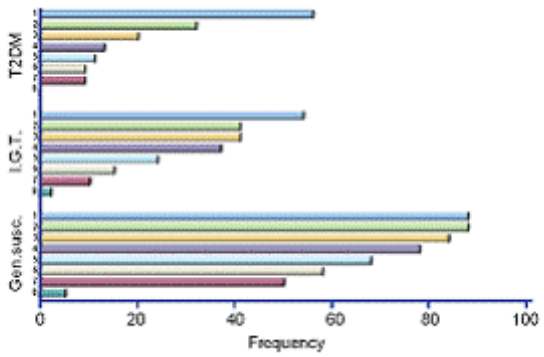


Figure 3. Number of visits conducted until May 23rd 2008, separately for each panel.

893 visits of 198 subjects are available with a mean of 4.5 visits per participant. An overview of the number of visits in each panel is given in FIGURE 3. Besides the base program, 86 volunteers (43%) completed 271 visits in the add-on program and 124 visits in the base program. 81 subjects (41%) with 467 visits exclusively participated in the base program, and 31 subjects (16%) with 31 baseline visits are still undecided about joining the add-on program.

56 subjects with T2DM (28%) with 150 visits, 54 subjects (27%) with IGT with 224 visits, and 88 subjects (44%) with potential genetic susceptibility with 519 visits are part of the study. 69 participants have already completed 7 visits. Seven subjects participated formally in an eighth visit as they cancelled a previous visit because of an acute inflammatory disease. The average number of repeated visits was 2.7 for the T2DM subjects, 4.1 for participants with IGT and 5.9 for the subjects with potential genetic susceptibility.

Figure 4 shows the frequency of CPC and noise measurement durations. 14 of the CPC measurements (5%) were missing and 17 (6%) had measurement durations of less than one hour. The incomplete data were mostly due to handling of the device by the study subjects. The CPC switches off in the case of vertical (tilted) position for more than 4 seconds and a manually restart is necessary. Unfortunately, not all subjects are able to restart the device on their own despite having instructions. We tried to dispose the producer of CPC for changing the software and make an automatically restart possible, but the negotiations were not successful. A preliminary analysis of the pilot phase has been presented at the EPA-GSF meeting in Montreat, NC, in spring 2007. First results from the pilot study indicate a good relation between personal exposure and the data of the central measurement site on an hourly basis depending on time spent indoors and ventilation habit of the study subjects. However, these analyses are limited to only few people (n=5) and the results of the main phase are yet to come.

24 noise measurements (9%) were absent. Only one measurement took less than one hour. The reasons for the missing noise measurements are defective microphones and errors in data storage.

Relative humidity and air temperature were missing only in one case, respectively. All other measurements took longer than five hours.

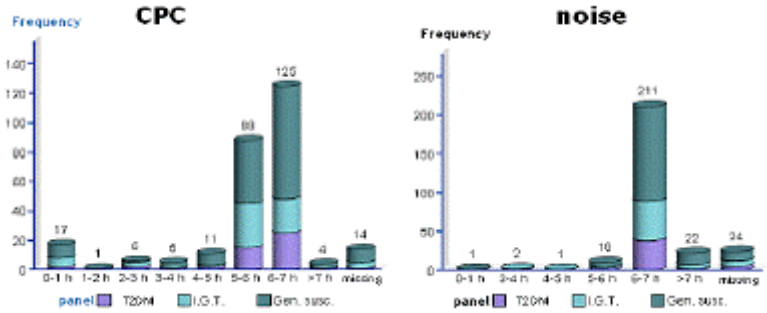


Figure 4. Frequency of measurement durations split by panel.

268 ECG measurements were conducted, while three measurements could not be conducted because of missing flash cards. This happened in the beginning of the main phase, now there is a sufficient number of flash cards available. 250 (93%) of the 268 ECG recordings were valid, in eleven cases (4%) the flash card was empty or unreadable, incorrect data were recorded for three ECG measurements (1%), and for four measurements (1%) the evaluation of the ECG remains to be done. The ECG data for the year 2007 is currently checked for plausibility. 261 measurements of the endothelial function (ED) were carried out. Two ED measurements could not be conducted because of technical problems and 5 participants refused the measurement (8 visits). Out of the 261 conducted measurements, 6 ED recordings (2%) had to be excluded because of inadequate quality.

Participant characteristics

Figure 5 shows the frequencies of age groups in the three panels. The medication intake of the subjects is shown in TABLE 1. For one person with IGT the information about the medication is still missing and will be gathered soon. Thus, the descriptive analysis includes only 53 subjects in this panel. Two genetic susceptible participants (2%) seem to be medicated with antidiabetic drugs. After completing data collection the assignment of all participants to the panels will be rechecked and changed if necessary

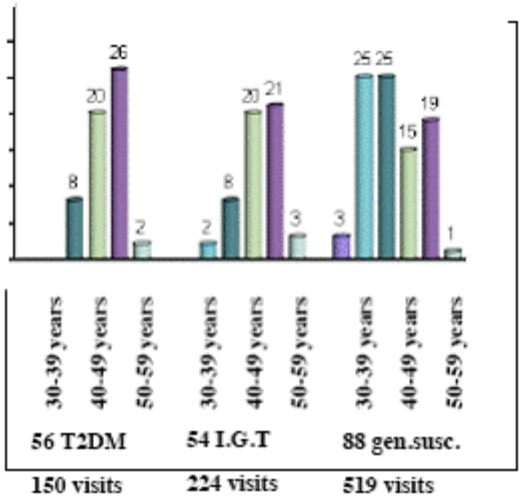


Figure 5. Frequencies of age groups per panel.

Table 1. Subjects' age, sex and medication intake by panel.

Characteristic	Subjects with T2DM (N=56)	Subjects with IGT (N=53)	Genetic susceptibles (N=88)	p-value for heterogeneity between panels
Male (%)	60.71	54.72	55.68	0.78[b]
Age [yrs][a]	68.40 (52.3-79.8)	67.20 (44.3-81.9)	56.40 (32.8-79.1)	<.0001[c]
Medication groups (%)				
Agents acting in rennin-angiotensin-system	53.57	35.85	23.86	0.0014[b]
Analgesics for system use	7.14	5.66	11.36	0.03[d]
Anti-Diabetics	53.57	0.00	2.27	<.0001[b]
Anti-inflammatory/ antirheumatic agents for systemic use	17.86	20.75	11.36	0.29[b]
Antithrombotic agents	30.36	26.42	14.77	0.06[b]
Beta blockers	39.29	33.96	17.05	0.008[b]
Calcium channel blockers	17.86	9.43	7.95	0.17[b]
Diuretics	50.00	39.62	15.91	<.0001[b]
Drugs with Acetylsalicylic acid	25.00	24.53	13.64	0.15[b]
Hormone replacement therapy for woman	3.57	11.32	5.68	0.02[d]
Impact on inflammation	48.21	39.62	18.18	0.0004[b]
Statins	35.71	20.75	14.77	0.01[b]

[a] mean (range); [b] chi-square test; [c] ANOVA; [d] fisher's exact test

Future Activities:

For the upcoming year the following activities are planned:

Complete field work in December 2008

Include an additional measurement device:

Subjects participating in the add-on program will be equipped with a special belt called Actibelt1. Its high-tech buckle carries high precision 3D-acceleration sensors that allow for a continuous, unobtrusive long-term monitoring of physical activity in daily life. The belt will be applied to support the collection of activity data and could be used as an alternative in further studies.

Genetic analysis with Affymetrix 1000k chip

Data cleaning

Draw up the analysis plan

Statistical analyses

Pilot project on wood smoke:

This project will provide pilot data on the contribution of wood smoke to fine particulate air pollution as well as on the role of wood combustion particles in modulating cardiac function.

References:

Daumer M et al. Steps towards a miniaturized, robust and autonomous measurement device for the long-term monitoring of patient activity: ActiBelt. *Biomed Tech.* 2007;52:149–155.

Publications:

Type	Citation	Document Sources
Presentation	Cyrys J. Personal sampling of ultrafine particles–Spatial and temporal correlation of ultrafine particles in an urban area–is there a need for modelling? Presented at the 5th EPA-GSF-Rochester Biennial Workshop, Montreat, NC, May 17, 2007.	*not available*
Presentation	Peters A. The burden of environmental pollutants on cardiac arrhythmia pathogenesis and mortality. Presented at the 12th Congress of the International Society for Holter and Noninvasive Electrocardiology, Athens, Greece, June 7, 2007.	*not available*

Presentation	Peters A. SNPs in the fibrinogen gene cluster modify fibrinogen response to ambient particulate matter. Presented at the University of Southern California Particle Center, USC, Los Angeles, June 1, 2007.	*not available*
Presentation	Schneider A. Results of the North Carolina Diabetes and the Environment Panel Study (DEPS). Presented at the 5th EPA-GSF-Rochester Biennial Workshop, Montreat, NC, May 18, 2007.	*not available*

Supplemental Keywords:

Human Clinical Studies of Concentrated Ambient Ultrafine and Fine Particles

Grant Number: R832415C003
Center: Rochester PM Center Abstracts
Center Director: Günter Oberdörster
Title: Human Clinical Studies of Concentrated Ambient Ultrafine and Fine Particles
Investigators: Mark W. Frampton, Mark J. Utell
Institution:
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: October 1, 2005 **through** September 30, 2010
Project Amount: $978,060.00
Report Start Date: October 1, 2006
Report End Date: September 30, 2007
RFA: Particulate Matter Research Centers
NCER Research Categories: Particulate Matter

Content

Objectives:

Core 3: Human Clinical Studies of Concentrated Ambient Ultrafine and Fine Particles

The overall objective of our current and planned studies is to determine the pulmonary and cardiovascular effects of exposure to ultrafine and fine particulate matter (PM). The clinical studies in healthy humans and susceptible individuals with diabetes proposed in this research core focus on the effects of ambient ultrafine and fine particles on three major determinants of adverse cardiac events: 1) blood coagulation induced by effects on platelets and circulating microparticles; 2) cardiac output; and 3) cardiac rhythm and repolarization.

Our overall hypothesis is that inhalation of ambient PM causes small but measurable changes in coagulation and cardiovascular function that help explain the cardiovascular effects of PM exposure. We further hypothesize that the cardiovascular effects are determined by the ability of PM to generate reactive oxygen and nitrogen species, and are more pronounced in subjects with type 2 diabetes. Inhaled ultrafine particles increase the burden of reactive oxygen species to the endothelium. Endothelial activation and vasoconstriction increase platelet adherence and release of thromboxane, activate and prolong the transit time of blood leukocytes, and deplete vascular nitric oxide (NO). Particles may also have direct effects on platelets and leukocytes. Vascular injury triggers release of procoagulant microparticles into the blood, and initiation of coagulation. In collaboration with the Vascular and Inflammation Facility Core, we measure the effects of inhaled ambient fine PM on platelet number, phenotype, and function, and

quantitate intravascular microparticles derived from platelets and endothelial cells. In collaboration with the Cardiac Core, we use noninvasive monitoring methods to measure exposure effects on cardiac output, rhythm, and repolarization. These studies take advantage of and extend upon our project funded by the National Institute of Environmental Health Sciences and the EPA, “Ultrafine Particle-Induced Oxidative Stress”, which focuses on effects on vascular function and NO.

In Rochester, New York 75 patients taking part in a cardiac rehabilitation program will be studied. Patients from an active cardiac rehabilitation program within the University of Rochester Medical Center are offered enrollment in the health effects study as they enter the Cardiac Rehabilitation program. These are patients who have had a recent coronary event such as myocardial infarction or unstable angina leading to coronary stenting. The program involves supervised, graded twice or thrice weekly exercise sessions for a total of 10 weeks. As part of the rehabilitation protocol, vital signs and a standard 12 lead EKG will be performed. In addition to the regularly electrophysiologically monitored exercise of the rehabilitation program, subjects will undergo continuously recorded Holter ECG recordings performed and analyzed by the Cardiac Core allowing evaluation of a series of ECG parameters at rest, during exercise, and during immediate post-exercise period. Venous blood samples will be obtained once per week and analyzed by the Vascular & Inflammation Core for acute phase reactants (fibrinogen and C-reactive protein) previously found to vary with ultrafine particle exposure, as well as complete blood counts. Concurrently, ultrafine particle number and particle mass will be measured continuously at a central measuring site in downtown Rochester. Other EPA Criteria Pollutants are also measured in eastern Rochester as well. In addition, one-third of the patients are being asked to do personal particle count monitoring in their car to and from the rehab facility and in their homes for 48 hours using a portable nuclei counter (TSI model 3781). Levels of ambient ultrafine and fine particles will then be associated with health data from the cardiac rehabilitation panel study.

Progress to Date:

Human clinical exposures to concentrated ambient ultrafine particles The Harvard ultrafine particle concentrator has been installed in a dedicated room in the Kornberg Medical Research Building at the University of Rochester Medical Center, and is fully operational. We have completed construction of a negative-pressure inhalation chamber, which is housed within our new exposure facility. We have initiated human clinical exposures to concentrated ambient ultrafine particles, using the concentrator and the exposure chamber. Installation and maintenance of the concentrator and exposure system requires close collaboration with Dr. Oberdörster, Research Core #1, and the Aerosol Generation & Analysis Core.

We have now completed a clinical study of healthy never-smoking subjects inhaling

concentrated ambient UFP using the concentrator. This study is co-funded by an NIEHS RO1 grant and this EPA center grant. Subjects are 20 healthy never-smokers, age 30 to 60 yrs, stratified by age and gender. Subjects are admitted to the Clinical Research Center the day prior to exposure, to minimize the potential influence of outdoor pollutant exposure in the hours prior to the experimental exposure. Physiologic measurements are made the day prior to exposure, and then 0, 3.5, 21, and 45 hours after exposure. Measurements include blood sampling for flow cytometry and soluble markers of inflammation and coagulation, pulmonary function testing, diffusing capacity for carbon monoxide, and flow-mediated dilatation of the forearm. The EPA Center is funding continuous cardiac monitoring, noninvasive measurement of cardiac output, and newly developed methods for analysis of platelet activation and circulating microparticles, using flow cytometry.

All 20 subjects have completed both exposures. Exposures have been successful from a technical standpoint, and no subject has experienced symptoms or problems with the exposures. Data analysis is underway. Figure 1 shows the mean (SD) concentrated particle number concentrations for each subject's UFP exposure.

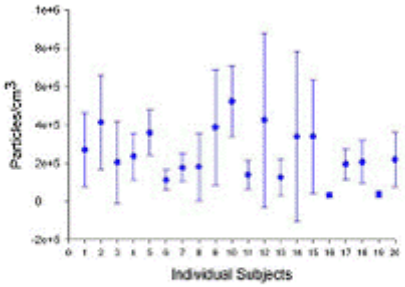


Figure 1. Mean (SD) particle number

There was considerable variability in exposure concentrations, as expected. Preliminary findings suggest UFP exposure resulted in small increases in diastolic and mean blood pressure immediately after exposure (Figure 2), without changes in heart rate.

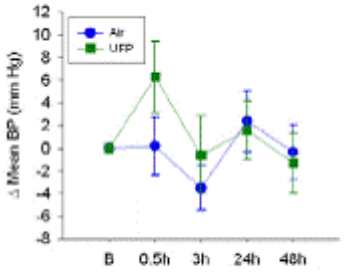


Figure 2. Change in mean

There were small reductions in the FEV1 and mid-expiratory flow rates after UFP exposure relative to air (Figure 3). Flow cytometry analyses, measurement of markers of endothelial and vascular effects, and ECG analyses are underway.

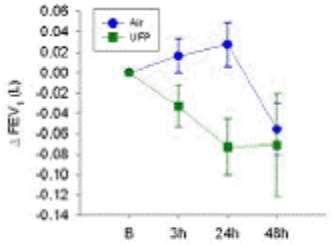


Figure 3. Change in FEV_1

2. Inhalation of carbon UFP in diabetics

We have completed our study of the effects of inhalation of ultrafine carbon particles in subjects with diabetes. Diabetics have vascular endothelial dysfunction which may increase their risk for adverse cardiovascular effects from airborne particles. Type 2 diabetics, age 30-60, without clinical cardiovascular disease and not on “statin” medications, were exposed to filtered air or 50 µg/m3 carbon UFP (count median diameter ~30 nm, GSD 1.8) by mouthpiece for two hours, in a randomized double-blind cross-over study. Exposures were separated by at least two weeks. Nineteen subjects completed the study.

A preliminary analysis of the findings was published in abstract form and presented at the 2007 American Thoracic Society International Conference. Compared with air exposure, UFP exposure increased platelet expression of CD40 ligand (CD40L) (Figure 4), a marker of platelet activation and a key molecule in the development of atherosclerosis.

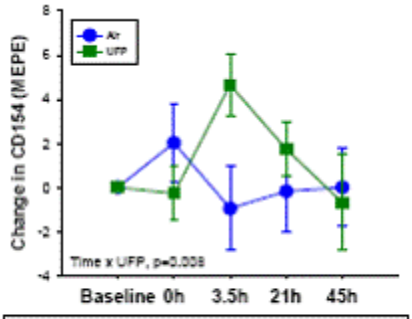


Figure 4. Change in platelet expression

UFP exposure also increased platelet-associated tissue factor (TF) and increased the number of microparticles expressing TF (Figure 5).

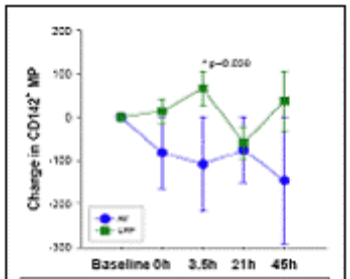


Figure 5. Change in number of microparticles expressing tissue factor (CD142) following exposure to air or UFP.

Changes occurred most consistently 3.5 hours after exposure, and effects were no longer seen 24 hours after exposure. There were no significant effects of UFP on platelet counts or platelet aggregates. The subjects with diabetes showed significant reductions in forearm flowmediated vascular dilatation in comparison with healthy subjects, as expected. Both the pulmonary diffusing capacity for carbon monoxide (an indicator of pulmonary vascular function), and forearm flow mediated dilatation (an indicator of systemic vascular function), decreased with UFP exposure to ultrafine particles compared with clean air exposure, but the differences were not statistically significant.

Analysis of continuous ECG recordings suggests delayed effects of UFP exposure on heart rate. Analysis of the interval between normal beats (NN interval), which is inversely related to heart rate, showed a decrease over time relative to air exposure. When heart rate was averaged over the duration of each multi-hour ECG recording interval, heart rate increased during the 48 hrs after UFP, relative to air exposure (Figure 6).

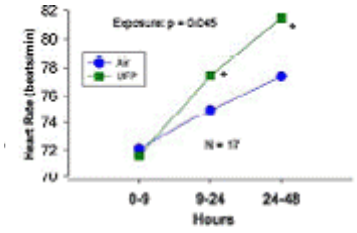


Figure 6. Average heart rate for 3 ECG recording intervals. 0-9 hrs

We concluded that inhalation of carbon UFP for 2 hours may transiently activate vascular endothelium and/or platelets in subjects with type 2 diabetes. This finding supports the hypothesis that exposure to ambient UFP may increase the potential for vascular thrombosis in patients with severe vascular disease or ulcerated atherosclerotic plaques. The reasons for the delayed increase in heart rate are unclear, but increased heart rate increases the oxygen demand of the heart, and

could adversely affect people with angina or coronary artery disease. We are currently working closely with the Biostatistics Core, using a 2-model analysis to examine effects of gender and age on the findings. This project also involved close collaboration with Research Core #1 and the Aerosol Generation & Analysis Core for UFP generation and exposure monitoring. Manuscript preparation is underway.

3. Ultrafine Particles Activate Platelets In Vitro

Inhaled UFP have the capability of entering pulmonary vascular endothelial cells and even the blood. One possible mechanism for activation of platelets, thus increasing risk for cardiovascular thrombotic events, may involve direct effects of UFP that have entered the circulation. The purpose of this study is to examine the effects of both laboratory-generated carbon ultrafine particles, and ambient UFP, on platelet activation in vitro. The following particles (and count diameters) were studied: copper (0.022 μm), commercial elemental carbon (P90, 0.014 μm), laboratory-generated elemental carbon (lab carbon, 0.030 μm), and diesel exhaust particles (DEP, 1.62 μm). Vortexed particle suspensions were added to whole blood from seven healthy never-smoking subjects and incubated for 30 minutes at 37ºC. Platelet expression of CD62P (p-selectin), platelet aggregates, and platelet-leukocyte conjugates were measured by flow cytometry. Dose-dependent increases in the expression of CD62P were most pronounced with copper and lab carbon. Lab carbon also significantly increased platelet aggregates and platelet-leukocyte conjugates. These findings indicate that UFP can activate platelets in vitro, and the activity varies with particle size and composition.

We have further examined effects of lab carbon UFP on platelet activation and conjugates in the presence of low concentrations of platelet agonists. Carbon UFP at 20 μg/ml significantly increased platelet p-selectin expression ($p = 0.04$), but did not further enhance pselectin expression in the presence of platelet agonists. There were no significant increases in platelet aggregates or microparticles. Carbon UFP at 20 μg/ml also significantly reduced the platelet count in the presence of all agonists except thrombin, suggesting that low concentrations of agonists enhanced the formation of platelet-leukocyte aggregates.

These studies suggest that UFP, to the degree that they gain access to pulmonary capillary blood following inhalation, have the potential to directly activate platelets and enhance the formation of leukocyte-platelet aggregates in the presence of low concentrations of platelet aggregates. This provides a further mechanism for the acute cardiovascular effects of PM exposure.

To date, 40 of the 75 patients have been enrolled. Thirty-six patients have completed the entire protocol; the patients include 22 men and 9 women; mean age = 64 years and range = 38 to 80 years)). Four patients are in various stages of the 10-week study. Five patients withdrew during the study for various reasons. We are

actively screening several potential patients. To make the ambient measurements of ultrafine particles relevant to subject exposures, we are enrolling subjects who live within 5 miles of the central monitoring site or the cardiac rehab center. Ultrafine particle exposures within and without the cardiac rehab center are being monitored continuously.

In order to meet the inclusion criteria, participants must have stable coronary artery disease, must be enrolled in the University of Rochester Medical Center rehabilitation program, and must live in the Rochester area. Participants have to be current non-smokers and should be in the study area during the entire study period. Participants have to be able too physically and mentally comply with the cardiac rehabilitation protocol. Patients with atrial fibrillation, pacemakers, bundle-branch blocks, type 1 diabetes and patients that are away from the study area for an extended period of time will not be included in the study. Annual approval for the protocol has been received from the University of Rochester Research Subjects Review Board (RSRB).

Particle Monitoring Intermediate Results:

Measurements of ultrafine particles are proceeding as proposed with continuous monitoring of ambient particles within and outside of the cardiac rehab center. The particle size distribution data from the Cardiac Rehabilitation Center (indoors and outdoors) and at the NYS DEC site are being collected to support the clinical studies of heart rate variability and inflammatory markers in the blood of rehabilitation patients. Fifteen patients have completed the take home monitoring, with 11 of those doing automobile monitoring.

Monitoring period for September 2006 through December 2007. 94% ultrafine particle monitoring rate was recorded at the rehab facility. 82% ultrafine particle monitoring rate was recorded at the DEC site. Average commute was 15 min. each way for the 11 patients with monitoring performed in their automobile.

Hourly Ultrafine Particle Count Concentrations (x103 p/cm3) (Sept. 06 – Dec. 07)

	Rehab Indoor	Rehab Outdoor	DEC Outdoor	Take home N=15	Auto N=11
Average	1.6	4.4	6.9	9.0	17.5
S.D.	1.0	2.5	3.6	7.0	8.2
% Reported	94.2	94.2	82.3	100	100

Hourly DEC Data (September 2006 – December 2007)

	PM2.5 ug/m^3	O_3 ppm	SO_2 ppm	CO ppm	Tem. °F	RH %	BP mmHg	Wind Spd.mph	Precip. Inches
Average	9.27	0.024	0.004	0.456	50.5	64.6	29.43	4.60	0.004
S.D.	7.68	0.013	0.004	0.164	11.1	19.7	0.28	2.33	0.018
% Reported	85.0	97.5	97.8	98.2	99.9	99.0	100	99.9	99.9

We are examining the relationship between the ambient ultrafine particle size distributions at the Rehabilitation Facility and the measurements at the NYSDEC site during a one-year period. The data have been collected and the analyses are underway. The goal is to examine the differences in ultrafine particle counts in 2 areas that are closely located.
Analytical Approach:

In our analytical plan, we will explore the relationship between closeness of home to roadway and traffic and clinical responses to ultrafine particles in the ongoing panel study. At present, we have zip codes and addresses of all patients enrolled in the study. We have identified the outcome variables and what the outcome variables will be adjusted for.
Currently we are exploring several models for the analysis.

1) General Strategy: We are developing the analytical plan with assistance from our biostatistical core. A key strength of this study is the availability of longitudinal measurements on each subject, corresponding to their successive visits to the rehabilitation center. This design feature allows each subject to be used as his or her own control. Differences between subjects may be eliminated by stratification, corresponding to the use of a fixed effects analysis of variance model, or by explicitly modeling subject effects as random. The latter approach is necessary for examining the influence of factors that do not change from one visit to another, for example the gender of the subject. The former approach will give greater statistical power to address the primary hypotheses of the study relating to the influence of the UFP concentrations, which do change from visit to visit. In the simplest case, the fixed approach corresponds to a simple paired t-test, comparing responses of the same subject during periods of relatively high and low exposure to ultrafine particles. More commonly analysis of covariance (PROC GLM) will be used, with the subjects entered as fixed effects and ultrafine particle exposure as a continuous variable. Models with subjects entered as random effects will be used to assess the generalizability of the findings to other populations. The random effects approach requires specification of the correlation structure of the data. All proposed analyses can be implemented in SAS using PROC MIXED and PROC GENMOD.

2) Multivariable Analysis: In view of the complex relationship between UFP levels and weather (which may independently influence cardiac responses) it will be necessary to examine multivariable models including effects of variations in temperature and relative humidity as well as calendar (e.g. day of week) effects. The

functional forms of the potential confounding effects will be explored using penalized spline methodology.

3) Sample size: The proposed net sample size of 75 subjects will allow detection of an effect size of 33% (ratio of mean difference to standard deviation. of difference) in a response variable with 80% power, using a two-sided test with alpha level = 0.05.

4) Progress to date: Data on cardiac responses including perceived exertion, changes in heart rate from pre-exercise to peak and to post exercise, associated changes in systolic and diastolic blood pressure, and in white blood count has been examined on the first 12 subjects to complete the program. Correlations have been assessed with measures of ultrafine particle exposure at the corresponding visit. The primary purpose of performing these analyses has been to check the data acquisition and management process, which requires merging of data from several sources. In the course of performing these analyses we have identified a number of data issues which have now been resolved.

Future Activities:

We are in the planning and approval stages of our next clinical protocol, examining concentrated UFP effects in people with type 2 diabetes. This study will provide a comparison with our just-completed study in healthy subjects, and with our findings in diabetics exposed to elemental carbon UFP.

We have applied for and received pilot study funds from the EPA Center to examine the effects of ambient ultrafine particles on blood dendritic cells (DC). The DC is a key controller of the allergic immune response, by processing inhaled allergens and foreign proteins and presenting antigens to T lymphocytes. We hypothesize that inhalation of ambient UFP contributes to the worsening of airways disease in people with asthma by altering DC maturation and function, driving the immune response to a Th2-type, or allergic, phenotype. In the last five subjects of our UPCON study, we have obtained blood before and 24 hours after exposure, begun to analyze peripheral blood DC phenotype and function, with the help of Drs. Stephen Georas and Marc Williams of the Pulmonary and Critical Care Division. We have submitted a protocol to the Research Subjects Review Board, and to the EPA, to study subjects with mild to moderate asthma. These subjects will inhale air or concentrated ambient UFP for two hours, and blood dendritic cells will be analyzed and compared with the findings in healthy subjects. In addition, lung function and exhaled breath markers of inflammation will determine whether dendritic cell effects correlate with airway effects. This pilot study is planned as the first effort in a new direction for the clinical core of the Center, examining the mechanisms for airway effects of PM in asthma.

For the in vitro studies of platelet activation, future experiments will explore the effects of ambient UFP collected from different sites, additional types of diesel exhaust, as well as the mechanisms for particle activation of platelets. We are collaborating with Dr. Phipps and the Vascular & Inflammation Core in developing these research strategies.

We will continue patient recruitment into the protocol. At the present time, our recruitment rates are moving ahead according to the proposed time line; we have completed enrollment of more than 50% of the proposed population into the study. We will continue particle measurements in homes and automobiles with a plan to sample these sites in approximately 25% of the enrolled population.

Publications:

Type	Citation	Document Sources
Abstract	Frampton MW, Stewart JC, Chen X, Pietropaoli AP, Taubman M, Utell MJ. Platelet and vascular effects in type 2 diabetics inhaling ultrafine carbon particles. *American Journal of Respiratory and Critical Care Medicine* 2007;175:A168.	*not available*
Abstract	Klein LW, Stewart JC, Oberdorster G, Frampton MW. Ultrafine particles activate platelets in vitro. *American Journal of Respiratory and Critical Care Medicine* 2007;175:A169.	*not available*
Abstract	Shah AP, Stewart JC, Klein LW, Utell MJ, Frampton MW. Effects of carbon ultrafine particles on platelet activation. *Proceedings of the American Thoracic Society* 2006;3:A551.	*not available*
Abstract	Shah AP, Stewart JC, Pietropaoli AP, Utell MJ, Frampton MW. Vascular effects of carbon ultrafine particle inhalation in diabetics. *Proceedings of the American Thoracic Society* 2006;3:A387.	*not available*
Presentation	Frampton M, Stewart J, Chen X, Pietropaoli A, Taubman M, Utell M. Platelet and vascular effects in type 2 diabetics inhaling	*not available*

	ultrafine carbon particles. Presented at the NIH/EPA Conference, The Role of Air Pollutants in Cardiovascular Disease, Research Triangle Park, NC, October 12-13, 2006.	
Presentation	Stewart JC, Speers D, Frampton MW. Blood leukocyte production of reactive oxygen species in people with type 2 diabetes. Presented at the International Society for Analytical Cytology, Montreal, Canada, May 2006.	*not available*

Supplemental Keywords:

Project-Specific Reporting Core 4

Animal Models: Cardiovascular Disease, CNS Injury and Ultrafine Particle Biokinetics

Date of Report: June 30, 2008

EPA Grant Number: RD 832415-1

Center: Rochester Particle Center – Core 4

Center Directors: Günter Oberdörster; Mark J. Utell

Investigators: Günter Oberdörster, Alison Elder, R. Phipps, J-P. Couderc, R. Gelein, D. Oakes, S. Eberly

University of Rochester

Project Period: October 1, 2005 – September 30, 2010

Period Covered by the Report: October 1, 2005 – June 30, 2008

Objective of Research:

The objective of the Core 4 studies is to correlate physico-chemical particle characteristics (from Core 1 measurements) with pulmonary and cardiovascular endpoints following exposure of animals to inhaled ambient concentrated ultrafine/fine particles, inhaled freshly-generated exhaust particles from low and ultralow diesel fuel, and intratracheally administered ultrafine and fine ambient particles from different sites and sources. Effects measurements will take into account endpoints determined in the epidemiological (Core 2) and clinical (Core 3) studies and coordinate mechanistic evaluations with Core 5 *in vitro* studies. In addition, effects on the CNS will also be assessed.

Progress Summary/Accomplishments:

Exposures of Rats to Freshly-Generated On-Road Aerosols

Humans with type II diabetes have been shown in recent epidemiological studies to be susceptible to the adverse health effects related to ambient particulate matter exposures. There are several animal models of diabetes, of which one is the JCR:LA-cp rat. Although these rats are not hyperglycemic (according to published literature and our results), the JCR cp/cp rats are obese, hyperlipidemic, hyperinsulinemic, and have atherosclerotic and ischemic lesions that are hallmark features of human type II diabetes. Heterozygotes or homozygous

normals (designated by JCR +/?) are not obese or hyperinsulinemic and do not exhibit the atherosclerotic lesions that the cp/cp rats do. Females of the same strain are similar to the +/? rats, which is why males are used exclusively in our studies.

One of the hypotheses being investigated in our studies in coordination with the Cores 2 and 3 projects is that some of the adverse health effects associated with exposure to ambient particulate pollution are causally related to inhaled ultrafine particles (UFP) and their gaseous co-pollutants. We have recently completed two rounds of studies using the JCR rats, one in which they were exposed to freshly-generated exhaust emissions aerosols (two fuel types) in a mobile laboratory and another where the rats were exposed to concentrated ambient ultrafine particle-containing aerosols using the HUCAPS system.

Due to the limitation on the total number of animals that could be exposed, we focused on genetic background and exhaust atmosphere as being the response modifiers. Exposures to low- and ultralow-sulfur Diesel fuel exhaust emission aerosols were conducted in compartmentalized whole-body chambers while the mobile laboratory was driven between Rochester and Utica (NY I-90). Rats exposed to ambient UFPs received either HUCAPS aerosols or filtered air. Endpoints related to lung inflammation, inflammatory cell activation, acute phase responses, and platelet activation were measured after exposure. As in the past, groups of rats were also implanted with radiotransmitters to continuously monitor changes in heart rate, blood pressure, temperature, and activity associated with exposure to exhaust emissions or clean, filtered air.

There was an obvious effect of obesity and insulin resistance on baseline lavage inflammatory parameters, namely that the JCR cp/cp (obese) rats had higher total cell numbers, percentages of PMNs, and lavage fluid protein content and LDH and β-glucuronidase activities than their lean litter mates. However, neither the emission nor the HUCAPS aerosols had any consistent effects on these parameters. We also measured several parameters in serum and lavage fluid related to inflammation and metabolism using a rat adipokine panel bead array. For most of the parameters, the obese cp/cp rats had higher levels than in the lean rats. Another observation was the there was a slight decrease in the number of circulating platelet microparticles in the obese rats that were exposed to either the

HUCAPS or exhaust emission aerosols. Finally, samples obtained from the concentrator study revealed that the JCR cp/cp rats are iron overloaded and that HUCAPS exposure exacerbates this. The specific implications of this iron overloaded state in JCR cp/cp rats in terms of responses to low-level ambient UFP exposure are not yet clear. A manuscript(s) reporting these findings are in preparation.

The evaluations of blood pressure, heart rate, and heart rate variability changes in the rats that were exposed to exhaust emission or HUCAPS aerosols are ongoing. We found a significant divergence in heart rate following on-road exposures between the air- and full exhaust-exposed rats that increased over time in the post-exposure period. More specifically, the heart rate continued to decline over time in the exhaust-exposed rats. The statistical analyses of these data are almost complete.

Exposures of R6/2 Mice to Concentrated Ambient Ultrafine Particle-Containing Aerosols

Based on previous studies from our group demonstrating that inhaled poorly-soluble laboratory-generated UFP travel to the brain (Oberdörster et al., 2002, 2004) and evidence that they cause oxidative stress and inflammation in those regions where particles accumulate (Elder et al., 2006), we hypothesized that ambient UFP can induce similar effects, particularly in an animal model that exhibits early-onset neurodegeneration (in this case Huntington's disease, HD; Mangiarini et al., 1996). Transgenic R6/2 mice express 105-150 polyglutamine (polyQ) repeats in the huntingtin protein (Htt) and are the best characterized and most widely used of the HD animal models. Nuclear inclusions of aggregated Htt are abundant throughout the whole brain in these mice and can be detected as early as three weeks of age (8). The mice exhibit subtle motor deficits as early as one month of age, which then lead to overt symptoms by two months; death occurs within three to four months (Carter et al., 1999; Lione et al., 1999; Murphy et al., 2000).

HD and other neurodegenerative diseases have in common abnormal protein folding and aggregation (e.g. Alzheimer's, Creutzfeldt-Jakob, Parkinson's). However, the significance of protein aggregation in these diseases is not entirely clear, as there is debate regarding whether the aggregated proteins are toxic or the misfolded monomers. Nonetheless, recent acellular

assays have shown that particles of different sizes, shapes, and surface chemistries can induce the unfolding and subsequent fibrillation (aggregation) of proteins (Linse et al., 2007). We hypothesized that the UFP present in ambient aerosols could be translocated to the brain, potentiate the aggregation of Htt, and accelerate neurodegeneration in exposed R6/2 mice.

We exposed the R6/2 mice, starting at 5-7 days of age, to concentrated ambient UFP-containing (HUCAPS) aerosols for 4 hrs/day, 5 days/week, for a total of 6 weeks in whole-body exposure chambers. About 1 week after the mouse pups were weaned, they were trained on an apparatus that allows an evaluation of locomotor function (Rotarod) and then re-tested every week through the end of exposure (a total of 4 evaluation points). Statistical analyses revealed that, unlike nontransgenic mice, the Rotarod performance of the transgenic mice declined over time. For those mice exposed to HUCAPS aerosols, the performance was significantly lower by the third week of testing, whereas for filtered air-exposed mice, performance did not drop significantly until the fourth testing week. The mice were euthanized 24-48 hrs after the last exposure, with one group being used for evaluations of lung inflammatory responses and one used to collect tissue samples for histopathological analyses. Serial coronal sections of brain tissue will be evaluated for striatal atrophy and huntingtin protein (Htt) expression and aggregation. Other tissues have also been saved so that we can examine Htt aggregation as a result of exposure in other tissues (e.g. lung, heart, pancreas).

Another group of pups will be born soon and they will be exposed to HUCAPS aerosols or filtered air for 6 weeks and then recovered for 4 weeks. As with the study described above, locomotor function testing using the Rotarod system will be done. In addition, their performance during the recovery period in a beam-break apparatus will be evaluated to assess additional aspects of locomotor function, such as traveled distance and jumping. The mice will be euthanized at the end of the 4 week-recovery and the same endpoints evaluated in brain tissue, lavage fluid, and blood.

Future Activities:

As mentioned above, we will be focusing on tissue analyses from the R6/2 mice that were exposed to HUCAPS aerosols. In addition, we have another 10-week study to complete (6 weeks of exposure with 4 weeks of recovery). We have saved several tissues from this study and hope to share them with other investigators who may be able to measure endpoints that we ourselves cannot. We will also continue our analyses of the heart rate variability data from the telemetered JCR rats that were exposed to freshly-generated emission aerosols and concentrated ambient PM. Lastly, in Fall, 2009, we will carry out a fourth on-road exposure study using the mobile emissions laboratory. We will test the specific hypothesis that exhaust filter technology will affect cardiovascular, pulmonary and CNS responses in rats with compromised cardiovascular systems.

Research Core 5: Ultrafine Particle Cell Interactions In Vitro: Molecular Mechanisms Leading to Altered Gene Expression in Relation to Particle Composition

Investigators: J. Finkelstein, R. Phipps, A. Rahman, D. Oakes, K. Prather

Objectives of Research

The experiments proposed within this project are designed to address specific mechanistic hypotheses regarding the interactions between inhaled ultrafine particles and specific pulmonary cell populations. The in vitro experiments in this core are intended to provide a mechanistic link and biological plausibility for the whole animal and controlled clinical (human) exposures, described in the other programs of this Particle Center. Our ability to use defined populations of cells and well characterized particles allow us to test specific hypothesis that arise from the *in vivo* studies described elsewhere. A particular focus of the in vitro studies is to attempt to identify mechanisms that may be involved in the enhanced susceptibility of cells from diabetics. Also by working with our particle characterization and analytical cores we will attempt to correlate cellular effects with composition that is related to specific sources.

Progress Report/ Accomplishments

1. Differential responses to particles by endothelial cells

As more of the physiological effects noted in our in vivo studies have pointed towards cardiovascular endpoints our emphasis on cellular models has shifted away from the typical pulmonary cell targets, namely alveolar macrophages and respiratory epithelium, to vascular cell populations. The main focus of our in vitro studies continues to be the endothelium but we continue to examine epithelial responses to PM for comparison and to test the validity of the cellular mechanisms of response in different cell types. An important direction of these experiments has been a more thorough

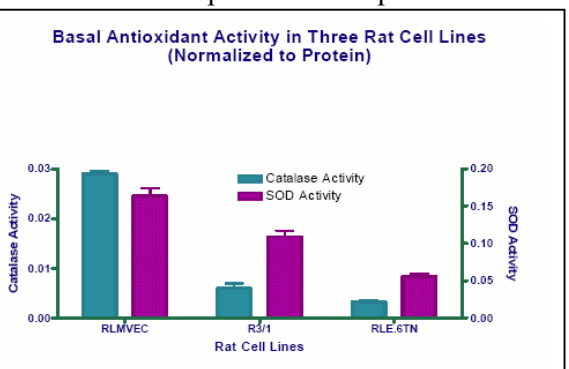


Figure 1 Comparison of Basal Antioxidant Activity in pulmonary Epithelial and Endothelial cell lines.

examination of the role of oxidant stress in the response to PM and the effect of cellular and exogenous antioxidants as protectors against PM induced cellular injury.

In this regard we have compared the activity of SOD and catalase in 3 different pulmonary cell lines cultured under identical conditions. Both catalase and SOD activity was found to be highest in the microvascular endothelial line RLMVEC. Both the type I (R3/1) and type II alveolar (RLE.6TN) lines had significant activity but catalase was found to be substantively lower in the epithelial cells. On the basis of this result one might predict that the endothelial cells would be most resistant to oxidant induced damage.

2. In vitro models for diabetes.

A major thrust of the current research of this PM Center is to investigate the proposed increased sensitivity of diabetics to the effects of PM. Our in vitro studies are designed to model this under controlled conditions. As we reported previously, one of the hallmarks of the diabetic is the increased blood glucose and we have shown that culture of vascular endothelial cells in high glucose alters both the basal and particle induced cytokine responses. Using this model we have begun to address the response of pulmonary cells to particles collected by a high volume sampler in Rochester. These would be similar to PM used in animal and human clinical studies carried out using the Harvard ultrafine particle concentrator. Using production of IL-6 as our benchmark may reflect a possible acute phaase response following particle inhalation and subsequent translocation to the vasculature. As shown in Figure 2 human respiratory epthelial cells exposed to collected ultrafine particles respond through increased production of IL-6. In contrast to previous work that focussed only on the production of NO and endothelium

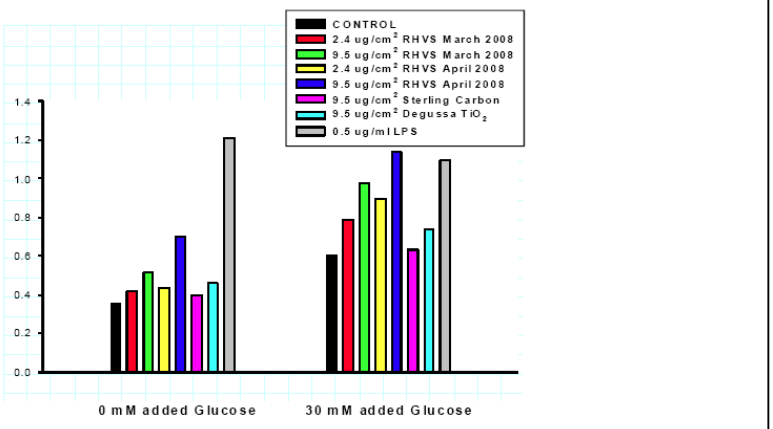


Figure 2 Production of IL-6 by pulmonary epithelial cells following treatment with PM collected with the Rochester high volume samples at different times. Cells were incubated with a fixed concentration of the various particles for 24 hrs. Culture media was harvested and assayed for IL-6 by ELISA. A second group of cells were similarly exposed after growing in 30mM supplemental glucose

epithelial cells maintained under conditions of hypergycemia actually produce increased amounts of IL-6. This is in contrast to results shown in Figure 3 where HUVEC were cultured under similar conditions. It is also evident that the ambient samples show a somewhat different response in these cells for this marker. In collaboraqtion with our Analysis core we will attempt to determine if differences in compostion can help explain the differences in activity.

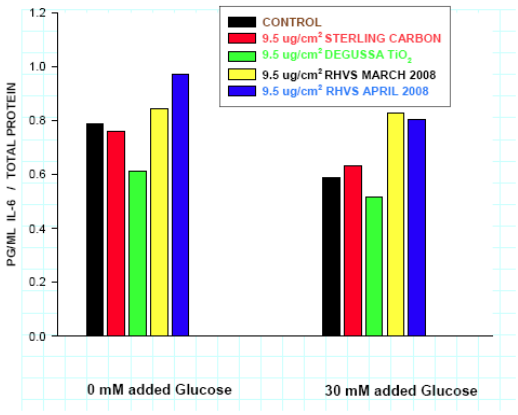


Figure3 Production of IL-6 by pulmonary endothelial cells HUVEC following treatment with PM collected with the Rochester high volume samples at different times. Cells were incubated with a fixed concentration of the various particles for 24 hrs. Culture media was harvested and assayed for IL-6 by ELISA. A second group of cells were similarly exposed after growing in 30mM supplemental glucose

One of the other questions raised in our proposed studies is the role of PM induced oxidative stress in the generation of cytokine or NO (Nitric Oxide) responses. The human clinical studies have been measuring vascular reactivity as a measure of response to inhaled PM. Included in that battery of outcomes was IL-6 and plasma Nitric Oxide (NO). To examine this response in a mechanistic manner we began to measure changes in both of these outcomes in cells that have had their antioxidant status altered by culturing with exogenous antioxidants. For thes initial studies we used both a soluble sulfhydryl agent , N-acetyl cysteine (NAC)and a lipophilic antioxidant butylate hydroxyanisole (BHA).

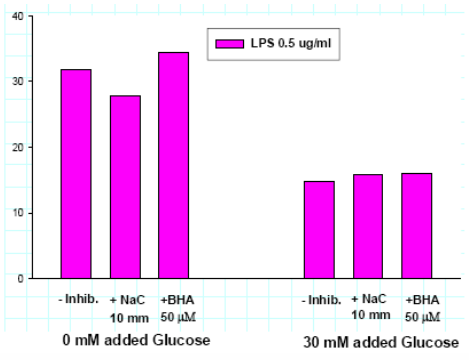


Figure 4 IL-6 production by HUVEC in culture after treatment with LPS: ***Effect of hyperglycemia and antioxidant pretreatment.*** RLMVEC cells grown with or without 30 mM supplemental glucose were culture for 24 hrs. in the presence of various antioxidants and then treated with LPS. Media was collected and IL-6 determined by ELISA.

Culture with NAC is know to increase intracellular –SH groups including glutathione while BHA as a lipophilic agent sequesters in cell membranes to trap lipid radicals. Our initial studies looked at cells stimulated by LPS which is know to stimulate the production of IL-6 by endothelial cells. Results in Figure 4 show that LPS stimulated IL-6 production was not altered by either antioxidant nor did glucose pretreatment interact with the antioxidants. Interestingly it did show that hyperglycemia inhibited LPS induced endothelial IL-6 production while stimulating PM induced IL-6 production by epithelail cells(see figure 2). This difference also emphasizes the need for examining multiple cell types when attempting to assess the effects of PM as there appears to be a degree of cellular specificity.. As shown in Figure 5 pretreatment with antioxidant did have some effect on PM induced IL-6 but this was limited to the NAC treatment and generally appeared due to an overall reduction of IL-6 rather than a specific effect on induction by PM.

We are currently investigating the mechanism of this suppression through effects on intracellular signaling pathways.

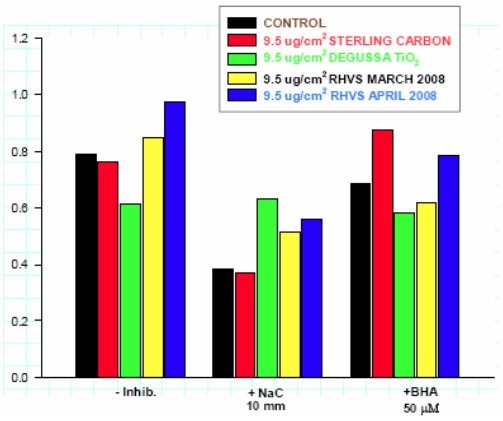


Figure 5 Effect of antioxidant pretreatment on IL-6 production by vascular endothelial cells.

Future Activities:

In the coming year we plan to continue to characterize the response of the microvascular endothelium to particles and begin to model for the effects of diabetes on these responses. Recent studies suggest that hyperglycemia can significantly alter the oxidative stress response in the epithelium and we plan to assess the effect of this treatment on cytokine production following particles. Also, in collaboration with Core 4 we plan to determine the effect of

serum isolated from diabetic animals, both PM exposed and controls, on the ability of vascular endothelial cells to be restimulated by concentrated PM. We also expect to extend these studies to cells of neuronal origin and possibly cardiac muscle. Measurement of prostaglandin production, and COX-2 activation will be evaluated with respect to its usefulness as a marker.

Also, in support of the *in vivo* projects, we will evaluate *in vitro* effects of particles of differing composition. We will continue to examine the cytokine response to ultrafine particles containing elemental carbon and iron and organic carbon compounds and begin studies of concentrated real world particles.