Cardiopulmonary Health Effects: Toxicity of Semi-Volatile and Non-Volatile Components of PM

Final Report

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Table of Contents

List of Figures

List of Tables

List of Inventions

None

List of Copyrighted Materials

None

List of Terms, Abbreviations and Symbols

Symbol or Abbreviation	Explanation
AAALAC	Association for the Assessment and Accreditation of Laboratory Animal Care
APHEL	Air Pollution Health Effects Laboratory, University of California, Irvine
apoE-/-	Mice in which the gene regulating apoE is knocked out
AQMD	Southern California Air Quality Management District
Arteriosclerosis	A chronic disease in which deposits of cholesterol and/or calcium cause abnormal thickening and hardening of the arterial walls with resulting loss of elasticity
Atherosclerosis	A stage of arteriosclerosis in which fatty deposits (atheromas) form inside the arterial walls, thus narrowing the arteries
Atherothrombosis	Disruption of an atherosclerotic plaque or lesion resulting in release of fragments that can block an artery
BAL	Bronchoalveolar Lavage
CAPs	Concentrated Ambient Particles
CO	Carbon Monoxide
CRP	C-reactive protein (acute phase protein which is a marker of systemic inflammation)
CVD	Cardiovascular disease
EC	Elemental Carbon
ECG	Electrocardiogram
GSH	Glutathione
HEPA	High Efficiency Particle Air filter

LDL	Low density lipoprotein
NYU	New York University
OC	Organic Carbon
PFO	Particle Free Organics
PM	Particulate matter
$PM_{2.5}$	Particulate matter less than 2.5 μm in aerodynamic diameter
PTFE	Fluorocarbon-based filter media
SS	Stainless Steel
UFP	Ultrafine Particles ($d_p \leq 0.18$ μm)
VACES	Versatile Aerosol Concentration Enrichment System
VCAM	Vascular Cell Adhesion Molecule

Abstract

The goal of this project was to determine whether or not the toxicity of ultrafine (UFP; particles ≤ 0.18 μm aerodynamic diameter) particles depends on the concentration and composition of semi-volatile and non-volatile fractions of the PM. We tested the hypothesis that adverse effects of exposure to these UFP, which are primarily emitted by combustion sources and are highly enriched in semi-volatile components, will be significantly attenuated by removal of those components from the aerosol. We used a unique mobile *in vivo* rodent exposure system in combination with a particle concentrator and thermal denuder to study the cardiopulmonary effects of UFP, before and after the removal of the semi-volatile components. The study used genetically modified (apoE-/-) mice that had impaired lipid metabolism and were therefore predisposed to the development of atherosclerotic-like plaques. Exposures were 6 hr/day, 4 days per week for 8 weeks and were conducted near the University of Southern California campus in central Los Angeles. Detailed chemical and physical characterization examinations of the concentrated ambient UFP (CAPs) and thermally denuded CAPs were conducted. The thermal denuder removed more than 60% of the particle-associated organic compounds (OC) but did not remove the non-volatile components such as elemental carbon (EC) or trace metals. Exposure to undenuded CAPs accelerated the development of atherosclerotic plaque in the apoE-/- mice, characterized by decreased arterial lumen diameters and increased incorporation of lipids in arterial walls. The lumen diameters and arterial wall lipid contents in apoE-/- mice exposed to thermally denuded CAPs suggested significantly less plaque development than in the mice exposed to undenuded CAPs and were not different from plaque levels in apoE-/- exposed to purified air, as controls. In addition, heart rate variability was decreased in the mice exposed to undenuded CAPs but not in the mice exposed to either purified air or denuded CAPs. In a separate experiment apoE-/- mice were exposed to air, undenuded CAPs and the particle free organic compounds (PFO) that were stripped from the CAPs in the thermodenuder and delivered to the exposure system. This study demonstrated that the

organic compounds, independent of the presence of particles, played an active role in the acceleration of plaque development. Cholesterol and low density lipoprotein-cholesterol (LDL) levels were relatively high in the apoE-/- mice, as would be expected. Exposure to undenuded CAPs, denuded CAPs and PFO all induced increased levels of both cholesterol and LDL in the serum of these mice, but only the undenuded CAPs and the PFO caused significant serum lipid peroxidation, which is a known contributor to plaque formation. We therefore conclude that the organic constituents of UFP contribute to the accelerated development of atherosclerotic plaque in arteries, lipid oxidation is an important mechanism of action in PM-induced coronary artery disease, and that removal of the organic compounds from PM greatly ameliorates plaque development associated with air pollutant exposure. These findings suggest that emission control measures that remove and sequester or destroy organic constituents of combustion generated aerosols could benefit public health because coronary artery disease is a leading contributor to heart-related deaths, which represents about 50% of deaths, annually, in California and other states as well.

Executive Summary

Background

Heart disease is the leading cause of death in the U.S. Recent data have indicated that exposure to air pollutants is a risk factor for cardiopulmonary diseases and may represent an important preventable contributor to both morbidity and mortality among populations living in polluted environments. The strong and relatively consistent epidemiological associations between cardiovascular morbidity and mortality may be related to PM-induced oxidative stress and/or inflammation. Epidemiological and in vivo exposure studies demonstrate that exposure to particles (fine and ultrafine) in close proximity to mobile source emissions may be more deleterious to health than are exposures to airborne particles more distant from these mobile source emissions. Our earlier studies [1, 2] of mice exposed to fine and ultrafine PM 50 m downwind of a freeway showed that these exposures had significant biological activity which was associated with elemental and organic carbon fractions of the aerosol.

Ultrafine particles are capable of inducing the greatest amount of pulmonary inflammation per unit of PM mass. This has been attributed to the physical and chemical characteristics of ultrafine particles, including, high particle number, high pulmonary deposition efficiency, and a surface chemistry involving a high surface area that can carry adsorbed or condensed toxic air pollutants (oxidant gases, organic compounds and transition metals). The specific mechanisms by which particulate matter (PM) exposure disrupts cardiac function and worsens cardiovascular disease (CVD) are not well understood. There is a growing body of knowledge that suggests that PM exposure can induce inflammatory changes in blood vessels and exacerbate atherogenesis leading to the development of atherosclerotic plaques and lesions. We initially hypothesized that PM exposure would increase free radical production, contribute to the induction of oxidative stress that could abnormally activate endothelial cells and induce vascular inflammation. This process could then lead to the accelerated formation of arterial plaques, which are a hallmark of atherosclerosis. However, preliminary studies [3]

demonstrated that if the particles were stripped of most of their organic constituents they also lost a substantial amount of their ability to elicit free radicals and they lost their oxidant potential. We therefore designed this study to test a new hypothesis that removal of organic constituents of PM would reduce the particle's ability to induce or accelerate atherosclerosis.

Specific Aims: The objective of this 5-year project was to determine how the atherogenicity of ultrafine particles depends on the concentration and characteristics of semi-volatile and non-volatile fractions of PM emitted from vehicles and other sources. We tested the hypothesis that the atherogenicity of near-source PM was due to nanoparticles that are composed largely of semi-volatile components and that biological activity will be attenuated by removal of those components from the aerosol. We used our *in vivo* rodent exposure system in combination with particle concentrator-thermal denuder technology [3-6] to separately study the cardiopulmonary effects of PM, before and after the thermal device was used to denude (i.e. remove the semi-volatile components from) the particles. Detailed chemical and physical characterizations of concentrated ambient PM (CAPs) and thermally denuded CAPs were conducted by Dr. Sioutas and colleagues.

Methods

The study was performed using mice that were genetically susceptible to the development of atherosclerosis (apoE-/- mice). Groups of 18 mice were each exposed to: (a) undenuded concentrated ambient ultrafine particles (CAPs; $PM_{0.18}$); (b) denuded CAPs; or (c) purified air for 4 days per week, 6 hours per day for 8 weeks. The average exposure concentration was about 58 $\mu g/m^3$, over the total of 192 exposure hours. We examined several factors relevant to mechanisms of atherogenesis and the development of cardiovascular heart disease. Plasma was assayed for total cholesterol and low density lipoprotein cholesterol concentrations and C-reactive protein, which is produced in the liver and is an acute phase protein that increases during systemic inflammation. Assays were also conducted for protein carbonyl content and lipid peroxidation as markers of

oxidative stress and glutathione (GSH), as a measure of antioxidant capacity. We measured the effects of exposure on cardiac function in a subset of the mice that were implanted with cardiographic transponders. Heart rate and heart rate variability (HRV) were determined and HRV was shown to decrease after exposure to undenuded PM. The initial study suggested an important role of the organic constituents of PM, notably significant reductions in atherogenicity and serum lipid peroxidation in animals exposed to denuded PM as opposed to undenuded PM. We therefore conducted a follow-on study to directly examine the effects of the organic compounds stripped from the denuded particles. To accomplish this, the thermal denuder was modified; the activated carbon adsorber was removed, a pre-fired quartz filter was added and apoE-/- mice were exposed to the resulting particle-free organic vapor (PFO), purified air and undenuded PM.

Results

The main findings of this study are that: (1) the VACES and the Dekati Thermodenuder can be used in tandem to deliver undenuded ultrafine ambient PM (UFP, $d_p \leq 0.18$ μm), denuded UFP and PFO (consisting of organic compounds stripped from the PM by the denuder) to genetically modified, apoE-/-, mice in a mobile rodent exposure system; (2) exposures to undenuded PM or to PFO accelerated the development of atherosclerotic plaques and induced decreases in heart rate variability (3) the organic constituents of UFP are important contributors to atherosclerotic plaque development and significantly accelerate the growth of arterial plaques after an 8 week exposure; (4) exposure to both organic and inorganic constituents of UFP raise serum concentrations of cholesterol and low density lipoprotein-cholesterol (LDL), but (5) exposures to denuded UFP (PM denuded of most organic constituents) did not promote serum lipid peroxidation while exposures to undenuded UFP or to PFO did promote serum lipid peroxidation.

Conclusions

This study has demonstrated that the semi-volatile PM fraction of ambient ultrafine particulate matter could be an important contributor to the development of atherosclerosis

and heart disease. PM exposure was also shown to increase serum levels of cholesterol and LDL-cholesterol, both of which are known risk factors for atherosclerosis and heart disease. In this study, exposure to undenuded UFP and to PFO also promoted the peroxidation of serum lipids while exposure to denuded UFP did not. Peroxidation of serum lipids, especially LDL, has been specifically implicated in atherosclerotic plaque formation. Removal of the semivolatile organics by a process of thermal denuding significantly mitigated the adverse effects of acceleration of atherosclerosis and reduced HRV induced by exposures to undenuded PM in a genetically modified animal model of atherosclerosis. We did not determine if these effects would be observed in unmodified, or wild type mice.

Chemical characterization of the ambient PM using a high resolution aerosol mass spectrometer showed that the UFP fraction, particles with diameters ≤ 0.18 μm, was more enriched in organic compounds that were less oxygenated, hence less polar, than particles with diameters ≥ 0.18 μm. Studies of mobile source emissions have shown that emissions from heavy duty diesel vehicles are enriched in UFP and that those UFP are characterized by low oxygenation levels which are less lipophilic and more nonpolar. This is important because organics that are nonpolar are better able to cross cell membranes than are more polar molecules and once inside the cell have the potential for toxic interactions with critical cell components.

An important corollary to these results is that they suggest that removal of UFP-associated semivolatile compounds could significantly mitigate adverse cardiovascular effects. Modern diesel engines are often provided with emission control technologies that remove effectively the non-volatile fraction, but not necessarily the volatile fraction. Some research has shown that removal of the non-volatile PM fraction can increase the concentration of the volatile fraction by enhancing nucleation of condensing organic vapors. This project has provided findings that improve our understanding of the mechanism of toxic action of freshly-emitted combustion aerosols and has identified organic constituents of ambient aerosols as being causally related to potential health effects. This information will also aid regulators and planners in developing air quality

mitigation strategies and land use guidance to better protect the health of California residents.

Cardiopulmonary Health Effects: Toxicity of Semi-Volatile and Non-Volatile Components of PM

Introduction

Heart disease is the leading cause of death in the U.S and is responsible for nearly 50% of mortality for all non-accidental causes. Recent data have indicated that exposure to air pollutants is a risk factor for cardiovascular disease and may represent an important preventable contributor to heart-related morbidity and mortality among populations living in polluted environments [7-9]. There are strong and relatively consistent mechanistic associations between cardiovascular morbidity or mortality and oxidative stress associated with inflammation [7, 10]. Ultrafine particles are capable of inducing pulmonary inflammation due to their physical and chemical characteristics[11], including, high particle number, high pulmonary deposition efficiency, and a surface chemistry involving a high surface area that can carry adsorbed or condensed toxic air pollutants (oxidant gases, organic compounds and transition metals). These PM components have been identified as having pro-inflammatory effects[12]. A seminal series of studies performed by Chen, Lippmann and colleagues at New York University (NYU) demonstrated that exposure to concentrated ambient fine ($PM_{2.5}$) particles in Tuxedo NY could exacerbate the development of atherosclerotic lesions in mice that were genetically predisposed to abnormal lipid metabolism [13, 14]. Studies conducted in Los Angeles demonstrated that UFP were more potent than fine (PM2.5) particles with respect to accelerating development of atherosclerotic plaques in apoE-/- mice [15, 16]. The specific mechanisms by which particulate matter (PM) exposure disrupts cardiac function and worsens cardiovascular disease (CVD) are still under investigation, however there is a growing body of knowledge that suggests that PM exposure can oxidize circulating lipoproteins and induce inflammatory changes in blood vessels leading to the development of atherosclerotic plaques and lesions [17]. Epidemiological and *in vivo*

exposure studies demonstrate that particles (fine and ultrafine) in close proximity to mobile source emissions can affect cardiopulmonary health to a greater degree than particles in the air more distant from the source [2, 18, 19]. Our earlier studies of mice exposed to fine and ultrafine PM 50 m downwind of a freeway showed that these exposures had significant biological activity which was associated with elemental and organic carbon fractions of the aerosol. When exposures were performed 150 m downwind of the freeway the biological activity was greatly diminished and there were no measurable exposure-related effects [1, 2]. Careful measurements in a roadway tunnel [20] and near a major freeway with heavy-duty diesel traffic [21-23] demonstrated that there were rapid shifts in aerosol size and composition within minutes of emission and that the volatility of the PM increases with decreasing particle size. We initially hypothesized that PM exposure would increase free radical production, contribute to the induction of oxidative stress thus abnormally activating endothelial cells thereby inducing vascular inflammation leading to the accelerated formation of arterial plaques which are a hallmark of atherosclerosis. Sioutas and colleagues developed the capacity to couple a thermal denuder to a Versatile Aerosol Concentration Enrichment System (VACES) [3]. The thermal denuder heats the aerosol to a specified temperature to evaporate and remove semi-volatile components, and then returns the aerosol to the original temperature. The VACES can increase the concentration of the processed aerosol by factors of 20 to 30 to provide adequate concentrations for performing acute *in vivo* toxicology exposure studies. Preliminary studies demonstrated that if the particles were stripped of most of their organic constituents they also lost a substantial amount of their ability to elicit free radicals and they lost their oxidant potential [3]. Using this VACES/denuder system, combined with our mobile rodent field exposure/cardiac monitoring unit, we were able to systematically examine the role of the semivolatile components of PM on heart function, plaque development and cardiac physiology. We therefore designed this study to test the hypothesis that removal of organic constituents of PM would reduce the particle's ability to induce or accelerate atherosclerosis. This research examined the link between particle-induced inflammation and the development of atherosclerosis in atherosclerosis-prone mice and used a thermal denuder coupled with a particle concentrator and mobile

exposure system to specifically address the question "Do semivolatile organic constituents of UFP play an important role in the PM-induced acceleration of atherosclerotic plaque development?" In addition, we examined oxidative stress and inflammation-associated biomarkers to determine the relative importance of these modes of action in the development or exacerbation of cardiovascular disease. Improved understanding of the roles of these specific modes of action could lead to improved techniques for preventing or treating heart diseases caused by environmental contaminants.

Methods and Materials

Experimental Techniques

Selection and Characteristics of the Animal Model

The transgenic mouse model of cardiovascular disease that we proposed to use in this study was developed from the C57BL/6 mouse. The C57BL/6 strain lacking the apolipoprotein E receptor (apoE-/-) has been shown to be particularly susceptible to cardiovascular effect but is also subject to adverse pulmonary effects from a variety of inhaled substances including O_3 [24], acid-coated carbon particles [25], ovalbumin [26] and concentrated pseudo-ultrafine particulate matter ($PM_{0.18}$) [15].

Animal Husbandry

Male apoE-/- 6 week old mice were purchased from a commercial supplier and housed two to a cage in AAALAC accredited animal housing facility at the Air Pollution Health Effects Laboratory (APHEL). All animals used in this study were apoE-/- mice. The mice that were implanted with telemetry devices were housed singly so that ECG parameters could be monitored while the mice were in the vivarium. Animals were provided with food and water ad libitum. Animals were transported to the exposure site [2] near the campus of USC in Los Angeles using a van and customized

transport/exposure modules. During transport the animals breathed filtered, purified air. Telemeter-equipped mice were monitored during exposures and while they were in the vivarium, but were not monitored during transport. On the average, mice were monitored about 20 hr per day. When ECG data were evaluated the data from the same time periods were evaluated on exposure and non-exposure days so the unmonitored transport time did not impact our analyses.

Ambient Particle Concentrator

Ambient particles with particle diameters smaller than 0.18 μm were concentrated using the Versatile Aerosol Concentration Enrichment System (VACES) which has been described in detail by Kim et al. [4, 5]. VACES consists of a size selective inlet, a saturator/chiller module that supersaturates the aerosol with water vapor causing fine and ultrafine particles to grow to a size that can be inertially separated using a virtual impactor, and a diffusion drier module that removes the excess water vapor and returns the aerosol to a size distribution that is very close to that in the unconcentrated ambient air. The system is mobile and capable of enriching the concentration of particles in the range of 0.03- 2.0 μm by up to a factor of 30 x ambient, depending on the output flow

rate [27]. The concentration efficiency falls off above 2.0 μm or below 0.03 μm.

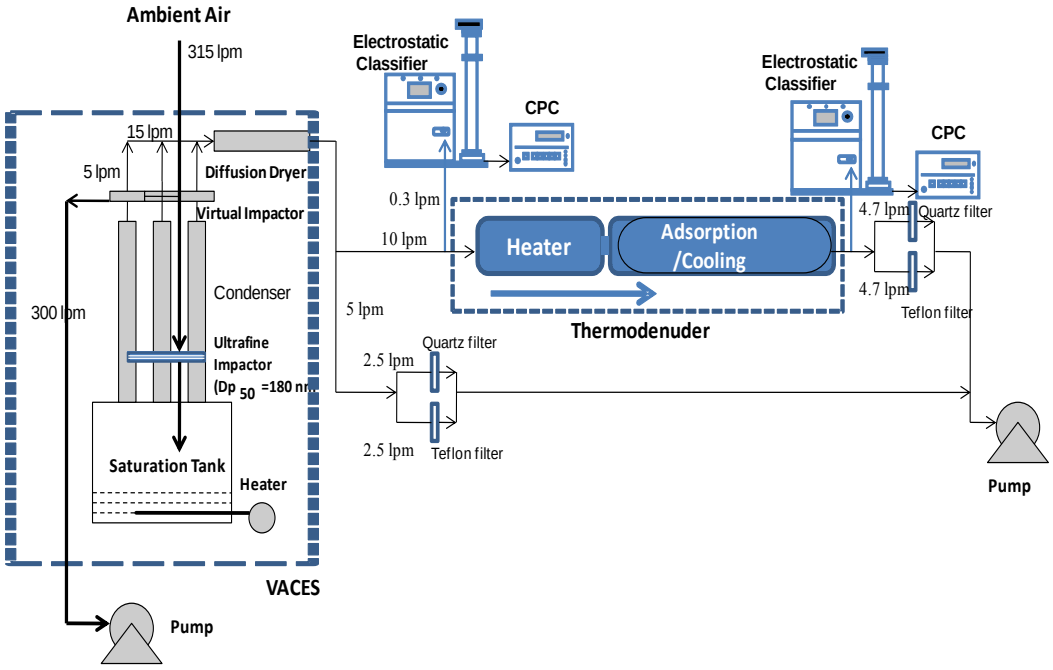

Figure 1. Exposure System for Comparing the Effects of Denuded and Undenuded Ambient Particles.

To determine the role of semivolatile components of ultrafine particles on cardiovascular disease, the VACES was coupled to a Dekati thermodenuder which is designed to remove volatile and semivolatile organic compounds from sample ambient particles [3]. This allowed us to compare the effects of denuded and undenuded particles on cardiac physiology and the development of atherosclerotic plaque. In a subsequent exposure, we modified the thermodenuder, by removing the activated carbon annular denuder and replacing it with a pre-fired quartz filter, to allow mice to be exposed to the vapor and semivolatile organic components in the absence of particles (PFO) and to allow us to compare the effects of undenuded particles on mice to effects of mice exposed to PFO.

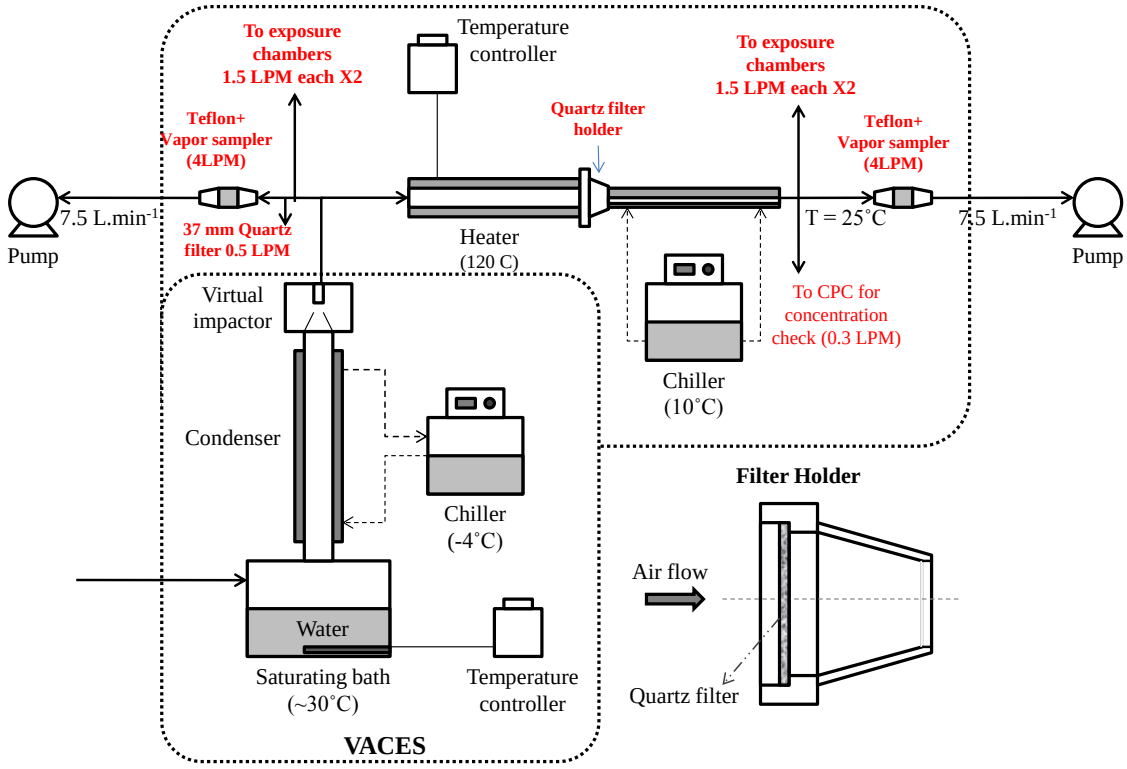

Figure 2. Schematic Diagram of VACES and Modified Dekati Thermodenuder for Comparing Particle Free Organic Vapors and Semivolatiles with Undenuded Ultrafine Particles.

Exposure Chambers

A whole-body exposure mouse chamber (Figure 3) was designed specifically for use with the VACES. Each stainless steel (SS) chamber (20 inches X 12 inches X 6 inches) was segmented into 18 cubicles (1 mouse per cubicle) separated by perforated SS sheets (0.078" hole diameter, 36% open and staggered, (McMaster-Carr, New Brunswick, NJ). Concentrated ambient particles (CAPs) were delivered through SS particle delivery tubes that distributed CAPs uniformly throughout the exposure chamber [28]. A raised sub-floor constructed from perforated SS sheet (0.25" hole diameter, 50% open, staggered) was used, which permitted urine and feces to fall to the bottom of the vat and kept the mice relatively clean. The exposure atmosphere was exhausted from below the sub-floor through 2 SS tubes, each 40 cm in length with 28 0.5 mm downward-facing holes. Absorbent sheets impregnated with an antibiotic to prevent fecal bacteria from generating

ammonia from urine were placed under the exhaust lines to absorb urine and to collect feces.

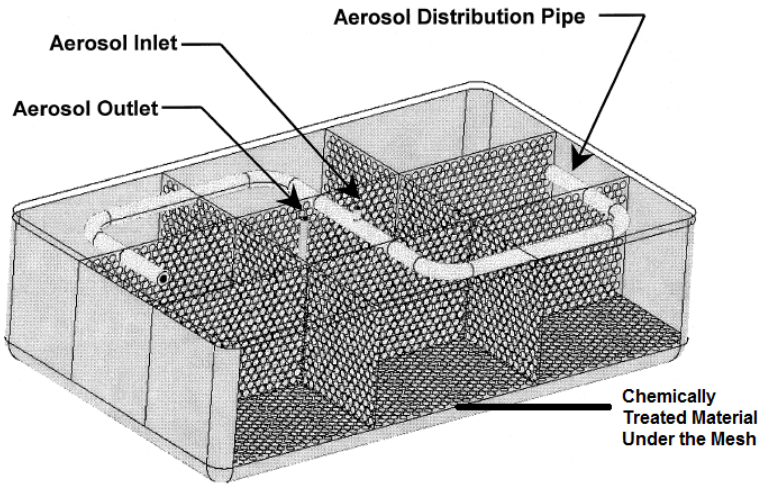

Figure 3. Drawing of the mobile exposure cage showing the rectangular stainless-steel pan (50 cm length × 27 cm width × 15 cm height), perforated stainless-steel floor, partitions, copper aerosol inlet and distribution pipe, and copper aerosol outlet. The aerosol return line below the floor is not shown. The aerosol inlet is designed to connect to the outlet of the VACES aerosol concentrator.

Exposures

During exposures, we monitored concentrations of ambient and CAPs particles. Samples were also collected on quartz filters that were pre-treated at 400°C to remove adsorbed organic compounds. These filters were composited on a weekly basis and analyzed for elemental carbon (EC) and organic carbon concentrations (OC). EC is a reasonable tracer for particles originating from diesels [29] and represents between 5 - 20% of the UFP in ambient samples. EC and OC were measured by a thermal photometric method on a fraction of the filter; the remaining fractions were stored (-80º C) and subsequently analyzed for organic and inorganic constituents. Particles were also collected for mass concentration and chemical analyses on pre-weighed fluorocarbon filters. Following collection, the filters were equilibrated overnight at constant humidity and weighed. These filters were submitted for ICP/MS analysis of elemental constituents, including Fe, V, Zn, Cr, Ni, Cu, Pb and Mn, among many others (the entire

list is seen in Table 1). These specific metals were identified because previous inhalation or *in vitro* studies have shown them to be potentially toxic [30-36]. We plan to examine possible links between these metals and health-related outcomes in the future. Some of the other metal and non-metal constituents that were measured may also be evaluated (e.g. As, Se).

In order to put the chemistry of ambient particles into even better perspective, we were able to perform a limited number of measurements using a High Resolution Time of Flight aerosol mass spectrometer (AMS), shown schematically in Figure 4.

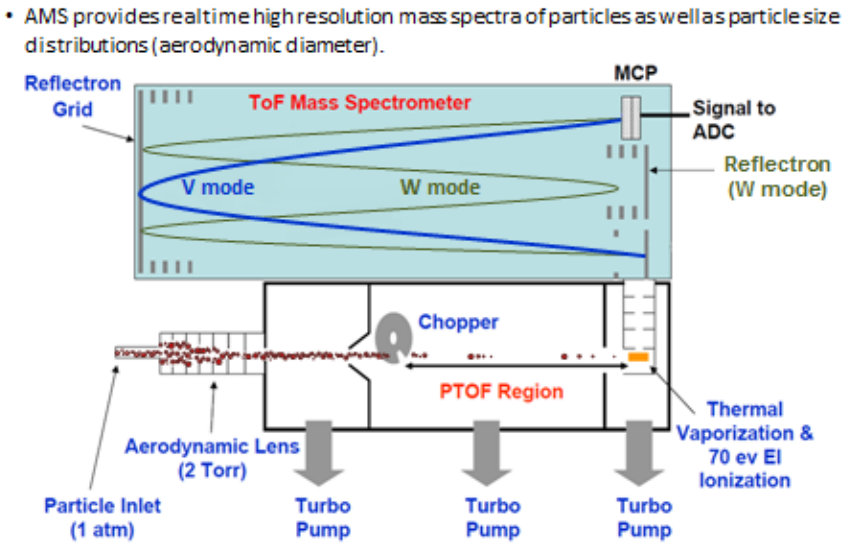

Figure 4. High Resolution Time-of-Flight Aerosol Mass Spectrometer

Particles enter the inlet and are gated into an aerodynamic lens that accelerates the particles through a chopper that limits the number of particles entering the particle time of flight (PTOF) region. Particle size can be determined by measuring the time of flight between the gate and the particle's impaction onto the thermal vaporization and ionization unit. The ionized vapor material is then accelerated and ionized to molecules

in the TOF Mass Spectrometer and mass to charge ratios are compared with an internal library to provide molecular identities.

Exposure Procedure

Animals were housed at UC Irvine (UCI), transported daily to Los Angeles while breathing purified air and exposed. At UCI the mice were housed in ventilated caging attached to an air purification system. The air purifier delivered filtered air at flows adequate to provide 15-20 air exchanges per hour in each ventilated cage unit. The vivarium was supplied with Class 100 filtered air using a laminar flow air purifier that consisted of a 1000 CFM blower, an oxidizing adsorbent canister containing permanganate-impregnated alumina spheres, and a high efficiency particle air (HEPA) filter system. Between exposures the animals were supplied with purified air, clean water and food, ad lib. During the first set of exposures, groups of 18 animals were placed into sealed, compartmentalized exposure chambers and were each exposed to undenuded concentrated ultrafine particles (CAPs) or to CAPs from which semivolatile components were removed using the Dekati thermal denuder (denuded CAPs) for 6 hours per day, 4 days per week for 8 weeks. During the second set of exposures, groups of 18 animals were each exposed to particle free vapor removed from the CAPs using the Dekati thermal denuder or to undenuded concentrated ultrafine particles for 6 hours per day, 4 days per week for 8 weeks. Control animals in each set of exposures received purified air under conditions identical to those of the animals exposed to CAPs. Temperature was monitored continuously during the exposures and held to 75 ± 5 °C. Animals were observed throughout the experiment for signs of distress (*e.g.* changes in grooming, food and water uptake, shaking). The Versatile Aerosol Concentrator for Exposure Studies (VACES) designed by Sioutas and colleagues at USC [4, 5, 27, 37, 38] was used for the PM exposures. This device has been used by UCI for exposures of mice near freeways in Los Angeles [39]. The animals were euthanized 24 hr after the last exposure on the eighth week of the study.

Bioassay and Data Analysis Methods

Blood: serum samples were collected from each animal from the descending aorta for cytokine and circulating biomarker levels.

Heart: A sample of the heart was collected and frozen for subsequent gene expression analyses. The remaining tissue was fixed for subsequent morphological study.

Arteries: arterial tissue was coronary arteries and the aorta for histological evaluations of plaque and biochemical assays for oxidative stress and plaque lipid analyses.

At sacrifice, the mice were euthanized with an overdose of pentobarbital and the heart and aorta perfused with 4% paraformaldehyde. The heart and thoracic and abdominal aorta were removed en bloc. Sections of the aorta were obtained from the proximal, central and distal areas, snap frozen and stored in liquid nitrogen. These samples were homogenized and used for assays of gene expression, and for analyses of biomarkers of vascular inflammation and oxidative stress. The remaining proximal and distal aorta segments were cut longitudinally. One section was frozen in O.C.T. embedding medium (Sakura Finetek, Torrence, CA) and reserved for subsequent laser capture microdissection and genomic and proteomic analysis. The remaining aorta sections were fixed in buffered formaldehyde and examined for total atherosclerotic lesion areas, lipid contents, and cellularity [40]. A section of the heart was removed, snap-frozen in liquid nitrogen and reserved for gene expression analysis. The remaining tissues were fixed in 4% paraformaldehyde. The fixed cardiac tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin for subsequent histological analysis.

Vascular and Cardiac Histology

All morphological assessments were done blind (i.e. without knowledge of the treatment group). Fixed tissues were embedded in paraffin and sectioned at 5 μm. Samples were stained (H&E) and assessed using an optical microscope.

Inflammatory and anti-inflammatory cytokines and markers of vascular inflammation

Sera were analyzed for inflammatory and oxidative stress cytokines and acute phase proteins using a state of the art multiplexed bead assay system (Luminex) as well as adaptations of more conventional enzyme linked immunosorbant assay systems (ELISA). The Luminex system uses fluorescently tagged beads to which antibodies to specific proteins are attached. After incubation with the sample, the beads are incubated with detection antibodies and then analyzed using a fluorescent flow sorting system (similar in working principle to flow cytometry) and the data are recorded and quantified using an online computer. Assays also included arterial disease biomarkers (total cholesterol, low density lipoprotein cholesterol (LDL) and C-reactive protein (CRP)) and arterial wall oxidative stress indicators (protein carbonyl content (PCC), glutathione (GSH), and lipid peroxidation). Total glutathione (GSH) was measured as an indicator of antioxidant capacity using an enzymatic recycling method [41]. Malondialdehyde (MDA) was measured as an indicator of lipid peroxidation using a colorimetric assay [42, 43]. Protein carbonyl content was measured with a fluorometric assay [44] as an indicator of protein oxidation.

Electrocardiographic (ECG) telemetry

The protocol for surgical implantation of telemetry devices (PhysioTel Telemetry system, Data Sciences International, St. Paul, MN) to measure biopotential (ECG tracings), temperature, and physical activity in mice has been previously described [45, 46]. Aseptic techniques were used throughout the implantation procedure.

ECG Data Analysis: The DataQuest A.R.T. system was used to detect, collect and analyze biopotential, body core temperature and activity telemetry signals from each animal. The acquisition program interfaced with a receiver that was tuned to each animal's implanted ECG telemetry device. At the start of our field study, data was sampled each day of exposure for 15 min. before, for 5 min every 30 min during the 6 hour exposure period and 15 minutes post-exposure. As the project progressed, we were able to expand the monitoring to include monitoring overnight while the animals were housed in the vivarium. The acquisition program automatically cycled through the

animals, and acquired data for 5 min out of every 30 min in groups of 4 mice at a time. ECG waveforms were stored on a dedicated computer for subsequent analysis and analyzed to determine heart rate variability (HRV). Changes in HRV may represent alterations in autonomic control of cardiac function [47]. Reduced HRV in humans can represent an adverse effect. Analysis of the ECG waveform was used to extract measures of HRV (the magnitude of variance explained (power) in the heart's rhythm across different frequency bands (spectra) of periodic oscillations in heart rate). Portions of these spectra reflect different autonomic influences on heart rate and blood pressure (BP) [48]. The high frequency (HF) band (1.5 – 5.0 Hz) of the heart period power spectrum has been used to estimate cardiac vagal control [49]. HRV in this band is linked to respiratory influences and has been referred to as "respiratory sinus arrhythmia" [50]. Heart period oscillations at lower frequencies (LF, 0.1 to 1.5 Hz) are less well understood. They may represent mixed sympathetic-parasympathetic and thermoregulatory influences [51-53].

Results

Concentration and Composition of Undenuded and Denuded Ultrafine PM

The results of compositional analyses of the denuded and undenuded particles are detailed in Table 1. The total undenuded particle concentration was 58 µg/m^3, however about 49% of the mass was lost after thermally denuding the aerosol at 120°C, i.e. the denuded particle mass concentration was 28.6 µg/m3. Organic carbon represented about 44% of the total mass of the undenuded particles (25.4 µg/m^3) and approximately 35% of the organic carbon mass was lost during the denuding process. The residual organic carbon however still represented about 31% of the mass of the denuded particles. These data are consistent with our results which showed that the mass of organic compounds stripped from the particles during the denuder process was increased as the temperature of the denuder was increased. Why did we select 120°C for the denuder temperature for this study? This was done because at that temperature we were able to demonstrate that there was no nucleation of new particles downstream of the heated zone of the denuder.

The concentrations of chemical components in the particulate phase, before and after denuding, were contrasted using a 2-sample t-test. As expected, elemental carbon was conserved during the denuder process, *i.e.*, EC concentrations before and after denuding were not significantly different ($p \geq 0.05$). Water soluble organic carbon was significantly reduced after denuding ($p \leq 0.01$). Ions (Cl^-, $SO_4^=$ and $NH4^+$) were conserved during the denuding process, however there were significant (40%; $p \leq 0.05$) losses of NO_3^-, which was not unexpected. The particles contained small amounts of polycyclic aromatic hydrocarbons (PAHs). While concentrations of phenanthrene, anthracene and fluoranthene were not significantly changed by the denuding process ($p \geq 0.05$), pyrene was reduced by 29% ($p \leq 0.01$) and 87% or more of the benzo-addition compounds of fluoranthene, pyrene and anthracene were lost ($p \leq 0.01$). There were small, mostly non-significant ($p \geq 0.05$) changes in the concentrations of alkali metals (Li, Na and K) after denuding. Concentrations of some of the common metals (Al, Cr,Fe, Co, Cu, Sn, and Pb) were slightly, but significantly reduced, but on the average, 55% was retained in the particle phase after denuding.

Table 1. Chemical Composition of Exposure Atmospheres (Undenuded s. Denuded)

		Particle Composition				Ratio of Denuded/Undenuded
		Total UFP (Undenuded)		**Thermal denuded (Denuded)**		
		Concentration Mean	Uncertainty S.E.	Concentration Mean	Uncertainty S.E.	
Total UFP, µg/m3		58.20		28.65		49%
ECOC, µg/m^3	OC	25.382	1.522	8.826	0.716	35% **[1]
	EC	1.472	0.371	2.008	0.392	136% ns
	TC	26.855	1.724	10.834	0.953	40% **
WSOC µg/m^3		5.701	0.219	2.322	0.142	41% **
IC, µg/m^3	Chloride	0.027	0.039	0.100	0.041	368% ns
	Nitrate	0.359	0.049	0.214	0.037	60% *
	Sulfate	1.959	0.206	2.401	0.250	123% ns
	Ammonium	1.372	0.145	1.171	0.126	85% ns
	Potassium µg/m3					
	Sodium µg/m3					
	Phosphate µg/m3			0.119	0.086	
H, ng/m^3	Naphthalene					

[1] ns = non significant ($p \geq 0.05$); * $p \leq 0.05$; ** $p \leq 0.01$

	Acenaphthylene					
	Fluorene					
	Phenanthrene	0.250	0.071	0.216	0.064	86% ns
	Anthracene	0.023	0.020	0.075	0.026	320% ns
	Fluoranthene	0.142	0.035	0.108	0.030	76% ns
	Pyrene	0.326	0.071	0.095	0.029	29% **
	Methylfluoranthene		0.018		0.018	
	9-Methylanthracene		0.018		0.018	
	Benzo(ghi)fluoranthene		0.018		0.018	
	Cyclopenta(cd)pyrene		0.018		0.018	
	Benzo(a)anthracene		0.018		0.018	
	Chrysene		0.018		0.018	
	1-Methyl chrysene		0.018		0.018	
	Retene		0.018		0.018	
	Benzo (b)fluoranthene	0.912	0.183	0.028	0.019	3% **
	Benzo (k)fluoranthene					
	Benzo(e)pyrene	0.765	0.154		0.018	0% **
	Benzo(a)pyrene	0.788	0.159		0.018	0% **
	Perylene		0.018		0.018	
	Indeno(123-cd)pyrene	0.096	0.027		0.018	0% ns
	Benzo(ghi)perylene	0.216	0.047	0.028	0.019	13% **
	Dibenzo(ah)anthracene	0.023	0.019		0.018	0% ns
	Picene		0.018		0.018	
	Coronene	0.204	0.060	0.022	0.031	11% **
	Dibenzo(ae)Pyrene					
Trace Element	Li7	0.162	0.020	0.289	0.045	179% *

	B11	3.681	0.096	2.137	0.094	58% **
	Na23	55.537	6.811	82.356	10.017	148% ns
	Mg25	20.678	2.057	14.998	1.904	73% ns
	Al27	256.878	17.390	106.785	9.682	42% **
	P31	12.968	1.024	9.416	1.354	73% ns
	S34	890.648	80.678	298.446	54.494	34% **
	K39	35.409	7.619	54.840	11.405	155% ns
	Ca44	39.716	4.899	85.710	14.913	216% *
	Sc45	0.009	0.005	0.011	0.005	125% ns
	Ti49	22.059	1.607	13.202	1.814	60% **
	V51	5.009	0.302	16.306	1.323	326% **
	Cr52	6.315	0.336	4.299	0.342	68% **
	Mn55	6.716	0.295	6.864	0.463	102% ns
	Fe57	320.185	14.725	175.638	14.212	55% **
	Co59	2.902	0.127	1.985	0.184	68% **
	Ni60	6.528	0.517	9.276	1.079	142% ns
	Cu63	17.661	0.695	11.119	0.809	63% **
	Zn66	16.620	1.413	24.472	2.867	147% ns
	As75	6.258	0.594	6.541	0.765	105% ns
	Se82	0.795	0.185	0.356	0.213	45% ns
	Rb85	0.119	0.012	0.180	0.035	151% ns
	Sr88	0.632	0.052	0.825	0.085	131% ns
	Y89	0.064	0.005	0.023	0.004	35% **
	Nb93	0.043	0.005	0.033	0.008	76% ns
	Mo95	3.304	0.255	2.643	0.448	80% ns
	Rh103	0.001	0.001	0.001	0.001	90% ns

	Pd105	0.017	0.008	0.013	0.010	78% ns
	Ag109	10.785	2.531	3.665	1.219	34% ns
	Cd111	0.094	0.011	0.135	0.028	144% ns
	Sn118	20.364	1.178	10.916	1.440	54% **
	Sb121	1.460	0.113	1.381	0.175	95% ns
	Cs133	0.007	0.002	0.010	0.004	150% ns
	Ba138	7.668	0.399	7.866	0.611	103% ns
	La139	0.402	0.023	0.252	0.018	63% **
	Ce140	0.392	0.017	0.242	0.020	62% **
	Pr141	0.019	0.001	0.010	0.002	52% **
	Nd143	0.059	0.005	0.028	0.004	46% **
	Sm147	0.013	0.003	0.008	0.001	61% ns
	Eu151	0.006	0.001	0.007	0.001	127% ns
	Dy163	0.006	0.001	0.003	0.001	54% ns
	Ho165	0.001	0.001	0.001	0.000	88% ns
	Yb173	0.003	0.001	0.001	0.001	41% ns
	Lu175	0.001	0.000	0.001	0.000	96% ns
	W184	0.209	0.009	0.200	0.009	95% ns
	Pt195	0.007	0.002	0.004	0.002	59% ns
	Tl205	0.005	0.001	0.008	0.001	173% ns
	Pb	7.264	0.401	4.856	0.384	67% **
	Th232	0.016	0.003	0.010	0.002	62% ns
	U238	0.010	0.001	0.006	0.001	59% *

High Resolution Aerosol Mass Spectrometer Results

Some results are shown in Figure 5. While these data were obtained for concentrated PM in Irvine, CA rather than Los Angeles, CA the observed results are quite consistent with the characterizations conducted on the LA aerosol [3].

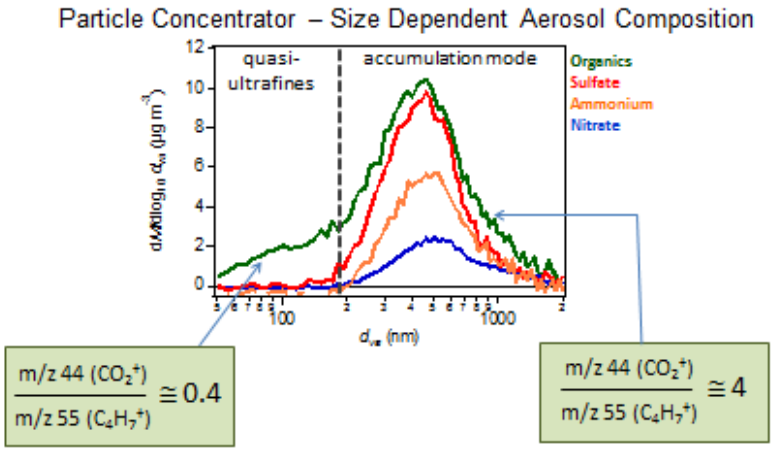

Figure 5. Particle Size Dependent Composition of Concentrated Ambient Particles.

The particles in the "accumulation" mode (≥ 0.18 µm diameter) are comprised of organic compounds, sulfates, nitrates and ammonium ions. The particles smaller than 0.18 µm diameter (the particle size range used in our comparison of denuded vs. undenuded particles) are more highly concentrated in organic compounds. Another interesting contrast is that the organic constituents of the accumulation mode particles are nearly 10 times more oxygenated (based on the mass/charge ratios for marker oxygenated and non-oxygenated ions) than are the organics from the ultrafine particles. This is consistent with studies of emissions from diesel engines which are reported to be major sources of ambient ultrafine particles.

As shown in Table 1, the denuder process significantly alters particle mass and composition. These changes are more apparent when one changes the denuder temperature. In Figure 6 changes that are observed when particles are denuded at 50°C, 100°C and 200°C, both in composition and oxidation potential, as measured using the dithiothreitol (DTT) assay method. The data are adapted from Verma et al. [54]

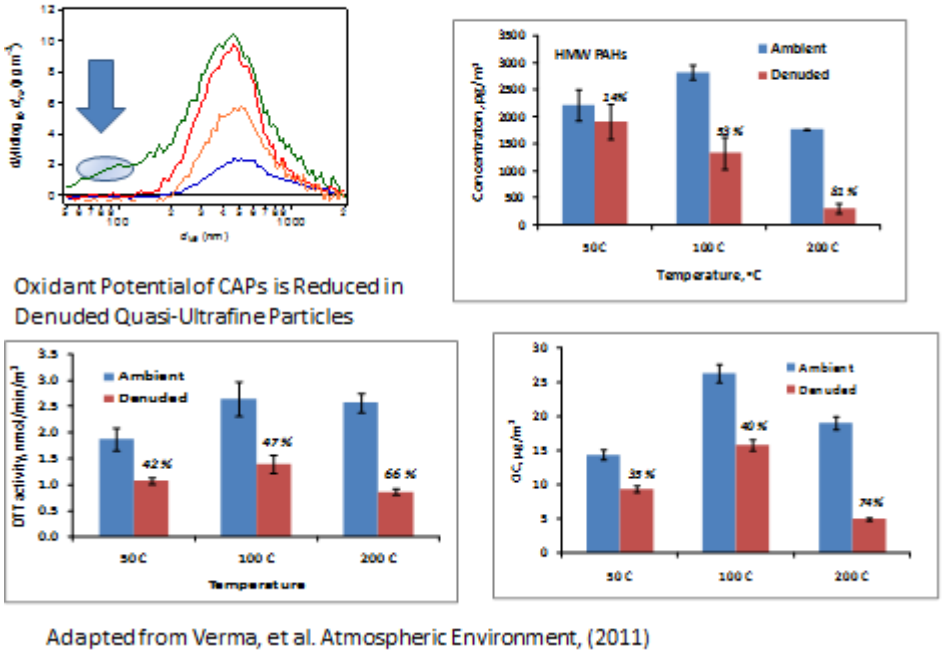

Figure 6. Progressive Losses of Organic Constituents and Oxidant Capacity with Increased Denuder Temperature

Atherosclerosis Development after Exposure to Denuded and Undenuded Particles

Figure 7 summarizes the total plaque areas in coronary arteries after exposure to air, undenuded concentrated ultrafine particles (total UF) and the non-volatile particles from denuded UF (NVUF). The upper panels show the average wall thickness of the aortic arch and A1 measured from histological cross-sections of each. The middle and lower panels show aortic plaque areas and lipid content also measured from histological sections with lipids stained by Oil Red-O.

Aortic arch wall thickness was significantly greater in mice exposed to undenuded PM compared to air exposed mice. There was no difference at the $p \leq 0.05$ level in aortic arch wall thickness between mice exposed to denuded PM and air. A1 wall thickness was not significantly different between the three exposure groups.

Plaque development, by plaque area and lipid content measures, was accelerated in the mice exposed to undenuded PM compared to either that in mice exposed to air or denuded PM. The difference was significant for the difference between denuded and undenuded PM exposures, but the differences between air and undenuded PM exposures were not significant at the $p \leq 0.05$ level.

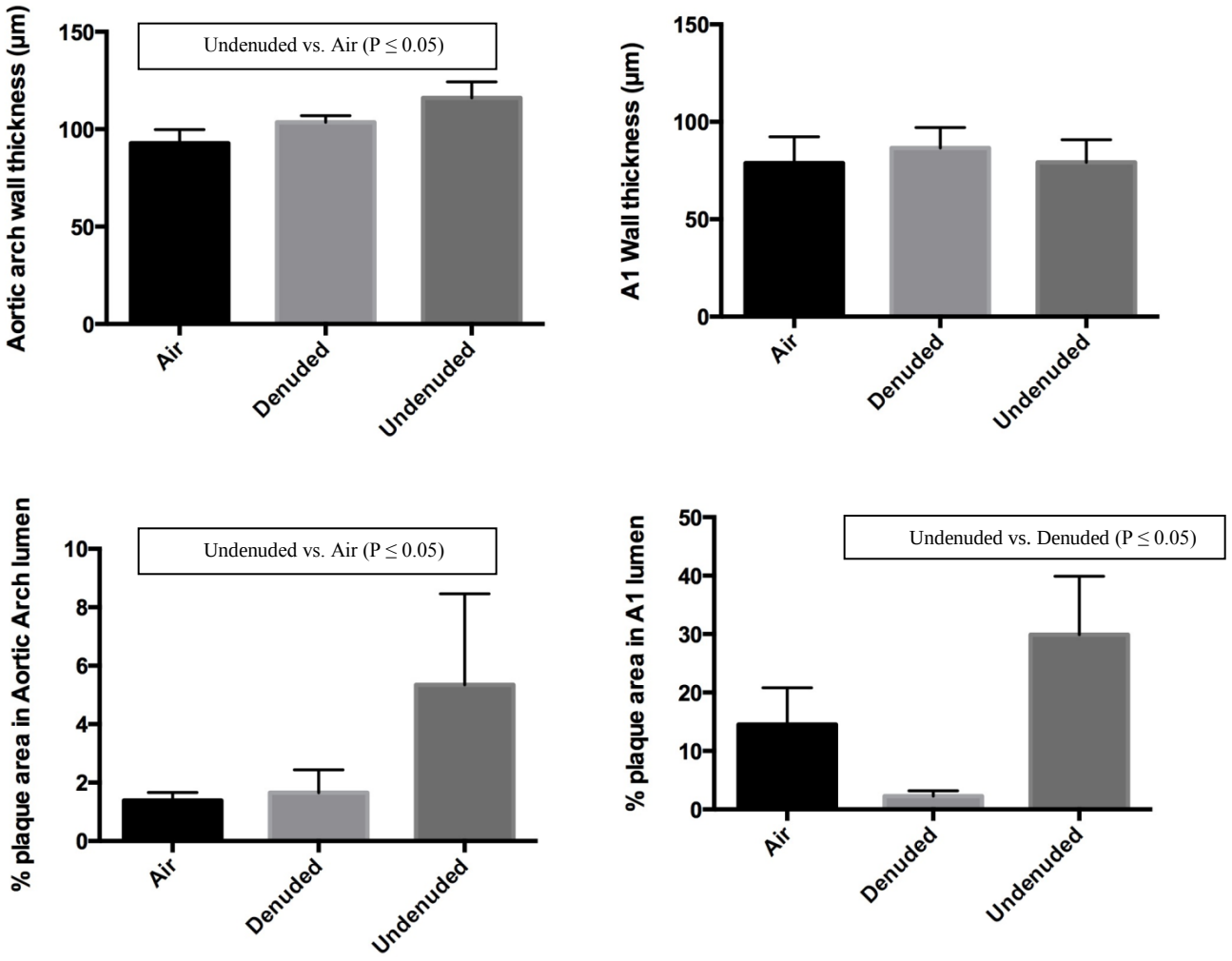

Figure 7. Arterial Plaque Development is Accelerated in Arteries from Mice Exposed to Concentrated UF Particles Compared to Plaque Development in Mice Exposed to Purified Air or the Non-volatile Particles Remaining After Thermal Denuding.

The panel on the left in Figure 8 are a measured lipid content of homogenized samples of the aortic arch from mice exposed to purified air, total (undenuded) PM and the non-volatile (denuded or NV) particles. There is a significant increase in lipid content in the mice exposed to undenuded PM

compared to that in mice exposed to denuded PM. The panels on the right are a series of stained histology sections of the aortic arch and the C1 coronary artery from mice exposed to air, denuded and undenuded CAPs. Nearly all the mice had some occlusion of the C1 artery, even those exposed to purified air. However the occlusion in mice exposed to undenuded CAPs was considerably more serious than for mice exposed to either air or undenuded CAPs.

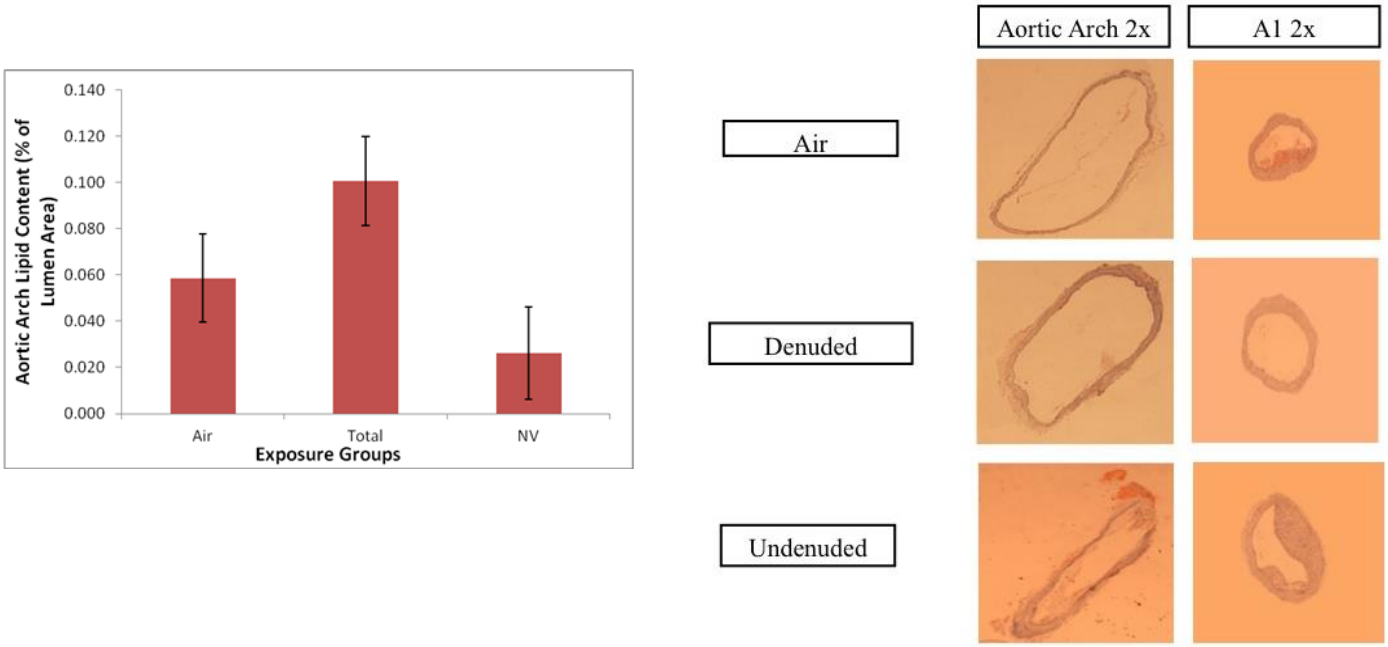

Figure 8. Lipid content of aortic arch samples (Air = Purified Air, Total = Undenuded PM and NV = Denuded PM. These can be compared with stained histology sections of the aortic arch and the C1 coronary artery from mice exposed to purified air, denuded and undenuded CAPs.

ECG Assessments

As mentioned earlier, the analysis of the power spectrum of changes in heart rate frequency is a useful measure of heart rate variability (HRV) which reflects an influence of the autonomic nervous system on heart work. In humans, the ratio between low and high frequency components (LF/HF ratio) of HRV spectra may represent a measure of sympatho-vagal balance. As shown in Table 2 the LF/HF ratio in mice exposed to concentrated, undenuded PM was significantly greater than the ratios in mice exposed to either purified air or denuded PM. The results were normalized with respect to baseline measurements in these mice measured during a 1 week "run-

in" period to compensate for individual group mean differences between the mice. These results suggest that, in addition to accelerating the development of arterial plaque, exposure to undenuded ambient particles induce changes in autonomic control of cardiac physiology.

Table 2. Effects of Denuded and Undenuded PM on Heart Rate Variability (Indexed as % Change From Baseline)

LF/HF Ratio (mean ± SE)			
Week	Purified air	Denuded	Undenuded
1	22.5±5	6.3±5	22.5±5
2	39.4±3	10.4±6	39.4±3
3	16.4±5	16.4±5	57.5±3
4	39.0±2	20.7±6	57.8±9
5	14.7±5	14.7±8	57.4±3
6	13.3±5	31.0±3	45.3±6
7	35.8±7	7.4±6	55.6±5
8	44.9±12	-8.4±40	44.9±12
Average ± S.E.	28.3 ± 4.5	12.3 ±4.1	47.6 ±4.4
One-Way Anova Results	I----------I Air vs Denuded $P \geq 0.10$		I----------I Denuded vs. undenuded $P \leq 0.01$

During the course of the study Dr. Sioutas and his team developed a unique modification of the thermodenuder which allowed us to directly test the hypothesis that the semivolatile components of PM were active toxins that promoted atherosclerotic plaque development. In the modified thermodenuder the activated carbon annular trap was replaced with a quartz filter

which removed all of the particles but permitted free passage to the volatilized organic compounds on a dynamic basis. Accordingly, apoE-/-mice were exposed to purified air, particle free organic (PFO) compounds that were stripped from the denuded particles and to undenuded particles. The HRV results are summarized in Table 3. Exposure to PFO produced a significant increase in the LF/HF ratio compared to both purified air-exposed and denuded PM-exposed mice. The undenuded PM also increased the LF/HF ratio compared to purified air, but the increase was not statistically significant at the $p \leq 0.05$ level.

Table 3. Effects of Particle Free Organic (PFO) compounds and Undenuded PM on Heart Rate Variability (Indexed as % Change From Baseline ± SE)

LF/HF Ratio			
Week	Purified air	PFO	Undenuded
1	-6.1±5	12.6±17.8	4.2±17.8
2	6.9±2	-0.2±16.6	6.9±10.5
3	14.5±10	26.75±19.0	-5.8±7.6
4	3.6±2	49.5±24.8	16.8±6.3
5	7.6±1	24.0±15.3	7.6±22.6
6	-0.5±5	33.7±19.7	18.8±12.3
7	5.4±1	51.1±27.2	18.2±18.3
8	-7.6±8	62.3±33.6	21.7±24.3
Average ± S.E.	3.0 ± 2.6	32.4 ± 7.4	11.1 ± 3.2
One-Way Anova Results	I----------I $p \leq 0.005$ I----------I $p \leq 0.05$ I----------I $p \geq 0.10$		

Biomarkers of Systemic and Vascular Inflammation

We examined several biomarkers that could cast light on potential mechanisms for the observed effects of PM exposures on atherosclerotic plaque development. ApoE-/- mice have aberrant lipid metabolism giving rise to increased levels of cholesterol and LDL-cholesterol. As shown in Figure 9, most of the serum cholesterol in these mice is associated with LDL-cholesterol.

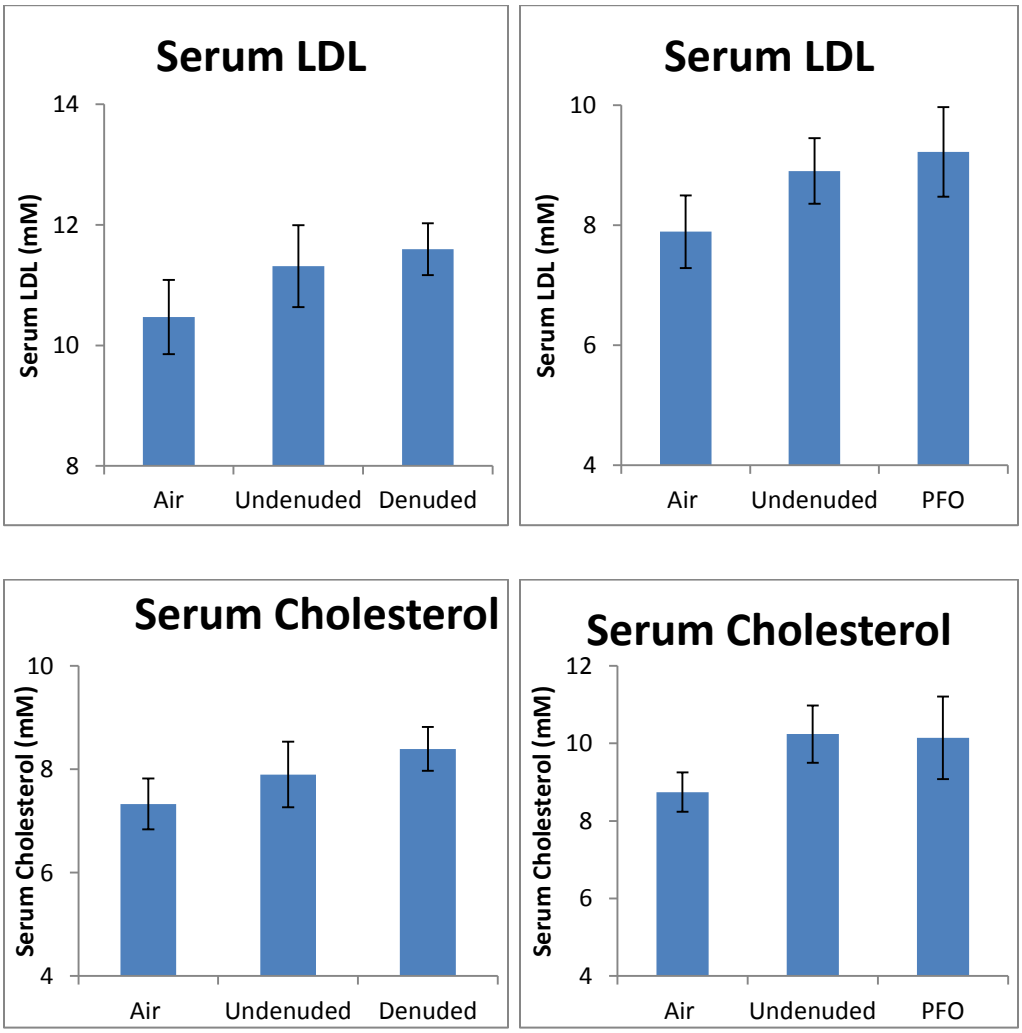

Figure 9. Effects of Undenuded Particles and Particle Free Organic Compounds on Total Cholesterol and LDL-Cholesterol (Upper and Lower Left n = 13; Upper and Lower Right n = 8)

It can however be see that there is a clear pattern of increased LDL and cholesterol in the sera of mice that were exposed to either undenuded PM or to the particle free organics that were removed from the PM by thermal denuding compared to the concentrations observed in mice exposed to purified air.

Two additional biomarkers were examined in sera from exposed mice, C-reactive protein (CRP), which is a marker of systemic inflammation, and total glutathione (GSH), which is an indicator of antioxidant defense capacity.

Table 4. Serum Levels of Glutathione and C-Reactive Protein (ng/mL)

	Undenuded vs. Denuded Experiment 1			**Undenuded vs. Particle Free Organics Experiment 2**	
	CRP	**GSH**		**CRP**	**GSH**
Purified air	**125 ± 2**	**12.8 ± 0.9**	**Purified air**	**131 ± 2**	**11.8 ± 1.5**
Undenuded	**126 ± 5**	**12.4 ± 0.9**	**Undenuded**	**139 ± 2**	**12.9 ± 0.8**
Denuded	**122 ± 3**	**12.1 ± 0.9**	**PFO**	**132 ± 4**	**13.4 ± 0.9**

Mean concentrations of CRP and GSH were consistent across the groups and were not affected by the exposures to PM in this study. However there is an indication that oxidative stress might be a factor. As seen in Figure 10, exposure to denuded PM did not increase lipid peroxidation in mouse sera, compared to that in mice exposed to purified air. However there was a consistent increase in lipid peroxidation in sera from mice exposed to undenuded PM or to the PFO. We did not have sufficient sample to examine whether or not LDL was oxidized.

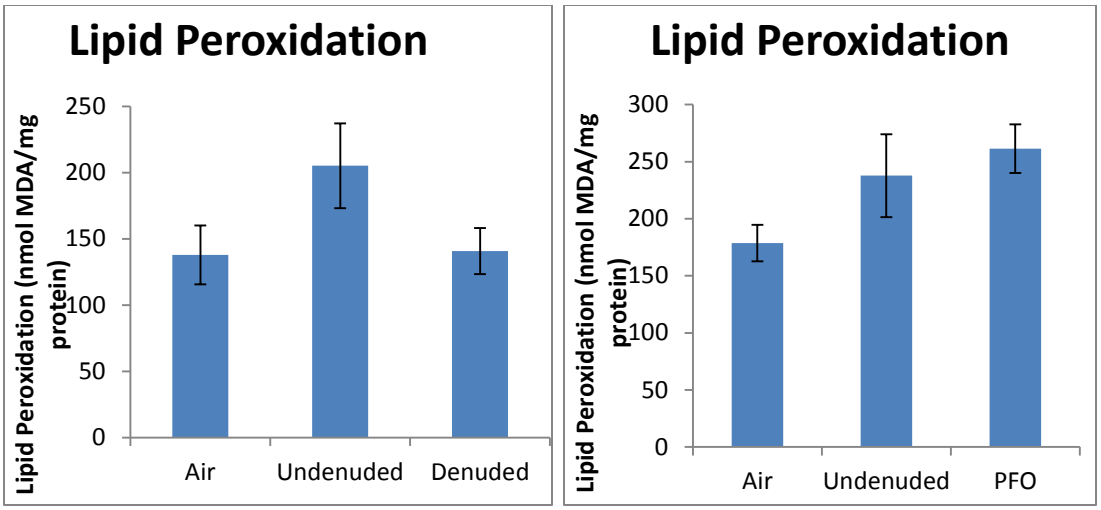

Figure 10. Serum Lipid Peroxidation Levels in Mice Exposed to Either Denuded PM, Undenuded PM and Particle Free Organics (PFO)

Conclusions

The findings of this study are that: (1) the VACES and the Dekati Thermodenuder can be used in tandem to deliver undenuded ultrafine ambient PM (UFP, $d_p \leq 0.18$ μm), denuded UFP and particle free organic PM constituents (PFO) to genetically modified, apoE-/-, mice in a mobile rodent exposure system; (2) the organic constituents of UFP are important contributors to atherosclerotic plaque development and significantly accelerate the growth of arterial plaques after an 8 week exposure; and (3) exposure to both organic and inorganic constituents of UFP raise serum concentrations of cholesterol and low density lipoprotein-cholesterol (LDL), but exposures to UFP that were denuded of most organic constituents did not promote serum lipid peroxidation while exposures to undenuded UFP or to PFO did promote serum lipid peroxidation.

This study has demonstrated that the semi-volatile PM fraction of ambient ultrafine particulate matter is an important contributor to the development of atherosclerosis and heart disease. PM exposure was also shown to increase serum levels of cholesterol and LDL-cholesterol, both of which are known risk factors for atherosclerosis and heart disease. In this study exposure to undenuded UFP and to PFO also promoted the peroxidation of serum lipids

while exposure to denuded UFP did not. Peroxidation of serum lipids, especially LDL, has been specifically implicated in atherosclerotic plaque formation. In fact, serum levels of oxidized LDL are increased in individuals with exposures to traffic-related air pollution [55] and oxidized LDL contributes to formation of lipid-laden macrophages (foam cells) and promote the induction of foam cell formation, inflammatory cytokines secretion [56]. Removal of the semivolatile organics by a process of thermal denuding significantly mitigated the adverse effects of PM exposures in a laboratory animal model of atherosclerosis.

Chemical characterization of the ambient PM using a high resolution aerosol mass spectrometer showed that the UFP fraction, particles with diameters ≤ 0.18 μm, was more enriched in organic compounds that were less oxygenated, hence less polar, than particles with diameters ≥ 0.18 μm. This is important because organics that are nonpolar are better able to cross cell membranes than are more polar molecules. Additionally, studies of mobile source emissions have shown that emissions from heavy duty diesel vehicles are enriched in UFP and that those UFP are characterized by low oxygenation levels.

An important corollary to these results is that they suggest that removal of UFP-associated semivolatile compounds can significantly mitigate adverse cardiovascular effects. Modern diesel engines are often provided with emission control technologies that remove effectively the non-volatile fraction, but not necessarily the volatile fraction. Some research has shown that removal of the non-volatile PM fraction can increase the concentration of the volatile fraction by enhancing nucleation of condensing organic vapors. This project has provided findings that improve our understanding of the mode of toxic action of freshly-emitted combustion aerosols and has identified organic constituents of ambient aerosols as influencing potential health effects. This information will also aid regulators and planners in developing air quality mitigation strategies and land use guidance to better protect the health of California residents.

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