FINAL REPORT

Effects of Inhaled Fine Particles on Lung Growth and Lung Disease

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Disclaimer

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

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Statement of Significance

One of the most provocative and potentially important of the outcomes of the Children’s Health Study (CHS) conducted by the University of Southern California for the ARB was the finding of reduced lung function growth associated with exposures to NO₂, acid vapor, fine ambient particles and elemental carbon (Gauderman et al. 2004a; Gauderman et al. 2002b). Pulmonary function deficits, measured as the percentage of children with clinically significant depression (i.e. <80% of age adjusted expected level) of forced expiratory volume at one sec. (FEV1.0) increased with increasing community pollutant concentration levels. The children were followed to 18 years of age, by which age, most lung growth is complete. One can therefore speculate that any deficits might not be repaired after that time. Because the pollutants in ambient air that were associated with the development of lung function deficits were inter-correlated, it was not possible to definitively attribute the health effects to one or more causal agents. It remains unknown whether the lung function deficits seen in these children will manifest in chronic health effects in adult life. These questions can be addressed using animal exposures and a mobile exposure system that was developed and tested using ARB support. This study sought to investigate the relationship between fine particulate matter exposure and impaired lung function development in an animal model, and the possibility that these changes might persist after the termination of exposure.
Abstract

The Children’s Health Study (CHS), conducted by the University of Southern California, has reported significant associations between reduced lung function growth and exposures to nitrogen dioxide (NO$_2$), acid vapor, ambient particles less than 2.5 microns in diameter (PM2.5), and elemental carbon. The primary objective of this study was to use an animal model to test the hypothesis that chronic PM2.5 exposure during the period of rapid lung growth and development can lead to reduced growth in pulmonary function that is related to oxidative stress and tissue injury. We studied changes in the development of the mouse lung and lung function during chronic exposure to concentrated ambient PM2.5 using a mouse model. The mice were exposed from 3 weeks to 11 weeks of age, i.e. after weaning to the point where the rate of lung growth is slowed. The study also evaluated whether observed deficits in lung function persisted for up to two weeks after exposure was terminated. Mice that were exposed to concentrated ambient fine particles (CAPs) for eight weeks had reduced pulmonary function, measured as increased respiratory resistance, that persisted for up to 2 weeks after the termination of exposure. We have preserved tissue, blood, and bronchoalveolar lavage fluid samples for later analyses to investigate the relationship between pulmonary function deficits and alterations in lung structure, biochemical mediators of oxidative stress and inflammation, as well as alteration in gene expression that might be associated with lung development. We also examined the associations between particle chemical composition, particle physical characteristics and particle concentrations and observed changes in pulmonary function. There were two sets of exposures; the first exposures were performed at ‘high’ concentration (PM2.5, 243 $\mu$g/m$^3$; number concentration, 93,000 particles/cc) and the second exposures were at ‘low’ concentration, (PM2.5 56 $\mu$g/m$^3$; particle number 83,000 particles/cc). Both studies produced significant increases in resistance. Particle mass concentrations in the low study were nearly 1/5$^{th}$ that in the high study, however the particle number concentrations
were nearly the same, suggesting that high concentrations of ultrafine particles were present during both sets of exposures. There were methodological differences between the two sets of exposures; however the results suggest that the ultrafine components of PM2.5 may be more strongly associated with the observed decreases in resistance in the PM-exposed developing lung than are larger-sized particle components.

**Executive Summary**

**Background**

The Children's Health Study (CHS), which was conducted by the University of Southern California for the ARB, has reported that NO₂, acid vapor, fine ambient particles and elemental carbon exposures during the period of lung growth and development in Southern California children were associated with impaired lung function growth, increased school absences, and exacerbated asthma (Kunzli et al. 2003). An earlier study by Frischer and colleagues had shown that long term exposure to ambient ozone (O₃) was associated with reduced pulmonary function growth (Frischer et al. 1999), however O₃ was not associated with pulmonary function growth deficits in the CHS (Gauderman et al. 2004a). In reviewing the CHS data, Tager suggested that the decrease in some measures of lung function growth might also have been associated with summertime levels of SO₂, NO₂ and PM10 (Tager 1999).

**Specific Aims**

This study had 5 specific aims:

1. Examine effects of eight week exposure to a high concentration of concentrated PM2.5 on the development of lung function and capacity in mice exposed from the time that they are weaned through 11 weeks of age (adulthood).
2. Examine the role of oxidative stress in PM-induced lung injury and pulmonary function decrements in Nrf2-/- mice.
3. Determine whether the effect of exposure to concentrated PM2.5 from weaning to adulthood on pulmonary function decrements persists once exposure ends.
4. Obtain tissue, blood, and bronchoalveolar lavage samples to examine effects of PM2.5 exposure during the period of lung growth and development on expression of growth and development-related genes in the lungs.

To accomplish these specific aims we used an animal model; mice were exposed to concentrated ambient fine particles (CAPs) during the animal’s period of rapid growth and development (weeks four through twelve). The particle concentrator does not concentrate gases or vapors. The concentrator removes up to 70% of nitrogen oxides and most of ambient ozone. Because the concentrator particle inlet is size-selective, this study could specifically address the question of whether PM2.5 exposure was responsible for lung function growth deficits, with little interference from other covariable air pollutants.

**Results**

Exposure of mice to CAPs at high concentration (mean concentration 243 μg/m$^3$) supports the conclusions of the CHS study; namely that PM2.5 exposure could lead to decreased pulmonary function growth (increased resistance and decreased compliance) in animals exposed from weaning to adulthood. The study also showed that the changes persisted up to a week after exposure ended. Animals exposed to a lower CAPs concentration (mean concentration 56 μg/m$^3$) during the same age range showed a similar increase in pulmonary resistance but did not show the reduction in compliance that was observed in the high concentration study, suggesting that PM2.5 concentration is a factor in influencing lung function growth. Overall, the results of this study suggest that PM2.5 exposure can adversely influence lung function growth, since other pollutants were removed, or at least greatly reduced, by the operation of the particle concentrator.
Discussion

We were unable to completely address all of the objectives of the project. Studies with the genetically modified mice were only partially successful because the animals did not breed well, and proved to be too frail to withstand the exposure and pulmonary function measurement protocols. They were also more variable in their pulmonary function responses than had been anticipated. In addition, due to a Governor’s Executive Order putting all state contracts on hiatus for three months, an experiment had to be terminated prior to its completion, and then repeated once the hiatus was lifted. Because of this, monies were not available to perform all planned analyses. Tissue samples for histology, and blood and bronchoalveolar lavage fluid for analysis of oxidative and inflammatory mediators, and for gene array analyses have been stored for subsequent analyses when funding becomes available. In addition, the hiatus contributed to the reduced effort for the planned experiment using the Nrf2-/- mice because funding was not available to support continued maintenance and breeding of these mice.

Conclusions

The major conclusion of this study is that PM exposure, independent of other pollutants, can impede the development of pulmonary function in a growing mammal and that deficit persists for at least two weeks after the termination of exposure. There were two sets of exposures; the first exposures were performed at ‘high’ concentration (PM2.5, 243 μg/m³; number concentration, 93,000 particles/cc) and the second exposures were at ‘low’ concentration, (PM2.5 56 μg/m³; particle number 83,000 particles/cc). Both studies produced significant increases in resistance. Particle mass concentrations in the low study were nearly 1/5th that in the high study, however the particle number concentrations were nearly the same, suggesting that high concentrations of ultrafine particles were present during both sets of exposures. Although there were methodological differences between the two sets of exposures, the results suggest that the ultrafine components of PM2.5 may be more strongly associated with the
observed decreases in resistance in the PM-exposed developing lung than are larger sized particle components.

**Background**

Longitudinal studies of children in Europe (Brunekreef et al. 2002; Horak et al. 2002) and the United States (Gauderman et al. 2004a; Gauderman et al. 2002a) have reported that exposure to air pollution is associated with reduced growth of lung function. The results of these longitudinal studies were consistent with those from earlier cross-sectional studies (Detels et al. 1987; Tashkin et al. 1994). These population-based studies established that pulmonary function losses were associated with advancing age and also with exposures to several components of air pollution, including particles with aerodynamic diameters less than 10 µm (PM10) and particles with aerodynamic diameters less than 2.5 µm (PM2.5). Dockery et al. also showed that continued exposure to air pollution led to accelerated loss of lung function in adults (Dockery et al. 1985). Other components of ambient air pollution were also associated with pulmonary function losses, and those components were also correlated with PM10 and PM2.5 making it difficult to determine whether the observed effects were related to the air pollution mixture as a whole, or to one or more individual pollutants.

Several studies have examined the effects of air pollution exposure on lung growth and lung function development in children. Jedrychowski and colleagues reported a significant association between residential sulfur dioxide and suspended particulate concentrations and poorer gain of pulmonary volumes in children living in Poland (Jedrychowski et al. 1999). Their findings suggested that residential air pollution may be a part of a chain of reactions leading to retardation in PF growth during a child’s preadolescent years. However, there was no control for environmental tobacco smoke in the children’s residences, which might have been a significant contributor to inducing PF deficits (Jedrychowski et al. 2005). Kunzli et al. reported that the Children’s Health Study (CHS) found that PM2.5 exposure during the period of lung growth and development in Southern California children was associated with impaired lung
function growth, increased school absences and exacerbated asthma (Kunzli et al. 2003). An earlier study by Frischer and colleagues showed that long term exposure to ambient ozone (O$_3$) was associated with reduced pulmonary function growth (Frischer et al. 1999), however O$_3$ was not associated with PF growth deficits in the CHS (Gauderman et al. 2004a).

Gauderman and associates recruited 1,759 children (average age, 10 years) from schools in 12 southern California communities and measured their lung function annually for eight years (Gauderman et al. 2004a). The children resided in communities that represented a wide range of ambient exposures to ozone, acid vapor, nitrogen dioxide, and particulate matter. Linear regression was used to examine the relationship of specific components of air pollution in the communities in which the children resided and the child’s forced expiratory volume in one second (FEV1) and other spirometric measures. The communities with the lowest levels of air pollution included Lompoc, Santa Maria and Atascadero, communities along the Central Coast of California. The communities with the highest levels of air pollution included Riverside, San Dimas and Upland, California. Over the eight-year period, deficits in the growth of FEV1 were associated with exposure to nitrogen dioxide (NO$_2$; P=0.005), acid vapor (P=0.004), PM2.5 (P=0.04), and elemental carbon (EC; P=0.007). Exposure to pollutants was associated with clinically and statistically significant deficits in the FEV1 attained at the age of 18 years. The results of this study indicated that exposure to elevated levels of air pollution can lead to clinically significant deficits in pulmonary function as children reach adulthood. The pulmonary deficits increased with increasing community pollutant concentration levels. An additional analysis (Avol et al. 2001) showed that children from this study population who had moved to areas of lower PM10 while they were still growing showed increased growth in lung function and subjects who moved to communities with a higher PM10 showed decreased growth in lung function suggesting that repair was possible if exposures were mitigated during a child’s period of lung growth and development. However, pulmonary function deficits
continued to be observed in the children that continued to reside in high pollution areas and were followed to 18 years of age, when most of their lung growth was complete. Gauderman et al. (Gauderman et al. 2004a) speculated that any pulmonary function deficits evident at age 18 might not be repaired after that time, although no studies have investigated this to date. There is evidence that decreased growth of lung function in childhood is a predictor of chronic lung disease in later adult life (Shaheen 1997; Tager 1999), thus the CHS study results suggest that as these children grow older they might be at elevated risk of developing chronic lung diseases.

One potential mechanism for the observed decrease in lung function growth is that chronic exposure to PM causes oxidative stress in the lungs and injury to lung tissues. The resulting inflammation can lead to permanent tissue changes that can alter lung development. In susceptible adults (e.g., asthmatics, COPD patients), exacerbation of pulmonary inflammation may be a central mechanism by which PM can exert toxicity. PM contains components that release, or can elicit, free radicals and reactive oxygen species (ROS). These components include metals, endotoxins and reactive hydrocarbons, such as polycyclic aromatic hydrocarbons, quinines and carbonyl compounds (Tao et al. 2003). *In-vivo* PM exposures induce release of inflammatory cytokines and chemokines (e.g. IL-1 and TNF-α) resulting in neutrophil (PMN) influx to the lung and release of ROS by activated macrophages and PMNs. *In-vitro* exposures of critical cellular targets (e.g. alveolar macrophages, epithelial cells, and PMNs) to PM demonstrate PM- and oxidant-dependent responses consistent with the *in vivo* data. *In-vitro* data also demonstrate that lung epithelial cells, primed by inflammatory mediators, show enhanced cytokine production after exposure to PM, and that this response can be blunted in the presence of an antioxidant (N-acetyl-cysteine), which supports the hypothesis that oxidants, and perhaps oxidative stress, are key to the mechanism(s) for at least some of the effects of PM on the lung (Stringer and Kobzik 1998).
There is evidence at the molecular level linking oxidative stress to lung function deficits. Gilliland et al. (Gilliland et al. 2002) analyzed DNA from buccal cell specimens obtained from children enrolled in the CHS and found that polymorphisms in glutathione-S-transferase (GST) M1, GSTT1, and GSTP1 genes were associated with deficits in forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) growth. They concluded that GSTM1 and GSTP1 genotypes were associated with lung function growth in school children. GST is an enzyme that, in part, mediates levels of an endogenous antioxidant, glutathione. Variation in a key locus of the GST gene could potentially result in the expression of a GST enzyme molecule that is less biologically active than normal, and thereby reduce the efficacy of the antioxidant defenses in individuals with this modified gene. However, not all of the individuals that exhibited reduced pulmonary function growth in the Gilliland et al. studies had genetic GST polymorphisms, suggesting that other factors are also involved (Gilliland et al. 2002).

Previous studies have shown that brief inhalation by C57BL/6 mice of PM2.5 particles (300 μg/m$^3$) for 6 h caused significant ($P \leq 0.05$) increases in steady-state messenger RNA (mRNA) levels of a number of nuclear factor -kappaB (NFκB)- associated and/or -regulated genes, including tumor necrosis factor-alpha and β (TNF-α and TNF-β), interleukin-6 (IL-6), interferon-gamma (IFNγ), and transforming growth factor-beta (TGFβ) (Shukla et al. 2000). It is well established that inhaled particles release, or cause to be released free radicals and other oxidants that induce oxidative stress and injure lung tissue, induce apoptosis and alter lung permeability (Bucchieri et al. 2002; Ghio et al. 1999; Schins and Borm 1999). Thus, oxidants associated with PM or components of PM can cause inflammation and injure tissues in the lung.

This study examined the effects of concentrated ambient fine particles (CAPs$^{2.5}$) on the functional and morphological development of the mouse lung as a model for the development of the human lung. The advantage of the mouse model is
that, like humans, there is a rapid growth in the post-weaning, ‘juvenile’ and ‘adolescent’ stage. Unlike humans, mice continue to grow in body size and to some extent in lung size after becoming adults; however, growth after adulthood is quite slow. After 7 weeks of age overall growth in the mouse is very slow and there is a marked decrease in the rate of lung growth. In fact, some decline in characteristics such as functional residual capacity occurs after about 8 weeks of age (Mitzner et al. 2001). Thus, the mouse model, while not perfect, was a reasonable choice for examining the effects of air pollution exposure on PF development.

Biological removal of oxidant metabolites is facilitated through increased expression of phase II detoxifying genes under the control of Nrf2 transcription factors; the so-called antioxidant response element (ARE). Thus, oxidant injury repair is blunted in Nrf2-deficient mice. Genetically modified mice with specific knockouts along the Nrf2 signaling pathways (Nrf2-/-) can be used to test mechanistic hypotheses regarding the roles of inflammation and oxidative stress and to address the question of whether impairment of the ARE response adversely affects lung structure and lung function development (Biswas and Chan 2009).

The Nrf2-/- mice used in this study were derived from the C57BL/6 strain. The C57BL/6 strain is the basis for most of the commercially available transgenic mouse models. Although other mouse strains such as the A mouse might have greater intrinsic susceptibility to ozone (Leikauf et al. 2000; Leikauf et al. 2001), the C57BL/6 mouse exhibits greater susceptibility to PM-induced adverse effects than does the A mouse (Ohtsuka et al. 2000b), and genetically modified C57BL/6 strains are expected to be even more sensitive. We also used normal, or wild type (WT), C57BL/6 mice to evaluate the effects of impaired anti-oxidant defenses on the development of PM-induced lung injury and pulmonary function deficits.
Objectives and Hypotheses

The primary objective of this study was to test the hypothesis that chronic PM2.5 exposure during the period of rapid lung growth in mice will cause pulmonary function deficits, and that these deficits will be associated with oxidative stress and inflammation in the lung. The secondary hypothesis was that pulmonary function deficits elicited by chronic exposure during the period of rapid lung growth will persist after the termination of PM2.5 exposure. A plausible reason for the differences in lung function growth between the CHS children growing up in the Central Coast compared to those growing up in the Riverside area is the difference in PM2.5 concentration between these areas. Accordingly, this study compared responses of animals exposed at high and low concentrations of CAPs.

To accomplish these objectives, we conducted repeated inhalation studies using the versatile aerosol concentration enhancement system (VACES), which is a well characterized mobile particle concentrator (Kim et al. 2001a; Kim et al. 2001b). Mice were exposed to high and low concentrations of concentrated ambient fine particles (CAPs2.5). The VACES nominally concentrates ambient particles approximately 20-fold, however the concentration can be modified by diluting the output of the VACES to achieve the target concentrations shown in Table 1. The VACES also effectively reduced the concentrations of oxidant gas co-pollutants (e.g. O₃ or NOx) by 70 to 100%, so that the risk of potential confounding by these compounds is lowered, making attribution of observed effects to PM2.5 exposure more certain.

We selected Riverside as the initial study site because CHS participants residing in Riverside and nearby Mira Loma had the largest percentage of children with clinically significant FEV₁ deficits (Gauderman et al. 2004a). We used a log-probability distribution to estimate the 80th percentile concentration of ambient PM2.5 using AQMD and CHS annual average data for Riverside to select the target PM2.5 concentrations for the study, also taking into account
the operating characteristics of the VACES. Our target (Table 1) for the high concentration study was based on expected ambient PM2.5 concentrations of 7.5 to 15 μg/m³ and a VACES concentration efficiency of 20. The low concentration was estimated using a VACES concentration efficiency of 10.

Table 1. Target Concentrated Ambient PM2.5 Concentrations

<table>
<thead>
<tr>
<th>Site</th>
<th>Conc. (μg/m³)</th>
<th>Conc. (μg/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riverside</td>
<td>150-300</td>
<td>75</td>
</tr>
</tbody>
</table>

The endpoints used in this study included markers of inflammation, production of pro-inflammatory and anti-inflammatory mediators, histological examinations for evidence of changes in lung growth or development and pulmonary function measurements. The physical and chemical composition of the particles was determined, and collected particles have been reserved for *in-vitro* studies to evaluate the potential of these particles to produce free radicals and induce changes in cell signaling factors related to tissue growth or development.

**Specific Aims**

1. **Examine effects of high concentration chronic PM exposures on the development of lung function and capacity in mice exposed from the time that they are weaned through 11 weeks of age.** C57BL/6 WT mice were exposed to purified air or concentrated ambient PM for 4 hr per day, 5 days per week from Post-natal (PN) Week 3 (just weaned) to PN Week 11 (growth plateau). Cohorts of mice were removed after 1, 3 and 8 weeks of exposure for pulmonary function measurements and tissue preparation.

2. **Examine the role of oxidative stress in PM-induced lung injury and pulmonary function decrements in Nrf2-/- mice.** Nrf2 -/- mice were exposed to purified air and to CAPs₂.₅ using the same exposure protocol. Endpoints and bioassays were as described for Aim 1. We anticipated that lung function and
l lung size would be reduced in the particle-exposed mice and that lung function decrements in the Nrf2 -/- mice would be greater than those in the wild-type mice. Unfortunately, these mice were very fragile and the stresses associated with the exposure and measurement protocols caused high mortality in both air and CAPs-exposed animals, so only limited data were obtained.

3. **Determine if the effects of chronic PM exposures on pulmonary function decrements are persistent.** Mice were exposed to purified air or CAPs$_{2.5}$ from 3 weeks of age to 11 weeks of age, followed by exposure to purified air for one week. Endpoints sampled included pulmonary function measurements to evaluate persistence of pulmonary function deficits, and collection of tissue, blood, and bronchoalveolar lavage samples for subsequent analysis.

4. **Obtain biological samples to subsequently examine effects of chronic PM exposures on expression of growth and development-related genes in the lungs of mice.** Lung tissues from the mice exposed in experiments under Aims One, Two, and Three were frozen in liquid nitrogen and stored at -80°C for subsequent examination of gene expression in genes known to be involved in lung growth and development. Even though these studies involved inbred mice, there can be a range of susceptibility and some individual mice within a group will respond to environmental agents to a greater degree than others. Due to circumstances outside our control, we were unable to complete work on this objective. In addition, it was important to complete the analyses of pulmonary function data before undertaking these assays. The tissues are frozen, and will be analyzed at such time as funds become available. The current plan is that tissue from mice exhibiting significant pulmonary function changes (responders) will be contrasted with tissues from those showing no changes in pulmonary function with respect to changes in expression of genes related to inflammatory response, tissue repair and antioxidant production. We will also contrast the expression of these genes in mice exposed to purified air to that in mice exposed to concentrated PM2.5.
5. **Contrast differences in lung function and lung growth as a function of particle concentration and particle composition.** C57BL/6 WT mice were exposed to a lower concentration of CAPs_{2.5} using the same protocol as described for Aims 1 and 3. Endpoints were as described above.

**Experimental Techniques**

**Selection and Characteristics of the Animal Model**

The C57BL/6 mouse strain used in this project is the same genetic background strain used for producing the Nrf2 -/- mouse. The wild type, normal C57BL/6 mouse has been shown to be particularly susceptible to adverse pulmonary effects from a variety of inhaled substances including O_3 (Kleeberger et al. 1990), acid-coated carbon particles (Ohtsuka et al. 2000a) and ovalbumin (Morokata et al. 1999a; Morokata et al. 1999b), and demonstrates robust findings of pulmonary injury.

**Animal Husbandry**

Animals were housed two to a cage in an ALAAAC accredited animal housing facility at the Air Pollution Health Effects Laboratory (APHEL) at the University of California, Irvine (UCI). Animals were provided with food and water *ad libitum*, according to guidelines and suggestions of the UCI campus veterinarian, and were housed in cages ventilated with filtered, purified air. The animals were maintained at APHEL to avoid transmission of disease from other investigators’ animals to ours. There was no evidence of respiratory infection or disease in the mice used in this study.

**Ambient Particle Concentrator**

Ambient PM2.5 particles were drawn through a size selective inlet and concentrated using the Versatile Aerosol Concentration Enrichment System (VACES), which has been described in detail by Kim et al. (Kim et al. 2001a; Kim
et al. 2001b). VACES consists of 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 a factor of 20 times ambient. The enrichment factor can be modified by altering the ratio of the bypass and output flow rates (Kim et al. 2002). The efficiency of concentration begins to fall off for particles above 2.0 μm or below 0.03 μm in aerometric diameter. Consequently, animals were exposed to PM primarily between 0.03 and 2 μm in aerometric diameter.

**Exposure Chambers**

A whole-body exposure mouse chamber was designed specifically for use with the VACES. Each chamber consists of a 5-gallon (20 inches X 12 inches X 6 inches) stainless steel (SS) tray, and contains 24 cubicles (1 mouse per cubicle) separated by perforated stainless steel sheets (0.078” Hole Diameter. 36% Open, Staggered, (McMaster-Carr, New Brunswick, NJ). A raised sub-floor, constructed from a perforated stainless steel sheet (0.25” Hole Diameter, 50% open, staggered) permitted urine and feces to fall to the bottom of the vat and kept the mice relatively clean. Absorbent sheets impregnated with an antibiotic to prevent fecal bacteria from generating ammonia from urine were placed under the exhaust tubes to absorb urine and to collect feces. The top of the cubicles was covered with perforated plastic sheets (0.078” Hole Diameter. 36% Open, Staggered McMaster-Carr, New Brunswick, NJ) to confine the animals to a single cubicle. The system was tested and found to supply reasonably uniform particle distributions to the individual mouse cubicles (Oldham et al. 2004).

Aerosol is delivered through six stainless steel particle delivery tubes, each 22 cm in length with fifteen 0.25 mm holes that were 13.5 mm apart. The particle delivery tubes were bolted to the Plexiglas top to distribute concentrated ambient
particles (CAPs) evenly throughout the exposure chamber. The exposure atmosphere was exhausted from below the sub-floor through two stainless steel tubes, each 40 cm in length with 28 0.5 mm downward-facing holes.

**Exposures**

The VACES concentrates the mixture of fine and ultrafine particles that comprises PM2.5. In the Los Angeles Basin ultrafine particles originate mostly from vehicular emissions, and account for over 90% of the number-based particle concentrations in PM2.5. Recent studies at the Southern California Supersite (funded by the U.S. EPA) report that the mass median diameter of elemental carbon (EC), a surrogate of vehicular emissions in Los Angeles, was in the range of 0.15-0.20 µm (Kim et al. 2002). Moreover, median ultrafine particle diameter in the inland valleys near the city of Riverside, CA (receptor areas of the Los Angeles Basin) was in the range of 0.09-0.15 µm during the summer months. EC, especially from diesel engine exhaust, is a potentially important carrier of many toxic organic compounds and has been associated with a variety of adverse health effects (McCreanor et al. 2007; Neumeyer-Gromen et al. 2009; Zhang et al. 2009). Thus our exposures using the VACES should capture most of the potential toxicity of Southern California PM2.5.

Exposures were conducted at the University of California, Riverside (UCR), which was identified as a high PM2.5 exposure community in the Children’s Health Study. PM2.5 concentrations at Riverside are among the highest in the U.S. In addition, detailed data are available from the South Coast Air Quality Management District (SCAQMD) on the chemical and physical characteristics of the pollutant components present at this site.

SCAQMD pollutant data for Riverside included 1-hr averages of fine particle counts and mass concentrations for PM2.5, PM10, and concentrations of criteria pollutant gases including O₃, NO₂, CO and SO₂. In addition, CPC and DustTrak
particle monitors were used to measure real time particle number concentrations and particle mass concentrations, respectively, during the exposures. We supplemented the above with data on elemental and organic particle composition provided by Dr. Suzanne Paulson (UCLA).

Particles were collected for mass concentration and chemical analyses on pre-weighed fluorocarbon filters. Following collection, the filters were equilibrated overnight at constant humidity and weighed. By operating the VACES under modified flow conditions, or by mixing the concentrated particles with purified air, we were able to produce particle concentrations over a substantial range. Air was drawn through a 2.55 µm cutoff low pressure drop impactor and particles less than 2.5 µm diameter entered a stack located at the same height as the sampling inlets at the adjacent SCAQMD monitoring site. The residence time in the stack was about 3 sec and diffusion losses during transit were small. The output of the VACES (see operational description above) was drawn into exposure chambers allowing the mice to be exposed. The concentration of particles and the particle number concentrations were measured at the inlet to the exposure chamber to document the exposures.

Samples collected on pre-fired quartz filters were composited on a weekly basis for estimating elemental carbon (EC) and organic carbon (OC) concentrations. EC is a reasonable tracer for particles originating from diesel engines, and represents between 5 - 20% of the UPF at the Riverside site. EC and OC were measured using a thermal photometric method on a fraction of the filter; the remaining fractions are stored (-80º C) for future analyses.

Analysis of Hydrogen Peroxide and Metals (Paulson Lab, UCLA)

The Paulson analyses were funded from other sources in conjunction with our ongoing health effects studies in Riverside CA. Particle sampling for the H$_2$O$_2$ and metals analyses were performed at the same time as the lung growth high concentration exposures.
**H$_2$O$_2$ analysis**

Collected particles were extracted into the aqueous phase by immersing filters into a known volume (4 mL for whole filters) of extraction solution for 2 hrs, after which the extraction solution is analyzed for H$_2$O$_2$ using an HPLC/fluorimeter as described below. The standard extraction solution (SS 3.5) was made from 18 MΩ water with 0.1 mM disodium ethylene-diamine-tetraacetic acid (Na$_2$EDTA), adjusted to pH 3.5 using 0.1 N H$_2$SO$_4$. Extraction solutions with dithiothreitol (DTT) were made by adding a volume of stock DTT (1×10$^{-3}$ M) solution to SS 3.5, resulting in 1×10$^{-6}$ M DTT in SS 3.5. The concentrations of quinones were generally below 1 ng/m$^3$ in ambient aerosols (Chung et al. 2006), which corresponds to a maximum concentration of 5 ×10$^{-8}$ M in extraction solutions under our experiment set-up. Thus, DTT at a concentration of 1×10$^{-6}$ M, should serve as an abundant electron donor.

Peroxides in particles were measured by HPLC-fluorescence, which has been described in detail elsewhere (Arellanes et al. 2006; Hasson et al. 2001; Hellpointner and Gab 1989). Briefly, sample filters were conditioned in 4 ml stripping solution for 2 h to extract peroxides in particles. Peroxides were separated on a C-18 reversed-phase column (Alltech) with stripping solution as the mobile phase. Directly after separation, the eluate was introduced into a thermostated reaction coil, where the reaction of H$_2$O$_2$ with para-hydroxyphenylacetic acid (POHPAA) catalyzed by Type II horseradish peroxidase took place (Figure 1). A schematic of the measurement system is shown below (Figure 2). The resulting dimer of POHPAA shows a strong fluorescence at an excitation wavelength of 320 nm and an emission wavelength of 400 nm:
Figure 1. Chemical reaction for the measurement of H2O2

Figure 2. Schematic of HPLC-fluorescence detector for hydroperoxides.

Multipoint calibration of the HPLC was performed once a week. Fresh standards of H₂O₂ with concentrations in the range of 10⁻⁶-10⁻⁸ M were prepared by serial dilution of a 0.3% stock solution (Aldrich), which was titrated against I₂-Na₂S₂O₃ every week. In brief, iodometric titration is a red-ox reaction in which hydrogen peroxide first oxidizes iodide (I⁻) in the presence of Mo(IV). The thiosulfate is titrated in until the bright blue endpoint is determined.

\[ \text{H}_2\text{O}_2 + 3 \text{I}^- + 2 \text{H}^+ \rightarrow 2 \text{H}_2\text{O} + \text{I}_3^- \]  
(2)

\[ \text{I}_3^- + 2 \text{S}_2\text{O}_3^{2-} \rightarrow 3 \text{I}^- + \text{S}_4\text{O}_6^{2-} \]  
(3)

A H₂O₂ standard was injected every day to check changes in the enzyme activity and the performance of the HPLC. The concentrations of H₂O₂ were determined
by comparison of the integrated fluorescence peak areas with those of H$_2$O$_2$
standards.

**Mass and Metal Measurements**

Aerosol mass concentrations were determined by weighing samples on a
microbalance (1 μg precision, ME 5, Sartorius Inc., Goettingen, Germany) in a
temperature (22-24 °C) and relative humidity (RH 40–45%) controlled room.
Filters were allowed to equilibrate to the RH in the room and weighed before
loading into filter holders or for blank analysis, and again before determining their
loaded mass. Particle mass concentrations were determined from the mass
change on the filters and the total volume of air sampled. For virtual impactor
samples, we collected two sets of filters with identical impactors. One set was
analyzed for hydrogen peroxide within several hours, and the other later for mass
and transition metals.

The concentrations of 14 trace elements, aluminum, calcium, magnesium,
potassium, sodium, silicon, iron, copper, lead, manganese, nickel, selenium, zinc
and vanadium (Al, Ca, Mg, K, Na, Si, Fe, Cu, Pb, Mn, Ni, Se, Zn, and V), were
determined by inductively coupled plasma atomic emission spectroscopy (ICP-
AES, Perkin-Elmer, TJA RADIAL IRIS 1000). Sample filters were extracted under
similar conditions as used for the H$_2$O$_2$ analysis, in 4 ml nitric acid solution (pH
3.5, HNO$_3$) for 2 hours to determine soluble metals likely to contribute to H$_2$O$_2$
generation. The resulting clear extracts were further acidified with 1 ml of 25%
HNO$_3$ so that submitted samples had a pH matching the ICP-AES matrix (5%
HNO$_3$). The extracts were transferred to high density polyethylene (HDPE)
bottles for ICP-AES analysis. AA multiple element stock solution, containing 20
ppm each of Si, Na, Fe, Al, K, Ca and 10 ppm each of Mg, Zn, V, Pb, Mn, Cu, Ni,
Se, was prepared from 1000 ppm individual element standards (CPI
International). Standards for metals with concentrations in the range of 0.01-1.2
ppm were prepared by serial dilution of the stock solution.
Exposure Procedure

Animals were allowed to acclimate for at least one week in chambers with HEPA filtered air before any experimental procedures were started. Animals were exposed to actual ambient or to concentrated ambient fine particles (CAPs) at the UCR field location using the mobile exposure trailer previously described. Between exposures the animals were transported to UC Irvine in a specially configured van in which the animals were continuously supplied with purified air. They were housed in a ventilated cage system supplied with purified air, and given clean water and food, ad lib. During exposures, the animals were placed into sealed, compartmentalized exposure chambers that were connected to the outlet of the VACES system. The animals were exposed to fine concentrated ambient fine particles (CAPs) for 5 hours per day, 4 days per week for 8 weeks. Control animals 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 exposure period for signs of distress.

Pulmonary Function Measurements

Plethysmography

*High concentration study – groups evaluated at single time points:*

Pulmonary function was evaluated in anesthetized, freely breathing tracheotomized mice. Isoflurane was administered via inhalation for anaesthesia. The tracheal cannula was attached to an integrated pneumotachometer that measured tidal flow via a differential pressure transducer. A saline-filled tube was inserted into the esophagus to the level of the midthorax and coupled to a pressure transducer to measure transpulmonary pressure (Figure 3). The use of a tracheal tube avoids measurement of changes in the upper airways, an important variable in mice, which are obligatory nose-breathers.
Respiratory parameters measured included pulmonary resistance, dynamic compliance, maximum and minimum expiratory flow, tidal volume and respiratory rate. These parameters provided not only classical determination of pulmonary function, but also more detailed insight into pulmonary mechanics. Pulmonary resistance reflects both narrowing of the conducting airways and parenchymal viscosity (Glaab et al. 2007). Dynamic compliance primarily reflects the elasticity of the lung parenchyma, but it is also influenced by surface tension, smooth muscle contraction, and peripheral airway inhomogeneity. These respiratory indices were continuously measured, recorded, and stored by a software system (Hugo Sachs Elektronik) that has been previously described and utilized in our laboratory (Glaab et al. 2004). This method was used in our first study in which mice were exposed at high concentrations.

**Low concentration study – repeated measures exposures:**

An improved method for measuring pulmonary functions was developed and used in the second experiment in which mice were exposed to low concentrations of concentrated PM2.5. They were anesthetized with ketamine (100 µg/g body wt) and xylazine (8 µg/g body wt) in saline via intraperitoneal injection. (Brown et al. 1999; Macdonald et al. 2009), and placed into a whole body plethysmograph to monitor respiratory function during both baseline pulmonary function tests. Sufficient time was allowed for the mouse breathing pattern to stabilize before methacholine (MCh) challenges were begun. A detailed description of the pulmonary function apparatus and protocol has been previously published (Glaab et al. 2004). Briefly, each challenge lasted for 2-min, and doses of MCh were separated by 5-min intervals. Pulmodyn software (designed by Fraunhofer ITEM in cooperation with HSE-Harvard Apparatus, March-Hugstetten, Germany) used measurements from pressure transducers to calculate pulmonary resistance (RL) and dynamic compliance (Cdyn), as well as a variety of other pulmonary function endpoints.
Analyses of Lung Lavage and Tissue Specimens

**Bronchoalveolar Lavage (BAL).** Mice were anesthetized by intraperitoneal injection of sodium pentobarbital. The abdominal aorta was severed, and a polyethylene catheter was placed in the trachea and tied in place. An incision was made in the diaphragm to allow lung expansion during the lavage. Lungs were lavaged by introduction of 0.025 ml/g body weight 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffered Hanks Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} through a tracheostomy tube, followed by withdrawal of the fluid. The process of introduction and withdrawal was repeated three times and 0.5 ml of lavage fluid was recovered in a 1.5 ml polypropylene centrifuge tube. The lavage was repeated two additional times; fluid from the last two lavages was
pooled in a separate centrifuge tube. The lavage samples from each animal were centrifuged at 800 x g for 10 min.

Lung lavage cell number was determined by hemocytometry. Viable cells were differentiated from non-viable cells by trypan blue exclusion. An aliquot representing 100,000 cells was placed on a microscope slide using a cytocentrifuge, and was stained using a Wright-Giemsa procedure. Differential cell counts were made by microscopically examining a minimum of 300 cells per sample.

Lung lavage fluid was frozen for later analysis for markers of lung injury such as total protein, cytokines and chemokines involved in pulmonary inflammation and oxidative stress (i.e., IL-1β, IL-6, IL-8, TNF-α, PDGF, and TGF-β) using a Luminex bead array system. Frozen lung tissues were stored at -80°C for subsequent differential gene expression analyses using microarray technology, as described below.

Macrophages recovered from the lavage fluid were examined to determine degree of particle loading in the exposed mice. These data provided an index of macrophage ability to phagocytose and clear particles deposited in the respiratory tract.

**Bioassay and Data Analysis Methods**

**Blood:** serum samples were collected from the blood of each animal via cardiac puncture.

**Lungs:** one side of the lung was lavaged and frozen for gene expression analysis. The other side of the lung was fixed in buffered formaldehyde at a fixed pressure for subsequent morphometric analysis.
Lung Histology

The trachea and right lung were fixed by airway instillation of 4% paraformaldehyde at a pressure of 20 cm H$_2$O, and the tissue transferred to 70% ethanol after 24 hours. The total volume of the fixed lung was measured by liquid displacement. The right upper lobe, right middle lobe, and trachea were embedded in glycol methacrylate. Semi-thin sections (2 µm thick) were stained with hematoxylin and eosin for histopathology. All morphological assessments were done blind. Assessments were made using NIH Image software, which is a public domain image processing and analysis program developed at the Research Services Branch (RSB) of the National Institute of Mental Health (NIMH), part of the National Institutes of Health (NIH). The software is available at http://rsb.info.nih.gov/nih-image/Default.html.

Statistical Analyses

Data were analyzed using analyses of variance (ANOVA). Differences in group mean values were tested for significance using the Tukey Multiple Comparison test.

Results (by Objective)

Objective 1. Examine effects of high concentration chronic PM exposures on the development of lung function and capacity in mice exposed from the time that they are weaned through 11 weeks of age. Particle mass and number concentrations from this experiment are summarized in Table 2.

<table>
<thead>
<tr>
<th>Mass Concentration</th>
<th>Filtered Air</th>
<th>5 ± 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE (µg/m$^3$)</td>
<td>PM2.5</td>
<td>240 ± 44</td>
</tr>
<tr>
<td>Particle Count</td>
<td>Ambient</td>
<td>10,750 ± 2095</td>
</tr>
<tr>
<td>Mean ± SE (particles/ cm$^3$)</td>
<td>PM2.5</td>
<td>93,500 ± 12000</td>
</tr>
</tbody>
</table>
Sixty-four male C57BL/6 mice were exposed 4 days/week for 5 hours/day at various points of their growth. Mice were sacrificed at 1, 3, and 8 weeks of exposure, and 1 week post exposure (Fig. 4). Within 24 hours of exposure, pulmonary function was measured, and BAL fluid, blood, lung and heart tissue were collected to measure biological responses to CAPs exposure.

Figure 4. Timeline of High Concentration Study. Baseline represents values measured in unexposed mice at the time of pulmonary testing. Measured body weights for air and fine CAPs-exposed groups at time of measurement are contrasted with values provided by the supplier. The number of mice in each test group is shown.

Animals were exposed to an average CAPs concentration of 243 µg/m³, which is higher than the initial target concentration (see Table 1). The VACES was operated at an average enrichment factor of approximately nine times ambient PM2.5 level. This exposure concentration is several times the national ambient air quality standard, but is within the range occasionally observed on high smog days at the Riverside site. Differential cell counts of the BAL demonstrated a steady-state level of particles in the lung based on what appear to be
carbonaceous inclusions in alveolar macrophages of BALF (Fig. 5). In macrophages collected from animals sacrificed one week post-exposure, the number of inclusions approached the baseline level. This result suggests that the majority of the particles had been cleared from the lung by 1 week post exposure.

![Graph showing numbers of inclusions in macrophages recovered from BALF after high concentration exposures to air or fine CAPs. Numbers of inclusions are relatively constant in macrophages during the exposures and macrophages from CAPs-exposed mice have significantly more inclusions. Levels in air and CAPs-exposed mouse macrophages are not different 1 week post exposure suggesting that long term clearance was not impaired. *p < 0.05.](image)

Figure 5. Numbers of inclusions in macrophages recovered from BALF after high concentration exposures to air or fine CAPs. Numbers of inclusions are relatively constant in macrophages during the exposures and macrophages from CAPs-exposed mice have significantly more inclusions. Levels in air and CAPs-exposed mouse macrophages are not different 1 week post exposure suggesting that long term clearance was not impaired. *p < 0.05.

Pulmonary function data from CAPs-exposed C57BL/6 mice demonstrate a small decrease in dynamic compliance relative to purified air (Fig. 6) after 1 and 3 weeks of exposure, as well as one week after exposure was terminated, but not
after 8 weeks. The differences at the individual time points were not statistically significant; however the data suggest a pattern of decreased compliance associated with CAPs exposures. An increase in pulmonary resistance (Fig. 7) in the CAPs-exposed group was observed after 1 and 8 weeks of exposure, and this increase both persisted and was greater 1 week post-exposure. The changes are consistent with a loss of pulmonary function in the CAPs-exposed mice compared to the mice exposed to purified air.

**Dynamic Compliance**

![Dynamic Compliance Graph](image)

**Figure 6.** Dynamic compliance measured after high concentration exposure to fine CAPs or to air. There is a pattern of decreased compliance in CAPs-exposed mice compared to air-exposed mice but the individual group means are not significantly different. Baseline represents values for dynamic compliance in unexposed mice, age 4 weeks.

We did not make measurements of forced expiratory volumes (FEVs) or forced vital capacities (FVCs) that would have been analogous to measurements made in human volunteers. We did, however, measure the maximum expiratory flow
during each breath. As shown in Figure 8, maximum expiratory flow was slightly but not significantly decreased 1 week post exposure. The observed increases in resistance and decreases in compliance and maximum expiratory flows are consistent with findings of decreased in pulmonary function in USC's CHS (Gauderman et al. 2004b), although the specific pulmonary functions measured in the CHS were not the same as those made in the mice in the present study. These findings are also consistent with pulmonary function measurements made in freely breathing asthmatic adults exposed to ambient air pollution (Abbey et al. 1998).

**Figure 7.** Respiratory system resistance measured after high concentration exposure to fine CAPs or to air. There is a pattern of increased resistance in CAPs-exposed mice compared to air-exposed mice but the individual group means are not significantly different except for measurements made 1 week post-exposure. Baseline represents values for resistance measured in unexposed mice, age 4 weeks. *p < 0.05
Figure 8. Changes in maximum expiratory flow (MEF) as a function of respiratory resistance and dynamic compliance. Increased resistance would be expected to cause decreases in MEF and presumably forced expiratory volumes, as well. Baseline represents values in unexposed mice, age 4 weeks. *Significantly different from control (p ≤ 0.05)

**Particle Composition**

Paulson and colleagues analyzed PM2.5 in parallel with the exposures during the high concentration study. In addition, she also determined coarse (PM2.5-10) and ultrafine (PM 0.1) particle compositions. These data are presented in Table 3.
Table 3. Summary statistics for absolute concentrations of soluble elements in coarse, fine, and ultrafine particles at the Riverside Site (Ultrafine, \(d_p\leq0.18 \, \mu m\); Fine, \(d_p\leq2.5 \, \mu m\); Coarse, \(2.5\leq d_p\leq10.0 \, \mu m\); where \(d_p\) is mass median aerodynamic particle diameter)

<table>
<thead>
<tr>
<th>Element</th>
<th>Coarse</th>
<th></th>
<th></th>
<th>Fine</th>
<th></th>
<th></th>
<th>Ultrafine</th>
<th></th>
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</thead>
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<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>EF*</td>
<td>Mean</td>
<td>S.D.</td>
<td>EF*</td>
<td>Mean</td>
<td>S.D.</td>
<td>EF*</td>
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<td>(H_2O_2)</td>
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<td>0.153</td>
<td>0.223</td>
<td>0.204</td>
<td>0.234</td>
<td>0.028</td>
<td>0.022</td>
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<td>0.061</td>
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<td>1.000</td>
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<td>K</td>
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<td>Fe</td>
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</tr>
<tr>
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<tr>
<td>Se</td>
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<td>0.003</td>
<td>0.011</td>
<td>0.007</td>
<td>0.012</td>
<td>0.007</td>
<td>0.005</td>
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</tr>
<tr>
<td>Pb</td>
<td>0.007</td>
<td>0.004</td>
<td>0.002</td>
<td>0.009</td>
<td>0.008</td>
<td>0.009</td>
<td>0.003</td>
<td>0.002</td>
<td>0.075</td>
</tr>
</tbody>
</table>

*EF: Enrichment Factor relative to Al

The coarse particles are enriched (EF ≥ 1.0) with Na, Mg, Ca and Al, elements associated with the earth’s crust and sea spray (Arhami et al. 2009). The elemental enrichment in Ultrafine (UF) relative to Coarse (C) particles (EF UF/EF C) shows that K, possibly from biomass burning, V and Ni, probably from fuel oil combustion, and Pb and Se, possibly associated with secondary sulfate aerosols (Kim et al. 2005) are indicative of specific emission sources that should be investigated further as being related to adverse PM health effects.

Exposures at Low PM Concentrations

Objective 5: The original plan for this project was to perform the low concentration exposure study in Lompoc, CA. However, there was a hiatus in
the project due to a Governor’s Executive Order that halted all work on the project for several months. This made it impracticable to perform the low concentration exposure study in Lompoc, and so it was performed in Riverside, CA at the same location as the high concentration study. During these exposures ambient concentrations were considerably lower than during the high concentration study, and it was not necessary to dilute the VACES output as had been originally proposed. Fourteen male C57BL/6 mice were exposed to CAPs 4 days/week for 5 hours/day for 7 weeks and then maintained in purified, filtered air for an additional 2 weeks. Particle mass and number concentrations are summarized in Table 4.

**Table 4. Low Concentration Particle Mass and Number Concentrations**

<table>
<thead>
<tr>
<th>Mass Concentration</th>
<th>Filtered Air</th>
<th>6 ± 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE (µg/m³)</td>
<td>PM2.5</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>Particle Count</td>
<td>Ambient</td>
<td>7,700 ± 1020</td>
</tr>
<tr>
<td>Mean ± SE (particles / cm³)</td>
<td>PM2.5</td>
<td>83,500 ± 760</td>
</tr>
</tbody>
</table>

The ambient mass concentrations were lower than during the previous run so that the average CAPs exposure concentration was 56 µg/m³, significantly lower than the concentrations in the high concentration study. However the particle number concentration was only about 11% lower than that of the high concentration study, suggesting that the mice in this study were exposed to nearly as high a particle number concentration as the mice in the high concentration study, even though the PM2.5 mass concentration was considerably lower. This indicates that exposure to ultrafine particles was similar between the two experiments.

Mice were intubated and pulmonary function was measured at 1, 3, and 5 weeks of exposure, and 2 weeks post exposure using the revised protocol described
above. This protocol differed from the one used for the high concentration exposure study, in that the same mice were studied in a repeated measures design, allowing each mouse to be its own control. This reduced the number of animals required for the study. The pulmonary function measurements and outcomes were essentially the same as reported in the high concentration study.

Figure 9. Timeline of Low Concentration Study. Baseline represents values measured in unexposed mice at the time of pulmonary testing. Measured body weights for air and fine CAPs-exposed groups at time of measurement are contrasted with values provided by the supplier. Numbers of mice examined are shown.

Dynamic Compliance (Figure 10) did not change consistently for the CAPs-exposed group, in contrast with the decrease seen in the mice exposed at high concentration, over the course of the study. While the air group had higher average compliance than the CAPs group at baseline and after 2 weeks of exposure, this tendency was reversed at 5 weeks and at 2 weeks post-exposure.
Figure 10. Dynamic Compliance (Low Concentration Study) measured in mice during and after exposure to air or to fine CAPs. CAPs exposure did not induce any consistent, or statistically significant, changes in compliance. Baseline represents values for dynamic compliance in unexposed mice, age 4 weeks.

Pulmonary resistance, as shown in Figure 11, was increased by CAPs exposure relative to purified air exposure at both 2 and 5 weeks. At two weeks post-exposure, pulmonary resistance was significantly increased (p < 0.05) in the CAPs group compared to the air group. Consistent with the high concentration exposure study, the effects of CAPs exposure on resistance appear to persist at least for a period of one to two weeks after exposures end.
Figure 11. Respiratory Resistance (Low Concentration Study) measured in mice during and after exposure to air or to fine CAPs. CAPs exposure consistently increased resistance compared to the air-exposed group and was significantly increased 2 weeks post exposure. Baseline represents values for unexposed mice, age 4 weeks.

*p < 0.05

The baseline data for maximum expiratory flow (Figure 12) show that the groups were not exactly matched on this parameter, in that the maximum flow was initially greater in the PM group than in the purified air group. However, after 5 weeks of exposure this tendency reversed and the CAPs group had lower maximum expiratory flow than the air group. The differences were not
statistically significant and did not persist once CAPs exposure ended.

![Max Expiratory Flow](image)

**Figure 12. Maximum Expiratory Flow (MEF, Low Concentration Study)** showed no consistent pattern of exposure-related responses. Baseline represents values unexposed mice, age 4 weeks and MEF was significantly lower than baseline at 2 weeks Post for both the CAPs and Air-exposed mice.

**Exposure of Nrf2-/- Mice**

Because many of the effects of inhaled contaminants are associated with oxidant-induced responses we investigated whether mice in which the antioxidant response system was impaired by knocking out the Nrf2 gene would demonstrate larger responses than wild type mice. Nrf2-/- mice have been shown to be much more sensitive to oxidant-related adverse effects in multiple organs (Araujo et al. 2008; Li et al. 2004). We evaluated the effects of inhaled CAPs on pulmonary function in these animals.
These mice were exposed under conditions similar to those used in the high concentration study. There were significant difficulties in conducting the study. Notably, the mice bred slowly and only a few mice were available at any given time, hence the exposures were actually conducted over extended time frames so that an adequate number of mice could be exposed for 8 weeks. These animals proved to be quite frail. Several animals failed to survive the intubations (we believe that issue has been overcome with our new protocol). As mentioned above, this project was interrupted due to Governor’s Executive Order that stopped work on state-funded contracts. In addition to other disruptions, the hiatus contributed to the reduced effort for the experiment using the Nrf2-/- mice because funding was not available to support continued maintenance and breeding of these mice.
As shown in Figure 13, the Nrf2-/- mice did show a response to the CAPs exposure (decreased compliance, increased resistance, decreased maximum inspiratory and expiratory flows). They also had a decreased respiratory rate although the tidal volumes were unchanged.

**Discussion**

As discussed previously, we hypothesized that one possible reason for the differences in lung function growth in CHS participants living in the Central Coast region of California compared to those growing up in the Riverside area.
was the difference in the long-term average PM2.5 concentration between these sites. Thus, the primary objective of this study was to test the hypothesis that chronic concentrated PM2.5 exposure during the period of rapid lung growth in mice would lead to pulmonary function deficits, and that these deficits would be associated with oxidative stress and inflammation in the lung. The secondary hypothesis was that pulmonary function deficits elicited by chronic exposure during the period of rapid lung growth would persist after the termination of concentrated PM2.5 exposure.

Accordingly, this study sought to determine in a mouse model whether pulmonary function growth was influenced by semi-chronic PM2.5 exposure during the period of rapid lung growth in a dose dependent fashion. Initially we planned to use both wild type and genetically modified (Nrf2-/-) C57BL6 mice. We were able to include only a small number of the Nrf2-/- mice in our experiments because the mice had difficulty tolerating the stress related to the exposures and to the pulmonary function measurements. The data we were able to obtain suggest that the Nrf2-/- mice followed a similar lung growth pattern as the normal mice in our study, although their pulmonary function responses were more variable.

We addressed the study objectives by exposing mice to CAPs or purified air from about 3 weeks to 11 weeks of age, the period of rapid lung growth and development in the mouse, and while mice do not actually stop growth at 11 weeks, their growth rate is much slower after that point. Animals exposed to the high concentration of CAPs clearly demonstrated a pattern of decreased pulmonary function, measured as decreased dynamic compliance and increased pulmonary resistance that persisted for at least 1 week after the termination of exposures. Although the specific pulmonary functions measured were different than those reported in the CHS, our observation of decreased pulmonary function with CAPs exposure is consistent with findings from the CHS (Gauderman et al. 2004b). It is difficult to determine whether these pulmonary
function decrements will persist into later life or will increase susceptibility to chronic lung disease or respiratory infections later in life.

It is significant that the high level exposure did not impair the lung’s mechanisms for clearing deposited particles both during exposure and once the exposure ended, as shown by the relatively constant number of alveolar macrophages with carbonaceous inclusions observed in the lungs of animals sacrificed during the exposures, and the drop to baseline levels at one week post-exposure (Figure 5).

The effects of PM2.5 on pulmonary function may be concentration dependent since the high concentration exposures resulted in a somewhat more consistent pattern of increased resistance and decreased compliance, maximal flows and respiratory rate when measured 1 week post exposure. In contrast, the low concentration study showed increased resistance but inconsistent changes in compliance.

While the particle mass concentrations were very different between the high and low concentration experiments (243 µg/m³ vs. 56 µg/m³), the particle number concentrations were similar (93,500 particles/cc vs. 83,500 particles/cc) suggesting that ultrafine particle concentrations were similar for the two exposure studies. The exposure protocols for the high and low concentration studies were not identical, and so any direct comparisons of the results need to be considered carefully. However, given the great difference in the exposure mass concentrations and the similar particle number concentrations, the finding that both exposure protocols produced similar increases in respiratory resistance suggests that some component of the ultrafine particles may have contributed to the pulmonary resistance increases that were observed in both exposure groups.
A major accomplishment of the study was the development and implementation of a new methodology that allowed us to make repeated measurements of pulmonary function in mice as young as 4 weeks of age. The method enabled us to perform repeated pulmonary function tests on the same animals so that each could be its own control. This allowed us to follow changes in individual animals over the course of our study, and strengthened the study design by reducing the influence of variability in responses between animals. Evaluation of resistance and compliance as functions of age (i.e. comparing the values for resistance and compliance measured in air-exposed mice in the low and high concentration studies) in this project indicated no significant biases introduced by the new technique. Thus, use of the technique in the future studies will allow greater efficiency.

The pulmonary function responses of the Nrf2-/- mice were similar to those observed in wild type mice exposed to CAPs under comparable conditions, however this finding is based on a small data set and more analyses are needed.

Two of the original aims included evaluations of inflammatory and oxidative stress mediators, and genetic interactions that might be important in the effects of inhaled particles on lung growth. Samples were frozen and stored, but could not be completed within the framework of the present study after the project was reinstated after the hiatus. The samples are available to be analyzed at such time as monies become available.

**Conclusions**

The USC Children’s Health Study observed that children exposed to higher levels of PM2.5 air pollution during the life period when the lungs are still developing demonstrated reduced growth in pulmonary function compared to
children exposed to lower levels of PM2.5 pollution. We examined one hypothesis suggested by the CHS, namely that exposure to high levels of PM2.5 air pollution would lead to reduced lung function growth. We exposed mice to filtered air or to fine CAPs during the period of rapid growth between 3 and 11 weeks of age.

To facilitate this study, we developed a technique for making repeated pulmonary function measurements in young mice from 3 weeks to 12 weeks of age so that repeated measures study designs could be used in this and future studies.

Our studies at high concentration support the CHS study findings; semi-chronic exposure of juvenile mice to a high concentration of PM2.5 can lead to decreased pulmonary function growth. Increased respiratory resistance was also evident up to 2 weeks after the exposures ended, suggesting a persistent effect.

The ambient PM2.5 mass concentrations during the low concentration study were much lower than used in the high concentration study. Because ambient PM2.5 concentration during the exposure period was lower than in previous years, we were unable to attain the target mass concentration, even with the concentrator operating at full efficiency. Particle number concentrations were very similar for both the high and low studies. Animals exposed to the low concentration of CAPs showed a pattern of pulmonary function growth that was less consistent than that seen in the high concentration study, although there was evidence of reduced lung function growth even at the low PM2.5 concentration used in this study. This suggests that PM2.5 may be able to alter lung function development during childhood, and that the magnitude of the effect is concentration dependent. The average particle number concentrations during the low concentration study were slightly (~11%) lower than those during the high concentration study even though the mass concentrations were significantly (~76%) lower, indicating that the exposures to ultrafine particles
during the two studies might be similar. This might explain why there were significant reductions in respiratory resistance in both studies.

**List of Abbreviations**

d_p Mass median aerodynamic particle diameter

DTT dithiothreitol

EDTA ethylene diamine tetraacetic acid

HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

CAPs Concentrated Ambient Particles

PM2.5 Particles with median aerodynamic diameter ≤ 2.5 µm

UF Ultrafine Particles (d_p ≤ 0.05 µm)

F Fine Particles (d_p ≤ 2.5 µm)

C Coarse Particles (2.5 ≤ d_p ≤ 10 µm)

CHS Children’s Health Study (USC)

BALF bronchoalveolar lavage fluid

H_2O_2 hydrogen peroxide

ANOVA analysis of variance
Nrf2  Nuclear factor E2–related factor-2 (Nrf2) is a transcription factor that orchestrates antioxidant and cytoprotective responses to oxidative and electrophilic stresses.

SE Standard Error
References


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