Effects of Cache Valley Particulate Matter on Human Lung Cells

Todd L. Watterson
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EFFECTS OF CACHE VALLEY PARTICULATE MATTER ON HUMAN LUNG CELLS

by

Todd L. Watterson

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY in

Toxicology

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UTAH STATE UNIVERSITY
Logan, Utah

2012
ABSTRACT

Effects of Cache Valley Particulate Matter on human lung cells

by

Todd L. Watterson, Doctor of Philosophy
Utah State University, 2012

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Department: Animal Dairy and Veterinary Science

During wintertime temperature inversion episodes the concentrations of particulate air pollution, also defined as particulate matter (PM), in Utah’s Cache Valley have often been highest in the nation, with concentrations surpassing more populated and industrial areas. This has attracted much local and national attention to the area and its pollution. The Cache Valley has recently been declared to be in non-attainment of provisions of Federal law bringing to bear Federal regulatory attention as well. While there is epidemiological evidence indicating that PM is detrimental to public health, there is much less information indicating by which biological and molecular mechanisms PM can exert harm. This study was undertaken to better understand the mechanisms by which ambient PM collected in the Cache Valley can be harmful to human lung cells. Cache Valley PM was found to be mildly cytotoxic only at concentrations that were much greater than physiologically achievable, and such concentrations were difficult to obtain with the limited amounts of captured ambient PM. The limited cytotoxicity was despite apparent PM-induced pro-apoptotic signaling such as caspase-3 upregulation, and
activation of caspase-12 and calpain. Cache Valley PM was found to be stressful to cells, triggering endoplasmic reticulum stress and the unfolded protein response. Cache Valley PM was also found to be inflammogenic leading to activation of pro-inflammatory transcription factors, increases in the release of pro-inflammatory cytokines and chemokines, as well as the upregulation of the activating receptors of these cytokines. The proinflammatory effects and absence of apoptosis, despite pro-apoptotic signaling of the Cache Valley PM on human lung cells appeared to stem from increased activation of the central pro-growth protein Akt with subsequent inactivation of the tumor suppressor P-TEN. These findings have indicated novel mechanisms of PM-related cellular stress and inflammation contributing needed information on what may be underlying mechanisms of PM associated illnesses.

(274 pages)
PUBLIC ABSTRACT

Effects of Cache Valley Particulate matter on human lung cells

Todd L. Watterson

During the wintertime, residents of the Cache Valley are very aware of the poor air quality that occurs during cold-air inversion episodes. If one somehow avoids the radio and television announcements as well as the electronic roadside signs indicating poor air quality and encouraging less driving, one need only to look upward toward the mountains that surround the valley and notice the lack of daytime visibility. This lack of visibility and health warnings are due to particulate air pollution or particulate matter (PM). PM is only one of many types of regulated air pollution but is the one that occurs most in the Cache Valley. Mathematical studies done with large populations in other cities, or epidemiological studies, have associated higher concentrations of PM with early death from a variety of causes. The deaths are not caused by the air pollution; they are the same causes of death that affect everyone such as heart attack, stroke, cancer, etc. The PM is associated with populations of people dying sooner from those causes. How that occurs no one really knows. The epidemiological studies cannot determine how so other studies must be done to see what biological mechanisms are involved in PM causing harm. The research conducted here is an example of such studies. Using locally collected PM and human lung cells these studies were conducted to determine some potential mechanisms by which PM can cause harm. The PM did not readily kill the cells used here, which made the investigation more in-depth. However, the PM did cause stress in the cells. The PM caused the cells to produce proteins that are involved in signaling to other cells messages involved in the inflammatory process. The PM also caused the cells
to undergo a special type of stress called endoplasmic reticulum (ER) stress. The PM caused the cells to activate internal signaling pathways that lead to the above-mentioned inflammation and cell growth as a likely adaptation to the stress. The findings of this study involving ER stress and activation of some of the cellular pathways were original and are mechanisms shared by diseases that have been associated with early death and PM exposure. While these findings add valuable information, there remain a large number of questions pertaining to how PM can exert harm in cells, the lung, and across the body.
ACKNOWLEDGMENTS

I would like to thank foremost Roger Coulombe for his guidance and mentorship, and the Marriner S. Eccles Foundation, the Utah Water Research Laboratory, the Center for Integrated Biosystems at Utah State University, and the Utah Agricultural Experiment Station for support. Many thanks are due to committee members, Drs. Jeff Hall, Randy Martin, Phil Silva, Bryan Stegelmeier, and Anhong Zhou, as well as former committee members who assisted in the failed plant project Drs. David Hole, Richard Wang, and Ann Aust. I would like to thank Dr. Ya Jun Wu for his assistance with Arabidopsis. I am grateful for Dr. John R. Stevens for the helpful suggestions on statistical analyses; and Lorinda Anderson, Dr. John Veranth, Gary Yost, and Chris Reilly of the University of Utah for performing the original IL-6 ELISA and providing information, input and protocols, respectively. I would like to thank undergraduate researchers Jared Sorenson and Brett Hamilton for their contributions. Mostly I want to thank my wife, Sarah, for supporting and putting up with me through this folly, and my son Evan who may have contributed to my delaying completing this, but has been the greatest thing to happen to me in my life. I also thank fellow graduate students Terry VanVleet, Pat Klein, John Guarisco, Sumit Rawal, and Tyler Cranny for camaraderie and moral support and good friend and mentor Lance Thomas.

Todd L. Watterson
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CHAPTER 1

REVIEW OF PERTINENT LITERATURE AND RESEARCH AIMS

History of particulate air pollution regulation

The public health narrative concerning particulate air pollution or particulate matter (PM) is best told as a combination of politics, epidemiology, and research through following the history of the regulation of PM. The first environmental law concerning PM in the modern industrialized world was the British Clean Air Act of 1956 (Greenbaum 2003). This act was passed following findings of a 1954 report by the United Kingdom Ministry of Health that investigated a London incident in December 1952 where PM concentrations were in excess of 1000 µg/ml and concluded that the incident was strongly associated with increased mortality from respiratory and cardiovascular conditions among the elderly (Greenbaum 2003; Krewski et al. 2003). The incident was caused by stagnant cold weather conditions that trapped pollutants at ground level (Krewski et al. 2003). On April 30, 1971 the United States Environmental Protection Agency (EPA) instituted primary standards for total suspended particulate (TSP) of 260 µg/m³, a 24 hour standard not to be exceeded more than once per year with an annual mean of 75 µg/m³ (National Center for Environmental Assessment [Research Triangle Park N.C.] 1996). Following contentious public debate and the first substantial legal challenges to EPA rulemaking (Greenbaum 2003), the EPA replaced TSP as the principle indicator of PM with a size differentiated standard of PM$_{10}$ (particulate matter with a 50% “cut-point” of 10 µm aerodynamic diameter). The 24 h standard was 150 µg/m³ with one exceedance per year and an annual average standard of 50 µg/m³. The 10
µm diameter was selected for attention since it is a size capable of entering the thoracic region of the respiratory system, the significance of which is discussed later in this review (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Later the EPA decided to examine particles finer than PM$_{10}$ due to the ability of the smaller diameter particles to enter the respiratory system and settled on particulate matter less than 2.5 µm in diameter (PM$_{2.5}$). The 2.5 µm “cut-point” for selecting between coarse and fine PM was originally used due to the availability of dichotomous samplers capable of selecting to a size of 2.5 µm (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). After evaluation of research involving PM$_{2.5}$, the EPA proposed and eventually adopted a new set of standards for PM$_{2.5}$. After a public review process that was very contentious involving questions concerning the strength of the epidemiologic studies, the lack of a plausible biologic mechanism, and potential exposure measurement error, the EPA adopted a new standard of PM$_{2.5}$; a 24 h average of 65 µg/m$^3$ and annual average of 15 µg/m$^3$, with seven exceedances allowed per year (Greenbaum 2003). The standards are found in Table 1-1.

In 1996 the EPA relied heavily upon two major studies justifying the new standards, the Harvard Six Cities Study which showed the greatest hazard ratio with early all cause mortality of 1.75 (1.32-2.32 95% confidence interval, CI) among currently smoking men (Dockery et al. 1993) and the American Cancer Society (ACS) study (Pope et al. 1995) which showed a ratio of 1.17(1.09-1.26 95% CI) ratios with only cardiopulmonary and lung cancer but not all cause mortality. The relative risks were slight, albeit significant. These findings were quite important since previous studies
demonstrated the mean effects on mortality associated with air pollution were essentially the same for PM and gases and were “invariant with respect to particle diameter” indicating that there was not a difference between particle diameters and proposed PM$_{2.5}$ regulations lacked support (Lipfert and Wyzga 1995). The EPA’s reliance upon the ACS and Six Cities studies brought intense attention upon the two studies (Krewski et al. 2003). In response to the demand for the data from the two studies to be made public given concerns about the reliance of the EPA on those studies, and public and political questions concerning the data, Harvard University commissioned the Health Effects Institute (HEI) in MA to conduct independent analysis of the Six Cities study. Later the American Cancer Society (ACS) eventually joined the project resulting in independent analysis of both studies (Higgins et al. 2003). The findings of the reanalysis project concluded that there may have been flaws in the cohort studies including a strong influence of educational attainment on PM associated risk wherein risks were reduced in the populations with greater educational attainment, but the original studies were supported in their conclusions and their findings reinforced with more sophisticated modeling (Dockery et al. 2003; Krewski et al. 2003; Lipfert 2003). These reanalyses resulted in increased confidence in the cohort studies which have been used for justification of subsequent regulatory initiatives. For a comprehensive summary of the reanalysis project and excellent suggestions for regulators and decision makers, volume 66 of Toxicology and Environmental Health Part A is recommended reading, particularly the article by Lipfert (Lipfert 2003).

The estimated costs for the regulations were $48.8 billion in year 2000 dollars (Hahn et al., 2003) making the 1996 PM national ambient air quality standards (NAAQS)
potentially the most expensive environmental regulation in history (Fumento 1997). Multiple lawsuits were filed culminating in the Supreme Court decision (Whitman v the American Trucking Associations 531 U.S. 457 2001) where the court upheld the NAAQS for PM and ruled that the Clean Air Act requires the EPA to set health based standards without consideration of costs, while costs can be considered by the EPA and the states in the implementation of the set standards (Hahn et al., 2003). By way of comparison, the European Union (E.U.) implemented two-stage standards for 2005, a 24 h average of 50 µg/m³ with thirty five allowed exceedances per year and an annual average of 40 µg/m³. For 2010, a 50 µg/m³ 24 h average with seven annual exceedances and an annual average of 20 µg/m³ was instituted (Greenbaum 2003). The United Kingdom concluded that a PM\textsubscript{2.5} standard is not required (Greenbaum 2003). For this reason, most studies from the E.U. do not differentiate between PM\textsubscript{2.5} and PM\textsubscript{10}. In this work the term PM includes PM\textsubscript{10}, PM\textsubscript{2.5}, and PM\textsubscript{1} unless otherwise stated.

The lack of underlying biological mechanisms and lack of correlation from human epidemiological studies and animal studies was acknowledged by the EPA, and PM research centers were established for conducting additional research to address these matters (Lippmann et al. 2003).

Risk Assessment

In the process of enacting most public health and environmental regulation, the regulatory agencies conduct risk assessments. Risk assessment is defined as “the systematic scientific characterization of potential adverse health effects resulting from human exposures to hazardous agents or situations” (Foustman 1996). Risk assessment
generally involves the steps of hazard identification, dose response assessment, and exposure assessment (National Research Council [U.S.]. Committee on Risk Assessment of Hazardous Air Pollutants 1994). This review is not intended to be a risk assessment but will summarize the literature along similar steps in order to convey a coherent message about the intricate relationship of the complex environmental contaminant PM and human health.

Hazard Identification

Hazard identification is the process of identifying contaminants suspected to pose health hazards and determining the concentrations of the contaminants, and the specific forms of toxicity (National Research Council [U.S.]. Committee on Risk Assessment of Hazardous Air Pollutants 1994). The EPA uses epidemiologic studies in hazard identification because they provide relevant information involving observations of human beings (Risk Assessment Task Force 2004).

A short review of the epidemiology and the criticisms thereof is helpful in understanding how PM can exert harm in individuals and be a public health concern. Also, the controversy and lawsuits involved in the regulation of PM centered on the quality and nature of the epidemiologic research of the time. The epidemiologic studies used in establishing the air quality criteria document (AQCD) for PM are generally divided into morbidity and mortality studies. Morbidity studies examined the associations of PM on health endpoints such as hospital admissions, medical visits, reports of respiratory distress, low birth-weight, alterations in cardiovascular functions, and blood coagulation (Zanobetti et al. 2004). Mortality studies examine the associations between PM and non-accidental death. Such studies “provide the most unambiguous
There are multiple types of epidemiological studies, four of which were considered for the establishment of the AQCD: ecologic, time series, cohort, and case control (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). With ecologic studies the focus is upon groups and the units of analysis are populations and no individual level analysis is performed (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Ecologic studies have numerous methodological problems that limit causal inference (Morgenstern 1995) such as the lack of information on individual confounders like smoking (Brunekreef 2003). A time series study is a collection of sequential observations made over time (Chatfield 2004). These studies are deemed more informative since they allow examination of associations between changes in health outcomes and changes in indicators of exposure (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). A cohort is a group of people who share a common condition or experience. A cohort study usually examines two groups and the incidence of disease/death is studied in the groups over time (Brunekreef 2003). While cohort studies may be retrospective, prospective studies allow for optimal study design, recruitment of participants that represent the target population, and collection of individual-level data. Cohort studies are considered to possess the greatest inferential strength (Brunekreef 2003; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Case control studies are retrospective studies where the outcome has already occurred and allows the investigators to pick cases where low incident effects have occurred. The groups where
the outcome has occurred are compared with groups that have not been affected. The controls must be selected independent of their exposure status (Coggon et al. 2003; Motulsky 1995; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Another type of study is the meta-analysis, where previous independent studies are statistically examined in order to provide a better estimate of treatment effects and explain the heterogeneity between the individual studies (Egger et al. 1997).

Epidemiological studies generally present their findings as relative risks (RR), odds ratios (OR), hazard ratios (HR), or attributable risks (AR). The relative risk is the ratio of the probability of an endpoint (disease or death) occurring in a group following an exposure divided by the probability of the same endpoint occurring in an unexposed group (Coggon et al. 2003; Motulsky 1995). RR are used in prospective studies, with the confidence intervals calculated by a number of methods (Motulsky 1995). A relative risk of 0.0-1.0 indicates the outcome risk decreases with exposure, relative risk greater than one indicates the risk increases. A relative risk of 1.75 indicates that an exposed population was 1.75 times more likely to suffer (or benefit) from the studied factor (Motulsky 1995). Odds ratios are calculated by dividing the odds of an endpoint in an exposed population by the odds of the endpoint in an unexposed population (Motulsky 1995). Odds ratios are similar to, and close approximations of, relative risks (Coggon et al. 2003; Motulsky 1995). Odds ratios are used in case control studies rather than RR, since researchers in case control studies select cases based upon endpoint status and no information is available about the incidence or risk of the endpoint (Motulsky 1995). Hazard ratios are part of survival analysis with the measured endpoint being death, and are essentially relative risks (Motulsky 1995). The attributable risk is the disease rate in
exposed individuals, minus the rate in unexposed individuals. It is related to relative risk and is thought to be more relevant than the RR in regulatory decision making (Coggon et al. 2003).

In establishing the 1996 AQCD the EPA examined multiple aspects of the epidemiological research including exposure metrics, well defined study populations, meaningful endpoints, appropriate statistical analysis, whether confounders are properly controlled, and if the findings were biologically plausible (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Perhaps the most important and difficult of the above aspects of the epidemiological research is whether the health endpoint measurements were meaningful. In an award-winning article for Science, that was ironically published in 1995 prior to the controversial 1996 AQCD, Gary Taubes interviewed a number of epidemiologists concerning the manner in which weaker epidemiological studies have lead to an “epidemic of anxiety” among the public through an “unholy alliance” between epidemiology, the journals, and the press (Taubes 1995). Epidemiologists, including the well known Richard Doll, were cited as stating relative risks should be greater than three or four for paper publication or causal inference, and epidemiologists were divided on whether weak associations were considered convincing with repetition (Taubes 1995). While PM studies were not mentioned in the article, it is informative to see the strength (or lack thereof) of the PM studies and why the implementation of PM standards was and still is so contentious.

Of the multiple studies reviewed by the EPA in issuing the 2002 NAAQS, the greatest RR for all cause mortality was 3.01 for SO₂ in non-smoking females. The RRs for PM did not exceed 2.0. While the RRs were low, the HEI reanalysis affirmed the
appropriateness of the statistical analysis, and provided information on the role of confounders. Further studies affirmed that there is a risk to public health from PM (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Although the effects of air pollution on health may have been shown to be detectable and significant, they are of a lesser magnitude than many other health concerns (Hayes 2003). Nevertheless, PM regulations are in place and will be examined every five years in the United States due to the classification of PM as a criteria pollutant. Further investigations of PM and its potential harmful effects and underlying mechanisms are ongoing.

Short-term exposure

Daily alterations in PM have been associated with alterations in daily mortality. This effect was monitored in Chicago, IL (Cook County) and Salt Lake City, UT (Salt Lake County) with meteorological confounders (Styer et al. 1995). Similar associations with alterations in daily mortality were observed in multiple analyses of the six cities of Watertown, MA, Kingston-Harriman, TN, St. Louis, MO, Steubenville, OH, Portage, WI, and Topeka, KS (Klemm et al. 2000; Laden et al. 2000; Schwartz et al. 1996). Short term PM exposure also was shown to be associated with early mortality in nine counties in California (Ostro et al. 2006).

Long-term exposure

Long term PM exposure studies in multiple cites have demonstrated associations between PM concentrations and early mortality. Such mortality involves illnesses such as cardiopulmonary disease (RR= 1.3), cardiovascular disease (RR= 1.36), respiratory
disease (RR= 1) lung cancer (RR= 1.14) and other cancers (RR=1.14) (Krewski et al. 2005). Of particular importance is the risk of cardiovascular disease, the leading cause of death in the United States (Brook et al. 2004; Dockery 2001). Studies subsequent to the ACS and Six Cities studies have also demonstrated associations with long term PM exposure and mortality (Pope and Dockery 2006), with the greatest effect (HR= 1.76) seen with PM associated cardiovascular events (not just mortality) in women (Miller et al. 2007). Critics have pointed out that the effects observed in the Miller et al. study are likely acute and not truly long term, a point conceded by the authors (Brook and Rajagopalan 2007).

Morbidity studies have demonstrated PM associated increases in hospital admissions and stays, although there are strong regional differences in the duration of the hospital stays, which indicate geographic behavioral differences may confound the analysis (Lipfert 1993b). Reasons for hospital admissions include cardiac arrhythmia (Santos et al. 2008), headache (Szyszkowicz 2008), influenza, bronchitis, rheumatic fever, asthma, hypertension, and allergic conditions (Lipfert 1993b).

The EPA is required to identify subpopulations or special populations that are at greater risk of environmental harm (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Subpopulations observed to be under greater risk from PM exposure include, but are not limited to, persons with congestive heart failure (Maynard et al. 2003), persons with “dirty” jobs or occupations that were deemed to be “dirty” by a team of industrial hygienists and exposure assessors (Siemiatycki et al. 2003), children (Barraza-Villarreal et al. 2008), the elderly (Aga et al. 2003), and poor people (Lipfert 2004).
Many of the PM related health risks are stronger at low exposure concentrations than higher exposure concentrations indicating that there is no safe exposure level threshold for PM (although sulfate appears to have one) and a non-linear response relationship (Abrahamowicz et al. 2003; Bayer-Oglesby et al. 2005; Schwartz et al. 2002). The lack of threshold makes the task of risk assessment of low exposures difficult (Kroes et al. 2005).

Dose response relationship

Many of the epidemiologic studies attempted to address a dose response relationship between PM exposure and the morbidity/mortality endpoints that the researchers were examining. Most of the concentration response relationships are for short term exposures and depend upon the smoothing models used. Some early studies that established an association between PM and mortality lacked a good dose response relationship (Pope et al. 2002), however in argument, most relationships approximated linear responses (Pope and Dockery 2006). The concentration response relationship for long term PM exposure has not been widely explored. Given the effects of small changes in PM concentrations on mortality are small, the results of many epidemiologic studies are presented as changes in mortality over 10 µg/m³ of PM (Dominici et al. 2005; Pope and Dockery 2006).

Exposure Assessment

Exposure assessment involves the determination of the type, source, magnitude, and duration of contact with the harmful agent of interest (Foustman 1996). This must be done on a population and individual basis. Often exposures take place over time and in
multiple locations, making it difficult to accurately estimate exposures (Zeger et al. 2000). Personal exposure studies have demonstrated that ambient PM$_{2.5}$ concentrations and personal exposure measurements poorly correlate (Meng et al. 2005). Personal exposure of indoor PM is greater than outdoor PM, especially to PM components of biological origin (Dominici and Burnett 2003; Maynard and Cohen 2003) (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). In fact, ambient PM$_{2.5}$ is a poor indicator of personal PM exposure (Meng et al. 2005).

Additional exposure research is being investigated by the EPA and associated research groups in order to gain better insight into how PM affects individuals and populations (Lippmann et al. 2003). Many of the first studies involving PM and health effects are to be interpreted carefully as many of the earlier studies involve single air monitoring stations for PM$_{10}$ for a wide geographical area (Dockery et al. 1993; Pope et al. 1999; Schwartz et al. 1996). This does not allow for adequate measurement of personal exposure for PM$_{10}$ and affects the population exposure estimates as well. Fine particulates linger in the atmosphere and spread over geographical distances, while coarser particulates settle quickly and must be measured geographically closer to the population (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Many studies indicated that the finer PM$_{2.5}$ has a greater association with early mortality than PM$_{10}$ or that the PM$_{2.5}$ component of PM$_{10}$ is responsible for PM$_{10}$ associated effects (Dominici et al. 2006; Klemm et al. 2000; Pope and Dockery 2006).

Dosimetry

Dosimetry involves the study of the amounts and distribution of PM deposited in the respiratory tract, how it can be moved to other sites in the respiratory tract, and
potentially to distal organ systems (Lippmann et al. 2003). The pulmonary system can be divided into three regions for the purposes of dosimetry studies (Figure 1-1): the extrathoracic, the tracheobronchial, and the alveolar. The extrathoracic comprises the airways of the head and the larynx. The tracheobronchial involves the trachea, the main bronchi, the bronchi, and the bronchioles. This region involves multiple branch points. The alveolar region is where gas exchange occurs (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Mechanisms of Particle Deposition

Particle deposition in the respiratory system may occur through four dominant mechanisms: inertial impaction, gravitational sedimentation, Brownian diffusion, and interception. All four are though to operate simultaneously (Balashazy et al. 2003). A fifth potential mechanism is electrostatic precipitation (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Particle size is the major determinant of by which mechanism impaction occurs (Balashazy et al. 2003; Darquenne and Prisk 2004; National Center for Environmental Assessment [Research Triangle Park N.C.] 1996). Inertial impaction occurs when particles impact airway surfaces following sudden changes in the direction of airflow. The extrathoracic and tracheobronchial areas dominate as sites of inertial impaction due to high air velocities. For humans the flow rate in the trachea is 60 l/min, however, following 3-4 subsequent bifurcations the flow rate is reduced to 7.5 l/min (Balashazy et al. 2003; National Center for Environmental Assessment [Research Triangle Park N.C.] 1996, 2003). The higher velocities create secondary reverse flow patterns in the vicinity of the carinal ridges. Smaller particles in the 0.2 µm range, a diameter representative of radon attached to
indoor aerosols and cigarette smoke, stay within the air flow, but larger particles in the 5 µm range (representative of urban or anthropogenic air PM) overcome the reverse air flow and deposit onto the carinal ridges (Balashazy et al. 2003). For particles greater than two µm in diameter, impaction is a significant mechanism of deposition (National Center for Environmental Assessment [Research Triangle Park N.C.] 1996).

The second mechanism of particle deposition, gravitational sedimentation, occurs when the acceleration of gravity acting on the particle achieves balance with air resistance and the particle settles out of the airstream coming in contact with the airway surfaces. While gravitational forces affect all particles, those with a diameter greater than 1µm are affected to the greatest extent (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Through Brownian diffusion, the third mechanism, small diameter particles (<1 µm) undergo random bombardment with air molecules resulting in contact with the airway surface (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Along with gravitational sedimentation, this mechanism is thought to predominate in the alveolar (or acinar) region of the lung although another mechanism called “convective stretching and folding” has been proposed to also occur in this region, explaining impaction studies performed in low gravity environments (Darquenne and Prisk 2004). Convective stretching and folding occurs when:

reciprocal motion of the air in the airways wraps the streamlines around each other during tidal breathing… not unlike that of the stretching and folding of pastry, with the effect being both to cause initially close streamlines to diverge from one another and to bring previously widely separated streamlines into close apposition. (Darquenne and Prisk 2004, p. 2083).
The fourth mechanism is interception or deposition by physical contact with the surface of the airway. This mechanism is most important in deposition of fibers, with fiber length having the greatest influence on where that deposition occurs (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Electrostatic precipitation, the fifth hypothesized mechanism occurs when particles acquire charges from collisions with air ions and are either like-charge repulsed into the airway or attracted by charges on the airway wall. The effect of charge on particle deposition is inversely proportional to particle size and airflow rate and is thought to affect workplace exposures and cigarette smoke while having a minimal effect on the deposition of urban PM (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

As stated, particle size is the greatest determinant of the mechanism of particle deposition; however, the issues concerning locations within the pulmonary system of particle deposition are complex, lacking clear areas of size dependent deposition. As particles get larger (from 1 to 10 µm), the probabilities of deposition are greater due to the stronger effects of inertial impaction. The areas of greatest deposition are carinal ridges (Figure 1-2) in airway bifurcations (Balashazy et al. 2003). Deposition depends upon many factors such as lung size, particle size, tidal volume, oral or nasal breathing, and breathing rate (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). The greatest numbers of human lung cancers are found in the lobar, or secondary, bronchi; an effect likely due to enhanced deposition in those areas (Lippmann et al. 1980). Recognizing the complexity of deposition patterns and the need for additional research, the EPA pointed out key aspects of particle deposition. Ultrafine
particles in the median of the accumulation mode (0.3 to 1.0 µm) have the lowest depositional fraction in the extrathoracic and tracheal bronchial regions. For alveolar deposition and ultrafine particles, the greatest deposition is for particles between 0.02 and 0.03 µm. For alveolar deposition of larger particles, the peak fractional deposition is between 2.5 and 5 µm. This deposition is greatly reduced if inhalation is nasal rather than oral. Peak fractional deposition in the tracheobronchial region is between 4 and 6 µm. As particle size decreases below 0.1 µm, the total particle deposition increases, with the pattern of deposition increasing in the extrathoracic region (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). A major complicating factor is that dry aerosols of multiple compounds will take up water and grow in size within the warm humid environment of the lung (Lippmann et al. 1980). Given the complexity of particle deposition, the understanding of size resolved particulate deposition and clearance is not complete and is the subject of further research (Lippmann et al. 2003). As a corollary to deposition, the cell types that receive the greatest particle deposition are currently unknown and the determination thereof is also an area of current EPA sponsored research (Lippmann et al. 2003). While it is tempting to simplify deposition as PM$_{10}$ deposits in the conducting airway and PM$_{2.5}$ deposits in the lower tract and alveoli (Baeza-Squiban et al. 1999), the reality of deposition is much more complex.

Clearance

Upon deposition, particles contacting the respiratory surfactant film are “wetted and displaced toward the epithelium” interacting immediately with proteins, other biomolecules, and opsonins which enhance uptake by phagocytes (Kreyling et al. 2006;
Peters et al. 2006. “The processes by which deposited particles are removed from the surface of the respiratory tract” define particle clearance (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Clearance from the respiratory tract does not mean clearance from the body (Witschi and Last 1996). The mechanisms of clearance depend upon particle qualities and location of deposition. Clearance from the extrathoracic region occurs through dissolution and absorption into the blood, mucociliary transport, sneezing, and nose wiping and blowing. Clearance in the tracheobronchial region takes place through mucociliary transport, endocytosis by macrophages and epithelial cells, coughing, and dissolution and absorption into the blood or lymph. Clearance from the alveolar region involves endocytosis by macrophages and epithelial cells, as well as dissolution and absorption into the blood or lymphatic system (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). A material cleared by mechanical means moreso than dissolution is said to be insoluble (Schlesinger 1995), and coughing, sneezing, nose wiping and blowing are the only mechanisms that truly remove the particles from the body (Witschi and Last 1996).

Mucociliary clearance, by definition, involves the cilia driven transport of mucous. The direction of this movement depends upon the pulmonary region. For example, the movement is forward in the anterior region of the nose to where particles can be removed by wiping, sneezing, or blowing (Schlesinger 1995). Mucous flows toward the oropharynx in the tracheobronchial region (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). The movement of mucous can approach 1-5 mm/min, although mucocilliary transport is sensitive to inhaled irritants (Lippmann et al. 1980). Oxides of sulfur can accelerate mucocilliary clearance at low
concentrations (5 ppm) and slow it at high concentrations (300 ppm) (Lippmann et al. 1980). Reduced mucociliary transport is a component of bronchitis (Lippmann et al. 1980), an illness associated with an increase of 7% in children with each 10 µm/m³ increase in PM (Hertz-Picciotto et al. 2007).

The mucociliary system is the primary route of clearance of particle laden macrophages from the respiratory tract, although the macrophages can also migrate through the alveolar epithelium into the alveolar lumen or the interstitium (Schlesinger 2000). Free PM that penetrates the interstitium is taken up by macrophages and removed through the interstitial or lymphatic pathway (Lippmann et al. 1980; Schlesinger 1995).

In the alveolar region, the first step of uptake of PM by alveolar macrophages occurs rapidly (within 24 hr), unless the particles are cytotoxic or very large (Schlesinger 1995). The number of macrophages increases depending upon the number of particles deposited (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). However, the clearance through phagocytosis is less rapid than by mucociliary transport, which is why particles trapped in the alveolar region are retained longer (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Particles are also taken into the epithelium with smaller particles directly penetrating epithelial membranes (Witschi and Last 1996). These particles typically end up in the interstitium (Schlesinger 2000).

Particles deposited in the extrathoracic region are typically cleared from anterior to posterior region by mucociliary clearance in 10-20 min with non-uniform mucosal flow rates (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003; Schlesinger 1995). Mucociliary flow rates vary greatly in the tracheobronchial
region, resulting in greater retention times. Older research has shown that within 48 h of deposition, 99% of particles are cleared from the tracheobronchial region. The remaining 1% can have half lives (the author uses the term half times) of 80 days or more (Schlesinger 1995). However, there are conflicting studies that demonstrate that sizable fractions of deposited PM remain in the tracheobronchial region over 24 h following deposition with remaining particles exhibiting half lives (the term half times was also used by the authors) over 150 days (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

As previously stated, particles deposited in the alveolar region are retained much longer than those retained in areas with mucociliary clearance (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Alveolar clearance is classified as being multi-phasic with the rapid phase having half times from 2-6 weeks, and the slower phase with half times from months to years (Schlesinger 2000). This retention was one of the compelling reasons behind the EPA establishing standards for PM$_{2.5}$ (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Clearance from the lung can be slow compared to the clearance from other organs. This was demonstrated in a study performed in hamsters where animals were harvested at 30 min, seven days, 300 days, and 365 days following exposure to an initial dose of 1.32 µm particles. Of the initial exposure $31.5 \pm 7.6\%$ of the total retained dose of was found in the lung. This percentage increased to $83 \pm 4\%$ total retained particles in the lung after seven days, $92.1 \pm 1\%$, after 30 days and $97.18 \pm 0.7\%$ 200 days post inhalation indicating that the other measured organs had more effectively cleared the
particles than the lung (Ellender et al. 1992). Clearance is affected by the diameter of the particle, especially when comparing fine and ultrafine particles. Clearance of ultrafine particles (20 nm diameter) is less than that for fine (250 nm) despite the particles being administered at the same gravimetric dose and the particles forming similar sized agglomerates (Oberdorster et al. 1994). Both particle types had the same retention in the alveolar space, but the ultrafine particles were more greatly translocated to the pulmonary interstitial spaces and retained for longer periods of time. Sub-chronic inhalation of fine and ultrafine particles resulted in reduced alveolar macrophage mediated clearance. Variances in retention half times range from 66 days for control rats, 117 days for rats exposed to fine particles, and 541 days for ultrafine exposed rats. This likely explains the longer inflammatory response mediated by the ultrafine particles (Oberdorster et al. 1994). One week exposure to diesel engine exhaust, a primary source of some PM, has been shown to impair the clearance of intra-tracheally instilled Pseudomonas aeruginosa in mice, leading to enhanced infection and pathogenesis of the inhalable bacterium (Harrod et al. 2005).

Retention

How particles interact with the pulmonary system depend not just upon coming in contact with cells, but where this contact occurs and how long they maintain contact. Studies of particle retention determine this. Retention has been defined as deposition minus clearance or the dose deposited minus the amount cleared (Oberdorster et al. 1994). Particle diameter is a major factor in PM retention with sub-micron sized particles exhibiting vastly different characteristics. Long-term retention on the bronchial region is negligible for particles greater than or equal to 6 µm- it increases with diminishing
diameter to where 80% of 30 nm particles are retained long term (Kreyling et al. 2006). Studies have shown that ultrafine particles are more capable of entering the alveolar and interstitial spaces (Oberdorster et al. 1994). Ultrafine particles are less likely to be actively taken up by lung macrophages (Kreyling et al. 2006), likely due to the lack of receptor ligands that trigger phagocytosis (Peters et al. 2006). Because of these reasons, ultrafine particles are less likely to be removed from the lung by bronchialveolar lavage (Kreyling et al. 2006), a mechanism that is effective at removing micron sized particles (Ellender et al. 1992).

Another unique capability of ultrafine particles is their ability to end up in the central circulation and distal organs. A few mechanisms have been proposed. One of the more interesting mechanisms is that ultrafine PM bind to proteins forming complexes that are not much larger than the protein itself therefore the fate of the particle is the ultimate fate of the protein (Kreyling et al. 2006). Another mechanism is that PM is transported to organs via erythrocytes in the central circulation. PM has been shown to rapidly enter erythrocytes though a passive mechanism (Peters et al. 2006). The hypotheses are not exclusive and utilize many of the same studies outlining their respective hypotheses. Ultimately, un-cleared particles of all sizes accumulate in the lung, only ultrafine particles accumulate in distal organs (Kreyling et al. 2006) and the effects of the ultrafine particles at the distal organs have not been elucidated (Peters et al. 2006).

Size and Composition of PM studied

In the epidemiology, there have been differences in the relative rates of mortality; with the northeastern U.S. cities having larger relative mortality rates than the northwestern cities. This may be explained by the composition of the PM, however
insufficient compositional analysis has been performed to make this determination (Dominici and Burnett 2003; Krewski et al. 2005). The epidemiological evidence greatly demonstrated a stronger association between fine and coarse PM leading to the lack of additional PM$_{10}$ standards with the new NAAQS (Table. 1-1). As seen with dosimetry, the particle size is a major factor in the study of PM, with the composition also likely contributing to the effects of PM.

The major distinction of how PM is classified that of diameter, where PM is divided into two categories: fine mode and coarse mode. These definitions are useful in that the sources of fine and coarse are different, allowing differing mitigation strategies (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Fine mode is defined thus:

Fine-mode PM is derived from combustion material that has volatilized and then condensed to form primary PM or from precursor gases reacting in the atmosphere to form secondary PM. New fine-mode particles are formed by the nucleation of gas phase species, and grow by coagulation (existing particles combining) or condensation (gases condensing on existing particles). Fine particles are composed of (a) freshly generated particles, in an ultrafine or nuclei mode, and (b) an accumulation mode, so called because particles grow into and remain in that mode. (National Center for Environmental Assessment [Research Triangle Park N.C.] 1996, 2003).

Coarse mode is defined:

Coarse-mode PM, in contrast, is formed by crushing, grinding, and abrasion of surfaces, which breaks large pieces of material into smaller pieces. They are then suspended by the wind or by anthropogenic activity. Energy considerations limit the break-up of large particles and small particle aggregates generally to a minimum size of about 1 μm in diameter. Mining and agricultural activities are examples of anthropogenic sources of coarse-mode particles. Fungal spores, pollen, and plant and insect fragments are examples of natural bioaerosols also suspended as coarse-mode particles. (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

While the terms PM$_{10}$ and PM$_{2.5}$ have been used to describe fine and coarse PM, this is not completely accurate since PM$_{2.5}$ can contain coarse mode particles. Fine mode
particles are also divided into accumulation mode (between 0.15 and 0.5 µm formed by condensation and coagulation) and Aitken mode (between 0.0015 and 0.04 µm formed by nucleation, coagulation, and condensation) particles (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). The EPA considered a 1 µm cutpoint rather than 2.5 µm, but found it was not as applicable throughout the nation. Examples were given demonstrating the complexity of fine mode cutoff points using PM from Phoenix, AZ, where the coarse mode extends below 1µm and Los Angeles, CA, where in humid conditions the fine mode can exceed 2.5 µm in droplet form. The cutpoint of 2.5 µm was decided to be optimal at the time. The new divisions of fine particles mentioned above have had no regulatory impact as of yet (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

The diameter difference is most beneficial when studying dosimetry as well as atmospheric transport. As can be seen in Table 1-2, coarse particles tend to settle out of the air and have a reduced atmospheric half life and travel distance. There are a variety of epidemiologic studies that demonstrated that proximity to highways and freeways has an increased risk of early mortality, or a risk that nearly doubled with in 100m of a freeway or 50m of a major urban road (Pope and Dockery 2006). Given the wide disbursement of PM$_{2.5}$, it could be more likely that PM$_{10}$ or a gaseous co-pollutant is involved in the increased risk from living close to highways. PM$_{10}$ generated from the wear of studded snow tires on different pavement types proved to be more potent than other road dusts (Veranth et al. 2004) and ambient particles (Chapter 2), in human lung epithelial cells, at inducing TNF-α release from BEAS-2B airway epithelial cells (Lindbom et al. 2006). Some co-pollutants have been examined in studies with enhanced
chemical speciation analysis, with CO showing promise, but CO was not an explanatory variable with circulatory deaths (Klemm et al. 2004).

The chemical composition of PM is very complex presenting one of the most difficult chemical mixtures to study (Keeler et al. 2005). PM does not easily dissolve into solution regardless of the solvent, with just one fraction, the organic carbon, possibly composing thousands of different compounds (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). The most active components of PM “remain a matter of speculation” (Lippmann et al. 2003). “The ambient aerosol is a mixture and its components differ in terms of effect, source, and amenability to control” (Maynard and Cohen, 2003 p. 1500). Of the measurable components, sulfates and organic carbon predominates. Only about 10-20% of the organic compounds can be identified, due to analytical limitations and the presence of bio-polymers and humic-like substances. Trace metals typically compose less than 1% of PM, except in industrial areas, where they compose up to 2%. Prior to the phase-out of leaded gasoline, Pb concentrations were approximately 0.1-1 µg/m³. These concentrations are now in the ng/m³ range. Iron is now the most abundant trace metal but is only in the tenths of a µg/m³ range in the industrial areas. Zn is the second most abundant at < 0.1 µg/m³, and Ni and V are at <10 ng/m³ (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

General composition of PM is highlighted in Table 1-2. Large regional variations of the composition of PM exist. Sulfate is the PM_{2.5} component with the lowest measurement error and composes approximately 38% of PM_{2.5} in the eastern United States and only 11% in PM from the western United States. Crustal material composes
roughly 52% of Eastern coarse mode PM and up to 70% of Western PM. Organic compounds can account for 21% of PM$_{2.5}$ in the eastern United States and 39% of the PM$_{2.5}$ from the western United States although the uncertainty with organic compounds is great. In an EPA report the component “unidentified material” ranged from 23% in the East to 0% in the West (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

A description of the instrumentation used in chemical analysis is beyond the scope of this review but includes ion chromatography, energy dispersive x-ray fluorescence spectrometry, neutron activation analysis, atomic absorption spectrophotometry, inductively coupled mass spectroscopy, scanning electron microscopy, and thermal optical reflectance. These sophisticated methods are unable to detect and differentiate compounds the EPA labels as “bioaerosols” (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Bioaerosols include intact, as well as components of, bacteria, viruses, fungi, pollen, grain dusts, and shed components of insects, birds, and mammals (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). These bioaerosols are thought to play a major role in the health effects of PM, especially the PM that does not have a high metal content (Becker et al. 2005a; Becker et al. 2002; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Epidemiological studies have been conducted to determine which component is responsible for early mortality. One (Laden et al. 2000) attempted to attribute the mortality effects of PM$_{2.5}$ to combustion products such as sulfates and dismissed the role of crustal elements. This is interesting since crustal material involves silica, a known
human respiratory carcinogen (IARC 1993). Sulfates are confounders when studying PM associated early mortality (Krewski et al. 2003), and studies have produced inconsistent results that sulfates are responsible for PM associated health effects (Green and Armstrong 2003; Reiss et al. 2007). In toxicological studies, biological responses to sulfates have only been documented at very high doses, which are unlikely to be achieved in ambient PM exposure (Schlesinger 2007).

Despite nitrates being major components of PM in the Western US much less information is available for nitrates. There is little to no evidence of nitrates causing adverse health effects (Schlesinger 2007). It was stated that “acute exposures to airborne sulfate and nitrate salts have never succeeded in shortening the lives of any laboratory animals or other subjects” (Green and Armstrong 2003). The instability of ammonium nitrate makes its measurement difficult and often unreliable, especially with collectors using glass filters (Lipfert 1993a). A major epidemiological study in California, where nitrates predominate the PM, found lesser effects than other epidemiological studies from other parts of the country, effects hypothesized to be due to less toxic nitrates, healthier California residents, or geographic confounding (Ostro et al. 2006). Nitrate as an air pollutant is more of a concern as an environmental pollutant as it deposits onto soil and water contributing to acidification in a manner similar to sulfate. Nitrate aerosols are also believed to interfere with the nitrogen cycle (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003; World Health Organization. Regional Office for Europe. 2000).

While miniscule in terms of overall mass, bioaerosols such as bacterial lipopolysaccharide (LPS) have been determined to be major contributors to PM mediated

An excellent review (Valberg 2004) pointed out that the predominant chemicals such as nitrate, sulfate, and carbon are incapable of causing mortality by themselves. The major implications are that PM is more toxic than the sum of its parts, the affected populations are more susceptible to the effects of PM than anticipated, or that PM mass is not responsible for the early mortality. A suggested possibility is that PM acts as a measurable surrogate for other less or non detectable pollutants (Valberg 2004). Such a hypothesis is in line with the hypothesis that PM is a composite measure of pollution that is associated with the composite outcome of mortality (Klemm *et al.* 2004). All are reasonable hypotheses that remain to be adequately tested.

Potential Biological Mechanisms of PM Induced Harm

As stated previously, the lack of plausible biological mechanism of PM induced harm has made the establishment of PM regulations problematic and has been acknowledged by researchers and regulators alike. The epidemiological studies are useful in aiding researchers in determining where to address their efforts. An important first step was the determination of whether PM merely accelerates the death of those already ill. This “harvesting hypothesis” can be examined epidemiologically. If PM exposure leads only to the early demise of those already dying, there would be short periods of compensatory death reduction following PM exposures. Studies undertaken to explore this hypothesis have not been able to support it (Pope and Dockery 2006).
although, the elderly have greater relative risks from PM exposure than the general population (Aga et al. 2003). Additional potentially useful information is the lag from PM exposure to mortality. The length of the lag time used in a study can effect the outcome of the study, with shorter lag times showing diminished results (Aga et al. 2003). The implication is that acute PM exposure has measurable health effects for days following the exposure, which is consistent with systemic rather than local effects.

Epidemiological studies have been conducted to ascertain the mechanism(s) by which PM causes early mortality. Early studies hypothesized that PM would interfere with oxygen transport, but the data did not support the hypothesis (Dockery 2001). Studies addressing the hypothesis that PM alters autonomic function revealed that PM exposure alters heart rate variability (Magari et al. 2001). PM exposure also has been associated with increases in cardiac arrhythmias, although the mechanisms by which these occur are “unclear” (Bhatnagar 2004). Long term PM exposure has also been associated with deep vein thrombosis, although ambient temperature and hormone therapy in women were confounding factors (Baccarelli et al. 2008). PM exposure has also been associated with worsening of asthma (Neukirch et al. 1998) and COPD (Lagorio et al. 2006) through enhanced inflammation and redox metabolism. PM also worsens cystic fibrosis (CF) symptoms, presumably through the reduced lung antioxidant load found in CF patients (Kelly et al. 2003), although endoplasmic reticulum stress likely plays a role as well (Kerbiriou et al. 2007). While epidemiological studies have been beneficial for establishing the association with PM and early mortality and have provided some evidence for disease progression, the mechanisms are not yet elucidated (Pope and Dockery 2006), requiring toxicological studies (Lippmann et al. 2003).
Toxicological studies

Many, if not most, particle studies do not actually involve ambient PM. Surrogate particles such as reserve oil fly ash (ROFA) (Gao et al. 2004; van Eeden et al. 2001), dusts (Veranth et al. 2006; Veranth et al. 2004), LPS (An et al. 2002; Becker et al. 2005a; Becker et al. 2005b; Endo et al. 2005; Gilmour et al. 2004; Haddad and Land 2002; Prince et al. 2004; Reynolds et al. 2005; Shan et al. 2006; Veranth et al. 2004; Yang et al. 1999), synthetic particles (Agopyan et al. 2003; Churg et al. 2005; Dick et al. 2003; Oberdorster et al. 1994; Sayes et al. 2007; Veronesi et al. 2003), carbon black (Becker et al. 2005c; Boland et al. 1999; Dick et al. 2003; Li et al. 1997; Pozzi et al. 2003; Ramage and Guy 2004; Yang et al. 1999) silica (Becker et al. 2005c; Ovrevik et al. 2004; Sayes et al. 2007; Yang et al. 1999), diesel exhaust particles (DEP) (Becker and Soukup 2003; Boland et al. 1999; Dagher et al. 2005; Hashimoto et al. 2000; Hiura et al. 1999; Hiura et al. 2000; Jung et al. 2007; Juvin et al. 2002; Koike et al. 2004; Ma and Ma 2002; Nam et al. 2006; Ritz et al. 2006; Saber et al. 2006; Takizawa et al. 2003; Yang et al. 1999), and even volcanic dust (Becker et al. 2002) have been used. These surrogates can comprise portions of ambient PM (Braun et al. 2008; Pope et al. 1999), are workplace, as well as environmental contaminants (Ma and Ma 2002), and are easily obtained or commercially available.

ROFA contains metals that are hypothesized to be responsible for its effects (Saldiva et al. 2002). It “is remarkable in its capacity to induce lung injury” mostly though the metal vanadium, which is actually rare in PM, rendering ROFA a suboptimal PM surrogate (Ghio et al. 2002). LPS or endotoxin, is a bacterial lipopolysacharide involved in a wide variety of PM mediated effects (Becker et al. 2002; Schins et al. 2004)
and is a component of PM that is high in areas with intensive livestock activity (Schulze et al. 2006). LPS alone may not be the PM component entirely responsible for many PM mediated effects; however, it is an effective measurable surrogate for bacterial components or bioaerosols. The presence of LPS likely indicates other bacterial components, which are more difficult to detect, are present in the PM. Synthetic particles and carbon black are used in studies where uniform particle size, charge, and reactivity are needed. Silica is a component of PM of crustal origin (Table 1-2). DEP contain polycyclic aromatic hydrocarbons (PAH), are generally submicron in diameter, and are a component of urban PM (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). DEP may act as an adjuvant in the TH-2 response (Nam et al. 2006). DEP have rough surfaces that may adsorb other compounds (such as components of biological origin), which may also be responsible for the effects of DEP (Pozzi et al. 2003). Any of the surrogates are useful for specific hypotheses but are limited in that they do not approach the complexity of ambient PM and by themselves are unable to explain the full effects of PM.

In order to study ambient PM, it must be captured. The method of PM capture and measurement is dictated by the EPA and is described in great detail in the federal register (40 CFR part 50 Appendices B, J, and L). Essentially, a measured amount of ambient air is pulled into a covered housing through a filter during a set duration. The filter undergoes moisture equilibration, must have an efficiency of 99% of 0.3 µm diameter particles, and is weighed prior to and after PM collection. The collection range is from 2 to 750 µg/m³. For precision, the coefficient of variation is to be 3.0% for a single analyst and 3.7% for inter-laboratory precision. The accuracy is “undefined
because of the complex nature of atmospheric particulate matter and the difficulty in determining the ‘true’ particulate matter concentration” (40 CFR part 50 Appendix B 5.1). There are multiple inherent sources of error, including airflow variation, air volume measurement, unavoidable loss of volatiles, artifactual PM formation from gases onto the filter, humidity, filter handling, PM deposited onto the filter when the collector is not active, errors in timing, and the recirculation of sampler exhaust (40 CFR part 50 Appendix B 6.0-6.9). Often researchers obtain PM captured by governmental organizations or other researchers (Dye et al. 2001; Frampton et al. 1999; Soukup et al. 2000). There are a few reference PM such as Standard Reference Material (SRM) 1648 from St. Louis, MO (Becher et al. 2007; Li et al. 2005; Li et al. 2006) and Ottawa dust (EHC-93), the second of which has been partially characterized, including elemental analysis, which unfortunately has not greatly contributed to an understanding of the mechanisms of PM induced effects. LPS activity in EHC-93 also has been measured and likely is at least partially responsible for the effects seen with EHC-93 (Becker and Soukup 1999; Chauhan et al. 2005; Fujii et al. 2001; Ishii et al. 2005).

Once captured, the manner in which PM is utilized to administer to animals/cells differs. In some studies the particles are removed from the dried collection filters by brushing off the dry PM following a four month collection interval (Dagher et al. 2005). Other studies use organic solvents rather than water for particle extraction (Jalava et al. 2008). The most common procedure for the removal of PM is immersion of the filter into water followed by agitation or sonication, and the aqueous PM is usually concentrated by drying or lyophilization and resuspended to a preferred concentration (Becker et al. 2005a; Becker et al. 2005b; Becker and Soukup 2003; Frampton et al.)
The use of aqueous suspensions is logical since ambient particles contain water which influences the size, light scattering and aerodynamic properties of the particles as well as influences the deposition of PM onto surfaces, airways, and sampling equipment. Water also provides a medium for the reactions of dissolved gases, but is not an air pollutant itself (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Both water soluble and solvent extractable fractions elicit responses (Jalava et al. 2008; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Some studies remove the insoluble portion of the particles themselves by centrifugation prior to concentration (Frampton et al. 1999). It has been recognized that all studies must be viewed with a cognizance of possible alterations of physiochemical characteristics due to collection, storage, and resuspension of the particles (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).

Some studies use concentrated ambient particles (CAPs) for direct inhalation studies, enabling researchers to expose humans or animals to concentrations of PM higher than those found in ambient air. The mechanism of the capture of CAPs is well described elsewhere (Harder et al. 2001). The limitations of CAPs include the inability of older equipment to concentrate particles less than 0.1 µm, and the inability of all concentrators to capture gaseous PM components that may include combustion related products (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003).
In vivo studies

Human volunteers

Studies conducted in human volunteers have given conflicting results, but in aggregate the effects of PM on healthy persons has been minimal. In healthy human volunteers, concentrated ambient particles from 23-311 µg/m³ were unable to affect lung function as measured by spirometry, with blood fibrinogen increased in a dose independent manner. The only effect with a dose response relationship was an increase in neutrophils in bronchoalveolar lavage fluid (Holgate et al. 2003), an effect mostly dependent upon total particle number rather than the nature of the particle (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Other studies using CAPs in healthy volunteers (aged 18-40) found only small increases in neutrophils in CAPs exposed individuals with negligible effects in macrophage function, and immune activation (Harder et al. 2001). The same study mentions that individuals administered Utah Valley PM had increases in CD-4 positive lymphocytes in a manner that cannot be attributed to total soluble metals in the PM (Harder et al. 2001). Another study using healthy volunteers treated with Utah Valley PM showed differences in IL-1β, TNF-α, IL-8 and neutrophils in the bronchoalveolar lavage fluid (BALF) (Ghio and Devlin 2001). In young healthy male volunteers working for the California Highway Patrol (CHiPs) in patrol cars, PM induced alterations in heart rate variability, increases in inflammatory, pro-thrombotic, and helmolytic responses (Riediker et al. 2004). The enhanced effects observed in the CHiPs volunteers over those administered CAPs may be due to gaseous PM components not concentrated in creation of CAPs, the presence of other gaseous co-pollutants not captured in PM collectors/monitors, or both. A study
with DEP, both a combustion product and a submicron particle, in human volunteers showed that DEP altered brainwave patterns, although inflammatory markers were not examined (Cruts et al. 2008). However, the same brainwave effects can be elicited by exercise, sleep deprivation, caffeine, and alcohol (Valberg et al. 2008). Healthy volunteers exposed to DEPs did not exhibit consistent alterations in heart rate variability (Peretz et al. 2008). Of interest with DEP is that there is very limited evidence that DEP exposure in highway and occupational settings is carcinogenic despite its containing a number of carcinogens, an effect attributable to lack of bioavailability (Hesterberg et al. 2006). A study with East German PM that was high in metals and low in LPS content, was interesting in that neutrophil numbers were not significantly altered with treatment, but monocyte numbers were (Schaumann et al. 2004). A study that used CAPs on healthy and asthmatic volunteers showed increases of ICAM-1 in both, and significant increases in IL-6 only in asthmatic volunteers. However, systolic blood pressure increased in the asthmatic volunteers receiving the sham filtered-air treatment (Gong et al. 2003). Studies using antioxidants as have given mixed results. A study using children with asthma living in the Mexico City metro area (breathing high concentrations of PM and ozone), given vitamin C and E supplementation demonstrated a potential protective effect in the children with the most advanced asthma (Romieu et al. 2002). Similar results have been seen in volunteers with high occupational exposures to air pollution with vitamin C alone not exerting a protective effect (Kelly et al. 2003). There is also evidence for a slight effect of ambient PM on fetal health, although the studies are inconsistent (Glinianaia et al. 2004).
Animal models

Many studies using animal models have been performed in the study of inhalation, deposition, and clearance, and form the basis of much of the information previously presented. Studies with PM and animal models have also provided information into the effects of exposure to other pollutants as well as PM- and the effects of PM on living organisms compromised by diseases. Animal studies have also provided information on mechanisms and systemic effects of PM and have attempted to address which PM components are responsible for detrimental health effects.

Co-exposure

Animal models have provided useful insight into co-exposure with PM and other pollutants. In a study examining the effects of ozone and PM via inhalation found that PM exacerbated the effects of ozone as measured by uptake of \(^{(3)}H\)-thymidine in rats (Vincent et al. 1997). An additional inhalation study with PM and ozone in rats found that the two co-pollutants affected the endothelin system in an additive manner, with upregulation of matrix metalloproteinase (MMP)-2, a potential marker of tissue damage, only in animals exposed to both pollutants (Thomson et al. 2005). A more recent study using ozone and the PM surrogate carbon black demonstrated that there are large discrepancies in the responses of mice to the PM and ozone based off genetic differences and sympathetic/parasympathetic responses, so care must be used in comparing rodent co-pollutant studies (Hamade et al. 2008). The co-pollutants carbon monoxide (CO) and CAPs were also examined in a rat inhalation model with some rats pretreated to give a left ventricular myocardial infarction (Wellenius et al. 2004). There was no clear evidence the cardiovascular effects of PM were modified by simultaneous PM and CO
exposure as determined by measuring alterations in heart rate and ventricular premature beats (Wellenius et al. 2004). Another study used EHC-93 intratracheally instilled into ozone pretreated rats to determine the effects of PM on the pulmonary system in a model with lung inflammation measuring cytotoxicity, protein in the bronchialveolar lavage fluid, mRNA, and serum chemistry activity. Ozone pretreatment was necessary for a PM mediated increase in the activity of angiotensin converting enzyme. Ozone treatment did not alter the ability of PM to cause changes in released TNF-α concentrations at lower ozone concentrations, but did affect the ability of PM to trigger higher concentrations of TNF-α release, and ozone pretreated animals had reduced concentrations of TNF-α in the days following instillation. There were no effects on IL-6, possible due to IL-6 being a more acute marker. Plasma fibrinogen was significantly elevated only in animals receiving the highest (5 mg PM) dose of PM independent of ozone. There were no effects of treatment on the mRNA of multiple markers in cardiac tissue, indicating no direct cardiac effects (Ulrich et al. 2002).

Disease simulation

Animal studies also allow for explorations into some of the mechanistic effects of PM by providing opportunities for simulation of disease states that are associated with PM and early mortality. In rats where the coronary artery was occluded, PM decreased heart rate and further increased ventricular arrhythmia while the PM had minimal effects on sham operated rats. The authors proposed serum endothelins, proteins which constrict blood vessels and contribute to hypertension, which were elevated in PM treated rats of sham operated and occluded rats, as a potential mechanism of PM induced harm (Kang et al. 2002). Elevated serum endothelin was found in children exposed to high
concentrations of PM (Calderon-Garciduenas et al. 2008), a population unlikely to have suffered ventricular occlusion. Animal models of chronic bronchitis had greater inflammatory changes when exposed to CAPs than the normal cohort, with such inflammation occurring in a small volume of the alveolar fraction (Saldiva et al. 2002). Another study used ultrafine carbon black, rather than ambient PM, and RSV exposed mice and found synergistic upregulation of the chemokines MIP-1α, and MCP-1 (Lambert et al. 2003). Intratracheally instilled PM resulted in the standard increases in neutrophils in the lungs and also increases in Toll-like receptor (TLR)-4 and CD14 in a rat hypertensive model (Gilmour et al. 2004). TLR-4 is the major receptor for LPS, and CD14 is a receptor that presents LPS to TLR-4 (Schulz et al. 2002) and has a soluble form which is an inducible acute phase protein (Bas et al. 2004). In studies using bronchitic rats exposed to CAPs there were discrepancies in lung damage, effects possibly due to compositional differences in the CAPs between Boston, MA area and Research Triangle, NC, PM (Tao et al. 2003). Effective, although inexact, rodent models of COPD have been developed, but little published research has been conducted on PM in these models (Wright et al. 2008).

PM and infections

Animal studies with PM and infections have shown that rodents treated with PM preceding infection with aerosolized bacteria died much sooner than the rodents not exposed to PM (Tao et al. 2003). DEP have been shown to reduce bacterial clearance (Harrod et al. 2005), which may be the mechanism by which PM enhances bacterial infection, although particle mass loading interfering with the ability of macrophages to phagocytose bacteria may be another mechanism (Tao et al. 2003).
A study attempting to distinguish the origin of the PM found that PM from high traffic areas was more potent than other PM instilled intratracheally into spontaneously hypertensive rats (Gerlofs-Nijland et al. 2007). This study supports the epidemiological findings of increased PM associated mortality within short distances of busy roads and the differences between healthy volunteers exposed to CAPs and the CHiPs volunteers exposed to ambient highway PM.

Studies of PM sizes, components, and mechanisms

Some rodent studies have pointed toward metals as the causative component of PM in adverse health effects. One study examined the effects of Mount St. Helens ash (a PM that does not contain metals), two doses of oil derived combustion particles containing zinc and nickel, the same combustion particles without the solid material, and a zinc-only group that was administered equivalent zinc concentrations as was contained in the oil derived combustion PM (Kodavanti et al. 2008). In inducing cell infiltration into BALF and myocardial lesions the high dose PM was most effective. Interestingly the number of cells in the BALF was reduced in 16-week exposures compared to 8-week exposures. In inducing cardiac mitochondrial DNA damage, the zinc-only dose had the greatest effects. The zinc-only and the lowest concentration of PM were the only treatments significantly inhibiting aconitase activity, an indicator of oxidative stress (Kodavanti et al. 2008). The authors speculated that PM mediated cardiac effects in their study, and in general, were due to chronic pulmonary inflammation (Kodavanti et al. 2008).

A study examining the effects of Utah Valley PM from the year prior, the year of, and year after the closure of the Geneva steel mill showed that PM from the years prior to
and after the plant closure stimulated greater lung pathology after 24 but not 96 h post
instillation in rats exposed via intratrachial instillation. The particles from the years the
plant was open had more sulfates, cationic salts, and metals than the PM from the year of
the plant closure, although the metal concentrations were “exceedingly low” compared to
other ambient TSP samples analyzed in the lab (Dye et al. 2001). Some early studies
strongly attributed the effects of PM to iron (Jimenez et al. 2000; Li et al. 1997), mostly
based off treating the PM extracts with the iron chelator, deferoxamine. However, more
recent in vivo studies discuss the contribution, rather than responsibility, of iron (Smith et
al. 2006). Other recent studies have indicated that iron only contributes to PM mediated
effects in the presence of hydrogen peroxide (H₂O₂), a well documented activator of lung
phagocytes and oxidant (Witschi and Last, 1996) and has minimal effect otherwise
(Imrich et al. 2007). In another study copper, not iron, was hypothesized to be
responsible for the effects of TSP from Utah Valley (Kennedy et al. 1998). In light of the
variability of many studies using metals and metal chelators the current, often cited,
hypothesis is that metal distribution rather than mass determines the biological effects
attributable to metals (Tao et al. 2003).

Other studies give more importance to biological components such as LPS. The
role of biological components is emphasized by multiple studies that demonstrated that
DEP did not induce lung inflammation alone, but did exacerbate LPS inducible lung
inflammation (Inoue et al. 2007; Inoue et al. 2006). Often for in vitro experiments,
macrophages are primed by LPS prior to PM treatment in order to enhance responses
(Imrich et al. 2007). LPS had a similar effect with coal combustion ash enhancing the
pro-inflammatory effects of the ash in mice, although in a manner that cannot be
explained by LPS alone (Finnerty et al. 2007). This effect has also been observed with road dust PM and human lung cells (Veranth et al. 2004). LPS is only one of a wide variety of bacterial components capable of remaining intact beyond the death of the organism (Swarbrick and Boylan 1988) that likely interact with cellular receptors and contribute the effects of PM.

While there is conflicting evidence as to whether metals, carbon containing molecules, or biological components of PM are the major contributors to the effects of PM, many studies indicated that free radical generation is a mechanism of the effects of PM (Tao et al. 2003). However, as for virtually all aspects of PM related research, there are some divergent findings as to the role of radicals. The addition of the thiol antioxidant, N-acetylcysteine greatly reduced the inflammatory response to Boston, MA area CAPs, although it only partially reduced the accumulation of oxidized proteins in rats (Rhoden et al. 2004). Differences between CAPs and un-concentrated ambient PM in their ability to induce TNF-α release from rat alveolar macrophages were highlighted by the differential ability of N-acetylcysteine to inhibit TNF-α release. N-acetylcysteine inhibited CAP mediated TNF-α release but not ambient PM mediated TNF-α release (Rhoden et al. 2004). One study using ultrafine PM found that the ability of a particle type to generate free radicals was not necessarily an indicator of the ability to trigger inflammation (Zhang et al. 1998).

An insightful rodent study demonstrated that oxidative stress induced by PM may not be due to oxidants in the PM, but due to autonomic signals, as evidenced by the elimination of PM induced oxidative stress by the muscarinic receptor inhibitor glycopyrrolate and the antihypertensive β-adrenergic receptor antagonist atenolol.
(Rhoden et al. 2005). Also of interest, is that there is an autonomic anti-inflammatory mechanism where acetylcholine reduces the inflammatory effects of LPS in rats (Borovikova et al. 2000).

Studies comparing intra-tracheally instilled PM with carbon black (200-25 nm) and ultrafine carbon black (20 nm) in rats demonstrated that PM was more potent than carbon black, but much less so than ultrafine carbon black, at inducing neutrophil numbers in the lung. There were no significant alterations in glutathione and reduced glutathione in the lungs of PM treated rats (Li et al. 1997). Neutrophil influx into the lung is often greater with smaller particles administered on a mass-basis because the total number of particles is usually the greatest factor for neutrophil influx (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Comparative studies with ambient PM found the coarse PM to be consistently more inflammogenic than fine and ultrafine particles, independent of the city of origin (Happo et al. 2007). Others attribute the collection location, mainly from near highways, to be the most relevant to the biological effects of ambient PM in spontaneously hypertensive rats (Gerlofs-Nijland et al. 2007). In another study comparing PM of differing diameters in mice administered PM intratracheally, regular and significant increases in BALF protein concentration only occurred with the coarse PM 24 h post-treatment (Happo et al. 2007).

Some interesting *in vivo* studies demonstrate the particular ability of ultrafine PM to penetrate the central circulation. Some hypothesize that such penetration is responsible for the systemic effects of PM (Nemmar et al. 2004). Brain inflammation was increased in mice treated with fine and ultrafine particles from Los Angeles, CA. which may have been due to NF-κB activation (Campbell et al. 2005). Brain
inflammation and damage resembling that of Alzheimer’s has been seen in dogs exposed to Mexico City air, which would include many more harmful pollutants than PM (Calderon-Garciduenas et al. 2004). One potential mechanism of the Alzheimer’s like pathology is that the PM or other pollutant induces endoplasmic reticulum stress (Siman et al. 2001; Yoshida 2007).

Although the hypothesis that the systemic effects of PM are due to direct interaction with the particles carried by the central circulation is interesting and merits additional research, the hypothesis that systemic PM mediated effects are due to signaling rather than direct particle interaction has greater experimental support (Ulrich et al. 2002). This is thought to occur though acute or chronic inflammation and cytokine signaling, increases in blood pressure and coagulation, alterations in heart rate variability, as well as other mechanisms (Becker et al. 2005c; Dagher et al. 2005; Griffin 2006; Harder et al. 2001; Hetland et al. 2004; Hodge et al. 2005; Ishii et al. 2004; Kamimura et al. 2003; Lindbom et al. 2006; Magari et al. 2001; Osornio-Vargas et al. 2003; Qureshi et al. 1998; Seagrave et al. 2004; Taneja et al. 2004; Ulrich et al. 2002).

As with all toxicological research, the appropriateness of animal models for extrapolation to humans must be examined. For example, animal models are questioned when comparing interactions of PM with surfactin, due to large variations between differing animal species as well as between humans and animals (Kendall 2007). Rats and humans have large differences in responses, especially in lung tumorigenesis. Particles induce tumors in rats when particle deposition overwhelms clearance, a condition known as particle overload. Particle overload induced carcinogenesis is not dependent upon the chemical makeup of the particle itself (Valberg et al. 2006).
Differences such as airway branching (which is more complex in humans), nasal inhalation (rodents are obligate nose breathers), exertion while inhaling (rodents are often at rest when exposed to PM), clearance times (alveolar retention half times of 60-80 days in rats, but up to two years in humans) and the use of filtered versus ambient air (humans rarely breathe filtered air), all must be considered, as well as the usual dose extrapolation issues when using animal studies for extrapolating the human effects of PM (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). For these reasons, computer models are used in recent inhalation and deposition studies (Balashazy et al. 2003; Lippmann et al. 2003; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). Doses used in most in vivo PM studies are 10-100 times the daily PM mass in most cities in the United States. “These high experimental doses must be considered when extrapolating the effects of PM observed in animal models to real-world effects” (Tao et al. 2003).

In conclusion the in vivo studies have shown that PM causes local and systemic inflammation. Generally, coarse PM is more inflammatory than fine, with ultrafine also being more inflammatory than fine PM. This inflammation may or may not be due to metals, bioaerosols, and free radicals. The modest effects of PM found in the in vivo studies involving human volunteers and animal models are entirely consistent with the low incidence of health effects found throughout the epidemiological studies (Tao et al. 2003). Often a lack of a clear dose response is observed, and there are large differences from humans to the model in vivo species.
In vitro studies

In vitro studies are “particularly important in risk assessment” in that they are rapid, inexpensive and provide information on the mechanisms by which chemicals exert harm (Faustman and Omenn 1996). In vitro studies supply information about the biological link to PM exposure and health (Dominici and Burnett 2003). It is hoped that the mechanistic findings of in vitro studies can then be applied to advance in vivo research.

Exposure

With all in vitro studies, the appropriate exposure is difficult to determine. While extrapolation from cell culture based studies to living systems “will always be uncertain and artificial,” relevant exposures are required for applicability (Phalen et al. 2006). Researchers are divided over whether particles should be administered in a total concentration basis (µg/ml) or on a deposition (µg/cm²) basis. The latter may approximate how deposition occurs in vivo however it assumes that administered particles will sink to the cells and not remain suspended in the media in which the cells are growing, assuming adherent cells grown on the bottoms of flasks. Some research has shown that the particles tend to aggregate (Churg et al. 2005), which may lead to some cells receiving a much greater concentration of PM than what was measured in the suspension. A majority of studies use “relatively high, and arguably unrealistic, doses of PM in order to measure detectable effects in single cell types, a strategy necessitated by the lack of effects seen at concentrations that approach likely real-world exposures” (Tao et al. 2003). Exposures of 50 µg/ml PM in vitro have been justified as possible real-world exposures by EPA researchers (Becker et al. 2005b). This justification assumed an
ambient PM concentration of 50 µg/m³ and an exercising ventilation rate of 30 L/min for one h and an epithelial volume of 20-40 ml. I was unable to replicate the calculation with the information provided and the calculation appeared to rely upon all of the particles inhaled over the time period depositing in the lung epithelium, despite the above mentioned studies that show such deposition is unlikely. It did not appear that any clearance was considered. Other researchers (Phalen et al. 2006) using multiple models and computer simulations present multiple scenarios where surface deposition can approach 15.58-85.5 µg/cm², which is much higher than the surface area equivalent of 25 and 12.5 µg/ml (10.7 and 5.35 µg/cm²) used in the studies in chapters three and four. Surface area measurements are used by some researchers but cells must grow suspended in liquid, can grow up the sides of culture surfaces, and cells are not two dimensional. Also, some predictive models have been shown to be off from in vivo deposition studies with rats in exposure chambers that have shown much less deposition than expected (Lippmann et al. 2003).

PM with infections

Cells can be infected with viruses and treated with PM in an attempt to elucidate mechanisms of PM mediated effects concurrent with infection. In one study, simultaneous exposure of PM and respiratory syncytial virus (RSV) had no effect upon the phagocytic ability of hamster alveolar macrophages. PM reduced the ability of RSV to initiate IL-8, and IL-6 release, independent of the sequence of exposure of RSV and PM. Both PM and RSV increased TNF-α release independently and together compared to untreated, however there was no additive or synergistic effect. PM exposure also led to reduced viral load in a manner consistent with the hypothesis that PM induced immune
response has an antiviral effect (Kaan and Hegele 2003). An earlier study using a co-culture of BEAS-2B cells and isolated alveolar macrophages also found a reduced ability of the cells to uptake the RSV in the presence of PM (Becker and Soukup 1999). As with most research pertaining to mechanistic PM studies, much remains unexplained as to how PM alters the pathology of viruses and additional research is needed (Ciencewicki and Jaspers 2007).

Studies of PM sizes, components, and mechanisms

*In vitro* studies have been undertaken to determine whether size and specific chemical components are more responsible for the effects of PM and address the mechanisms by which the effects occur. Unlike the epidemiologic findings, *in vitro* (as well as *in vivo*) studies where coarse and fine PM were compared, a majority found that coarse PM was more potent in many measured effects (Becker *et al.* 2005a; Becker *et al.* 2005b; Becker *et al.* 2002; Becker *et al.* 2005c; Becker and Soukup 2003; Hetland *et al.* 2004; Monn and Becker 1999; Pozzi *et al.* 2003) but not all (Choi *et al.* 2004). Others show dichotomous responses. In a study where coarse particles induced the greatest responses in most of the observations (cytokine release, COX-2 upreguation) the upregulation of the antioxidant heme oxygenase was greater in fine PM treated cells (Becker *et al.* 2005c). Other comparative studies have shown mixed effects with coarse PM from one region of Mexico being more cytotoxic (with a 72 h incubation) than the fine PM from the same region in murine monocytes, with the fine and coarse PM from another region of Mexico City having no significant effects (Osornio-Vargas *et al.* 2003). Other researchers have found that solvent extracts of fine PM were much more potent than those of the coarse at inducing cytotoxicity and DNA damage in rat fibroblast cells.
Coarse particles induce greater cytokine release from both macrophages and epithelial cells than both fine and ultrafine PM (Becker et al. 2005a; Becker and Soukup 2003).

With larger particles, chemical composition may contribute, but with smaller particles it is hypothesized that chemical composition is not as important as surface area in the effects of PM (Risom et al. 2005). Others argue that particle size is important for apoptosis, but composition and surface reactivity are most important for inducing inflammation (Schwarze et al. 2007). Multiple studies have demonstrated that ultrafine particles induce greater harm in vivo and in vitro than fine particles of the same material, presumably through the ultrafine particles enhanced ability to translocate from the alveoli into the interstitial space (Dick et al. 2003). Some studies with ultrafine particles attribute the degree of lung injury in vivo to the particles’ ability to generate free radicals rather than due to size or surface area (Dick et al. 2003). The generation of free radicals has been a prevailing, but not conclusive, hypothesis of PM induced damage (Jung et al. 2007; Schaumann et al. 2004; Upadhyay et al. 2003).

In vitro studies have demonstrated that PM does indeed differ in its effects depending upon the area in which it is collected. One study attempted to address the variability between PM diameter and location of collection by collecting multiple PM samples with multiple mean diameters from a variety of E.U. cities. RAW 264 murine macrophages were used and the differing PM were administered at the same concentrations of 15, 50, 150, and 300 µg/ml. Overall, coarse (2.5-10 µm) particles were more inflammatory and sub-micron particles (0.2 µ) were more cytotoxic. The most inflammatory PM was the coarse from Barcelona, Spain, and Athens, Greece, PM
thought to contain traffic related components, while the most cytotoxic was wintertime PM from Prague, Czech Republic, a PM with a high combustion derived component (Jalava et al. 2007). Fine PM from Rome, Italy, was shown to be more hemolytic than coarse on an equal mass basis, but not in terms of PM surface/volume (Diociaiuti et al. 2001). In a study comparing ambient PM$_{2.5}$ and PM$_{10}$ from Rome, Italy as well as ultrafine carbon black on RAW264.7 cells, PM$_{10}$ was significantly more potent than PM$_{2.5}$ at producing cytotoxicity and inflammatory markers (Pozzi et al. 2003). Interestingly, the fine particles contained greater concentrations of carbon and metals with the PM$_{10}$ having greater amounts of silicates. While the silicates may in part be responsible for the enhanced effects of the PM$_{10}$, the authors attribute the differences to bacterial LPS. Pretreatment of both PM fractions with deferoxamine had no effect on the capability of the PM to stimulate arachidonic acid release indicating a lack of contribution by iron (Pozzi et al. 2003). In studies that compared responses from ambient PM and surrogate compounds often the PM gave greater results. One study found that silica, ROFA, and volcanic ash, at 30 µg/ml, were unable to stimulate cytokine release in human alveolar macrophages while the same concentration of an urban air dust preparation did. The PM surrogates were able to stimulate cytokine release only when contaminated with environmentally relevant numbers ($10^3$-$10^4$) of Staphylococcus, Streptococcus, and Pseudomonas. This effect appears to be thorough the toll-like receptors (TLR) 2 and 4. LPS typically signals through TLR-4 with TLR-2 being very promiscuous at recognizing components of foreign pathogens (Becker et al. 2002). The effects of PM and environmental bacteria on the alveolar macrophages were through a CD14 mediated mechanism (Becker et al. 2002). In an interesting study, ambient PM
was cultured in an attempt to determine the species of bacteria associated to the PM. Thirty percent of the colonies formed were gram negative *Pseudomonas* and seventy percent were gram positive. Viable fungal spores of *Penicillium, Caudiosporium,* and *Alternarium* were identified. Live and ultraviolet light killed bacteria were tested for their capacity to induce cytokine release from human alveolar macrophages with no significant difference between live and ultraviolet light killed (Becker et al. 2002). All the bacteria were able to induce IL-6 release, but the ability of the gram negative bacteria to do so was attenuated by the endotoxin neutralizing polymixin B (Becker et al. 2002). The attachment of dead bacterial components to PM is important in that particle deposition results in recognizable pathogen components being placed into areas that bacteria would not normally deposit by themselves (Becker et al. 2002). Other researchers hypothesize that LPS is responsible for the inflammatory effects of PM in macrophage like RAW 264 murine cells (Salonen et al. 2004).

Other studies examining potential components have yielded interesting findings. In recent study, sea salt and soil components were believed to be the components responsible for the effects of coarse PM on RAW 264 cells (Jalava et al. 2008). Some studies examined the mineral particles dismissed by the epidemiological studies showing that not only the mineral particles are pro-inflammatory, but the effects are mediated by NADPH oxidase and the free radicals are formed by the cells themselves rather than by the particles (Becher et al. 2007). Another interesting study indicates that engine load alters the components of DEP and their effects on human lung cells (Madden et al. 2003) indicating that fuel/engine modifications may lead to a reduction in the toxicity of DEP and whatever contribution DEP has upon ambient PM. As with the epidemiological
studies, *in vitro* studies have shown that there are regional differences in the effects of PM. Unlike the epidemiological studies, the majority of studies do not show fine PM to be the most potent at exerting effects.

One basic measure of *in vitro* effects is cytotoxicity. While cytotoxicity assays vary greatly and are affected by a variety of factors (Riss and Moravec 2004), the observation of cell death allows for the investigation of mechanisms of cell death such as apoptosis which is not difficult to detect when it occurs (Cohen 1997; Sun *et al.* 1999; Thorburn 2004). If apoptosis is triggered, the various mechanisms by which it occurs can be readily determined (Hengartner 2000; Hiura *et al.* 2000; Tsokos 2004), which can lead to an understanding of the mechanisms of PM mediated harm. With PM, reported cytotoxicity is minimal and often discrepant. Studies examining cytotoxicity used a variety of methods, and a variety of PM, giving a variety of results. For example in BEAS-2B cells, the PM from the Utah Valley before, during, and after the Geneva Steel closure was cytotoxic as measured by morphological changes and lactate dehydrogenase (LDH) release, only with a very high 500 µg/ml concentration of PM from the year after the plant was reopened (Frampton *et al.* 1999). These findings are especially interesting since the PM from the year prior to the mill’s closure did not induce measurable cytotoxicity, but was most effective at generating reactive oxygen species (ROS) in a cell free system (Frampton *et al.* 1999). Another study using the same Utah Valley PM instilled into healthy human volunteers, as well as alveolar macrophages from the same volunteers (Soukup *et al.* 2000) found no significant effects among the volunteers and only the PM from the year prior to the mill closure had significant effects on the macrophages as measured by the trypan blue dye exclusion method (Soukup *et al.* 2000).
The differences between these studies can possibly be attributed to differences in cell type, or non-metal constituents of the PM such as LPS. However other studies suggest differences in the metal content cannot fully account for the differences in cellular responses (Tao et al. 2003). Another study with a maximum concentration of 200 µg/ml showed no cytotoxicity in NHBE and BEAS-2B cells by Utah Valley PM from any year as measured by LDH release (Wu et al. 2001). Vermont PM showed no cytotoxicity in murine lung epithelial cells, as measured by the incorporation of a cell permeable fluorescent dye (Shukla et al. 2000). PM cytotoxicity was mild in keratinocytes with 48 h incubations and concentrations greater than and equal to 40 µg/ml required for cell death (Ma et al. 2004). One study measured cytotoxicity of PM on L132 human lung embryo cells via the colony forming method, and determined LC$_{10}$ and LC$_{50}$ values of 18.84 and 76.36 µg/ml (Dagher et al. 2005). Concentrations up to 100 µg/ml of motorcycle exhaust particles were not cytotoxic according to the MTT assay in human breast cancer MCF-7 cells (Wang et al. 2002). A study illustrating and emphasizing large discrepancies in the results from five different PM surrogates in vivo and in vitro, also demonstrated discrepant results from the MTT and the LDH cytotoxicity assays (Sayes et al. 2007). Although the authors did not emphasize the discrepancy, it illustrates that PM studies can differ in more than just PM type, cell type, and laboratory conditions.

That PM and PM surrogates are inflammatory is one of the few areas of agreement among PM studies, with multiple studies showing PM mediated inflammation. Only a fraction are referenced below (Dagher et al. 2005; Donaldson et al. 2001; Hetland et al. 2005; Ishii et al. 2004; Lindbom et al. 2006; Ma and Ma 2002; Okeson et al. 2003; Osornio-Vargas et al. 2003; Ramage and Guy 2004; Ramage et al. 2004; Reibman et al.
2002; Rhoden et al. 2004; Soukup and Becker 2001; Tao et al. 2003; Veronesi et al. 2003). The capacity of PM to induce inflammation is most often determined through cytokine release from the cells or upregulation of cytokine mRNA. mRNA results should be interpreted with caution. The cytokine mRNA is not the signaling molecule that induces an inflammatory response, the extracellular protein is. Also, pro-inflammatory cytokines such as TNF-α and IL-1 require proteolytic cleavage prior to release (Armstrong et al. 2006; Cohen 1997). Multiple studies using PM and surrogates have demonstrated discrepancies from mRNA upregulation and cytokine release (Frampton et al. 1999; Nam et al. 2006). Early studies comparing ambient PM with the PM surrogate carbon black found the ambient PM to be more potent at inducing inflammation as determined by neutrophil influx and LDH in the BAL fluid (Li et al. 1997). Some in vitro studies have been able to point out that particles themselves can directly affect the mechanism(s) of the assays. DEP has been shown to directly bind IL-8, reducing the amount available for detection by ELISA, and potentially concentrating the chemokine and increasing its localized effects when the IL-8 laden particles contact cells (Seagrave et al. 2004). Nanometer sized particles have a similar effect on IL-6 (Veranth et al. 2007). These in vitro studies have not only confirmed the inflammogenic nature of PM but have also enabled a greater understanding of the difficulties involved in PM research.

In order to understand the mechanisms of how PM induces the release of cytokines, often pro-inflammatory extracellular signaling proteins, much research has focused upon the cellular signaling mechanisms involved, including the well documented inflammatory signaling transcription factor NF-κB. The NF-κB family is composed of 6 different proteins (p65, RelA, RelB, cRel, p50/p105, and p52/P100) that exist as homo or
heterodimers while in an inactive state. These dimers are tightly bound by IκB which inhibits their activity. NF-κB is activated through the classical or canonical mechanism which involves the phosphorylation, ubiquination, and eventual degradation of IκB or the more recently discovered direct processing of p100/p105, which occurs through TLR receptor signaling often, but not always, involving the MyD88 receptor (Hayden and Ghosh 2004). The mechanisms that activate NF-κB include TNF related receptors, IL-1 related receptors, T cell receptors, B Cell receptors, CD40, and TLRs (Hayden and Ghosh 2004). PM studies examining NF-κB signaling have yielded interesting and varied results. PM from London and Edinburgh has been shown to activate NF-κB through a non-classical (Liao et al. 2004) mechanism with a 4 h incubation in A549 cells that does not involve IκB phosphorylation and a contribution by iron (Jimenez et al. 2000). However, using Vermont PM and murine lung epithelial cells NF-κB activation was shown to be independent of iron, as indicated by the pretreatment of PM with deferoxamine being ineffective at altering NF-κB activation. The addition of catalase did significantly alter NF-κB activation indicating peroxides may be involved and the NF-κB activation is likely due to oxidant production from frustrated phagocytosis (Shukla et al. 2000). A study using DEP and murine keratinocytes demonstrated that DEP activates NF-κB at 6-12 h following treatment. Akt was activated in the absence of PTEN phosphorylation and likely through the SAPK/JNK pathway. The DEP mediated Akt activation was attenuated by the inhibition of phosphatidylinositol 3-kinase (Ma et al. 2004). LPS is a known activator of NF-κB (Liu et al. 2009) and may be involved in how PM activates NF-κB, however, a study using the reference PM EHC-93 where LPS was neutralized, the PM was able to activate NFκB following 2 h incubations, although iron
was required. Another interesting finding was that the researchers were unable to find alterations in NF-κB activity when PM treatment was concurrent with colchicine, which interferes with microtubule activity (Churg et al. 2005). Other researchers hypothesize that NF-κB is activated by Heat shock protein (Hsp)-70, itself an inflammatory and tumorigenic protein (Becker et al. 2005a; Garrido et al. 2006; Radons and Multhoff 2005) shown to upregulated in PM exposed cells (Becker et al. 2005a; Ramage and Guy 2004). NF-κB is chronically activated in macrophages and bronchial epithelial cells of asthmatic persons, independent of PM exposure (Shukla et al. 2000), and PM mediated NF-κB activation may explain the observed exacerbation of asthma symptoms (Neukirch et al. 1998).

Other important cell signaling pathways include mitogen activated protein kinases (MAPK). MAPK regulate cell proliferation, motility, differentiation, and survival, often through MAPK activated protein kinases (MK) (Roux and Blenis 2004). There are five groups of MAPKs, extracellular signal regulated kinases (ERKs) 1 and 2, ERKs 3 and 4, ERK 5, c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), and p38. ERKs 1 and 2 and p38 activate the MKs which include ribosomal S6 kinases (Roux and Blenis 2004). JNK and p38 MAPKs respond to cellular stress such as osmotic, UV irradiation, hypoxia, ischemia and cytokines (Pearson et al. 2001; Roux and Blenis 2004), and have received the most attention in PM research. As with most PM research there have been interesting and conflicting results. High concentrations (50, 100, or 200 µg/ml) of Utah Valley PM were able to induce ERK and MEK phosphorylation, but not p38 or JNK phosphorylation following 30 min exposures in NHBE cells, indicating the role of receptors in PM mediated activity (Wu et al. 2001). In human pulmonary artery
endothelial cells, the reference PM SRM 1648, was able to activate p38 following 20 min of treatment but not JNK (Li et al. 2006). JNK may indicate a species specific response as an in vivo rodent study using ultrafine PM from Los Angeles, CA, found no changes in phosphorylated p38 and IκB, but did find phosphorylation of JNK (Kleinman et al. 2008) and another study using DEP and murine keratinocytes also found JNK activation (Ma et al. 2004). Using a variety of inhibitors to influence silica mediated IL-8 release from A549 human alveolar carcinoma cells, Øvrevik et al. (2004), found p38 but not JNK phosphorylation and hypothesized that there are two distinct pathways involved in silica mediated IL-8 release. Similar separate pathways are likely involved with PM mediated effects.

Other mechanistic studies have examined whether PM must penetrate into cells to elicit responses. Some studies have shown that PM penetrates the cell, thereby triggering its effects though internal signaling (Calcabrini et al. 2004). Some have demonstrated that oxidant stress and NF-κB activation is subsequent to the uptake of particles by epithelial cells (Shukla et al. 2000). PM surrogates have activated vanilloid receptors which are internal receptors (Agopyan et al. 2003; Veronesi et al. 2003). Different chemistries at particle surfaces change the relative adsorption of the particle, with oxygen content being the most important determinant (Kendall et al. 2004). Other studies have demonstrated that contact with PM surfaces is all that is required for PM to exert cellular effects, which provides evidence that surface cellular receptor activation drives the observed effects of PM (Churg et al. 2005). The inflammatory potential of PM is possibly due to interactions of the PM surface with the cellular membrane (Diociaiuti et al. 2001) indicating a receptor mediated inflammatory response.
An interesting mechanistic study was performed using concentrations of motorcycle exhaust particles (50 µg/ml) on MCF-7 breast cancer cells and demonstrated induction of benzo[a]pyrene hydroxlyase activity though induction of CYP1A1 and CYP1B1 (Wang et al. 2002). Such induction mimicked the effect of benzo[a]pyrene and TCDD mediated CYP induction (Wang et al. 2002). A similar effect has been observed with ambient PM and another breast cancer line (Roepstorff et al. 1990) and in HepG2 cells with ambient TSP (Nakama et al. 1995). While the cell types are not those that would normally come in contact with ambient PM, these studies demonstrate that induction of phase I and phase II enzymes may be contributory mechanisms of PM mediated effects.

Some studies have found that metals and ROS are likely involved in PM mediated effects. A study using DEP and EHC93 ambient PM looking for evidence of airway remodeling on rat tracheal explants found that the effects of the PM on pro-collagen gene expression were attenuated by the iron chelator deferoxamine and the oxidant scavenger tetramethylthiourea. The effects of PM on transforming growth factor (TGF)-β gene expression were unchanged by deferoxamine but reduced to be proportionate with control by tetramethylthiourea indicating that ROS are involved in TGF-β upregulation but not necessarily those created by iron. Both the TGF-β and pro-collagen upregulation were mediated through the NF-κB transcription factor (Dai et al. 2003). The study used tracheal explants which required a tremendously high treatment concentration (500µg/cm²) to elicit effects, and the PM was autoclaved, potentially eliminating active LPS. The authors attributed the effects of EHC-93 ambient particles to iron and free radical formation since the iron chelator deferoxamine and the antioxidant
tetramethylthiourea inhibited NF-κB activation. However, such attribution to iron is likely due to the researchers autoclaving the PM and treating it with polymixin B to eliminate LPS effects prior to treatment (Churg et al. 2005). Another study examining the role of metals demonstrated that treating Utah Valley PM with chelex resin to remove metals resulted in significant reduction of IL-8 release from BEAS-2B cells. There were other non-metal PM chemical constituents removed by the treatment. However, when the metals were added back to the PM (at even 2x the removed concentration) there was not a significant increase in IL-8 release. Also, when the cells were treated with metals alone there was not a significant increase in IL-8 release. These discrepancies indicate that metals require other PM constituents to exert effects or maintain differing valence states in the PM (Molinelli et al. 2002). A review of the literature has shown the generation of ROS does not correlate with apoptosis nor the ability to induce inflammation, indicating alternative mechanisms at play (Schwarze et al. 2007).

The use of antioxidants has given interesting insights into the contribution of radicals to the effects of PM. In study measuring PM mediated alterations in Ca^{++} flux in murine macrophages and human monocytes, researchers found that the antioxidant nacystelyn inhibited Ca^{++} flux only in human monocytes and not the murine macrophages. The Ca^{++} flux was not PM concentration dependent and the antioxidant had no effect on TNF-α release (Brown et al. 2004). DEP induced IL-8, IL-1β, and GM-CSF was reduced by treating human lung epithelial cells with the potent phase II enzyme inducer sulforaphane, although dimethyl sulfoxide (DMSO) had a similar effect (Ritz et al. 2006). In a study using LPS primed rat alveolar macrophages treated with PM, CAPs, and H_{2}O_{2}, researchers obtained interesting results with differing antioxidants. The H_{2}O_{2},
OH, and hypochlorous acid scavenging antioxidant dimethyl-thiourea greatly reduced TNF-α release while the H2O2 specific catalase had only partial effects. Of greater interest is that N-acetylcysteine, a glutathione precursor, was unable to block ambient PM mediated TNF-α release while it did block the release of TNF-α from CAPs treated cells (Imrich et al. 2007). This may indicate that CAPs exert effects through differing mechanisms than PM and may not be suitable surrogates for ambient PM. None of the enhanced cytokine release was attributed to iron even though iron was present in the PM and CAPs (Imrich et al. 2007).

In studies examining PM mediated oxidant formation, little is done to make the extremely difficult distinction between radicals originating from the particles or from the cells themselves (Tao et al. 2003). The generation of oxidants is important in the respiratory (or oxidative) burst generated by immune cells (in an Akt and NADPH-oxidase dependent mechanism (Chen et al. 2003)) in order to kill pathogens. However radicals are also vital components of signal transduction and are involved in cellular survival (Chiarugi et al. 2003; Forman and Torres 2001; Kuwabara et al. 2008). The generation of ROS may also be signaling for the cytoskeletal modifications that are required for the internalization of the particles (Calcabrini et al. 2004).

Essentially, a greater understanding of some of the mechanisms of PM effects has been obtained with in vitro research. As with in vivo studies multiple in vitro studies have lacked a concentration or dose dependent effect (Brown et al. 2004; Choi et al. 2004; Diociaiuti et al. 2001; Kleinman et al. 2008). There are also inherent difficulties with extrapolating in vitro results to human health (Veranth et al. 2004), and there are inconsistencies in the findings. PM triggered inflammatory responses at multiple
concentrations used in the above cited studies, whether this is metal, biological, size, charge, shape, mass, or solubility mediated has yet to be determined. Overall, the understanding of the effects of PM on some mechanisms is greater than when air pollution was classified by whether it was a reducing pollution or not (Witschi and Last 1996), but there are still many conflicting studies and hypotheses. Most importantly, a single specific component of PM has yet to be identified as the responsible agent for the effects of PM (Schwarze et al. 2007). In 2001, researchers from the EPA stated “There is neither a consensus on a plausible mechanism nor is there any agreement on which component of PM is responsible for biologic activity” (Ghio and Devlin 2001); that statement holds true today.

Cache Valley PM

The Cache Valley and the inhabited area of Cache County that falls within the valley has been declared to be a non-attainment area for 24 h PM$_{2.5}$. During the period of January 10-17, 2004, the PM$_{2.5}$ concentrations broke records for the state of Utah. The less reliable (not adjusted for humidity) instantaneous value for the evening of January 15 2004, hit 182 µg/m$^3$, which would have exceeded the 160 µg/m$^3$ January 1, 2000 in Fresno, CA measurement which was considered the national record for circumstances not involving large wildfires (Malek et al. 2006). The 24 h humidity adjusted official reading for the day was still a very high 132.7 µg/m$^3$ (USEPA 2006) a state record and high for the nation on that day (Malek et al. 2006). The single greatest factor in obtaining those high concentrations of particulates is the weather. Wintertime inversions can occur in the Cache Valley where high pressure and no precipitation seal cold polluted air under a
layer of warmer air. The cold air inversions lead to the creation of PM and the trapping and concentration of the PM to unhealthy concentrations (Malek et al. 2006). Contributing weather conditions include a covering of snow on the ground that reflects sunlight, low surface pressure with a blanket of high pressure above, low air temperature, low wind, low precipitation, and high relative humidity (Malek et al. 2006).

Early studies demonstrated that the PM in Cache Valley is a secondary pollutant and is principally composed of nitrates, although there are no primary sources of nitrate air pollution in the valley (Mangelson et al. 1997). Later studies have essentially confirmed those findings and have also shown that most of the particle mass is by submicron particles (Malek et al. 2006; Silva 2004, Silva et al. 2004, Silva et al. 2007). While nitrates predominate (approximately 50%), other major components include organic carbon (21%), ammonium (18%), sulfate (6%), and, with low concentrations of elemental carbon (1-3%), metals in general, and iron (5.32 ± 4.50 nmol/m³) which is a tracer of soil dust indicating crustal contribution to PM (Mangelson et al. 1997; Silva et al. 2007). The organic carbon is a complex component of which vehicular emissions and wood burning only partially explain the origin. The sulfates, at least partially, are assumed to be from diesel emissions (Silva et al. 2007). A study performing trace element analysis revealed Si, K, Ti, Mn, (which are mostly soil derived) Ca, Cu, Zn, Pb, and Br were detectable but not quantifiable and were reported relative to a Fe in the air/Fe in the soil ratio (Mangelson et al. 1997). Ca, Cu Zn, Pb, and Br were enriched relative to Fe with leaded gasoline likely being the explanation given for Pb and Br, with no explanation for the other elements (Mangelson et al. 1997). Ammonium nitrate is the single greatest component to Cache Valley PM which composes 50% of total particle
mass on low PM days and up to 80-85% total particle mass concentration during inversions (Silva et al. 2007). These nitrate particles are small accumulation mode (.03 to 1.0 µm) particles that are formed through acid-base chemistry involving gaseous ammonia (mostly from agriculture) combining with gaseous nitric acid to form solid ammonium nitrate (Malek et al. 2006; Silva et al. 2007). This reaction requires cold temperatures and high humidity (Silva et al. 2007). It is not a phenomenon unique to the Cache Valley, but occurs frequently in the Western United States during the winter (United States Environmental Protection Agency. Office of Air Quality Planning and Standards. and United States. Environmental Protection Agency. Air Quality Trends Analysis Group 2008) and in areas with intensive agriculture (Edgerton et al. 2006; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003; Turkiewicz et al. 2006). An interesting finding from multiple studies using separated collector sites is that the PM concentrations are homogenous throughout the valley (Martin and Koford 2005; Silva et al. 2007). Given that most of the PM is secondary, being formed from gases and resulting in accumulation phase particles that are widely dispersed, it is reasonable that parts of the Valley that share similar weather patterns should share similar PM profiles.

BEAS-2B Cells

A wide variety of cell monoculture techniques have been used for in vitro studies. For PM research, lung epithelial cells have been widely used. As mentioned previously, airway epithelial cells are sites of PM contact. Airway epithelial cells phagocytose particles are involved in inflammation (Baeza-Squiban et al. 1999), and are hypothesized
to be contributing cells that send inflammatory and chemotactic signals that lead to a systemic inflammatory response; that includes stimulating the bone marrow to release polymorphonuclear leukocytes (Ishii et al. 2004). One of the most often used cells is the bronchial epithelial cell line BEAS-2B. BEAS-2B cells are an SV-40 large T-antigen immortalized cell line that is a derivative of normal human bronchial epithelial cells (NHBE). NHBE cells demonstrate early senescence prompting the development of the BEAS-2B line (Reddel et al. 1988). There are few PM studies using NHBE cells, with most of those, the researchers isolated their own NHBE from human volunteers (Becker et al. 2005a; Becker et al. 2005b; Becker et al. 2005c). Where commercially available NHBE cells were used, BEAS-2B cells were also used and the cell lines gave similar results (Agopyan et al. 2003). In the first published studies involving BEAS-2B cells they were shown to retain features of NHBE, form an epithelium on de-epithelialized rat tracheas implanted into mice (Reddel et al. 1988) and undergo squamous differentiation (Ke et al. 1988). Early studies stated that BEAS-2B cells were not tumorigenic (Reddel et al. 1988), however, subsequent studies from the same lab have shown BEAS-2B cells to be very weakly tumorigenic, a rarity with SV-40 immortalized cells (Reddel et al. 1993). BEAS-2B cells constitutively express a number of surface proteins involved in the inflammatory response, have inducible chemotactic responses, and respond to cytokines (Atsuta et al. 1997). BEAS-2B cells have normal phase II activity but reduced phase I, likely from the loss of cytochrome P450s (Mace et al. 1994; Nichols et al. 2003). The ROS producing system is also intact in BEAS-2B cells which can produce NO and superoxide (Felley-Bosco et al. 1995; Souici et al. 2000).
BEAS-2B cells have been championed for the study of carcinogenesis, and many of its related pathways have been extensively studied (Gerwin et al. 1992; Mace et al. 1994; Nichols et al. 2003; Reddel et al. 1993; Souici et al. 2000). BEAS-2B cells have been used as normal reference for cancer biomarker studies, due to the minimal chromosomal alterations they possess (He et al. 2004), as well as studies examining genetic targets of chemoprevention (Ma et al. 2003). Mutations in BEAS-2B cells have been induced by various carcinogens (Felley-Bosco et al. 1995; Hussain et al. 2001; Souici et al. 2000). Also, the tobacco-specific carcinogen N-nitrosamine-4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone (NNK) transformed BEAS-2B cells which exhibited increased epidermal growth factor receptor (EGFR) expression (Lonardo et al. 2002). The native p53 in BEAS-2B is inactive (Gerwin et al. 1992; Van Vleet et al. 2006), but the cells react to exogenous wild type p53 (Gerwin et al. 1992). Although the p53 in BEAS-2B cells is inactive, the cells are capable of undergoing apoptosis (Agopyan et al. 2003; Nakajoh et al. 2003; Nichols et al. 2003; Van Vleet et al. 2006). BEAS-2B cells respond in a similar fashion to A-549 and primary human tracheal epithelial cells to the apoptosis inducing protein elastase, although the BEAS-2B cells responded to lower concentrations (Nakajoh et al. 2003). Knowledge of BEAS-2B cells extends to observations that PM surrogate soil dusts and vanadium metal as well as the cells’ ability to respond to LPS can be changed by altering the growth media and culture flask size (Veranth et al. 2008).

For these reasons, BEAS-2B cells are widely used for the in vitro study of the cellular effects of PM and PM surrogates (Agopyan et al. 2003; Becker and Soukup 1999; Chang et al. 2005; Frampton et al. 1999; Huang et al. 2003; Jung et al. 2007;
Lindbom et al. 2006; Molinelli et al. 2002; Nakajoh et al. 2003; Pohjola et al. 2003; Spannhake et al. 2002; Veranth et al. 2007; Veranth et al. 2006; Veranth et al. 2004).

Research Goals and Hypotheses

The unique situation of locally generated and accumulated pollutants makes Cache Valley PM an excellent choice for the study of the effects of environmental pollutants. Since the PM is produced locally, it makes an excellent PM for the study of local regulatory efforts, and for the study of mechanisms involved in acute PM exposures. This laboratory has experience in documenting the effects and mechanisms of inhalable compounds in human lung cells (Van Vleet et al. 2006). Given the problems with plant transformations and this opportunity, it was deemed appropriate to determine some of the cellular mechanisms by which the local PM affects cells. This study was undertaken to add to the needed body of knowledge concerning the overall mechanisms of PM induced harm. The first hypothesis was that Cache valley PM would exert inflammatory and apoptotic effects on BEAS-2B human lung cells and that I would be able to evaluate some of the mechanisms of those processes. A benefit is that this study also provides markers for comparative analysis of PM as regulatory efforts are performed and PM components are altered. Therefore this study was conducted to determine if Cache Valley PM induces apoptosis and is pro-inflammatory in the widely used BEAS-2B cell line. PM has been shown to be pro-apoptotic in some cells (Agopyan et al. 2003; Choi et al. 2004), and apoptosis is thought to be a major contributor to respiratory and cardiovascular disease (Agopyan et al. 2003; Iliodromitis et al. 2007; Martin et al. 2005). The mechanisms and pathways of PM induced apoptosis are not adequately explained.
Using the proper markers, a researcher may determine the pathways of PM induced apoptosis (extrinsic, extrinsic, etc.) and possibly gain insight into which type of PM component is responsible. The inflammogenic nature of PM is well known, but the mechanisms by which the inflammation is induced are not completely understood. Findings of the preliminary study indicated that apoptosis is not a mechanism of Cache Valley PM induced harm, but endoplasmic reticulum stress might be. Therefore the hypothesis of the second set of experiments was that Cache Valley PM would cause endoplasmic reticulum stress in human lung cells. Many of the results of the first two could be explained by Akt activation, therefore the hypothesis of the third set of experiments was that Cache Valley PM would activate the cellular protein Akt.

References


antigen genes retain the ability to undergo squamous differentiation. *Differentiation* 38, 60-6.


Yang, H. M., Barger, M. W., Castranova, V., Ma, J. K., Yang, J. J., and Ma, J. Y. (1999). Effects of diesel exhaust particles (DEP), carbon black, and silica on macrophage


Table 1-1. Regulation of ambient particulate air pollution in the United States

<table>
<thead>
<tr>
<th>Year</th>
<th>TSP 24 h</th>
<th>TSP Annual</th>
<th>PM$_{10}$ 24 h</th>
<th>PM$_{10}$ Annual</th>
<th>PM$_{2.5}$ 24 h</th>
<th>PM$_{2.5}$ Annual</th>
</tr>
</thead>
<tbody>
<tr>
<td>1971</td>
<td>260$^1$</td>
<td>75</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>1987</td>
<td>n/a</td>
<td>n/a</td>
<td>150$^1$</td>
<td>50</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>1997</td>
<td>n/a</td>
<td>n/a</td>
<td>150$^1$</td>
<td>50</td>
<td>65$^2$</td>
<td>15$^3$</td>
</tr>
<tr>
<td>2006</td>
<td>n/a</td>
<td>n/a</td>
<td>150$^1$</td>
<td>n/a</td>
<td>35$^4$</td>
<td>15$^3$</td>
</tr>
</tbody>
</table>

$^1$Arithmetic average. One exceedance allowed per year.

$^2$Not to be implemented for five years.

$^3$The three year average of the annual weighted average should not exceed this number.

$^4$The three year average of the 98th percentile of the 24 h concentrations should not exceed this number.
TABLE 1-2. Comparison of ambient particles, fine particles (ultrafine plus accumulation-mode) and coarse particles.

<table>
<thead>
<tr>
<th></th>
<th>Ultrafine Processes:</th>
<th>Fine Accumulation Processes:</th>
<th>Coarse Formation Processes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation</td>
<td>Combustion, high-temperature processes, and atmospheric reactions</td>
<td>Condensation</td>
<td>Break-up of large solids/droplets</td>
</tr>
<tr>
<td>Formed by:</td>
<td>Nucleation</td>
<td>Coagulation</td>
<td>Mechanical disruption (crushing, grinding, abrasion of surfaces)</td>
</tr>
<tr>
<td></td>
<td>Condensation</td>
<td>Reactions of gases in or on particles</td>
<td>Evaporation of sprays</td>
</tr>
<tr>
<td></td>
<td>Coagulation</td>
<td>Evaporation of fog and cloud droplets in which gases have dissolved and reacted</td>
<td>Suspension of dusts</td>
</tr>
<tr>
<td>Composed of:</td>
<td>Sulfate</td>
<td>Sulfate, nitrate, ammonium, and hydrogen ions</td>
<td>Suspended soil or street dust</td>
</tr>
<tr>
<td></td>
<td>Elemental carbon</td>
<td>Elemental carbon</td>
<td>Fly ash from uncontrolled combustion of coal, oil, and wood</td>
</tr>
<tr>
<td></td>
<td>Metal compounds</td>
<td>Large variety of organic compounds</td>
<td>Nitrates/chlorides/sulfates from HNO₃/HCl/SO₂ reactions with coarse particles</td>
</tr>
<tr>
<td></td>
<td>Organic compounds</td>
<td>Metals: compounds of Pb, Cd, V, Ni, Cu, Zn, Mn, Fe, etc.</td>
<td>Oxides of crustal elements (Si, Al, Ti, Fe)</td>
</tr>
<tr>
<td></td>
<td>with very low saturation vapor pressure at ambient temperature</td>
<td>Particle-bound water</td>
<td>CaCO₃, CaSO₄, NaCl, sea salt</td>
</tr>
<tr>
<td>Solubility:</td>
<td>Probably less soluble than accumulation mode</td>
<td>Largely soluble, hygroscopic, and deliquescent</td>
<td>Largely insoluble and nonhygroscopic</td>
</tr>
<tr>
<td>Sources:</td>
<td>Combustion</td>
<td>Resuspension of industrial dust and soil tracked onto roads and streets</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atmospheric transformation of SO₂ and some organic compounds</td>
<td>Suspension from disturbed soil (e.g., farming, mining, unpaved roads)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High temperature processes</td>
<td>Construction and demolition</td>
<td>Uncontrolled coal and oil combustion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ocean spray</td>
<td>Biological sources</td>
</tr>
<tr>
<td>Atmospheric half-life:</td>
<td>Minutes to hours</td>
<td>Days to weeks</td>
<td>Minutes to hours</td>
</tr>
<tr>
<td>Removal Processes:</td>
<td>Grows into accumulation mode</td>
<td>Forms cloud droplets and rains out</td>
<td>Dry deposition by fallout</td>
</tr>
<tr>
<td></td>
<td>Diffuses to raindrops</td>
<td>Dry deposition</td>
<td>Scavenging by falling rain drops</td>
</tr>
<tr>
<td>Travel distance:</td>
<td>&lt; 1 to 10s of km</td>
<td>100s to 1000s of km</td>
<td>&lt; 1 to 10s of km (small size tail, 100s to 1000s in dust storms)</td>
</tr>
</tbody>
</table>

Adapted from (National Center for Environmental Assessment (Research Triangle Park N.C.) 2003).
The human respiratory tract. The upper region or extrathoracic region includes the posterior nasal passage where larger particles are trapped through nasal breathing, the nasal pharynx, the oral pharynx, and the larynx. The tracheobronchial region is the source of the BEAS-2B cells used in this work. It has significant branching and at such branches the greatest concentration of deposited particles occurs. The trachea branches into the main bronchi, which branches into the bronchi, which branches into the bronchioles. Inertial impaction predominates in this region. The alveolar region involves the terminal bronchioles, the respiratory bronchioles, and the alveoli. In this region Brownian diffusion and gravitational sedimentation predominate. Figure adapted from (National Center for Environmental Assessment (Research Triangle Park N.C.) 2003).
Figure 1-2. Modeled particle deposition in airway bifurcations (generations 3-4), at a respiratory minute volume of 30 L/min that imitates non-strenuous physical activity. A and B are the daughter airways. The larger 5 µm particles, which are more subject to the forces of impaction, deposit onto the top, bottom, and the central rounded carinal ridge. The smaller 0.2 µm particles deposit along the middle-sides of the daughter branches. Used with permission (Balashazy et al. 2003; J Appl Physiol).
CHAPTER 2
EFFECTS OF PM$_{2.5}$ COLLECTED FROM CACHE VALLEY UTAH ON GENES ASSOCIATED WITH THE INFLAMMATORY RESPONSE IN HUMAN LUNG CELLS

Abstract

In January 2004, the normally picturesque Cache Valley in Northern Utah made national headlines with the highest PM$_{2.5}$ concentrations in the nation. Epidemiological studies have linked exposure to particulate air pollution in other locations with stroke and Alzheimer’s disease and to early mortality from all causes, cancer, and cardiopulmonary diseases. To determine potential effects of these particles on human health, human bronchial epithelial cells (BEAS-2B) were cultured with PM$_{2.5}$ collected from various locations in the Cache Valley for 24 h. These particles were slightly cytotoxic, but more potent than NH$_4$NO$_3$, the major chemical component of Cache Valley PM$_{2.5}$. Gene expression analysis of PM$_{2.5}$-exposed cells was performed using microarray and quantitative RT-PCR. Among other genes, PM$_{2.5}$ exposure induced genes and proteins involved in the inflammatory response. Most notably, PM$_{2.5}$ exposed cells showed significant gene level up-regulation of activating receptors to interleukins-1 and 6 (IL-1R1 and IL-6R), as well as concomitant increases in protein. Increases in IL-1 receptor associated kinase-1 (IRAK) protein were observed. PM$_{2.5}$ exposure resulted in release of IL-6, as well phosphorylated STAT3 protein providing evidence that PM activates the

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IL-6/gp130/STAT3 signaling pathway in BEAS-2B cells. IL-20 and major histocompatibility complex peptide class-1 (MICA) were up-regulated and cleavage of caspase-12 was detected. In total, our results indicate that Cache Valley PM$_{2.5}$ produces the up-regulation of important cytokine receptors and is able to activate both IL-1R and IL-6R-mediated signaling pathways in human lung cells. These observations are generally consistent with the adverse effects associated with inhalation of fine particulate matter like PM$_{2.5}$.

Introduction

The Cache Valley in Northern Utah/ Southeastern Idaho has an area of 3050 km$^2$ with a 2004 population estimate of 100,000 (USCB 2006). The valley floor averages 1430 m above sea level and is surrounded by steep mountain ranges with elevations as high as 3042 m (Malek et al. 2006). During winter months, this narrow valley is susceptible to shallow temperature inversions where high barometric pressure traps cold air within the valley basin. Such meteorological phenomena lead to the accumulation of pollutants, namely particulates, resulting in episodic increases in particulate concentrations, such as the reading of 132.5 µg/m$^3$ PM$_{2.5}$ that occurred in January 2004 that was described as “worst in the nation” (Malek et al. 2006).

While only partially chemically characterized, secondary nitrate salts, specifically NH$_4$NO$_3$, are important components of Cache Valley PM$_{2.5}$ (Martin and Koford 2005) which are formed from the primary pollutants NOx, principally from automobile exhaust, and ammonia from livestock excreta. This reaction is catalyzed by cold temperatures, high humidity, volatile organic compounds (VOC), and the presence of other reactive
compounds. Nitrate salts may be coupled to an organic component (< 25%) which may enhance condensation onto the particle. Cache Valley PM$_{2.5}$ also contain sulfites and crustal components, the latter accounting < 6%; of particular toxicological significance is that the majority (> 80%) of these fine particles have a mean geometric diameter of the particles are < 1 µm in diameter (Mangelson et al. 1997; Martin and Zhu 2004; Silva 2004, Silva et al 2004). The mass concentration and chemical makeup of Cache Valley PM$_{2.5}$ has been shown to be spatially and temporally consistent during the winter (Martin 2004; Martin 2005). Unlike in other Utah urban areas where some fine particles are crustal elements drifting from nearby deserts (Pope et al. 1999), Cache Valley particles are generated locally.

Epidemiologic studies have associated exposure to fine particulates with early mortality (Klemm et al. 2000; Schwartz et al. 1996; Schwartz et al. 2002) by stroke (Hong et al. 2002), cancer (Dominici et al. 2005), cardiopulmonary (Krewski et al. 2005; Pope et al. 2002), and cardiovascular disease (Delfino et al. 2005; Ulrich et al. 2002). Particulate air pollution has also been associated with hospital admissions for cardiovascular and respiratory diseases (Dominici et al. 2006). Short-term exposures to air pollution are sufficient for increases in mortality (Ostro et al. 2006). While no epidemiologic studies examined the effects of particulate air pollution in Cache Valley, studies examining particulate air pollution in other Utah urban centers have associated PM exposure with early mortality (Pope et al. 1999) from a variety of cardiovascular and other diseases (Pope and Dockery 2006).

In humans, the bronchial epithelium acts as a physicochemical barrier and plays a crucial role in initiating and augmenting defense mechanisms and is responsible for
signaling a systemic response (Mills et al. 1999). Of particular relevance to this investigation is that airway epithelial cells exposed to PM in vivo and in vitro and are known to initiate and amplify the inflammatory response by secreting a number of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8 (Dick et al. 2003; Veranth et al. 2006). It was therefore hypothesized that Cache Valley PM$_{2.5}$ has the potential to elicit inflammatory or cytotoxic responses in human bronchial epithelial cells. In this study, the effects of Cache Valley PM$_{2.5}$ were examined on some inflammatory and cytotoxic mechanisms in cultured human bronchial epithelial (BEAS-2B) cells.

Materials and Methods

Chemicals and reagents

BEAS-2B cells were a gift from Dr. Katherine Macé (Nestle Research Centre; Lausanne, Switzerland). BEAS-2B cells are classified as bio-safety level-2 (BSL-2) and appropriate precautions were observed. Trypsin/EDTA, HEPES buffered saline solution (HBSS), and trypsin neutralizing solution (TNS) were from Cambrex, (Walkersville, MD). LHC-9 cell growth media came from Invitrogen (Camarillo, CA). FastStart TaqMan Probe Master mix and hydrolysis probes were from Roche (Roche, Indianapolis, IN). Interleukin-1 receptor type 1 (IL-1R1) and interleukin 6 receptor (IL-6R) primary antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-IRAK-1 primary antibody was from Affinity Bioreagents (Golden, CO). Biotinylated protein ladder, anti-rabbit and anti-biotin secondary antibodies, IFN-α treated HeLa cell extract, caspase-12, caspase-3, TNF-α, and Phospho-STAT3 primary antibodies were obtained from Cell Signaling (Beverly, MA). CaspACE Colorimetric caspase-3/7 activity
detection kit was from Promega (Madison, WI). Millipore Ultrafree DA spin columns (Millipore, Billerica, MA), and Corning well cell culture cluster 96-well plates were obtained from Fisher Scientific (Pittsburgh, PA). Quick Start Bradford Protein Assay was from Bio-Rad (Hercules, CA). The limulus amebocyte lysate (LAL) assay QCL-1000 was from Cambrex (East Rutherford, NJ).

**PM$_{2.5}$ collection and treatment**

Samples of ambient PM$_{2.5}$ were collected from one urban (“Smithfield”) and two rural (“Evan’s Farm,” and “Cornish”) Cache Valley locations, following the Federal Reference Method outlined in the U.S. Code of Federal Regulations (40 CFR 50, 2006). The official particulate concentrations for the county reported to the EPA came from the Utah Department of Environmental Quality Division of Air Quality and were measured in nearby Logan, Utah. The collectors used were an Airmetrics MiniVol (Eugene, OR) set to collect PM$_{2.5}$ (the collector can collect PM$_{10}$ and PM$_{2.5}$ but not simultaneously) for the rural locations or an RAAS-1000 PM$_{10}$/PM$_{2.5}$ sampler (Anderson, Atlanta, GA) which separate atmospheric particulate matter into the desired size fractions via a USEPA-designed WINS impactor was used to collect PM$_{2.5}$ onto Whatman 47 mm PTFE Air Monitoring Membrane Filters (Model No. 7592-104). The instruments were programmed to operate for 24-h periods (midnight-to-midnight) at flow rates of 5 Lpm (rural) and 16.7 Lpm (urban). Each filter was stored in individual plastic Petri dishes in a room temperature desiccator until equilibrated to within ± 2.5 µg before and after collection. Filters were handled only with Teflon-coated, stainless steel forceps. The collectors were cleaned prior to seasonal set up, and the impactor removed and cleaned.
following episodes of high PM. PM was collected from Nov.-Jan. 2004, Jan. 2002 and 2003. Unless otherwise stated, the filters used were from the Smithfield location.

For extraction, filters were weighed then placed into 50 ml conical bottom Falcon tubes (Becton Dickenson, Franklin Lakes, NJ) and immersed in 30 ml autoclaved and filtered (0.1 µm) de-ionized H₂O. Tubes containing the filter and water were then sonicated for 90 min in a sonicator bath (Model 1200 Branson Ultrasonics, Danbury, CT). Tubes and filters were dried and weighed to determine the amount of PM₁₀ extracted. In most cases the filters were visibly darkened following collection and the appearance was unaltered following extraction. Following desiccation a film was observable on the bottom of the Falcon tubes. Extracted PM₁₀ was then re-suspended in 1 ml LHC-9 cell growth media and used immediately or stored at 4°C until used (generally within 16 h). The extraction efficiency of PM₁₀ from the filters was routinely 75-80%. Filters were discarded following extraction.

Cytotoxicity, endotoxin detection, and apoptosis assays

Cytotoxicity was measured using a variation of the MTT method (Mosmann 1983) as described in (Van Vleet et al. 2002) with the exception that 36 µl of 0.1N HCl in isopropanol was added to dissolve crystals and neutralize phenol red rather than DMSO. The reactions were read on a microplate reader (Labsystems Multiskan MCC/340 Thermo-electron, Pittsburgh, PA). Staurosporine (1 µM) or caffeine (250 µM) (Van Vleet et al. 2006) were used as positive controls. Cytotoxicity of a range of PM₁₀ (5.5 – 1101 µg/m³) collected on separate occasions and locations were tested, as well as that of NH₄NO₃, the principal component of wintertime Cache Valley PM₁₀. Eight
replicates of each concentration were tested. Differences from control were
determined using Holm-Sidak one way analysis of variance using SigmaStat (SPSS,
Chicago, IL).

The introduction of artifactual endotoxin was examined PM$_{2.5}$ extraction water,
cell growth media, and a blank, extracted PTFE filter using the LAL assay kit. Samples
of collected particulate material at two concentrations (1.2 µg/ml PM$_{2.5}$ and 5.3 µg/ml
PM$_{10}$) were also examined.

Caspase-3 protein and activation were determined using the CaspACE
colorimetric assay kit according to the manufacturer’s instructions.

Cell culture

Cells were seeded into 10 ml of LHC-9 in T-75 flasks and incubated at 37°C/5%
CO$_2$ with media changes the following day and alternate days thereafter. Cells from the
same passage were counted and aliquoted to 6.15x10$^4$ cells per flask, grown to
approximately 70% confluence then cultured 24 h with a range of PM$_{2.5}$ concentrations
(0.13 - 3.90 µg/ml). After exposure, media was removed, cells washed with 5 ml HBSS,
detached with 5 ml 0.25mg/ml trypsin/EDTA, and pelleted in TNS.

Microarray RNA analysis

Following cell harvest, total RNA was extracted using the RiboPure RNA
isolation kit (Ambion, Austin, TX) according to the manufacturer’s instructions including
the optional DNAse incubation and purification step. RNA was then quantified
spectrophotometrically ($A_{260}$) (Model ND-1000 spectrophotometer Nanodrop,
Wilmington, DE), then analyzed for gene expression by microarray. Total RNA from
flasks treated with differing concentrations of PM$_{2.5}$, or media control for 24 h was submitted to an on-campus core facility where it was analyzed for quality (Bioanalyzer, Agilent, Palo Alto, CA). Gene expression was examined using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Array images were analyzed using Array Assist (Stratagene, La Jolla, CA) software applying the PLIER algorithm. Genes of interest were limited using exposure/control ratios of > 2 (Koike et al. 2004). Genes were further limited by selecting those that exhibited a > 2 ratio across all treatments using Microsoft Excel (Redmond, WA) and were linked to known proteins using NetAffx software (Affymetrix, Santa Clara, CA). While a variety of genes met these criteria, genes related to inflammation, stress response, cell death, or those that were associated with inhalation of particulate air pollution were selected. Verification of the array data was performed using quantitative RT-PCR (qRT-PCR).

qRT-PCR analysis

Total RNA from PM$_{2.5}$-exposed and control cells was isolated as described above. One µg total RNA was used to transcribe cDNA using the 2.5 µM anchored-oligo(dT)$_{18}$ primer with the Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN). A control cDNA synthesis reaction lacking reverse transcriptase was run for each reaction. cDNA was then stored at -20°C until used for qRT-PCR. HPLC-purified intron spanning primers were designed using the Universal ProbeLibrary assay design center (Roche, Indianapolis, IN) and ordered from Integrated DNA Technologies (Coralville, IA). Primers and probes are given in Table 1. All PCR reactions were run using the following cycling parameters: an initial melting and Taq antibody deactivation step at 95°C for 10 min followed by 40 cycles of a melting step at 95°C for 15 sec and a 60°C
annealing/extension step for 1 min. Optimal primer concentrations for all examined genes were determined by running a 50-700 nM equimolar primer concentration range with a constant 1µl cDNA in a 25 µl reaction using the FastStart TaqMan Probe Master mix. Reactions were separated on a 2% agarose gel (EC Minicell Primo, Fisher Scientific, Pittsburgh, PA) at 150V for 20 min. PCR product images were analyzed and archived using a Model 920 imager and Labworks software (UVP, Upland, CA). After the optimal primer concentration was selected, the PCR product was excised from the gel and purified using Millipore Ultrafree DA spin columns for sequencing and use as a standard. PCR product standard DNA was quantified spectrophotometrically (Thermospectronic Genesys 6, Fisher Scientific, Pittsburgh, PA) and copy concentration was determined using the molecular weight of the known amplicon DNA sequence as determined by OligoAnalyzer software (IDT, Coralville, IA). Standard DNA was diluted $10^3$ then serially diluted in triplicate to generate a standard curve. Quantitative reactions were done in triplicate including no cDNA and no reverse transcriptase controls using a DNA Engine Opticon 2 thermal cycler (Bio-Rad, Hercules, CA) using the above mentioned reaction conditions with the FAM read step occurring after the 60°C annealing/extension step. Each reaction was performed with 110 nM probe concentration and optimized primer concentration. Quantification was performed based upon the standard curve and calculated cycle threshold ($C_T$) values by the OpticonMonitor 2.02 software included with the instrument. Differences in mean values for treated vs. control were determined using Holm-Sidak one-way analysis of variance with SigmaStat software (SPSS, Chicago, IL).
Protein detection assays

Western Blotting was performed as previously described (Van Vleet et al. 2006). In brief, media from cell culture was removed, centrifuged, and frozen for subsequent analysis. Cells were washed with 5ml HEPES buffered saline solution and harvested via trypsinization. Cells were resuspended in 100 µl CHAPS cell extract buffer and total protein was quantified using the Quick Start Bradford Protein Assay (Hercules, CA). Equal protein concentrations were loaded onto gels, electrophoresed, transferred to nitrocellulose membranes, probed with the appropriate primary and secondary antibodies, and visualized using SuperSignal West Femto chemiluminescent substrate (Pierce, Franklin Lakes, NJ) using a Nucleotech Imaging workstation (Hayward, CA).

Densitometric analysis was performed using the Histogram function in PhotoShop CS (Adobe, San Jose, CA) (Woznicovaand Votava 2001). The software provided mean pixel intensity, standard deviations, and total number of pixels which were sufficient for one-way analysis of variance using the Holm-Sidak test using significance test at p<0.05. TNF-α release was examined using cells treated with higher concentrations of Cache Valley PM$_{2.5}$ (≤ 25 µg/ml) using the R&D Quantikine ELISA kit (Minneapolis, MN) according to the manufacturer’s instructions with 6 replicates per treatment. Measurement of IL-6 release into the media was performed as described (Veranth et al. 2006).
Results

PM extraction, cytotoxicity and endotoxin detection

Extraction of PM ranged from 70-670 μg from more than 10 filters. A prior ethanol wash resulted in post extraction filter weights that were less than the pre collection filter weights; hence extractions were done without an ethanol wash. Most of the extracted PM was used for cytotoxicity. For subsequent gene expression experiments cells were exposed to PM$_{2.5}$ at concentrations below those found to be cytotoxic. A single filter generally yielded sufficient PM for two independent experiments. Due to the influence of weather patterns on PM conditions (Malek et al. 2006) samples taken on different days were not pooled.

The Endotoxin concentrations in LHC-9 media, extraction water, and an extract of a blank collection filter were below the detection threshold (<0.1 Eu/ml) of the LAL assay. A 1.2 μg/ml sample of PM$_{2.5}$ also contained < 0.1 EU/ml endotoxin. Endotoxin above 0.1 EU/ml (0.15 EU/ml) was detected in a 5.3 μg/ml sample of PM$_{10}$ collected on the same day as the PM$_{2.5}$ (not used in this study).

Our data indicate that exposure to Cache Valley PM$_{2.5}$ resulted in minimal observable cytotoxicity in BEAS-2B cells, as determined by MTT. Figure 2-1A shows the concentration-dependency of cytotoxicity of PM$_{2.5}$ collected from an urban sampler in the city of Smithfield, Utah. Cells were cultured with varying concentrations of PM collected on Jan. 14, 2004, when the official ambient concentration was 116.9 μg/m$^3$ and with the highest concentration (1101.4 μg/ml) producing 39% inhibition. No significant differences in cytotoxicity were observed from PM$_{2.5}$ obtained from two different rural samplers on the same day and exposed to the same concentrations (data not shown).
Even though Cache Valley PM$_{2.5}$ was only modestly cytotoxic, it was substantially more potent than NH$_4$NO$_3$, when compared on a mass per volume basis (Fig. 2-1 B). NH$_4$NO$_3$ resulted in MTT conversion significantly above control at most concentrations with inhibition occurring only at the highest concentrations of 10 and 100mM (800.4 and 8004 µg/ml).

Gene expression analysis, IL-6 release, and protein activation

Selected up-regulated genes related to the inflammatory response determined by microarray analysis are presented Table 2-2. MICA, IL-20, IL-1R1, and IL-6R were selected for verification of mRNA up-regulation by qRT-PCR. In many cases, PM$_{2.5}$ exposure produced a significant up-regulation of these genes. For example, there was a significant up-regulation of mRNA coding for IL-1R1 in cells exposed to all but the lowest PM$_{2.5}$ concentration (0.69 µg/ml) (Fig. 2-2). Conversely, there also was significant up-regulation of IL-20 mRNA, but only in cells exposed to the lowest PM$_{2.5}$ concentration, while MICA was significantly up-regulated in concentration-dependent manner in cells treated with 1.37, 2.72, or 4.03 µg/ml PM$_{2.5}$ (Fig. 2-2). The lack of concentration dependency of the IL-20 response was repeatedly verified using 3 different reverse transcriptase (RT) reactions.

There was a significant up-regulation of IL-6R in cells cultured at all PM$_{2.5}$ concentrations (Fig. 2-2). These results were verified by reactions involving a minimum of two RT steps. To confirm that increases in expression were not due to differential reverse transcriptase activity, the content of single-stranded (ss) DNA after the cDNA synthesis step was determined. The control cDNA reaction contained more ssDNA than any of the treatments (1.1-1.9 X more in both RT reactions), thus any increases in mRNA
expression could not be attributed to increased cDNA from differential RT activity. Expression of IL-6R and IL-1R1 proteins were evaluated by western blotting to confirm that the increases in gene expression resulted in increased protein concentrations. There was an increase in IL-6R and IL-1R1 protein concentrations, which occurred at all PM$_{2.5}$ concentrations (Fig. 2-3 and 4). Because IL-1R1 is essential for IL-1 signaling (Sims et al. 1993) and involves interleukin 1 receptor associated kinase 1 (IRAK), the effect of PM$_{2.5}$ was examined on expression of IRAK protein. Microarray detected IRAK up-regulation in the unmodified (CHP) data set applying the same selection (>2) criteria, but not when data were transformed with the PLIER algorithm (data not shown). Western blots showed a significant up-regulation of IRAK protein levels in cells exposed to all concentrations of PM$_{2.5}$, and the expression pattern with respect to PM concentration appeared similar to that of IL-1R1 (Fig. 2-4).

Cells treated with PM$_{2.5}$ concentrations of 0.65, 1.3, or 3.9 µg/ml released significantly higher levels of IL-6 into the media (Fig. 2-5). There was no detectable TNF-α release into the cell media after 24 hPM$_{2.5}$ exposure. Subsequent Western blotting revealed the presence of the full length 32 kDa TNF-α precursor but not the 17 kDa mature TNF-α (data not shown). Because of the effect of PM on activation of the IL-6 signaling pathway, studies were performed to examine the expression of phosphorylated STAT3 protein in PM-exposed cells using a primary antibody specific for tyrosine 705-phosphorylated STAT3. Figure 2-3 shows that there was a clear concentration-dependent increase in levels of phosphorylated STAT3 in cells exposed to Cache Valley PM$_{2.5}$. The antibody detects doublet bands of 79 and 86 kDa.

Given the observation of PM-related up-regulation of genes associated with
apoptosis by microarray, the presence and activation of both caspase 12 and caspase 3 in control and PM-exposed cells was determined. Figure 2-6 shows that PM$_{2.5}$ exposure produced increased expression of the 60 kDa pro-caspase-12 protein as well as activation of caspase-12, as evidenced by the presence of the 25 kDa caspase-12 cleavage product compared to control cells. In contrast, while caspase-3 protein was significantly higher in PM$_{2.5}$ treated cells, there was no detectable activation of caspase-3 as shown by the lack of the 17 kDa cleavage product using Western blotting or by the use of an alternate colorimetric activity assay (Fig. 2-7).

Discussion

Laboratory research determining the potential adverse human health effects of fine particulate air pollution has been stimulated by an increasing number of epidemiologic studies in several geographic locations that have consistently associated exposure to this class of air pollutants to a variety of cardiovascular diseases (Krewski et al. 2005). Exposure to airborne particulates in nearby Utah and Salt Lake Valleys (80 and 120 miles south of Cache Valley, respectively) during similar meteorological episodes was associated with increases in early mortality and hospital admissions due to cardiovascular diseases (Pope and Dockery 2006). That many of the adverse effects associated with fine particle exposure are outside the respiratory tract may be due to signaling and signal amplification from lung cells, or from the ability of small particles to penetrate the central circulation or the brain (Nemmar et al. 2002; Nemmar et al. 2004; Oberdorster et al. 2004).

Our results demonstrate that while Cache Valley PM$_{2.5}$ was only modestly
cytotoxic in cultured human BEAS-2B cells, it induced up-regulation of several genes associated with the inflammatory response as well as increased STAT3 activation. The cytotoxicity of PM$_{2.5}$ was substantially greater than that of NH$_4$NO$_3$, a principal chemical component. In fact, low ($\leq 1$ µM) NH$_4$NO$_3$ concentrations exhibited a significant stimulatory effect on MTT reduction, which may be explained by increases in cytoplasmic conversion of MTT (Vistica et al. 1991) or in mitochondrial respiration (Gerlier and Thomasset 1986). Increased MTT reduction was shown to occur without an increase in cellular growth (Gerlier and Thomasset 1986). Minimal cytotoxicity of fine particulates in vitro was also observed in various cell types by other researchers (Monn and Becker 1999; Pozzi et al. 2003). While NH$_4$NO$_3$ may play a synergistic role in some of the other observed effects, it seems reasonable that the cytotoxic properties of Cache Valley PM$_{2.5}$ are not primarily due to this compound.

More significantly, the data showed that the cellular response to exposure to our locally-collected PM$_{2.5}$ appears to involve both IL-1R and IL-6R-mediated pathways. Interleukin-1 and IL-1R1 play essential roles in illnesses associated with PM exposure such as stroke, progressive neurodegeneration, mild asthma, chronic obstructive pulmonary disease (COPD), and coronary artery disease (Basu et al. 2002; Basu et al. 2005; Chung 2001; Kornman 2006). IL-1R1 and IL-1 are involved in Alzheimer’s disease (Basu et al. 2002; Griffin 2006) and play a crucial role in atherosclerosis (Chi et al. 2004) and cardiovascular disease (Lobbes et al. 2006). Particulate air pollution stimulates IL-1 release in human monocytes (Brown et al. 2004), in alveolar macrophages (van Eeden et al. 2001), and in patients with acute lung injury (Geiser et al. 2000). The potential for IL-1R activation in PM exposed cells was supported by
increased expression of its downstream signaling molecule IRAK. Increased IRAK expression would be expected to result in heightened IRAK-mediated kinase activity, known to affect a large number of downstream cascades and transcription factors (Bol et al. 2005; Janssens and Beyaert 2003; Takatsuna et al. 2003). Others also showed that PM exposure triggers upregulation of inflammatory receptors (TLR 2 and 4) associated with IL-1R that involve IRAK (Becker et al. 2005). Such findings are relevant in that the mechanisms for cytokine inhibition and activation are distinct. Cytokines require interaction with cell receptors to exert their effects. The effects of IL-1 are exerted exclusively through IL-R1, while inhibition of IL-1 occurs partially through binding with IL-1R2 (Rauschmayr et al. 1997). Cells responding to PM treatment by upregulating activating receptors rather than inhibitory receptors indicate that PM-mediated IL release may mediate inflammatory effects.

Likewise, increases in IL-6 and its activating receptor also are involved in PM-associated diseases. The IL-6 cytokine family is involved in a variety of cellular functions, which in addition to inflammation, include immunoglobulin production, cell growth and differentiation (Hirano et al. 2000). Interleukin-6R activation is directly involved in heart failure (Rivera et al. 2004) asthma, and pulmonary fibrosis (Jones et al. 2001). IL-6R also exists as a soluble receptor (sIL-6R) that enables IL-6 to potentiate effects in cells that would not normally respond to IL-6. Both differential splicing and proteolytic cleavage contribute to the production of sIL-6R although the cause is poorly understood (Heinrich et al. 2003; Heinrich et al. 1998; Jones et al. 2001; Muller-Newen et al. 1998). Particulate air pollution was found to produce vasoconstriction and activation of the ERK kinases (Li et al. 2005) which are regulated through IL-6-gp130-
STAT3 (Kamimura et al. 2003) as well as the epidermal growth factor receptor (Blanchet et al. 2004). That PM$_{2.5}$ caused IL-6 release in BEAS-2B cells has also been recently observed in this cell type (Veranth et al. 2006) as well as in human monocytes (Monn and Becker 1999), human alveolar macrophages (Suwa et al. 2002).

Interleukin-6 signaling activity, mediated only through binding with IL-6R (via gp130 receptor) (Heinrich et al. 2003; Heinrich et al. 1998; Jones et al. 2001), leads to activation of STAT3 by phosphorylation. Our observation of STAT3 activation indicates that PM$_{2.5}$ treatment not only leads to IL-6 release, but also activates the IL-6 signaling pathway. To our knowledge, this is the first report to link particulate exposure with STAT3 activation and potential IL-6 autocrine activity in vitro. IL-20, which was observed being upregulated, may also activate STAT3 as shown in human keratinocytes (Blumberg et al. 2001). IL-20 gene expression was only induced significantly at the lowest PM concentration (0.69 µg/ml) (Fig. 2-2), while phosphorylated STAT3 levels were altered in a PM$_{2.5}$ concentration-dependent manner (Fig. 2-4). An explanation as to why IL-20 transcription only occurred at the lowest concentrations over multiple experiments is not readily available. The copy number was low and these cells may not release it. While the promiscuity of the gp130 receptor (Dahmen et al. 1998; Jones et al. 2001) precludes the assumption that the observed STAT3 activation was solely due to IL-6 autocrine activity (Yeh et al. 2006), the increases in STAT3 phosphorylation were undoubtedly due to PM$_{2.5}$ treatment. Phosphorylated STAT3 was detected in the control cells. Phosphorylated STAT3 is constitutively expressed in some cell types (Kube et al. 2001) and this also may be case in BEAS-2B cells (Veranth et al. 2006). That Cache Valley PM$_{2.5}$ activated STAT3 illustrates the complexity of IL-6 signaling. While IL-6
activation is involved in the above-mentioned adverse health affects such as heart failure, IL-6 is also anti-inflammatory (Opal and DePalo 2000) and responsible in part for the beneficial health effects of exercise (Petersen and Pedersen 2005). STAT3 activation is the dominant mediator in the anti-inflammatory effects of IL-10 (Williams et al. 2004), is protective against hyperoxia in the lung (Lian et al. 2005), and is necessary for cardioprotection following cardiac hypertrophy (Butler et al. 2006). However autocrine IL-6/STAT3 activation is involved in adenocarcinoma pathogenesis (Yeh et al. 2006).

STAT3 is considered to be an oncogene (Bromberg et al. 1999) involved in a number of cancers (Hodge et al. 2005) and is a target for chemotherapy (Leeman et al. 2006). Given the association of PM with lung cancer mortality (Krewski et al. 2005), the ability of PM to increase activation of STAT3 and potentially other oncogenes is important and the subject of ongoing research. STAT3 activation has been shown to inhibit TNF-α production (Nishiki et al. 2004) and release (de Jonge et al. 2005). This may explain why this study and others (Veranth et al. 2004) were unable to detect TNF-α release in PM treated BEAS-2B cells. Western blotting revealed the full-length inactive form of TNF-α indicating that it is synthesized by these cells (data not shown). These cells have been shown to release TNF-α when treated with studded-tire wear particles (Lindbom et al. 2006) and following Streptococcus pneumoniae infection (Schmeck et al. 2006). It is possible that the inability to detect TNF-α may be due to the cytokine binding directly to the particles as occurs with diesel exhaust PM (Seagrave et al. 2004). However, the ELISA kit used is capable of detecting TNF-α bound to soluble receptors as well as free TNF-α.

In addition to IL-20, Cache Valley PM$_{2.5}$ induced the expression of MICA which
is a recognized response to stress and pathological conditions (Borchers et al. 2006). While not directly related to activation of IL-1R or IL-6, MICA expression further indicates induction of cellular stress by PM$_{2.5}$.

Particulate air pollution triggers calcium release into the cytoplasm (Brown et al. 2004) which ultimately activates caspase-12 (Nakagawa and Yuan 2000), which is also activated by the buildup of unfolded proteins in the endoplasmic reticulum (ER) under stress conditions (Zhang and Kaufman 2006). The ER stress response is associated with neurodegeneration, and the ability of PM to penetrate to the brain (Oberdorster et al. 2004) may partially explain PM associated neuropathology (Peters et al. 2006). The observed caspase-12 activation did not exhibit a strong dose response as measured by Western blot, however the response was consistently greater in PM treated cell lysate. Other PM studies that have utilized Western blotting have also shown marked differences from control while not exhibiting a strong dose response (Choi et al. 2004). To our knowledge, caspase-12 has not been previously examined in BEAS-2B cells. While the commercial murine polyclonal differs from those used in other studies, it detected the 25 kDa cleavage fragment (that follows cellular stress in lung cells such as respiratory sincitial virus infection) but not the 40 kDa fragment (Bitko and Barik 2001). The PM$_{2.5}$-dependent increase in the 60 kDa procaspase-12 as well as its 25 kDa cleavage product in addition to MICA up-regulation seem to support the hypothesis that these particulates induce cellular and ER stress which may include downstream events such as calpain activation, which are the subject of current investigation. Active caspase-12 is believed to ultimately lead to activation of the effector caspase, caspase-3 (Sun et al. 2000). While increases in the inactive, full-length, caspase-3 protein in PM$_{2.5}$ treated cells was detected,
as predicted by microarray analysis (Table 2-1), neither caspase-3 cleavage nor caspase-3 activity were observed. For activity to occur, full length caspase-3 must be cleaved to active zymogens (Susin et al. 1997). Lack of caspase-3 activation may be explained by the fact that activation of the IL-6 pathway was found to inhibit apoptosis (Hodge et al. 2005) and caspase-3 activation (Chen et al. 1999). The pro-survival signaling of the IL-6 pathway may override the pro-apoptotic signaling of caspase-12 in these cells.

Cache Valley PM$_{2.5}$ is chemically distinct from that in other Utah urban areas, such as the Salt Lake and Utah Valleys, urban areas characterized by greater population, vehicular traffic, and heavy industry. Particulates and dusts from those locales contain metals that have been the focus of several studies (Veranth et al. 2004; Wu et al. 2001). The iron content of Cache Valley PM$_{2.5}$ (0.82-17.73 nmol/m$^3$) (Mangelson et al. 1997) is substantially lower than in other studies where iron was considered the bioactive component of PM (Frampton et al. 1999; Molinelli et al. 2002; Upadhyay et al. 2003). The Cache Valley lacks the steel, smelting, and foundry industries of Utah Valley (Mangelson et al. 1997), and the intensity of motor vehicle use, and the desert dust of the Salt Lake Valley (Malek et al. 2006; Pope et al. 1999; USCB 2006). It is possible that fine particles from these Utah locations have common components, such as bacterial antigens. However, there is conflicting evidence on the importance of bacterial components such as lipopolysaccharide (LPS) in the in vitro toxicity of PM and dusts (Soukup and Becker 2001; Veranth et al. 2004).

It is important to point out that up-regulation of some of the genes detected by microarray in cells exposed to Cache Valley PM$_{2.5}$ (caspase-3, TOLLIP, IL-1R1, and
TLR-5) are also recognized cellular responses to bacterial components (Noulin et al. 2005). For example TLR-5 mediates mammalian defense responses to bacterial flagella (Smith and Ozinsky 2002). It is hypothesized that bacterial components play a major role in the cellular response to particulate air pollution (Becker et al. 2005). Furthermore, inhibition of CD-14, a receptor protein involved with toll-like receptor proteins significantly inhibited IL-6 release in alveolar macrophages (Becker et al. 2002). Low levels of LPS have been shown to induce IL-6 and IL-8 release from BEAS-2B cells (Schulz et al. 2002) despite an internalized TLR-4 LPS receptor via a mechanism that is not completely understood (Guillot et al. 2004). The quantity of LPS required for IL induction in these cells is unknown. Whether LPS alone or other components are responsible for the observed alteration of basal levels of IL-6 is unknown. While not detected in the PM$_{2.5}$ used in our study, LPS was found in PM$_{10}$ samples from the same Cache Valley locations. LPS has been shown to upregulate expression of IL-1R1 mRNA in mice (Pournajafi Nazarloo et al. 2003) and IL-6R in rats (Vallieres and Rivest 1999). Interleukin receptor up-regulation has been documented following exposure to gram-negative bacteria and may assist in angiogenesis (McCord et al. 2006). Receptor up-regulation has also been observed following viral infection and treatment with double stranded RNA (Tsuji et al. 2005; Zhou et al. 2006) Thus, undetectable bacterial or viral antigens in our PM$_{2.5}$ samples may have been responsible for some of the effects observed in this study.

In the present study, PM concentrations to which the BEAS-2B cells exposed were reasonable approximations of “real world” conditions. The lowest PM$_{2.5}$ concentrations showing gene-specific effects (0.65 μg/ml, equivalent to 0.08 μg/cm$^2$
given 75 cm² flask area in 10 ml media) were in the same order of magnitude as the theoretical particle deposition (0.01 μg/cm²) to epithelial cells in vivo resulting from a “high” environmental exposure as postulated by Jimenez et al. (2000). Many of the gene-specific effects of Cache Valley PM₂.₅ were detected at concentrations substantially lower than that in some other studies. This may not be due to inherent differences in particle effects, but to experimental protocols such as our use of larger flasks (75 cm³) and media volume (10 ml) to facilitate RNA and protein isolation, which resulted in particle dilution. Further, the sensitivity of the assays used with detection limits of < 30 copies of specific genes, and fg levels of protein (qRT-PCR, and Western blotting, respectively) revealed effects at low particle concentrations. The observed fold-changes in gene expression compared to control were similar to those in other studies using human lung cells exposed to higher particle concentrations (albeit using different RT-PCR protocols). Particle-dependent release of IL-6 seen in our study was comparable to that in BEAS-2B cells exposed to larger concentrations of soil dust particles (Veranth et al. 2004). In comparison with other studies on the effects of Utah PM in BEAS-2B cells, the nitrate rich Cache Valley PM₂.₅ appears more potent in inducing IL-6 release than the calcium rich dust collected from the Utah West Desert (Veranth et al. 2004) or the iron rich Utah Valley particles (Frampton et al. 1999). Such potency differences could have been due to differing culture conditions (plate coatings, media), differences in data presentation, or the presence of bacterial components. The primary sampling site in our study is located 1.6 km from a dairy, and higher ambient endotoxin levels are associated with areas of intensive livestock production relative to urban air (Schulze et al. 2006). In any event, our results clearly demonstrate that low concentrations of urban particulate can
elicit measurable and statistically-significant responses in vitro.

While caution should be used in attributing environmental relevance to our in vitro data, this initial study may have some value in determining potential adverse health risks associated with exposure to Cache Valley PM$_{2.5}$. The PM$_{2.5}$-induced activation of inflammatory cytokines observed is consistent with the cardiovascular and other diseases associated with exposure to urban PM. This study affirms an increasing body of evidence that IL-1 and IL-6 signaling play a role in the observed health effects of particulate air pollution.

References


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Figure 2-1. Cytotoxicity resulting from 24 h exposures to Cache Valley PM$_{2.5}$ in BEAS-2B cells as determined by the MTT assay. (A) Cytotoxicity of PM$_{2.5}$ samples collected in Smithfield UT Jan. 14, 2004 when the official State ambient PM$_{2.5}$ reading for the County was 116.9 µg/m$^3$(B) By comparison, NH$_4$NO$_3$ a principal component of Cache Valley PM$_{2.5}$, was substantially less cytotoxic than PM$_{2.5}$ in that it stimulated MTT metabolism at concentrations < 8 µg/ml (100 µM) with inhibition only occurring at the highest concentrations > 80.04 µg/ml (1000 µM). Multiple (n=16) assays were run, of which these are representative. Each point is mean (n = 8; ± SD). *Significant from control as determined by Holm-Sidak one-way analysis of variance on ranks (p<0.05).
Figure 2-2. Absolute RT-PCR quantification of IL-1R1, IL-20, MICA and IL-6R mRNA from BEAS-2B cells following a 24 h exposure to varying concentrations of Cache Valley PM$_{2.5}$. A minimum of 3 experiments were run with each concentration in triplicate. Each bar is mean (n=3; ± SD). *Significant from control as determined by Holm-Sidak one-way analysis of variance (p<0.05).
Figure 2-3. Expression of IL-6R and phosphorylated STAT3 proteins in BEAS-2B cells following 24 h exposures to various concentrations of Cache Valley PM$_{2.5}$ as determined by densitometric analysis of Western immunoblots of cell extracts as described in Materials and Methods. Representative of n=3 experiments. The IL-6 blot was loaded with 10 µg protein/lane protein while the STAT3 blot was loaded with 4.5 µg protein/lane. There was a significant increase in cellular expression of both proteins at all PM$_{2.5}$ concentrations. The phosphorylated Stat3 antibody only detects the protein when phosphorlyated at Tyr705. Stat3 positive control is an extract of interferon-α treated HeLa cells. In all cases, the double bands observed were consistent with the blots presented by the manufacturer. Each bar is mean band intensity ± SD. *Significant from control as determined by Holm-Sidak one way analysis of variance (p<0.05).
Figure 2-4. Expression of IL-1R1 and IRAK proteins in BEAS-2B cells following 24 h exposures to various concentrations of Cache Valley PM$_{2.5}$ as determined western immunoblots of cell extracts as described in Materials and Methods. Each lane in the IRAK and IL-1R1 immunoblots was loaded with 7 µg and 10 µg protein/well respectively. Expression of both proteins in cells exposed to all concentrations of PM$_{2.5}$ was significantly greater than in the control. Multiple (n=3) experiments were run, of which this is representative. Each bar is mean band intensity ± SD. *Significant from control as determined by Holm-Sidak one way analysis of variance (p<0.05).
**Figure 2-5.** Release of IL-6 from BEAS-2B cells following 24 h exposures to various concentrations of Cache Valley PM$_{2.5}$ as determined by ELISA assay as described in *Materials and Methods*. Each bar is the mean IL-6 concentration (n= 9; ± SD). *Significant from control as determined by Holm-Sidak one way analysis of variance (p<0.05).**
Figure 2-6. Expression of caspase-12 and cleavage of caspase-12 to the 25 kDa product induced in BEAS-2B cells following 24 h exposures to Cache Valley PM$_{2.5}$ as western immunoblots of cell extracts as described in *Materials and Methods*. Each lane was loaded with 10 µg/protein. All concentrations of Cache Valley PM$_{2.5}$ (0.65, 1.3, 2.6, and 3.9 µg/ml) induced cleavage of Caspase-12. All treatments of Cache Valley PM$_{2.5}$ also yielded a significant increase in the 60 kDa procaspase-12 with the exception of 1.3 µg/ml. Densitometry of the 25 kDa fragment includes the control lane which had an undetectable fragment for comparison. Multiple (n=4) experiments were conducted, of which this is representative. Each bar is mean band intensity ± SD. *Significant from control as determined by Holm-Sidak one way analysis of variance (p<0.05).
**Figure 2-7.** Caspase-3 expression and cleavage determined by western immunoblots (top) and caspase-3 activity determined by a colorimetric assay (bottom) in BEAS-2B cells following 24 h exposures to Cache Valley PM$_{2.5}$ as described in Materials and Methods. Gels were loaded with 5 µg protein/well. While all concentrations of PM induced expression of caspase-3 protein, there was no observable 17 kDa cleavage product indicative of activation of caspase-3 (top). Images were exposed to saturation to demonstrate the lack of cleavage. In the same way, there was no caspase-3 activation as determined by colorimetric activity assay (bottom). Controls include cells treated with the caspase inhibitor Z-VAD-FMK (ZVAD), 0.25µM staurosporine, and untreated cells. Results presented are the means of 2 different experiments with each treatment group done in triplicate. Each bar is mean absorbance (405 nm) ± SD. *Significant from control as determined by Holm-Sidak one way analysis of variance (p<0.05).
CHAPTER 3

URBAN PARTICULATE MATTER CAUSES ER STRESS AND THE UNFOLDED PROTEIN RESPONSE IN HUMAN LUNG CELLS

Abstract

Because of its presumed adverse health effects, particulate air pollution (PM) has received growing attention, but the cellular mechanisms by which PM exerts toxicity are not well elucidated. PM has been associated with early mortality from illnesses that share endoplasmic reticulum (ER) stress as a mechanism of pathogenesis. In this study, we examined whether PM would induce the unfolded protein response (UPR) which is a cellular response to ER stress. Coarse (PM$_{10}$) and fine (PM$_{2.5}$) PM was collected from a single location in Northern Utah’s Cache Valley during atmospheric inversions occurring in January 2002 and January of 2003. Extracts of PM samples were added (12.5 and 25 µg/ml) to cultured human bronchial epithelial (BEAS-2B) cells for 24 h. At these concentrations neither PM nor lipopolysaccharide (LPS) exhibited demonstrable cytotoxicity by the neutral red assay. However, PM elicited significant increases of UPR related posttranslational modifications, such as S6 ribosomal protein, Hsp27 and PERK phosphorylation and cleavage of ATF-6. PM exposure also resulted in significant increases in the UPR-associated proteins ATF-4, Hsp70, Hsp90, and BiP. PM also interfered with the export of Hsp70 from the cells in a concentration-dependent manner and resulted in release of C-reactive protein (CRP). Calpain was upregulated and

activated in PM-treated cultures, though these events were not pro-apoptotic. This study demonstrates that PM is capable of inducing ER stress and the UPR in vitro and may be a mechanism by which PM exerts toxicity.

Introduction

The normally-picturesque Cache Valley in Northern Utah gained unwanted national attention following measurements of some of the highest PM$_{2.5}$ concentrations in the United States (Malek et al. 2006; USA-Today 2005). Cache Valley PM is a secondary pollutant with the valley lacking major primary PM sources. Cache Valley PM is predominated by ammonium salts and the formation thereof is accelerated by low winter temperatures, and atmospheric inversions. The ammonium salts are derived by a reaction between NH$_3$ gas from livestock excreta and NO$_x$ primarily from automobile exhaust forming NH$_4$NO$_3$ as the principal component (Edgerton et al. 2006; Malek et al. 2006; Mangelson et al. 1997; National Center for Environmental Assessment [Research Triangle Park N.C.] 1996).

Inhalation of PM has been associated with early all-cause mortality in addition to cancer, ischemic heart disease, cardiac arrest, hypertensive disease, cerebrovascular disease, diabetes, pneumonia, influenza, and neurodegeneration (Peters et al. 2006; Pope et al. 2002; Pope et al. 2004a). While some of these diseases are outside the pulmonary system, they may share similar pathogenesis. For example, influenza, neurodegeneration, diabetes, and ischemia all involve endoplasmic reticulum (ER) stress and the accumulation of unfolded protein (DeGracia and Montie 2004; Zhang and Kaufman 2006). In eukaryotic cells, proteins are folded and assembled for transport in the ER;
homeostasis therein can be perturbed by a number of external stressors. The cellular adaptive response to accumulations of unfolded protein is called the unfolded protein response (UPR) (Zhang and Kaufman 2006).

In unstressed cells, the immunoglobin binding chaperone protein (BiP, formerly GRP78, or HspA5) binds to and inhibits the ER resident stress sensors, dsRNA activated protein kinase related protein (PERK), inositol-requiring enzyme-1 (IRE1), and activating transcription factor (ATF)-6. Under conditions of ER stress, unfolded protein accumulates in the ER, which bind to and sequester BiP, causing release of ATF-6, PERK, and IRE-1 allowing their activity (Rutkowski and Kaufman 2004).

Downstream events include dimerization and phosphorylation of PERK (Rutkowski and Kaufman 2004; Zhang and Kaufman 2006) and phosphorylation of elongation and initiation factor-2 (eIF2) resulting in reduced recognition of AUG initiation codons and attenuation of general protein synthesis (Liu and Kaufman 2003). Then ATF-6 migrates to the Golgi where it is cleaved to its active (50 kDa) form that induces the transcription of chaperones and foldases capable of evading PERK-mediated translational blockage, thereby increasing the protein folding capacity of the ER (Rutkowski and Kaufman 2004). Another consequence of PERK phosphorylation is the upregulation of ATF-4 which circumvents PERK mediated translational inhibition (Rutkowski and Kaufman 2004), causing transcription of genes involved in cellular adaptation to the accumulation of unfolded protein. These proteins also circumvent PERK-mediated translational reduction (Rutkowski et al. 2006). IRE1 induces proteins involved with ER associated protein degradation (ERAD) and feedback inhibition of PERK phosphorylation (Liu and Kaufman 2003; Rutkowski et al. 2006).
Although an adaptive response, the UPR can result in apoptosis if ER stress is prolonged (Zhang and Kaufman 2006). One mechanism involves the ATF-4/ATF-6-mediated upregulation of the transcription factor CCAAT/enhancer binding protein homologous protein (CHOP), which inhibits the transcription of the pro-survival Bcl-2 (Di Sano et al. 2006). An early hypothesized second mechanism of ER stress related apoptosis involves the release of calcium ions from the ER, which are rapidly taken up into the mitochondria potentially leading to the collapse of the inner mitochondrial membrane potential associated with the intrinsic apoptosis pathway (Rutkowski and Kaufman 2004), although massive release of calcium ions from the ER depends on prior transport of cytochrome c from the mitochondria to the ER (Boehning et al. 2003; Mattson and Chan 2003). Bax is likely the key component of ER related apoptosis through the mitochondria (Zhang and Kaufman 2006). However, other studies demonstrated that ER stress mediated apoptosis is independent of mitochondrial damage (Morishima et al. 2004). The UPR may also initiate apoptosis via the ER-bound stress specific caspase-12, which is released following ER stress, then cleaved following dimerization or cleaved by the calcium-dependent protease calpain. Cleaved caspase-12 cleaves caspase-9, which activates the executioner caspase, caspase-3 (Liu and Kaufman 2003; Rao et al. 2001; Rutkowski et al. 2006; Rutkowski and Kaufman 2004; Zhang and Kaufman 2006).

We demonstrated that Cache Valley PM$_{2.5}$ caused the upregulation and activation of caspase-12 in human bronchial epithelial (BEAS-2B) cells (see chapter 2). Because our results suggested a role of PM-induced ER stress, the present study was undertaken to
test the hypothesis that Cache Valley PM would cause ER stress and subsequent signs of the UPR in human bronchial epithelial cells.

Materials and Methods

Chemicals and reagents

BEAS-2B cells were a kind gift from Dr. Katerine Macé (Nestle Research Centre; Lausanne, Switzerland). LHC-9 cell growth media was from Invitrogen (Camarillo, CA). Antibodies to ATF-6, PERK, ATF-4 (CREB2), and phosphorylated PERK (phospho-PERK) were from Santa Cruz Biotechnology (Santa Cruz, CA). CHAPs cell extract buffer, dithiothreitol (DTT), Pathscan Multiplex Western Cocktail III containing antibodies to phospho-Stat1, phospho- SAPK/JNK, phospho-S6 ribosomal protein, phospho-heat shock protein (Hsp)-27 and Pin-1, and all other antibodies were from Cell Signaling Technology (Danvers, MA). QuickStart Bradford protein quantification assay kit was from Bio-Rad (Hercules, CA). Supersignal West Femto chemiluminescent substrate was from Pierce (Rockford, IL). Calpain activity assay kit was from BioVision (Mountain View, CA). Enzyme linked immuno-absorbance assay (ELISA) kits for C-reactive protein (CRP) and heat shock protein Hsp70 were from R&D Systems (Minneapolis, MN). Ultrapure TLR-4 grade lipopolysaccharide (LPS) from Salmonella minnesota lacking protein or DNA with TLR agonistic activity was from Alexis Biochemicals (San Diego, CA). The Limulus Amebocyte Lysate (LAL) QCL-1000 LPS detection assay was from Cambrex (East Rutherford, NJ). CellBIND cell culture plates were from Corning (Corning, NY).
PM collection and extraction and endotoxin detection

PM$_{2.5}$ collection was performed as previously described (see chapter 2) using a RAAS-1000 PM$_{10}$/PM$_{2.5}$ sampler (Anderson, Atlanta, GA). PM$_{10}$ was collected similarly using a PM$_{10}$ cyclonic separation head. The PM was collected in a single location in the Cache Valley during the wintertime when PM levels approached or exceeded the NAAQs standard, on January 3, 14, 17, 20, and 23 2002 and January 7 and 8 in 2003. The PM was extracted from the collection filter via sonication in 0.1 µm-filtered H$_2$O (sufficient immerse the filter), dried, weighed and resuspended in fresh media with sonication (see chapter 2). Endotoxin activity was detected in PM samples using the Limulus Amebocyte Lysate (LAL) QCL-1000 assay according to the manufacturer’s instructions, and measured as endotoxin (EU) units ml$^{-1}$ with the activity of the unknown sample measured against a standard curve of known values (performed in duplicate) that ranged from 0 to 1 EU/ml.

Cell culture and treatment

BEAS-2B cells from the same passage were seeded onto 6-well plates at approximately $7.2 \times 10^4$ cells/well in 2 ml LHC-9 media. Media was changed the day following seeding and every other day thereafter. Cells were grown until approximately 80% confluent and treated with either PM$_{2.5}$ or PM$_{10}$ suspensions (12.5 µg ml$^{-1}$ and 25 µg ml$^{-1}$) for 24 h. LPS (10 ng/ml$^{-1}$) was also used. Following treatment, media was removed and stored at -20°C. Cells were harvested by scraping and re-suspension into the appropriate buffer. While the PM collection dates varied, the fine and coarse PM used for cell treatment were collected on the same day to minimize any potential day-to-day compositional differences due to weather patterns, i.e. the fine and coarse PM used to
treat cells in one plate, were collected on the same day. Experimental replicates were performed using the same PM concentrations but from PM collected on a different day since an experiment often depleted the extracted PM from a filter. Cell culture supernatant was kept for ELISA analysis and stored at -20°C.

Cytotoxicity

Cytotoxicity of PM was determined by Neutral red (Hall et al. 1998; Van Vleet et al. 2002). Cells were grown to ~80% confluence, then treated with a range of concentrations of PM$_{2.5}$ and PM$_{10}$ suspensions (6.25, 12.5, 25, 50, 100 and 200 µg ml$^{-1}$) and compared to control. The uptake of the vital dye was measured by the absorbance at 540nm subtracting the absorbance at 630nm (Labsystems Multiskan MCC/340, Thermo Scientific Waltham, MA). Cytotoxicity of LPS (24 h. exposures at 10, 25, 50, 100, 200, 400, 800, and 1600 ng ml$^{-1}$) was determined in additional experiments. To confirm the functionality of the assay to detect cell death, cells were also cultured with staurosporine (1 µM) or caffeine (2.5 mM).

Western blotting

Isolation of cell lysates, Western blotting onto nitrocellulose membranes, image acquisition and archiving, and luminescent analysis were performed as described (see chapter 2) with the exception that for blots examining phosphorlyated protein, all buffers also contained 50 mM NaF. The lysate was collected, analyzed for total protein and frozen at -80°C until used. Cell lysate (10 µg of protein) was loaded onto each lane and electrophoresed for 1 h, blotted onto nitrocellulose membranes and incubated with secondary antibodies. Incubation times and antibody concentrations were in accordance
with the antibody manufacturers’ instructions. As the nitrocellulose membranes used for Western blotting were not amenable to stripping and re-probing with additional antibodies, blots were confirmed by replicate blots as well as independent experimental replicates. With the exception of the above-mentioned antibody cocktail, multiplex Western blots were not performed to minimize possible cross-reactivity.

CRP and Hsp70 determinations

After treatment, media was centrifuged (1325 xg) for 5 min and analyzed for CRP and Hsp70 content according to the manufacturer’s instructions. The Hsp70 sandwich ELISA was validated with LHC-9 media using the supplier’s spike-and-recovery protocol. All values were normalized to total protein values.

Calpain activity

Cells were lysed in 100µl extract buffer, removed by scraping, then placed on ice for 20 min. The lysate was centrifuged at 10,000 x g for 1 min and the supernatant was saved and protein concentration determined using the Bradford assay. The reactions were run (in triplicate) using 10µg protein/reaction with the exception of the active calpain positive control provided with the kit, which was added at 0.5 µl of an undetermined protein concentration per reaction. The reactions were carried out for 1 h at 37°C and fluorescence determined (VersaFluor, Bio-Rad, Hercules, CA) with 390 nm excitation and 510 nm emission filters.

Statistical analysis

Differences between treatment groups were determined with the t-test for comparison of two groups and one-way analysis of variance (ANOVA) with subsequent
multiple-comparison procedures Holm-Sidak as the first line test (Glantz 2005) for 3
or more groups. To minimize type II error due to excessive extraneous pair-wise
comparisons, the “versus control” feature of the Holm-Sidak procedure was utilized.
Nonlinear regression analysis for standard curves (ELISA, LAL) using the model
presenting the lowest PRESS statistic (Daly et al. 2005). All data were analyzed using
SigmaStat software (Systat San Jose, CA), with significance set at P=≤0.05.

Results

The ambient PM$_{2.5}$ and PM$_{10}$ concentrations ranged from 5.3 - 44.7 (mean 24.9)
µg/m$^3$ for PM$_{2.5}$ and 6-56 (mean 35.9) µg/m$^3$ for PM$_{10}$ during Jan. 2002 while those for
January 2003 ranged from 5.2–37.5 (mean 17.4) for PM$_{2.5}$ and 8-47 (mean 26.1) µg/m$^3$
for PM$_{10}$. Extraction efficiency was between 58-92% of the original collected PM$_{2.5}$ and
29-82 % of the PM$_{10}$. The amount of PM extracted into suspension ranged from 120-650
µg PM$_{2.5}$ and 70-810 µg PM$_{10}$.

Particles of both size classes were only cytotoxic at the highest concentration.
Differences in dye uptake between PM treated cells with that of control reached statistical
significance (P≤0.05) only at the highest concentration (200 µg/ml) tested (P=0.036 for
200 µg/ml PM$_{2.5}$, and <0.001 for PM$_{10}$ [Figure 3-1A]). Comparing differences between
PM$_{2.5}$ and PM$_{10}$ at the same concentration using the t-test, fine PM caused significantly
(P=≤0.001) less dye uptake than coarse but only at the 100 µg/ml concentration (Figure
2-1A). As LPS may be responsible for many of the cellular effects of some PM (National
Center for Environmental Assessment [Research Triangle Park N.C.] 2003) in areas of
intensive agricultural activity (Schulze et al. 2006), an examination of the effects of LPS
seemed appropriate. LPS was not cytotoxic at any of the concentrations tested with no significant difference (P=0.058) in dye uptake between control and any of the tested concentrations (Figure 2-1B).

Appropriate doses for *in vitro* PM studies using the bronchial epithelium has been a subject of debate (Phalen *et al.* 2006) and the same deposition studies (Balashazy *et al.* 2003) have been cited to support a wide range of exposure concentrations. A concentration of 50 µg/ml has been posited (Becker *et al.* 2005b) as possibly achievable in situations where ambient PM exceeds 50 µg/m³, which is well below 200 µg/ml where we observed cytotoxicity. Our choice of 25 and 12.5 µg/ml (10.7 and 5.35 µg/cm² in the 4.67 cm² well of a six-well plate for comparison with other studies where mass per surface area is used) is reasonable, and also afforded sufficient PM for experiments using a single filter. Preliminary testing revealed that these concentrations elicited various cellular responses (other than cytotoxicity). As cytotoxicity was not a good indicator of an appropriate LPS concentration, 10 ng/ml (4.3 ng/cm² in these plates) was selected, as it has been used in studies with BEAS-2B cells (Morris *et al.* 2006) and in other PM studies (Becker *et al.* 2002; Becker *et al.* 2005c; Chmura *et al.* 2008; Morris *et al.* 2006). Therefore, we were confident that PM and LPS concentrations used here did not induce cytotoxicity that might have confounded the detection of ER and UPR-related molecular events.

Lipopolysaccharide is known to induce the UPR, and has been postulated to be a major contributor to the inflammogenic properties of PM (Becker *et al.* 2005a; Skalet *et al.* 2005) Therefore determining the LPS content in our PM samples was of importance. The standard curve for LPS was best fit by a one-site saturation model \( y = B_{\text{max}} \frac{x}{(K_d + x)} \).
(B\textsubscript{max} = 3.88, K\textsubscript{d} = 0.62 PRESS = 0.0988) and ranged from 0.125 to 1 EU/ml. Ten and one ng/ml LPS exhibited activity greater than that of the upper quantitative limit of the assay (1 EU/ml). Linear extrapolation gave values over 17 EU/ml, but this likely underestimated the activity as maximum absorbance was approached. Given that the LPS activity would not be accurately quantified above this concentration, the 1 EU ml\(^{-1}\) activity was used for significance testing and was significantly higher (P = <0.01) than the LAL activity exhibited by both size classes of our PM samples (Table 3-1). The absorbance values for the low PM\(_{2.5}\) were within the 95% confidence interval of the lowest (0.125 EU/ml) point of the LAL standard curve, indicating that the value was near the limit of the quantitative range of the assay. Absorbance values obtained from 12.5 μg/ml of either PM\(_{2.5}\) (P = 0.90) or PM\(_{10}\) (P = 0.12) were significantly different from the low point of the standard curve as determined by t-test. Differences in LPS activity of the PM samples were statistically different dependent upon PM concentration rather than upon particle diameter (Table 3-1).

PM treatment resulted in increases in post-translational modifications related to ER stress. For example, phosphorylated (p)-PERK was significantly elevated in cells treated with PM\(_{2.5}\) and PM\(_{10}\) compared to that in control cells (P < 0.001). When p-PERK was normalized to a blot of unmodified PERK (pPERK/PERK) from the same cell lysates, all treatments were significantly higher than control. pPERK/PERK phosphorylation increased with concentration, and PM\(_{10}\) induced a significantly greater response than PM\(_{2.5}\) (P = <0.001). PM treatment had a significantly greater effect on p-PERK/PERK than that seen in cells treated with LPS. Likewise, expression of the other early UPR cellular sensor, the 50 kDa cleavage product of ATF-6 was increased in PM-
treated cells (P=<0.001) with concentration. ATF-6 cleavage was greater in cultures treated with the high PM concentration than that seen in LPS-treated cultures (P=<0.001).

A cocktail containing primary antibodies to phosphorylated Stat1 (Tyr\textsuperscript{701}), phosphorylated stress activated protein kinase (SAPK) and JNK (Thr\textsuperscript{183}/Tyr\textsuperscript{185}), phosphorylated S6 ribosomal protein (Ser\textsuperscript{235/236}), phosphorylated Hsp27 (Ser\textsuperscript{82}), and Pin-1 (as a loading control) was used in Western immunoblots. The different migration sizes of the proteins allowed for simultaneous detection of the five bands. Band intensities were normalized to Pin-1 levels (the loading control). Phosphorylated Stat1 was not detectable, and phosphorylated SAPK/JNK was detectable only after longer exposures which resulted in image saturation and high background, but there were no differences between lysates from treated and control cells (data not shown). Phosphorylation of S6-ribosomal protein was significantly higher than control (P=<0.01). The PM\textsubscript{2.5} treated cells showed the greatest response. Another marker of the UPR, Hsp27 phosphorylation was also significantly increased from control (P=<0.001). The responses for the fine treatments were concentration dependent while those of the coarse treatments were not. The lysates from the high PM\textsubscript{2.5} and both the PM\textsubscript{10} treated cells exhibited a greater response than the LPS treated cells (Fig. 3-2B).

Both fine and course PM elicited significant up-regulation of early ER stress chaperones, such as BiP the “master” regulator of the UPR (Zhang and Kaufman 2006) (Fig. 3-3). BiP was observed as a doublet indicative of covalent modification (Cell Signaling technical support, personal communication); both bands were included in the analysis. The increases in BiP were greater in cells exposed to the higher concentration of either size fraction of PM. BiP protein levels in the PM treated cells were below the
levels in the LPS treated cells. Cells exposed to both size classes of PM also exhibited significant up-regulation of the UPR-related chaperone Hsp70 compared to control cells. Increases in Hsp70 protein increased with concentration only in the PM$_{10}$-treated cultures (Fig. 3-3). LPS treatment elicited a greater response than PM of both size class and concentration. By contrast, levels of the UPR related chaperone Hsp90 were significantly up-regulated and increased with concentration with both PM size fractions. The response was consistently greater in PM treated cells than that seen in LPS treated cells (Fig. 3-3).

Likewise PM caused up-regulation of ATF-4 protein, which in the case of PM$_{2.5}$, increased with concentration. LPS caused a slight increase in ATF-4 compared to control. The ATF-4 protein levels were slightly elevated from the untreated control in the LPS and low PM$_{10}$ treated lysates, and the lysates from the high concentration from the coarse PM had significantly lower levels of ATF-4 than control. Levels of ATF-4 in the coarse PM treated cell lysates were significantly lower than those that received the LPS treatment (Fig. 3-3).

We were then interested whether the observed up-regulation of Hsp70 in PM-treated cells would be reflected in the appearance of this pro-inflammatory chaperone in the culture medium from PM-treated cells. As can be seen in Figure 3-4, PM exposure caused elevated levels of extracellular Hsp70 in the culture medium compared to control, but this effect was only statistically significant with the low concentrations of either PM fraction and with LPS. This pattern was inversely related to PM concentration response and differed from the alterations in intracellular Hsp70 (Fig. 3-4A). Given the association of ER stress with CRP release (Zhang et al. 2006), the effects of PM on
extracellular CRP release were examined. Cells exposed to all PM treatments resulted in increases from control. All treatments were significant from untreated control and none were significantly different from the LPS treatment or from each other.

Our previous observation that Cache Valley PM induced caspase-12 up-regulation and activation (see chapter 2) indicated that the cytoplasmic proteases calpains might be involved (Tan et al. 2006). Both calpains 1 and 2 were significantly up-regulated in BEAS-2B cell cultures exposed to both size classes of PM compared to control (Fig. 3-5A). With calpain-1 the lysates from the low concentration of the coarse fraction treated cells gave the greatest response which also surpassed the LPS treated lysate (P=<0.001). With respect to calpain-2 there was an increase with PM concentration with the high concentration of the coarse fraction giving the greatest response, but was significantly less than that of the LPS treated cells. Calpain activation is not dependent upon calpain protein levels, but upon the influx of calcium ions into the cytoplasm or by alterations in the sensitivity of calpain to endogenous calcium levels (Goll et al. 2003). Calpain activity were significantly (P=<0.001) increased with PM concentration (Fig. 3-5B) with PM\textsubscript{10} having the greater effect. Lysates from the high concentration PM\textsubscript{10} exhibited the highest increases in calpain activity, but this was not significantly different from LPS.

Calpain activation is generally pro-apoptotic (Goll et al. 2003) but since PM concentrations used in this study resulted in minimal cytotoxicity, various apoptotic markers were then examined. There was an increase of the 37 kDa. cleavage product of caspase-9 in PM treated cells compared to control, with (the effect of PM\textsubscript{2.5} was greater than PM\textsubscript{10}), but the 35 kDa. cleavage product that indicates full activation was not
detected in any treatments (Fig. 3-6). LPS treated cells showed reduced levels of caspase-9 compared to control.

PM exposure elicited significant increases of inactive full-length caspase-3 in all treatments with the exception of the low PM$_{2.5}$. There was no detectable PM-related increase in the active 17 kDa fragment of caspase-3. Compared to control, PM-treated cells also showed increases in the inactive, full length Bid, though not of the active fragment (Fig. 3-6). Interestingly, there were no detectable increases in phosphorylation of protein kinase-C delta (PKC-δ) in PM-treated cells, which might have been expected to increase along with pro-caspase-3 (Cataldi et al. 2002) (Fig. 3-6).

Discussion

Our data strongly suggest that ambient PM collected during atmospheric inversions in Cache Valley UT induce indicators of ER stress and the UPR in human bronchial epithelial cells. Similarly, diesel exhaust particles have been shown to induce indicators of UPR and ER stress, such as HSP 70, ATF-4, IL-6 IL-8 in BEAS-2B cells (Jung et al. 2007). Hallmarks of ER stress include posttranslational modifications such as ATF-6 cleavage and phosphorylation of PERK, S-6 ribosomal protein, and Hsp27 as well as upregulation of UPR related proteins BiP, Hsp70, Hsp90 and ATF-4. In the absence of ER stress, PERK is constitutively active; phospho-PERK increases in response to ER stress (Ranganathan et al. 2006; Rutkowski et al. 2006). The mechanisms of PERK activation are unknown, but may involve free-radical formation (Hayashi et al. 2003). However, the generation of radicals would have lead to phosphorylation of JNK (Shen and Liu 2006), which was not observed in our study.
PERK is activated by interferon-γ (Lees and Cross 2007) indicating the possibility
PERK phosphorylation may occur through a receptor-mediated mechanism. That LPS
treatment caused an elevation in unmodified PERK was unexpected.

The induction of phosphorylated S6 ribosomal protein, indicated P70S6-kinase
activation (Peterson and Schreiber 1998; Sun et al. 2007). Phosphorylation of Hsp27,
which was induced by PM exposure, is mediated by p38 MAPK activation in ER stress
(Ranganathan et al. 2006). Our results support the observation that ER stress induces
Hsp27 phosphorylation independently of PKC-δ (Ito et al. 2005). P-Hsp27 is modulated
by Stat-3, a transcription-factor we have shown to be activated by PM in BEAS-2B
cells(see chapter 2). Hsp27 activation enhances cell survival by inhibiting apoptosis and
mitochondrial injury and assisting in Akt activation (Havasi et al. 2008; Wu et al. 2007;
Zhang et al. 2007).

The observed PM-induced up-regulation of Hsp90 may be related to Stat-3-
activation as well as ATF-4 transcription (Chatterjee et al. 2006; Chapter 2). Hsp90
interacts with Akt to suppress apoptosis, and is a target of chemotherapeutic agents (Arya
et al. 2007; Bagatell and Whitesell 2004). Hsp90 also inhibits JNK activation and
caspase-9 association with the apoptosome (Arya et al. 2007).

Further evidence of UPR activation included increases in the ATF6 active
cleavage product, and increases in cellular chaperones Hsp70, and BiP. BiP is up-
regulated by enhanced protein stability, rather than by transcription (Rutkowski et al.
2006; Rutkowski and Kaufman 2004) which explains the subtle changes seen here. Up-
regulation of Hsp70, which is indicative of cellular stress and has been observed to be
induced by PM from other sources (Becker et al. 2005a; Ramage and Guy 2004). Hsp70
inhibits procaspase-3 cleavage to its active form, and like Hsp90, inhibits apoptosome formation and JNK activation (Arya et al. 2007). Up-regulation of Hsp90, phosphorylation of Hsp27, and increases in Hsp70 may explain why PM exposure did not induce apoptosis despite ER stress induction and calpain activation, two events normally considered to be pro-apoptotic. In addition to its chaperone ability, Hsp70 also possesses an extracellular immune-response signaling capacity similar to LPS (Asea 2005).

The role of PM in the inflammatory response is well established (Dagher et al. 2005; Sarnat et al. 2006; Seagrave et al. 2005) and has been postulated to be partially responsible for early-mortality (Pope et al. 2004b; van Eeden and Hogg 2002). While most studies have focused on the effect of PM on pro-inflammatory cytokine expression, the potential of Hsp70 mediated inflammatory signaling also seems reasonable. Hsp70 efflux occurs by active transport or by necrosis (Asea 2005; Asea et al. 2002; Vabulas et al. 2002); given the modest cytotoxicity observed, it is likely that Hsp70 is released by the former mechanism. Although supernatants from all PM-treated cell cultures had elevated extracellular Hsp70 compared to control, extracellular Hsp70 levels decreased from low to high PM concentrations (albeit only significantly with fine PM). That extracellular Hsp70 levels did not correspond with the observed increases in intracellular Hsp70 may be explained by the hypothesis that the energy intensive nature of protein folding (Zhang and Kaufman 2006) consumed intracellular ATP that would be needed in exporting Hsp70 from the cell. Another possibility is the increased need for the chaperone and apoptosis suppression activity of Hsp70 within the cell.

While a marker of cardiovascular insult, rather than a cause thereof (Hirschfield et al. 2005; Tennent et al. 2008; Timpson et al. 2005), CRP has also been observed in
PM$_{10}$-exposed A549 human lung cells (Ramage and Guy 2004). CRP is related to the UPR through the protein CREBH, although CREBH may not be expressed in BEAS-2B cells (Zhang et al. 2006). C-reactive protein release following PM insult is likely mediated by pro-inflammatory IL-6 (Anty et al. 2006) and may exhibit autocrine signaling (Veranth et al. 2007; see chapter 2). It is also possible that CRP release may simply be an indicator of in vitro stress induced by PM.

Unabated ER stress can lead to apoptosis, often through the activation of caspase-12 (Rutkowski and Kaufman 2004), which is believed to be activated by either dimerization following ER stress or by the cytoplasmic protease calpain (Rutkowski and Kaufman 2004). PM modulation of calcium homeostasis is very well established (Agopyan et al. 2003; Brown et al. 2007; Veronesi et al. 2003) and calcium flux can occur though mechanisms such as TLR activation (Adamo et al. 2004), cytokine signaling (Sporri et al. 1999), or ER stress (Zhang and Kaufman 2006). However the need for cytoplasmic calcium ions for calpain activation can be reduced by phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) (Goll et al. 2003) which inhibit apoptosis (Mejillano et al. 2001) and activate Akt (Ma et al. 2008). The observed calpain activation by PM seen here is consistent with both mechanisms.

This study appears the first to demonstrate that PM treatment can result in increased calpain activation which is the likely mechanism behind previously observed caspase-12 activation (see chapter 2). Of interest were the findings that PM and LPS treatment up-regulated of the large subunits of calpains-1 and 2, which indicated their ability to escape PERK-mediated translational blockage. Calpain upregulation is
associated with neurodegeneration (Guyton et al. 2005) and in the proliferation of small-cell lung-carcinoma (Grozio et al. 2007).

Despite the generally pro-apoptotic activation of calpain, the induction of apoptosis was not observed here. This is consistent with observations that the UPR can be an adaptive response to stress enabling continued survival or the inflammatory response (Rutkowski et al. 2006; Zhang et al. 2006). The relative lack of cytotoxicity of PM in BEAS-2B cells is in agreement with studies from our own (see chapter 2) and other laboratories (Monn and Becker 1999; Pozzi et al. 2003). Cytotoxicity was only observed at concentrations (200 µg/ml) well above a hypothetical potential human high exposure of 50 µg/ml (Becker et al. 2005b). The lack of LPS cytotoxicity was not surprising given that BEAS-2B cells are resistant to LPS compared to other cell types such as A-549 and phagocytic cells in cytotoxicity, and cytokine release, due to internal, rather than surface, TLR-4 receptors (Dijkmans et al. 1990; Guillot et al. 2004; Schulz et al. 2002). That PM was generally more potent in inducing ER stress than LPS, despite the fact that LPS treatment elicited a greater LAL activity, is in agreement with other observations that LPS is not primarily responsible for cytokine release by PM (Veranth et al. 2004). It also indicates that LPS is not likely responsible for the molecular events caused by PM, and that the receptor TLR-4 is not involved in subsequent cell signaling. There is a strong likelihood, however, that LPS may have a synergistic or additive effect with PM, as has been observed with PM surrogates (Arimoto et al. 2005; Finnerty et al. 2007).

There were few consistent differences between the two size classes of particulates with PM$_{10}$ eliciting greater responses in some cases and PM$_{2.5}$ in others. Any differences
in biological activity between the course and fine PM may have been due to possible
differing chemical composition of the particles or larger bacterial components that are
size excluded and thereby reduced in the fine fraction of the ambient PM.

Further studies to elucidate the mechanism of how PM induces ER stress are needed. We
previously showed that fine PM (see chapter 2) up-regulated to a greater extent than
down-regulated a broad spectrum of genes in BEAS-2B cells, suggesting that PM may
cause ER stress by inducing mRNA transcription. It is also possible that PM may affect
protein folding through a direct particle-protein interaction, as was recently shown to be
the case with nanoparticles (Fei and Perrett 2009). ER stress and the UPR can trigger cell
death in illnesses that are associated with early mortality and PM exposure; therefore ER
stress is a plausible mechanism of PM-mediated inflammation and adverse health effects.

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2- and 10-microm particles induces responses leading to apoptosis in human airway

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## Table 3-1. Endotoxin activity of the fine and coarse PM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>EU/ml</th>
<th>EU/µg PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM$_{2.5}$</td>
<td>Low</td>
<td>0.13±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.48</td>
</tr>
<tr>
<td>PM$_{2.5}$</td>
<td>High</td>
<td>0.28±0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.03</td>
</tr>
<tr>
<td>PM$_{10}$</td>
<td>Low</td>
<td>0.15±0.00&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.93</td>
</tr>
<tr>
<td>PM$_{10}$</td>
<td>High</td>
<td>0.28±0.01&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.08</td>
</tr>
<tr>
<td>LPS</td>
<td>10 ng/ml</td>
<td>&gt;1.0</td>
<td>N/A</td>
</tr>
<tr>
<td>LPS</td>
<td>1 ng/ml</td>
<td>&gt;1.0</td>
<td>N/A</td>
</tr>
<tr>
<td>Media</td>
<td>N/A</td>
<td>&lt;0.125</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Mean ± SD. Low=12.5 µg/ml, High=25 µg/ml, each concentration measured at n=3.  
<sup>a</sup>Significant from LPS, <sup>b</sup>Significant from differing concentration of the same PM diameter by one way ANOVA.
Figure 3-1. A) Cytotoxicity of PM$_{2.5}$ and PM$_{10}$ in BEAS-2B cells following 24 h exposures. B) Cytotoxicity of PM component LPS for comparison. Data are mean ± SE representative of 3 independent experiments. (n=8 for all treated cells, except 200 µg ml$^{-1}$ n=4; n=16 for control). aSignificant from control bsignificant from the same concentration of different PM diameter (P=<0.05).
Figure 3-2. Immunoblots (A) and luminescent analysis (B) of post-translational modifications related to the UPR in BEAS-2B cells after 24 h exposures to 12.5 µg ml$^{-1}$ (Low) and 25 µg ml$^{-1}$ (High) Cache Valley PM$_{2.5}$ and PM$_{10}$, or 10 ng ml$^{-1}$ of LPS. The ratio of phosphorylated PERK (Thr$^{980}$; p-PERK) to unmodified PERK was significantly greater than that in untreated cells. PM elicited significant increases in expression of the active 50 kDa cleavage product of ATF6 compared to control. Phospho–S6 ribosomal protein levels (normalized to the loading control Pin-1) were significantly increased from control. PM also increased phosphorylation of Hsp27 over untreated cells. Results are representative of three independent experiments. Data are mean ± SE. $^a$Significant from control, $^b$significant from differing concentration of the same PM diameter, $^c$significant from same concentration of differing PM diameter, $^d$significant from LPS as determined by one way ANOVA and Holm-Sidak multiple comparison analysis (P=<0.05).
Figure 3-3. Immunoblots (A) and luminescent analysis (B) of the effects of 24 h exposures of 12.5 (Low) and 25 (High) μg ml⁻¹ PM₂.₅, PM₁₀, or 10 ng ml⁻¹ of LPS on BEAS-2B cells on the UPR related chaperone proteins BiP, Hsp70, Hsp90 and ATF-4. Alterations in the endogenous levels of the central UPR protein BiP were detected at levels greater than control. Levels of Hsp70, Hsp90, and the ER stress inducible ATF4 were also increased. Data are mean ± SE as a percent of control and, representative of three independent experiments. aSignificant from control, bsignificant from other concentration of same PM diameter, csignificant from same concentration of other PM diameter, dsignificant from LPS as determined by one way ANOVA and Holm-Sidak multiple comparison analysis (P=<0.05).
Figure 3-4. The effects of PM treatment on the export of proteins related to inflammation and ER stress. A) ELISA of extracellular Hsp70 in the media from BEAS-2B cells treated with 12.5 (Low) and 25 (High) µg ml\(^{-1}\) PM\(_{2.5}\), PM\(_{10}\), or 10 ng ml\(^{-1}\) of LPS for 24 h. Results are combined from n=2 independent experiments with n=8 replicates in each treatment group. B) ELISA demonstrating that PM induced the release CRP into the media following 24 h exposures. Data are mean ± SE from the combined results of two independent experiments with seven replicates. 

- \(a\) significant from control, 
- \(b\) significant from differing concentration of the same PM diameter, 
- \(c\) significant from same concentration of differing PM diameter, 
- \(d\) significant from LPS as determined by one way ANOVA and Holm-Sidak multiple comparison analysis \((P<0.05)\).
Figure 3-5. The effect of PM on calpain expression and activity. A) Immunoblots and analysis of calpain-1 and calpain-2 large subunit levels following 24 h exposures to 12.5 (Low) and 25 (High) µg ml⁻¹ PM₂.₅, PM₁₀, or 10 ng ml⁻¹ of LPS for 24 h. Data are mean±SE as a percent of control, and representative of three independent experiments. B) Calpain activity in cell lysates following 24 h exposures. The positive control is protein lysate containing active calpain provided by the kit manufacturer. Data are mean relative fluorescent units (RFU) ± SE representative of three independent experiments. aSignificant from control, bSignificant from differing concentration of the same PM diameter, cSignificant from same concentration of differing PM diameter, dSignificant from LPS as determined by one way ANOVA and Holm-Sidak multiple comparison analysis (P=<0.05).
Figure 3-6. Immunoblots and luminescent analysis examining apoptotic markers from cell lysates in BEAS-2B cells exposed 24 h to 12.5 (Low) and 25 (High) µg ml⁻¹ concentrations of Cache Valley PM₂.₅ and PM₁₀. There were increases in protein levels but not in full activation of the caspases nor Bid. PM treatment did not result in increases in the phosphorylation state of protein kinase C-δ. Blots are representative three independent experiments and analysis data are mean ± SE as a percent of control. aSignificant from control, bsignificant from differing concentration of the same PM diameter, csignificant from same concentration of differing PM diameter, dsignificant from LPS as determined by one way ANOVA and Holm-Sidak multiple comparison analysis (P=<0.05).
CHAPTER 4

URBAN PARTICULATE MATTER ACTIVATES AKT
IN HUMAN LUNG CELLS

Abstract

The normally picturesque Cache Valley in northern Utah is often reported to have the worst particulate (PM) air pollution in the United States. Numerous epidemiological studies conducted elsewhere have associated PM exposure to a variety of cardiovascular diseases and early mortality. We have previously shown that Cache Valley PM (CVPM) is pro-inflammatory, through a variety of mechanisms involving the release of inflammatory cytokines, Unfolded Protein Response, ER stress, and C-reactive protein (CRP). This study was undertaken to determine whether Cache Valley PM (CVPM) would activate Akt, an upstream mechanism common to these events. Human lung (BEAS-2B) cells were treated with either fine (PM_{2.5}) or coarse (PM_{10}) particles (12.5 and 25 µg/ml) for periods up to 24 h. PM-exposed cells exhibited Akt activation as evidenced by phosphorylation at Thr^{308} and Ser^{473}. Events downstream of Akt activation such as NF-κB activation were observed at 1 and 24 h, but IκB phosphorylation occurred only at 24 h, indicating that mechanisms of PM mediated NF-κB activation are time-dependent. Akt and NF-κB related inflammatory cytokines IL-1α, and IL-6 and the chemokine IL-8 were upregulated in treated cells at 6 and 24 h. The calpain inhibitor

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1Previously published Portions of this work were presented at the Society of Toxicology Annual Conference, Baltimore, MD, March 2009. Coauthored by Todd L. Watterson, Brett Hamilton, Randy Martin, and Roger A. Coulombe JR (2011) Arch Toxicol. 2012 Jan, 86 (1) 121-35. Reprinted with kind permission of Springer Science and Business Media.
leupeptin limited Akt phosphorylation to Ser\(^{473}\) and reduced release of IL-1\(\alpha\), IL-6 and IL-8, indicating that calpain or similar protease(s) are involved in PM-induced activation of Akt and subsequent release of inflammatory cytokines. Our data indicate that PM activates Akt, which may play a role in the pro-inflammatory response to PM exposure.

Introduction

The normally picturesque Cache Valley in northern Utah frequently experiences the worst particulate air pollution (PM) in the United States, a situation exacerbated by winter atmospheric inversions that trap and concentrate pollutants (Edgerton et al. 2006; Lurmann et al. 2006; Malek et al. 2006; USA-Today 2005). Similar to PM such as that from the San Joaquin Valley of California, the single largest chemical component of Cache Valley PM (CVPM) is ammonium nitrate (NH\(_4\)NO\(_3\)), a secondary pollutant formed by atmospheric reactions between nitrogen oxides from vehicles, and ammonia gas from the excreta of dairy cows and other animals (Edgerton et al. 2006; Lurmann et al. 2006; Malek et al. 2006; Mangelson et al. 1997; National Center for Environmental Assessment [Research Triangle Park N.C.] 1996).

In other locations, exposure to PM is associated with early all-cause mortality, as well as to numerous cardiovascular and cardiopulmonary diseases, including ischemic heart disease, cardiac arrest, hypertensive disease, cerebrovascular disease, pneumonia, influenza, in addition to diabetes, neurodegeneration, and cancer although no such studies have been done in the Cache Valley (Peters et al. 2006; Pope et al. 2002; Pope et al. 2004a). Particulates are chemically diverse and elicit inflammatory and pro-oxidative
responses in a variety of cell types through differing mechanisms (Tao et al. 2003). U.S. Federal regulations have been implemented following epidemiological studies regulating 2 PM size fractions of which PM$_{2.5}$ appeared to have greater association with adverse health effects than PM$_{10}$ (Delfino et al. 2005; Schwartz et al. 1996), although some other studies indicate a significant health threat posed by the larger PM$_{10}$ (Becker et al. 2005c; Becker and Soukup 2003; Choi et al. 2004; Jalava et al. 2007; Monn and Becker 1999; Osornio-Vargas et al. 2003; Pozzi et al. 2003; Soukup and Becker 2001).

While little is known about the mechanisms by which PM may induce harm (Lippmann et al. 2003; Mossman et al. 2007; National Center for Environmental Assessment [Research Triangle Park N.C.] 2003; NHEERL, 2010), the inflammatory response has received the most attention (Delfino et al. 2005; Donaldson et al. 2001; Pope et al. 2004b; Schins et al. 2004). A potential mechanism for the pro-inflammatory action of PM is through activation of nuclear factor-kappa B (NF-$\kappa$B), a transcription factor stimulated in response to many pro-inflammatory agents (Dagher et al. 2007; Jimenez et al. 2000) which is activated by Akt (formerly known as protein kinase B). Another important component of inflammation is apoptosis suppression in cytokine-releasing cells, a process that also involves Akt (Abraham 2005).

Akt is a cytosolic serine/threonine kinase (Rane et al. 2003) required for cancer cell growth, promoting the survival of cancer cells exposed to chemotherapy and radiation-induced apoptosis (Brognard et al. 2001). Activated Akt is detectible in 90% of non small cell lung cancer cell lines and 100% of those derived from smokers (West et al. 2003). Akt contributes to lung inflammation and injury (Abraham 2003), promotes chemotaxis to fight bacterial infection (Abraham 2005), and is a pro-survival adaptation
mechanism following stress (Hu et al. 2004). Akt contributes to inflammation activating the pro-inflammatory transcription factor NF-κB playing a central role in survival by altering cellular metabolism and inhibiting apoptosis (Amaravadi and Thompson 2005; Chen 2005; Misra et al. 2006; Newcomb et al. 2008; Venkatachalam et al. 2008). Akt regulation is primarily through post-translational modification (Amaravadi and Thompson 2005). Prototypical Akt activation occurs though receptor mediated activation of phosphatidylinositol 3-kinase (PI3K). Activated PI3K produces 3-phosphoinositides (PIP₃), which bind to and induce a conformational change in Akt as well its translocation from the cytoplasm to the plasma membrane where it is phosphorylated. Akt is fully activated when phosphorylated at Ser⁴⁷³ and Thr³⁰⁸, a process inhibited by tumor suppressor phosphatase and tensin homolog (PTEN) which dephosphorylates PIP₃ (Cantley and Neel 1999). PTEN also limits the action of MDM2 allowing up-regulation of p53 activity (Brognard et al. 2001; Hajduch et al. 2001).

PTEN is inactivated by phosphorylation of its C-terminal tail (Vazquez et al. 2000), an event associated with a number of human tumors (Meek and Knippschild 2003).

We have previously demonstrated that ambient CVPM activates of Stat3, P70S6 kinase, calpain, and Hsp27, and concomitant increases of Hsp90 protein in human airway epithelial (BEAS-2B) cells (see chapter 2). Because these events are associated with Akt (Chatterjee et al. 2006; Peterson and Schreiber 1998; Rane et al. 2003; Tan et al. 2006; chapters 2-3), we hypothesized that CVPM would activate Akt and related proteins leading to the release of Akt-associated inflammatory cytokines.
Materials and Methods

Chemicals and reagents

BEAS-2B cells were a kind gift from Dr. Katerine Macé (Nestle Research Centre; Lausanne, Switzerland). Recombinant IL-1 was from R&D Systems (Minneapolis, MN). LHC-9 cell growth media was from Invitrogen (Camarillo, CA). IRAK-1 primary antibody was from Affinity Bioreagents (Golden, CO). All remaining antibodies, CHAPs cell extract buffer, and biotinylated molecular weight ladders were from Cell Signaling Technology (Danvers, MA). The Akt antibodies were not isoform specific but were able to detect total Akt or phosphorylated total Akt. Cytokine antibody array was from Quansys Biosciences (Logan, UT). Lipopolysachharide (LPS) from Salmonella minnesota was from Alexis Biochemicals (San Diego, CA). Restore Western Blot Stripping buffer was from Pierce (Rockford, IL). CellBIND 6-well cell culture plates were from Corning (Corning, NY). Nitrocellulose membranes were from Bio-Rad (Hercules, CA).

PM collection and extraction, Cell culture, and exposure

PM sampling, extraction, endotoxin detection, and culture of BEAS-2B cells was previously described (chapters 2 & 3). For time course studies, cells from the same passage were seeded onto separate plates and cell numbers adjusted accordingly (approximately 3.6x10^5 cells/well). Cells were grown until ~ 80% confluent then treated with fine (PM_{2.5}) and coarse (PM_{10}) at “low” (12.5) or “high” (25 µg/ml) concentrations. These concentrations are below a hypothetical “high” exposure of 50 µg/ml (Becker et al. 2005b) and represent potential “real world” exposures. In all experiments, LPS (10
ng/ml) was also used for comparison. None of the concentrations of PM or LPS used in this study are cytotoxic to BEAS-2B cells (chapter 3). Because Akt activation can occur rapidly (West et al. 2003), cells were harvested at 1 h and 24 h time points with cytokine release examined at 1, 6, and 24 h.

Western blotting and ELISA

Methods for isolation of cell lysates, western blotting, and luminescent analysis have been previously described in detail (Van Vleet et al. 2006; chapter 2) with the exception that PVDF membranes were used in this study rather than nitrocellulose. As PVDF allows for stripping and re-probing with additional primary antibodies, where appropriate, PVDF membranes were stripped for 15 min at room temperature. Membranes were re-incubated in chemiluminescent substrate for 5 min and reexamined to ensure proper stripping. Membranes were then re-probed with primary antibodies, and images were captured using an imaging workstation (UV Products, Upland, CA), and analysis was performed using the histogram function in Adobe PhotoShop CS (San Jose, CA) (Woznicova and Votava, 2001). The software provided mean pixel intensity, standard deviations, and total number of pixels sufficient for one-way analysis of variance by Holm-Sidak with significance set at p<0.05. Mean luminescent values of the selected bands obtained from Adobe PhotoShop (San José, CA) were normalized to control and to bands of unmodified protein or to β-actin on the same membrane. Gels were loaded with 10 µg protein/well. Media from treatments was saved for ELISA, which was performed on multiplex chemiluminescent ELISA plates (Quansys Biosciences, Logan, UT) according to the manufacturers’ instructions as previously described (Barnard et al. 2006; Gowen et al. 2006; Yuan et al. 2007). Curve fitting and
analysis was performed using SigmaPlot with the curve presenting the lowest PRESS statistic judged to have the best fit for interpolation (Daly et al. 2005).

Statistical analysis

One-way analysis of variance (ANOVA) was performed to examine differences between 3 or more groups, with post hoc. Holm-Sidak multiple comparisons analysis (Glantz 2005). Where type II error was suspected from multiple comparisons the “versus control” function of the Holm-Sidak analysis was utilized. Comparisons between two groups were performed using the t-test. SigmaStat software (SYSTAT, San José, CA) was used for all testing. P=<0.05 was judged to be significant.

Results

Akt requires phosphorylation at Thr^{308} and Ser^{473} for full activation, which was visualized on immunoblots. Akt was constitutively active (Fig. 4-1A) and cells exposed to PM had significant increases in Akt phosphorylation at both Ser^{473} and Thr^{308}, although phosphorylation at Thr^{308} appeared to be affected more than Ser^{473} (Fig. 4-1). After 1 h, only low concentrations of both PM size classes provoked significant Akt phosphorylation (P=<0.001 for both Thr^{308} and Ser^{473}) compared to control (Fig. 4-1A). However, at 24 h, Akt phosphorylation at both Thr^{308} and Ser^{473} was significantly (P<0.001) elevated in lysates from all PM treatments compared to control and to LPS-treated cells (Fig. 4-1B).

Further evidence of Akt activation was determined by observing the effects of PM treatment on two upstream proteins responsible for mediating Akt phosphorylation; phosphoinositide-dependent protein kinase 1 (PDK-1) which phosphorylates Akt at
Thr<sup>308</sup> (Williams et al. 2000) while in the process is phosphorylated itself at Ser<sup>241</sup> (Casamayor et al. 1999), and PTEN, a negative effector of Akt phosphorylation. Other proteins affected by activated Akt; glycogen synthase kinase (GSK) which is inactivated by Akt via phosphorylation at Ser<sup>9</sup>, and p53 whose levels are decreased by activated Akt (Limesand et al. 2006) were also examined. After either 1 (Fig. 4-2) or 24 h (Fig. 4-3) exposures to fine and coarse PM there was heightened PDK phosphorylation compared to control and LPS (P=<0.001), with the greatest responses at 1 h (Fig. 4-2) seen in cells exposed to low PM<sub>2.5</sub> and nearly equivalent responses with the fine PM after 24 (Fig. 4-3, P=0.242). At 1 h, the signal for p-PTEN was detectable but too faint to reliably analyze (Fig. 4-2), but significant (P=<0.001) increases in this protein were observed after 24 h exposures compared to control (Fig. 4-3). The greatest response for p-PTEN at 24 h was with the high concentration of coarse PM and LPS, which were nearly identical (P=0.5; Fig. 4-3).

Appearance of the inactivated p-GSK was increased in PM<sub>2.5</sub>, but decreased in cells exposed to PM<sub>10</sub> relative to control (P=<0.001) following 1 h (Fig 4-2). After 24 h treatments, p-GSK declined to levels below that of control (Fig 4-3).

Western blots of p53 showed PM-related increases in p53 from cell lysates exposed to both size fractions of PM compared to control after 1 h exposure (P=<0.001) (Fig 4-2). Low PM<sub>2.5</sub> caused the greatest increase in p53 which was significantly greater than the high PM<sub>2.5</sub> and both concentrations of PM<sub>10</sub> (P=<0.001). There was no significant difference between the two PM<sub>10</sub> concentrations (P=0.405) or from the high PM<sub>2.5</sub> to either low or high PM<sub>10</sub> (P=0.455 and 0.169, respectively). At 1 h the signal for the second of the doublet bands often seen by this particular antibody in lysates from
BEAS-2B cells (Van Vleet et al. 2006) was visible but not measurable as shown in Fig. 2 A. Following 24 h, p53, was observed as doublet bands with no significant alterations in treatment mediated response (P=0.97, luminescent analysis not shown Fig 4-3-B). The signal responses (212-238 luminescent pixels) were below that of saturation (246 luminescent pixels) indicating that the equivalent p53 response was not due to image overexposure. The lower band in the doublet, likely indicating a different phosphorylation state, (Van Vleet et al. 2006) was also examined with no significant differences between groups (P=0.067, luminescent analysis not shown). Akt mediated phosphorylation of PDK endothelial nitric oxide synthase (eNOS) on the Ser^{1177} site was examined but not detected following 1 and 24 h exposures (data not shown).

Given the documented ability of Akt to activate NF-κB (Misra et al. 2006), alterations in NFκ-B were examined as a marker of Akt activation. NF-κB is classically activated by phosphorylation and subsequent degradation of the inhibitory protein IκB, although degradation can also be mediated through calpain (Pianetti et al. 2001). Alternative NF-κB activity is through the constitutive processing of p105 to p50 and the inducible processing of p100 to give p52 (Hayden and Ghosh 2004). Therefore, both pathways of NF-κB activation were explored in PM-exposed cells.

Expression of constitutive IκB was clearly evident in all PM-treated cells after either 1 (Fig. 4-4) or 24 h (Fig. 4-5) exposures. 1-h exposures to PM did not result in the appearance of p-IκB, a measure of classical NF-κB activity (Fig. 4-4). Levels of inactive NF-κB p100 were slightly increased from control (P=0.018 for the high PM_{2.5}, P=<0.001, for the remainder), with PM_{10}-exposed cells giving the greatest response. There was
elevated expression of active p52, p50 and p105 (P=<0.001), in PM-exposed cells compared to control (Fig. 4-4).

At 24 h (Fig. 4-5), constitutive p-IkB (Ser\textsuperscript{32}) was detected in all cell lysates with significantly elevated levels in lysates from cells that were treated with PM and LPS (P=<0.001). Cells treated with PM\textsubscript{10} had the highest levels of p-IkB but no concentration effect was observed (P=0.589; Fig. 4-5). Interestingly, phosphorylation of IκB was not reflected in reductions of total IκB with only the lysates from LPS treated cells having significantly (P=0.031) lower levels of the protein than control. Lysates from cells treated with the lowest concentration of PM\textsubscript{2.5} had the highest levels of protein (Fig. 4-5). With the inducible active p52 protein, lysates from cells treated with PM demonstrated significant increases (P=<0.001) from control with the exception of the high PM\textsubscript{2.5} (P=0.43). The LPS-exposed cells expressed lower (P=<0.001) levels of p52 than that from control (Fig. 4-5). While the inactive p100 dimer was not detectable, there was increased expression of the inactive p105 dimer in PM-treated cells (P=<0.001 for all) compared to control. All PM treatments induced increases (P=<0.001) of the active p50 protein.

Cytokine release is regulated by activities of Akt (Strassheim \textit{et al.} 2004) and NF-κB (Hayden and Ghosh 2004) so the ability of CVPM to induce cytokine release was examined. IL-1\textalpha, IL-6 and IL-8 were detected and IL-1\beta, IL-2, IL-4, IL-10, interferon-\gamma, TNF-\alpha, and TNF-\beta were not. Cytokine release was detectable but not quantifiable 1 h post treatment but was measurable after 6 h (Fig. 4-6). At that time point, the fine (P≤ 0.004) but not the coarse (P ≥ 0.128) elicited significantly (P=0.00) greater IL-1\textalpha release than control. At 24 hr, only the low-fine exhibited significantly (P=0.002) greater IL-1\textalpha release.
release compared to control (Fig. 4-6). With IL-6 following 6 h exposures all treatments (P ≤ 0.005) excepting the low-coarse (P=0.174) induced significantly greater IL-6 levels than control with LPS eliciting a 4x greater response than that of the PM. Following 24 h all treatments exhibited significant increases from control (P=<0.001) with LPS inducing only a 40% greater response than the nearest PM induced response (Fig. 4-6). IL-8 release was not significantly affected by PM treatment after 6 h and only the high PM$_{2.5}$ elicited (and LPS) a greater response than control after 24 h (P=<0.001 each).

Previous studies have shown that CVPM treatment results in increases in extracellular Hsp70 (see chapter 3) which exhibits its signaling capacity through the Toll like receptor (TLR)/IL-1 receptor pathway (Vabulas et al. 2002). As there were PM mediated IL-1 release and alterations in NF-κB activity, which can also be activated through IL-1R1 activation we examined the central regulator of TLR-2,4/IL-1R the interleukin-1 receptor associated kinase-1 (IRAK) (Arcaroli et al. 2006). IRAK phosphorylation was not detected on Thr$^{387}$ which is required for IRAK activation (Kollewe et al. 2004), following 1 or 24 h of PM and LPS exposure (Fig. 4-7). IRAK phosphorylation at Ser$^{376}$ which is indicative of IRAK-4 activation (Koziczak-Holbro et al. 2007) likewise, was not detected (data not shown). Inactive IRAK was detected and was increased in PM treated cells, which is consistent with PM treatment (see chapter 2) and indicative of a lack of IRAK degradation (Yamin and Miller 1997). Antibody functionality was verified by treating cells with 25ng/ml IL-1α which caused IRAK phosphorylation at both Ser$^{376}$ and Thr$^{387}$ sites following 1 and 24 h treatment (data not shown).
In order to determine whether calpain activation might be involved in PM-mediated Akt phosphorylation (Tan et al. 2006b), cells were co-incubated with a non-cytotoxic concentration of 40 µg/ml leupeptin (Momiyama et al. 2006), a “relatively select” calpain inhibitor (Takahashi et al. 2006) for 24 h. Leupeptin is water soluble so does not need to be used with a vehicle that might itself alter some of the subtle pathways that PM alters such as PEG (Ono et al. 1999), ethanol (Carloni et al. 2004), and DMSO (Our laboratory, unpublished findings) altering calpain activity. Leupeptin eliminated detectable Akt phosphorylation at Thr$^{308}$, PTEN phosphorylation, PDK phosphorylation, and Gsk phosphorylation (data not shown). Leupeptin co-treatment had little effect upon the detection of Akt phosphorylation at Ser$^{473}$. Leupeptin co-incubations with PM and LPS exhibited significantly greater effects than leupeptin alone control (P=<0.001) except with the low concentration of the fine (P=0.296) PM (Fig. 4-8 A). Cytokine levels were examined in the conditioned media from the leupeptin plus PM and LPS treatments. For IL-1$\alpha$ the high PM$_{2.5}$ and both low and high PM$_{10}$ plus leupeptin treatments, achieved significantly different results from the leupeptin only (P=0.004, P=0.001, and P=0.001, respectively). Leupeptin plus the low concentration of fine PM and leupeptin plus LPS were not significant from leupeptin only (P=0.140, and P=0.09). In the case of IL-6 the leupeptin co-treatment eliminated the PM mediated increase from control of the PM$_{2.5}$ and the low concentration of the PM$_{10}$. Only the high (25 µg/ml) and LPS treatments exhibited significantly greater effects than control (P=0.001 and P=<0.001, respectively). Cells co-treated with both types of PM and leupeptin had slightly less IL-8 release than the cells treated with leupeptin only. This reduction was not significant (P=0.067 and P=0.122 for low and high PM$_{2.5}$ treated cells and P=0.252 and P=0.077 for low and high
PM$_{10}$ treated cells). Only the LPS plus leupeptin co-treatment group achieved a significantly greater response (P<=0.001) from the leupeptin only group (Fig. 4-8 B).

Discussion

To our knowledge, this study is the first to demonstrate that ambient PM activates Akt \textit{in vitro} with concomitant inactivation of the tumor suppressor PTEN. Previous studies have shown reserve oil fly ash altered related Akt genes in rat neonatal cardiomyocytes (Knuckles and Dreher 2007) and diesel exhaust particles (DEP) induced Akt activity in human umbilical vein epithelial cells (Sumanasekera \textit{et al}. 2007). In murine keratinocytes, DEP activated Akt in the absence of PTEN phosphorylation (Ma \textit{et al}. 2004). In another study, DEP (50 µg/ml) caused downregulation of Akt phosphorylation and concomitant apoptosis in A549 cells, events which were suppressed when these cells were stably transfected with recombinant human thioredoxin-1 (rh-Trx-1) suggesting a protective role of this protein against reactive oxygen species released from DEP (Kaimul Ahsan \textit{et al}. 2005). Diet-induced obese mice exposed to PM (1.6 mg/kg, intratracheal instillation and inhalation of 13 µg/m$^3$ for 6 h/day, 5 days/week, for 128 days) showed reductions in Akt phosphorylation (Ser$^{473}$) in intact aorta but no changes in Akt phosphorylation in epithelium-denuded aortic tissues (Sun \textit{et al}. 2009). Akt directly controls cellular metabolism via hexokinase and phosphofructokinase-2 and is involved in respiratory burst in neutrophils (Chen \textit{et al}. 2003) likely explaining previous findings of PM mediated oxidative burst (Soukup \textit{et al}. 2000) and PM mediated increases in MTT reduction (Pruett and Loftis 1990; see chapter 2).
The pro-inflammatory activity of Akt is primarily due to its ability to suppress apoptosis in cytokine releasing cells (Matute-Bello et al. 1997), a mechanism thought to contribute to acute lung injury (Abraham 2003). Akt suppresses apoptosis through mechanisms involving MDM2 activation and p53 degradation, inactivation of caspase-9 and Bad, stabilization of the anti-apoptotic protein XIAP, Bim down-regulation (Amaravadi and Thompson 2005), prevention of mitochondrial cytochrome-c release (Abraham 2003), and forkhead inactivation (Brunet et al. 1999). Akt activation is also a pro-survival compensatory action taken by cells following insult such as endoplasmic reticulum stress (Hu et al. 2004), ischemia (Mullenkal and Toledo-Pereyra 2007), sepsis, (Li et al. 2004), or ultraviolet light (Mallikarjuna et al. 2004). As we have shown CVPM to trigger ER stress in these cells (see chapter 3) it is possible that the Akt activation observed here is also a compensatory action.

The signals for NF-κB activation include Akt, TNF, and TLR network (Hazeki et al. 2007; Oda and Kitano 2006). NF-κB activation typically occurs through the phosphorylation and ubiquitin-mediated degradation of the inhibiting protein IκB-α although NF-κB activation via oxidative stress can bypass IκB-α degradation (Pianetti et al. 2001). PM has been demonstrated to activate NF-κB upon contact with cell surfaces via mechanisms that are independent of IκB degradation (Churg et al. 2005; Jimenez et al. 2000). The lack of IκB degradation with concurrent phosphorylation found in this study may be due to inhibition of proteosomal degradation, or to IκB upregulation, a recovery effect observed following LPS stimulation (Velasco et al. 1997). PM mediated enhancement of IκB-α phosphorylation observed in the present study are possibly associated with Akt (Abraham 2005) or via calpain (Pianetti et al. 2001; see chapter 3)
which activates Akt (Tan et al. 2006) and can be activated by PM (see chapter 3). The PM-induced appearance of processed p50 (NF-κB1) and p52 (NF-κB2) fragments is consistent with constitutive NF-κB signaling (Senftleben et al. 2001) and increases in the constitutive activation of Stat3 with PM exposure we previously observed (Nadiminty et al. 2006; see chapter 2). Of interest are the increases of NF-κB p105/p50, an effect observed with cell migration and tumor progression (Gao et al. 2006).

Akt signaling elicits inflammatory cytokine release (Wong et al. 2007) and is likely to be partially responsible for the release of inflammatory cytokines IL-6 and the chemokine IL-8 observed in this study, in agreement with previous studies using this cell type (Frampton et al. 1999; Veranth et al. 2004).

Chronic PM mediated IL-6 release may lead to atherogenesis via eNOS inhibition (Saura et al. 2006) and may be a possible mechanism for linking PM to cardiovascular mortality (Pope et al. 2004a) although it may require consistent PM exposure that may or may not occur with ambient conditions. IL-6 is constitutively released in these cells, has potential autocrine signaling, its release is enhanced by PM exposure (see chapter 2), and eNOS is expressed in the lung and lung cells (Higashimoto et al. 2005; Ten Broeke et al. 2006). Our inability to detect eNOS phosphorylation of at Ser1177 (Morrow et al. 2003) despite Akt activation, which activates eNOS (Ndiaye et al. 2005), supports this hypotheses of IL-6 inhibiting eNOS and warrants additional study. IL-8 is angiogenic contributing to tumor progression in lung cancer (Luppi et al. 2007; Yuan et al. 2005) but IL-8 autocrine signaling is unlikely here, as evidenced by a lack of observable STAT-1 phosphorylation examined previously (see chapter 3). While this is not the first study to detect IL-1α release from BEAS-2B cells (Griego et al. 2000) it appears the first to detect
PM mediated IL-1α release from BEAS-2B cells. The contribution of IL-1 to atherogenesis is well documented (Chi et al. 2004) and IL-1 contributes to tumor progression (Elaraj et al. 2006; Lewis et al. 2006). Of the cytokines not detected in this study, TNF-α release from BEAS-2B cells has been documented following exposure to studded-tire wear particles (Lindbom et al. 2006).

This evidence of increases of Akt activation raises additional questions concerning the mechanisms of PM induced pathogenesis. Akt is well known for its role in insulin signaling (Alessi et al. 1996) and insulin in the growth media (approximately 5 mg/L, Invitrogen Technical support) may be responsible for the basal levels of Akt phosphorylation observed here, as well as in other studies (Zhang et al. 2006). It would be interesting to see if PM mediated Akt activation might play a role in the observed association between PM exposure and diabetes prevalence (Pearson et al. 2010). The role of the TLR network in PM mediated effects has been examined (Becker et al. 2005a; Becker et al. 2002) and the TLR network is implicated in Akt activation (Ha et al. 2008; Hazeki et al. 2007; Li et al. 2004). The current study has demonstrated that PM mediated Akt and NF-κB activation is through an IL-1R1 independent mechanism as evidenced by the lack of PM inducible IRAK-1 phosphorylation. This is intriguing given that PM treatment resulted in increases in IRAK-1 protein levels (Fig.4-7). TOLLIP may be upregulated resulting in IRAK/MyD88 inhibition, or MyD88 may be activated by other receptors (Hayden and Ghosh 2004). IRAK dependent signaling represents a small portion of the large TLR signaling network and many other potential mechanisms of receptor mediated activation of Akt exist (Oda and Kitano 2006). Akt activation also occurs through receptor tyrosine kinases (RTKs) (Zhang et al. 2007), IL-6 (Chen et al.
1999), the janus kinase (Jak) /Stat pathway (Gross et al. 2006), G-protein coupled receptors (Kong et al. 2006), peroxisome proliferator activating receptors (Amaravadi and Thompson 2005), and calpain (Pianetti et al. 2001; Tan et al. 2006). The leupeptin co-administration experiments demonstrated that the PM mediated Akt activation is possibly due to calpain or a similar serine/cysteine protease(s). Leupeptin mediated calpain inhibition appeared to inhibit PDK-1 which phosphorylates Akt at Thr$^{308}$ (Vanhaesebroeck and Alessi 2000) or promoted its dephosphorylation (Gao et al. 2005), while not affecting PM mediated increases in integrin-linked kinase (Persad et al. 2001), MTOR (Sarbassov et al. 2005), or other unknown protein(s) with PDK-2 activity that phosphorylate Akt at Ser$^{473}$ (Vanhaesebroeck and Alessi 2000). The PM mediated calpain activation and subsequent Akt activation is possibly due to PM induced ER stress (see chapter 3).

There were no consistent differences between the responses to fine and coarse PM in this study. The slight differences observed, when present, were possibly due to a slightly different chemical profile generally present in differing PM diameter ranges (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003). In any event, toxicological differences between size categories in vivo are mostly likely a result of differential deposition patterns in the respiratory tract which are not possible to assess in an in vitro cell based system. There were time related differences between coarse and fine PM as evidenced by the 1 and 24 h exposures with pGSK, IκB, pPTEN, and p-Akt (Figs. 4-2 and 4-3). More rapidly activated pathways may be stimulated by the components of fine PM and the slower pathways by the coarse. Given that the longer 24
h exposure resulted in increased effects, individuals who have compromised clearance may be at greater risk for PM mediated harm.

The USEPA states, “Despite a strong consensus that exposure to PM induces adverse health effects [...] relatively little is known about the specific physical or chemical characteristics of the particles that cause these effects or the mechanisms through which the adverse effects are induced” (NHEERL 2010). Thus, the present study may be helpful in revealing molecular and cellular mechanisms by which PM exerts harm.

This study demonstrates that ambient PM enhances activation of Akt in human pulmonary epithelial cells *in vitro*, and this activation is likely compensatory to ER stress and other events. Activation of Akt involves PTEN inactivation and appears to depend upon calpain or other similar proteases, rather than receptor-mediated IRAK activation. In total, these studies support the hypothesis that the pro-inflammatory effects of PM may be linked to Akt activation.

**References**


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Figure 4-1. PM treatment resulted in marked increases in Akt phosphorylation in BEAS-2B cells. Western immunoblots (A-B) and luminescent analysis (C) examining the activation state of Akt (60 kDa.). Blots are of cell lysates treated with 1(A) or 24 h (B) exposures of 12.5 (low) and 25 (high) µg/ml PM\textsubscript{2.5} and PM\textsubscript{10} including 10 ng/ml LPS and untreated negative control Gels were loaded with 10 µg protein. Figures are representative of a minimum of 3 independent experiments. Results are presented as mean intensity normalized to control and unmodified Akt ± SE. \textsuperscript{a}Significant from control, \textsuperscript{b}significant from differing concentration of the same PM diameter, \textsuperscript{c}significant from same concentration of differing PM diameter \textsuperscript{d}significant from LPS (P=0.05).
Figure 4-2. PM treatment induced alterations of Akt related proteins in BEAS-2B cells following one h exposure. Western immunoblots (A) and luminescent analysis (B) examining the alterations of the phosphorylation states of PDK (58-68 kDa.), PTEN (54 kDa.), and GSK (46 kDa.), and the total levels of p53. Gels were loaded with 10 µg protein. Blots are of lysates from cells receiving 1 h exposures of 12.5 (low) and 25 (high) µg/ml PM2.5 and PM10 including a 10 ng/ml LPS positive control and untreated negative control. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa) ± SE.

Significant from control, b significant from differing concentration of the same PM diameter, c significant from same concentration of differing PM diameter d significant from LPS (P=<0.05).
Figure 4-3. PM treatment induced alterations of Akt related proteins in BEAS-2B cells following 24 h exposure. Western immunoblots (A) and luminescent analysis (B) examining the alterations of the phosphorylation states of PDK (58-68 kDa.), PTEN (54 kDa.), and GSK (46 kDa.), and the total levels of p53. Blots are of lysates from cells receiving 24 h exposures of 12.5 (low) and 25 (high) µg/ml PM$_{2.5}$ and PM$_{10}$ including a 10 ng/ml LPS positive control and untreated negative control. Gels were loaded with 10 µg protein. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa.) ± SE. aSignificant from control, bsignificant from differing concentration of the same PM diameter, csignificant from same concentration of differing PM diameter dsignificant from LPS at P=<0.05.
Figure 4-4. PM alters non-classical NF-κB activity in BEAS-2B cells after 1h incubation. Western immunoblots (A) and luminescent analysis (B) examining alterations of IκB (40 kDa.), NF-κB p100/52, and NF-κB p105/p50. Gels were loaded with 10 μg protein/well with the exception of the positive control lane which was a lysate of TNF-α treated HeLa cells provided by the antibody manufacturer loaded at 10 μl/well. Blots are of lysates from cells receiving 1 h exposures of 12.5 (low) and 25 (high) µg/ml PM\textsubscript{2.5} and PM\textsubscript{10} including a 10 ng/ml LPS positive control and untreated negative control. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa.) ± SEM. aSignificant from control, bsignificant from differing concentration of the same PM diameter, csignificant from same concentration of differing PM diameter dsignificant from LPS at P=0.05.
Figure 4-5. PM alters classical NF-κB activity in BEAS-2B cells after 24 h incubation. Western immunoblots (A) and luminescent analysis (B) examining alterations of IκB (40 kDa.), NF-κB p100/52, and NF-κB p105/p50. Gels were loaded with 10 µg protein/well with the exception of the positive control lane which was a lysate of TNF-α treated HeLa cells provided by the antibody manufacturer loaded at 10 µl/well. Blots are of lysates from cells receiving 24 h exposures of 12.5 (low) and 25 (high) µg/ml PM2.5 and PM10 including a 10 ng/ml LPS positive control and untreated negative control. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa.) ± SEM. aSignificant from control, bsignificant from differing concentration of the same PM diameter, csignificant from same concentration of differing PM diameter dsignificant from LPS at P=<0.05.
Figure 4-6. PM exposure causes the release of NF-κB related interleukins following 6 and 24 h exposures. Results are a combination of n=3 independent experiments with six replicates and are given in mean concentration (pg/ml) + SEM. aSignificant from control, bsignificant from differing concentration of the same PM diameter, csignificant from same concentration of differing PM diameter dsignificant from LPS at P<0.05.
Figure 4-7. PM signaling in BEAS-2B cells is not through an IRAK-1 dependent mechanism. Immunoblots revealed that IRAK-1 is not phosphorylated following treatment after 1 (A) or 24 h (B) treatment. This indicates that the IL-1 receptor pathway is likely not activated by the PM used in this study in BEAS-2B cells. This is despite PM mediated increases in IRAK levels. Western immunoblots of cell lysates treated with 1 or 24 h exposures of 12.5 (low) and 25 (high) µg/ml Cache Valley PM\textsubscript{2.5} and PM\textsubscript{10} including a 10 ng/ml LPS positive control and untreated negative control. Gels were loaded with 10 µg/ml total protein. Figures are representative of a minimum of 3 independent experiments.
Simultaneous leupeptin co-treatment alters PM mediated Akt activity and cytokine release. A) Co-incubation of the calpain inhibitor leupeptin with PM and LPS completely attenuated the ability to detect Akt phosphorylation at Thr$^{308}$, p-GSK, p-PTEN, and p-PDK as measured via Western Immunoblots. Only p-Akt (60 kDa.) at Ser$^{473}$ was detectable (compare with Figures 1-2). As phosphorylation of both sites on Akt is needed for full activity it indicates that calpain is required for PM mediated Akt activation. Western immunoblots were of cell lysates from BEAS-2B cells following 24 h coexposures of 12.5 (low) and 25 (high) μg/ml concentrations of Cache Valley PM$_{2.5}$, PM$_{10}$, 10 ng/ml LPS plus 40 μg/ml of the calpain inhibitor leupeptin (leu) and leupeptin only control. Gels were loaded with 10 μg/well. Figures are representative of a minimum of 3 independent experiments. B) Calpain inhibition induced changes in PM mediated cytokine release limiting the ability of most PM treatments, but not LPS to result in significantly elevated levels of IL-6 and IL-8 (compare with Figure 6). aSignificant from control, bsignificant from differing concentration of the same PM diameter, csignificant from same concentration of differing PM diameter dsignificant from LPS at P=<0.05.
CHAPTER 5

SUMMARY AND FUTURE WORK

This work was undertaken to develop a better understanding of the cellular mechanisms involved in PM mediated health effects. The original hypothesis, based off a reading of the literature, was that PM would illicit cytotoxic/apoptotic and pro-inflammatory effects in human lung cells. The chosen cell model was the well studied and widely used BEAS-2B human bronchial epithelial cell line. Part of the original hypothesis proved to be incorrect: PM was only modestly cytotoxic at concentrations that were not environmentally relevant. PM treated cells exhibited some pro-apoptotic signaling but not actual apoptosis. However, PM induced numerous pro-inflammatory responses. I later determined that the pro-inflammatory effects of PM likely explain the lack of cytotoxicity despite pro-apoptotic signaling.

This study demonstrated that PM treatment at hypothetical environmentally-relevant concentrations exhibited an increase in MTT conversion. Such an effect has been observed with the respiratory burst of neutrophils, which is an increase in free radical formation, oxygen uptake, and likely metabolic respiration (Pruett and Loftis 1990). This effect is likely due to biologically active components of the PM. The Neutral Red cytotoxicity assay supports this hypothesis given the absence of significant increases of dye uptake which would have indicated stimulated cell growth.

The cytokine receptor upregulation was a novel and interesting finding related to the inflammatory process. Cells do not respond to cytokines in the absence of receptors, and the expression of cytokine receptors governs the ability of a cell to respond to cytokine signaling (Parrish-Novak et al. 2002; Pestka et al. 2004; Sims et al. 1993).
Cytokines are generally short lived, transitory signaling molecules, and their inactivating receptors greatly outnumber the cytokine in the human plasma proteome (Anderson and Anderson 2002). The upregulation of the activating rather than the inactivating cytokine receptors likely prepares the cell for an inflammatory response.

As part of the preparation for an inflammatory response, immune cells undergo the UPR (Skalet et al. 2005). The findings of this study demonstrating that ambient PM can trigger the UPR in epithelial cells give insight to how PM can be stressful to cells without causing cytotoxicity. The novel finding of calpain activation is intriguing yet congruent with previous findings. Calpain activation is generally pro-apoptotic (Gil-Parrado et al. 2002; Goll et al. 2003), yet calpain activation can also be pro-inflammatory (Cuzzocrea et al. 2000). The best studied function of activated calpain is the cleavage of cyto-skeletal/cell membrane attachments (Goll et al. 2003), which is vital for cell motility (Gil-Parrado et al. 2002; Goll et al. 2003) and likely contributes to the effects of calpain on chemotaxis (Lokuta et al. 2003). Calpain is a calcium dependent protease, and calpain activation is likely due to cytoplasmic Ca\(^{2+}\) influx. However, there is some evidence that phosphatidylinositol (PI), phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)), and other phospholipids lower the Ca\(^{2+}\) concentration required for calpain activation (Goll et al. 2003). The evidence supporting this hypothesis is that the PM mediated calpain activation measured in Chapter 3 appeared concentration dependent, with differences between the PM fractions and with LPS. Concurrently, there were no alterations in the activation of the Ca\(^{2+}\) dependent protein kinase-C. PIP\(_3\) is involved in the cellular infiltration of *Pseudomonas aeruginosa* a pathogen found in PM (Becker et al. 2002; Veranth et al. 2004). PI and PIP\(_2\) as well as calpain activate the key pro-inflammatory anti-apoptotic protein Akt (Mejillano et al. 2001; Tan et al. 2006).
Akt is central to cell survival, cell proliferation (Choi et al. 2006; Misra et al. 2006), tumor progression (Testa and Bellacosa 2001), and is pro-inflammatory (Abraham 2005; Wong et al. 2007). Akt is responsible for alterations in respiratory metabolism (Amaravadi and Thompson 2005) and respiratory burst (Chen et al. 2003). The findings of the pro-survival, anti-apoptotic and pro-inflammatory effects of PM are all adequately explained by Akt activation.

With all good research, more questions are raised and avenues for further research created than are questions answered. For example, many of the findings of this work; (Hsp70 release, Hsp90 upregulation (Bagatell and Whitesell 2004), Akt activation (Testa and Bellacosa 2001), PTEN inactivation (Cantley and Neel 1999), IL-6 release (Hodge et al. 2005; Scheller and Rose-John 2006), IL-8 release (Luppi et al. 2007), calpain upregulation (Grozio et al. 2007), and activation (Pianetti et al. 2001), Hsp27 activation (So et al. 2007), and Stat3 activation- are associated with cancer. Cancer is one of the illnesses that has an association with early mortality and PM exposure (Krewski et al. 2005). While PM may not be carcinogenic, it may contribute to cancer’s pathogenesis. Many of the above effects have been observed in other studies. Further research could determine if the above-mentioned effects are indeed related to PM mediated enhancement of cancer progression or are artifacts of the immortalized cells.

Examination of the role of receptor tyrosine kinases (RTK) in measurable PM mediated effects would be beneficial in determining the mechanisms of PM mediated pathway activation and in determining which component(s) of the PM isolated in the manner used in this study are bioactive. Evidence for the possibility of RTK activation is the upregulation of caspase-3. The upregulation of caspase-3 in the absence of apoptosis without apparent alterations in the activation of protein kinase C (PKC)-δ (Cataldi et al. 2005).
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2002) is similar to the effects observed from α-tocopherol (Miyoshi et al. 2005). This effect is thought to be due to the transcription factor Sp1 (Miyoshi et al. 2005), which has been shown to be activated by various growth factors (Alessi et al. 1996; Citri et al. 2004).

Caspase-3 upregulation has also been shown to be upregulated following T-cell receptor activation (Sabbagh et al. 2005). This finding coupled with the evidence of respiratory burst activity (Akt activation and increases of MTT conversion) observed in this study leads to an intriguing hypothesis. The BEAS-2B epithelial cell line may share many of the receptors thought only to be possessed by immune cells and professional phagocytes.

Additional research could include the examination of the effects of PM on peroxisome proliferator activating receptors (PPAR). The microarray experiment conducted in Chapter 2 revealed PM mediated upregulation of PPAR. Subsequent studies have revealed that a PPAR agonist reduced LPS induced airway inflammation (Delayre-Orthez et al. 2008). Additional lines of research could include whether gangliosides are molecules involved in PM mediated signaling. The ganglioside asialo-GM-1 binds to bacterial flagella, mobilizing TLR-5 to the apical surface of epithelial cells leading to the release of IL-8 in an ATP independent mechanism (Adamo et al. 2004). TLR-3 is not located on the surface of the plasma membrane and activated by double stranded RNA (Oda and Kitano 2006) and double stranded RNA upregulates cytokine receptors (Tsuji et al. 2005), an effect outlined in Chapter 2, and can act independently of IRAK activation. This is an excellent receptor for study in PM mediated cellular effects.
A study suggests that mitogen activated protein kinases (MAPK) are central to PM mediated inflammation with JNK playing a major role (Kleinman et al. 2008). That study used concentrated ambient particles on apolipoprotein E knockout mice and found increases in phosphorylated JNK without increases in the phosphorylation of IκB and p38. I was unable to detect PM mediated increases in JNK activation but did find increases in IκB phosphorylation and Hsp27 phosphorylation, which is phosphorylated by p38 (Stathopoulou et al. 2006). The phosphorylation of p38 has been found in a number of PM studies and is believed to be due to PM mediated oxidative stress and is a pro-inflammatory marker (Li et al. 2005; Li et al. 2006; Reibman et al. 2002). This incongruence may be one of many experiments where in vivo and in vitro results do not agree, due to the differing particle types used or due to variations in the species used (knockout mice, rat tracheal explants, and human epithelial cells). Oxidants produce ceramides and lead to the degradation of Akt (Martin et al. 2002). Given that I was unable to detect alterations in SAPK/JNK and that Akt was activated with PM treatment with no obvious degradation (Chapter 4) it is unlikely that the PM used in these studies directly generates radicals as occurs with PM containing higher concentrations of metals. Any radical formation is likely due to the cellular responses to bacterial components on the PM. This emphasizes that the PM exerts it effects though receptors and signal transduction and additional research into which receptors are involved would be insightful.

Given that Cache Valley PM is mostly comprised of particles <1 µm in diameter and because similarly-sized particles have been shown to penetrate the central circulation and the brain (Nemmar et al. 2002; Nemmar et al. 2004; Oberdorster et al. 2004), studies to determine whether PM affects neurons and cardiac cells would be useful. The skin
also is an organ of contact for PM. There are commercially available \textit{in vitro} skin models that are required for dermal studies in the pharmaceutical and cosmetic industries that represent the skin quite well and are superior to the cell monoculture or co-culture used in most \textit{in vitro} lung models. Studies with PM and those models might yield interesting results. Skin proteins are also a major component of indoor PM (Fox \textit{et al.} 2008). Although indoor PM exposure is generally greater than outdoor PM exposures (National Center for Environmental Assessment [Research Triangle Park N.C.] 2003) there are fewer studies on indoor PM, with most research focused upon LPS (Long \textit{et al.} 2001). The effects of indoor PM, especially from near agricultural businesses (Reynolds \textit{et al.} 2005), on human lung cells and the biological mechanisms of indoor PM mediated effects are worthy of additional study.

The studies presented here have contributed to the general body of knowledge of potential molecular and cellular mechanism of PM induced harm, an area where insight is greatly lacking. They have provided many markers for determining differential effects of PM collected from differing locations as well as for the evaluation of pollution control efforts and are an excellent starting point for the continued study of our problematic local air pollution.


APPENDIX A

ATTEMPTED EXPRESSION OF CYP2K1 AND DETECTION OF P53 MUTATION IN HUMAN LUNG CELLS.

Abstract

Aflatoxin B₁ (AFB₁) is classified as a group 1 carcinogen due to evidence of carcinogenicity to humans. It requires enzymatic activation from its naturally occurring pro-carcinogenic form to a reactive form. That activation occurs after human or animal consumption and the reactive metabolite can bind to cellular macromolecules such as DNA. The cytochrome P450 (CYP) family of enzymes is the major contributor to AFB₁ activation. AFB₁ exposure is strongly associated with human hepatocarcinoma, is associated with mutations in the p53 tumor suppressor gene, and has been shown to mutate the same gene in vitro. The introduction of animal genes and foreign P450 into plants has been successfully performed therefore the possibility of inserting the gene for a cytochrome P450 that has high AFB₁ activating activity was investigated hoping to be able to have the plant metabolize the AFB₁ in planta prior to human or animal consumption. The cytochrome P450 selected was CYP2K1 from rainbow trout; a CYP with high metabolizing activity that would result in inactivated AFB₁ rather than less but still toxic metabolites. The gene product proved to be toxic to two different plant species and no viable plant transformants were produced. Concurrent experiments demonstrated that CYP2K1 did not fold properly when heterologously expressed in common protein expression systems. Additional research confirmed that AFB₁ does not specifically target p53 at the codon 249 hotspot in human lung cells.
Introduction

Following considerations in 1971, 1972, 1975, 1976, and 1987 the International Agency for Research on Cancer (IARC) classified the widespread mycotoxin Aflatoxin B₁ (AFB₁) as a group 1 carcinogen (carcinogenic to humans) in 1993 (IARC 1993a). Aflatoxins are a group of closely related compounds with AFB₁ being the most potent and the most prevalent (Williams et al. 2004a). The mechanisms of AFB₁ toxicity have been extensively studied. AFB₁ requires metabolic activation from its native form to a highly reactive 8-9 epoxide. The epoxide has endo and exo forms with differing activating enzymes preferring the formation of one over the other. The cytochrome P450 (CYP) enzymes perform a majority of this activation in the liver with CYP 3A4, CYP1A2, and CYP3A5 being the greatest contributors in human adults with CYP3A7 activating AFB₁ in babies (IARC 2002) and prostaglandin H-synthase and lipoxigenases being the major activating enzymes in the lung (Donnelly et al. 1996). The AFB₁ endo and exo epoxides react with other molecules which can result in damage or detoxification. Detoxification steps include glutathione-transferase mediated conjugation with glutathione forming endo and exo conjugates and hydrolysis to an 8, 9 dihydrodiol, which is often non-enzymatic with some controversy surrounding the role of epoxide hydrolase (Kelly et al. 2002). The dihydrodiols degrade to dialdehydes which then bind to primary amines forming protein adducts (IARC 2002). The activated 8, 9-exo epoxide is primarily responsible for DNA binding forming adducts preferentially at the N-7 position of guanine (Guengerich et al. 1998). Detoxification prior to metabolic activation does not occur through the cooking process with current practices involving ammoniation.
concurrent with high heat. Most exposure to Aflatoxin is dietary, but there is also exposure through occupational inhalation (IARC 1993a). Activated AFB₁ has been shown to bind to DNA in vitro and mutations in the p53 gene have been found in cases of hepatocellular carcinoma from areas of high dietary AFB₁ exposure, especially a G to T transversion mutation at codon 249 (IARC 1993a).

In the 2002 review of mycotoxins the IARC working group issued six conclusions and recommendations. Number two was for genetic engineering to offer new ways limit aflatoxin contamination of crops (IARC 2002). Therefore I undertook a high risk project attempting to insert an efficient AFB₁ metabolizing CYP into a plant to see if the plant would be able to metabolize and neutralize the AFB₁ prior to human or animal consumption. CYP2K1 (CYPLMC2) was selected as has high AFB₁ conversion activity and only metabolizes AFB₁ to the exo-epoxide creating no other metabolites such as AFM₁ (Yang et al. 2000).

The mechanisms for the introduction of foreign DNA into plants include many odd yet sophisticated techniques such as vortexing plant cells with DNA and silicone carbide whiskers (Dunwell 1999), gunpowder driven DNA coated particles (Daniell 1997), and electroporation of plant cells that have had their cell walls enzymatically degraded (Koscianska and Wypijewski 2001). However the most common method has been through the use of the soil bacterium Agrobacterium tumefaciens. The mechanisms of Agrobacterium mediated DNA transfer are complex involving the colonization of the bacteria on the plant, induction of DNA transfer systems, generation of the DNA transfer complex called T-DNA, transfer of the T-DNA into plan cell nuclei, and integration of the T-DNA into the plant genome. The transfer involves trans acting elements from
separate plasmids but essentially the DNA between two border sequences called the left border and right border are transferred into the plant DNA in a poorly understood random fashion. It is through placing genes of interest into plasmids containing these borders that enables the insertion of foreign genes into plants. (Chilton and Que 2003; del la Riva 1998).

Due to the time required for plant growth other experiments were undertaken. Given the ability to AFB1 to mutate DNA and the inhalation exposures that can occur, experiments were conducted to determine if AFB1 can mutate p53 in P450 expressing human lung cells at in exon 7 codon 249 as occurs with hepatocellular carcinoma (IARC 1993a). Additional work involved establishing that AFB1 induces apoptosis in the same human lung cells where the results are contained here (Van Vleet et al. 2006) and are not included in this manuscript. Other additional experiments included attempts to express CYP2K1 in order to be able to perform structure-function experiments and gain greater insight into its AFB1 metabolizing mechanisms.

Materials and methods

Chemicals and reagents

CYP2K1 cDNA in the NotI site of pSPORT1 was provided by Dr. Donald Buhler (Oregon State University, Corvallis, OR). All primers used in this study were obtained from Integrated DNA Technologies (Coralville, IA). Pfu Turbo DNA polymerase was from Stratagene (La Jolla, CA). pCWOR1+ E. Coli expression vector were obtained from Michael Waterman at Vanderbilt University (Nashville, TN). Rat NADPH P450 reductase was from Dr. Charles Kasper at the University of Wisconsin (Madison, WI).
The bicistronic pCWORI with human CYP1A2 and human NADPH p450 reductase was from Garold Yost at the University of Utah (Salt Lake City, UT) and originated from Fred Guengerich at Vanderbilt. The E. Coli expression vector pTRC99A was obtained from Irena Pikuleva at the University of Texas Medical Branch (Galveston, TX) and pSP19g10L was from Ronald Estabrook University of Texas Southwest Medical Branch (Dallas, TX). The E. Coli expression vector pGEX-KG was from Lance Thomas at Wake Forest University (Winston Salem, NC). Antibiotics ampicillin, tetracycline, streptomycin, and kanamycin as well as Extract-N-Amp plant DNA purification kits were from Sigma Aldrich (St. Louis, MO). Bacterial growth media mixes were from Becton Dickinson (Franklin Lakes, NJ). E. Coli strains XI-1 Blue, and BL-21, XI-10 gold, and BL-21 gold were from Stratagene (La Jolla, CA). E. Coli strains Top10, BL21 (DE3), and DH5α as well as Zero Blunt and PCRblunt cloning kits and agarose were from Invitrogen (Carlsbad, CA). E. Coli strain JM-109 was from Promega (Madison, WI). High Five insect cell line, pFASTBAC dual expression vector and Bac to Bac baculovirus expression system were from Invitrogen (Carlsbad, CA). HyQ TNM-FH insect cell growth media was from Hyclone (Logan, UT). QIAquick PCR purification kit was from Qiagen (Valencia, CA). Miniprep kits were from Qiagen (Valencia, CA) or Invitrogen (Carlsbad, CA). Ultrafree DA DNA gel extraction spin columns were from Millipore (Billerica, MA). Restriction endonucleases and DNTPs were from Fermentas (Glen Burnie, MD), Promega (Madison, WI), or New England Biolabs (Ipswich, MA). Agrobacterium tumefasciens LBA-4404 and pBI121 plant expression vector were from Invitrogen (Carlsbad, CA). Agrobacterium strain EHA105 was from Dr. John Carmen at Utah State University (Logan, UT). pBECKS plant expression vectors were kindly
provided by Dr. Alex McCormac at the University of Southampton (Southampton, UK). Plant expression vector pBI101 and the hypervirulent Agrobacterium strains EHA101, and EHA105 originally developed by Hood (Hood et al. 1993) were from Dr Daryll Dewald at Utah State University (Logan, UT). Murashige and Skoog (MS) plant growth media were from Sigma Aldrich (St. Louis, MO). Potting soil, and terra cotta pots were from Lowes (Mooresville, NC). Poly-pots and valuable Arabidopsis transformation expertise were kindly provided by Dr. Yajun Wu at Utah State University (Logan, UT). Aflatoxin B1 was from Sigma Aldrich (St. Louis, MO), DNA primers were from IDT (Coralville, IA). BCMV-1A2 human lung epithelial cells were a gift from Katherine Mace (Nestlé Research Centre, Lausanne Switzerland).

Plasmid construction

CYP2K1 cDNA was modified by the addition of unique restriction sites that enabled the directional cloning of the gene into the vector of interest. PCR reactions varied by primer pair (namely annealing temperature), but generally followed these steps. Initial melting step at 95°C for 120 sec, then 30 cycles of the following: melting step 95°C for 60 sec, annealing step various temperatures for 60 sec, and an extension step at 72°C for 60 sec.

For plant expression CYP2K1 cDNA was ligated into the expression vectors pBI121, pBI101, pBECKS19.5 and pBECKS19.Zsk. For Baculovirus mediated expression CYP2K1 and rat reductase were ligated into pFASTBacDual with CYP2K1 behind the poly promoter and the rat reductase behind the p10 promoter. For E. coli expression CYP2K1 was ligated into pCWORI+, pCW1A2, pSP19g10L, pTRC99A, pGEX-KG. Due to the length of the primers which included the restriction sites and for
E. Coli expression, additional modifications to the codons following the initial ATG, the melting and annealing times were extended to 60 sec rather than the customary 30 sec. Primers were added at a concentration of 0.2 µM each primer and the final deoxy nucleotide triphosphate (dNTP) concentration was 100 µM. The 2K1 DNA template concentration was 100ng/ml. The total reaction volume was 50 or 25 µl. The PCR reaction was electrophoresed in a 1% agarose gel for 30 min along with DNA size ladders. The gel was stained with ethidium bromide and visualized on a UV transilluminator. The proper sized band was then excised from the gel and purified using Utrafree DA spin columns. The DNA was then cut with the appropriate restriction enzymes using the concentrations, incubation times, temperatures, and buffers recommended by the manufacturers. Initial verification of insertion into the vectors was through the examination of the length of restriction fragments, but was later changed to PCR. Vectors were then sequenced to assure the intended sequences were in the plasmid and were not modified by the DNA polymerase. For plant transformation the 2K1 DNA was ligated into the BamHI site on pBI121 with the 5’ to 3’ orientation confirmed by restriction fragment analysis. CYP2K1 was inserted into pBECKS19.5 variants for plant transformation.

Plant growth and transformation

Virtually all Agrobacterium tumefaciens protocols for the transformation of tobacco are variations of the method of (Hoekema 1983) The variations used in this experiment were those of Topping and Gallois (Gallois and Marinho 1995; Topping 1998). The method of Agrobacterium mediated transformation of Arabidopsis thaliana was widely used floral dip method (Clough and Bent 1998).
The strain that was used in majority of the transformation experiments was LBA4404. When success was apparently unattainable the more virulent strain EHA105 strain was investigated. Due to the kanamycin resistance of the strain EHA101 it was not used, as kanamycin was the selectable marker contained in the pBI and pBECKS series of plasmids. Whichever method of transformation was used the time to determine if a transformation occurred ranged from 2-4 weeks.

Expression of CYP2K1 in E. Coli and insect cells

Expression if CYP2K1 was carried out according to the Bac to Bac expression system kit instructions with any subsequent modifications performed according to methods outlined here (Hood et al. 1998).

Initial attempts at expression of CYP2K1 in *E. coli* were conducted according to the method in the first manuscript demonstrating the ability (Barnes et al. 1991) with subsequent attempts following other methods (Barnes 1996; Gillam et al. 1993; Gillam et al. 1995; Gotoh 1992; Jenkins et al. 1998; Josephy et al. 1998; Sandhu et al. 1993; Waterman et al. 1995; Woyski and Cupp-Vickery 2001; Yun et al. 2006) with variations of plasmids, temperature, media, induction time, bacterial strain, cofactors, gene truncation, codon modification, etc.

Verification of transformation

Plant DNA was isolated using the Extract-N-Amp DNA purification kit and PCR analysis was performed using primers specific to *CYP2K1* or primers that amplified the region between the tDNA borders. The second set of primers was adapted in that the
potential for contamination was lessened as *CYP2K1* was a frequent target for PCR in the laboratory.

P450 spectral scan

Determination of CYP folding and was determined spectrally as described (Schenkman and Jansson 1998).

Microsome preparation

Microsomes were prepared from plants using the methods of Potter or, Chapella (Chiapella *et al.* 1995; Potter *et al.* 1995). Microsomes were harvested from insect cells using the method of Hood (Hood *et al.* 1998) and E. Coli spheroplasts were harvested according to the method outlined in Jenkins *et al.* (1998).

Activity assays

CYP2K1 metabolism of Aflatoxin was examined via the method outlined in (Van Vleet *et al.* 2001) which is a method of indirect measurement of the highly reactive short lived AFB\(_1\) epoxide through measuring AFB\(_1\) glutathione conjugates.

Cell culture and treatment

Culture and treatment of human lung cells were performed as described (Van Vleet *et al.* 2006).

Mutation detection

CYP1A2 expressing BCMV1A2 cells were treated with 1 µM AFB\(_1\) in DMSO or with DMSO (20 µl) alone for 24 h. AFB\(_1\) containing media along with unattached cells were removed, fresh media added, and media was changed every other day thereafter.
until cells reached confluence and were harvested. Cells in the AFB$_1$ treated flasks required 2 weeks to a month to reach confluence. Confluent cell cultures were harvested via trypsinization and genomic DNA was extracted, the concentration determined spectrophotometrically ($A_{260}$) and aliquots were frozen at -20°C. Aliquots were digested with HaeIII or its isochizomers to reduce the amount of unmutated codon 240 of p53 (Aguilar et al. 1993). Melting probe and PCR analysis was then performed as described (Bernard and Wittwer 2002; Millward et al. 2002).

Results

Plant Transformation

In no transformation situation with any vector or plant species were viable plants produced that had both kanamycin resistance and the CYP2K1 gene with wild type or modified sequences. An early attempt at tobacco transformation using pBI121 produced viable kanamycin resistant plants. However CYP2K1 DNA was not detectable in the plants. Subsequent examination of the vector revealed that the GUS cassette was not removed and the CYP2K1 DNA was not in the vector. The GUS cassette was the same size as the CYP2K1 and looked the same in a gel. PCR was then used for verification of all vectors thereafter. In a later situation some kanamycin resistant transformants were produced. However there were no detectable components of the vector except for the NPT II as determined by PCR and AFB$_1$ metabolism assay showed no activity in the tobacco microsomes. In the process of Agrobacterium mediated transformation the DNA next to the left border is transferred first and at times, the entire DNA segment located near the right border is not transferred. With the pBI vectors the NPTII which codes for
kanamycin resistance can be transferred without the transfer of the gene of interest since it flanks the left border sequence (McCormac et al. 1997). That disappointment prompted the switch from the pBI121 to the pBECKS19.5 vector where the vector is oriented to where the gene of interest is adjacent to the T-DNA left border (McCormac et al. 1997). There were no other instances in which kanamycin resistance was conferred to plants from a vector containing the CYP2K1 gene in well over 500 different attempts, neither with tobacco nor with Arabidopsis. Figure A-1 contains exemplars of tobacco transformation where Agrobacterium mediated transformation was unsuccessful with a vector containing CYP2K1 (Fig. A-1A) and successful with an empty vector (Fig. A-1B).

**E. Coli expression**

The concurrent attempts at expression of CYP2K1 in E. Coli were more successful only in that actual protein was expressed by the chosen expression systems. High amounts of protein were measured versus uninduced control. However the protein was in a non functional misfolded conformation as evidenced by large peaks at 420 nm in P450 spectral scans (Kim et al. 2005; Mao et al. 2008). In no case was there a large P450 peak indicative of proper CYP expression, nor was detectable CYP (AFB1 epoxidation) activity detected in isolated spheroplasts (data not shown). Truncation and removal of membrane spanning regions also did not result in correctly folded protein as has helped with other problematic P450s (Lamb et al. 2009).
**Baculovirus expression**

The eukaryotic insect cells and viral expression system also exhibited high amounts of protein detected in infected cells yet only misfolded P420 protein was observed (data not shown).

**Mutation detection**

Some initial melting curve analysis showed promise with shifts in the melting curve from AFB$_1$ treated DNA and that of control (Fig. A-2). However these shifts were not as great as those found the benchmark article (Millward et al. 2002) and subsequent DNA sequencing demonstrated that there was no mutation in codon 249, although it did not preclude mutation in nearby codons in exon 7.

**Discussion**

As this project was originally planned, I was not intended to undertake the plant transformations, those were to be done offsite by a collaborating scientist at Texas A&M. Our laboratory was to supply the cDNA and perform all of the tests of P450 functionality. Tragically the scientist who was to perform the transformations was diagnosed with cancer and for obvious reasons was unable to do so. In my naivety I thought that I could follow the simple protocols that would result in transformed plants with the plant transformation being a small component of the research. Unfortunately it was not so simple.

The laboratory that originally cloned CYP2K1 was able to successfully express CYP2K1 in insect cells leaving the 5’ untranslated region intact (Yang et al. 2000) however expression was extremely low and rainbow trout microsomes were used for most of the activity analysis in the manuscript. The lab dedicated subsequent attention to
E. coli expression (Donald Buhler, personal communication) which apparently was not completed prior to his retirement. The expression of non-cytoplasmic proteins has proven to be difficult with many proteins (Dunham and Hall 2009). With E. coli the early expression of CYP was somewhat serendipitous. The original hypothesis involved the mutation of the first seven codons to reduce secondary structure formation with the mRNA (Barnes et al. 1991). It was later determined the leader sequence between the promoter and the 5’ cloning sites was likely more relevant for expression (Barnes 1996) however many studies still use the modified “Barnes sequence” in unrelated CYPs as it worked well in previous attempts (Mosher et al. 2008). CYP2K1 is not the only protein to be expressed only as a P420, and some cannot be expressed at all (Mao et al. 2008). The evidence of unfolded protein was at first heartening as factors such as chaperones, deletion of membrane anchors, deletion of 5’-untranslated regions, and alternative expression temperatures might have helped, but did not. My unsuccessful attempts did demonstrate that CYP2K1 does not fold well when heterologously expressed.

The transfer of CYPs from one plant species to another have resulted in the production of cyanogenic metabolites that might lead to enhanced pest resistance in some plants (Bak et al. 2000) and enhanced herbicide resistance in others (Didierjean et al. 2002). The transfer of CYP2K1 containing T-DNA could have been into vital genes resulting in non-viable transformants (Zhang et al. 2008) but this would have happened over a large number of transformation attempts to always result in non-viable transformants while empty vectors did not. Given the random nature of Agrobacterium mediated DNA transfer it is unlikely that every case of foreign gene insertion was into a gene or genes required for viability. Improper targeting of the CYP2K1 protein could
have been toxic to the plants (Murray et al. 2002) but that did not seem to be an issue with other researchers inserting foreign CYPs into plants (Dueckershoff et al. 2005; Schoendorf et al. 2001; Shiota et al. 1994; Yamada et al. 2002). It is much more likely that stable expression of the gene or the protein product was toxic to the plants (Agarwal et al. 2006; Czako and An 1991) and “if the gene product to be expressed is toxic to the organism, it is impossible to establish stable transgenic lines carrying the chimeric gene” (Brand and Perrimon 1993). It is tempting to neatly tie in the findings of the lack of plant viability to high quantities of misfolded CYP2K1 protein. Accumulation of unfolded P450 protein possibly induced ER stress and apoptosis, which occurs in plants (Iwata and Koizumi 2005; Urade 2007) as well as mammals (Zhang and Kaufman 2004). Misfolded P450 has been shown to upregulate Kar2 (Zimmer et al. 1999) the yeast equivalent of BiP, the central protein involved in the unfolded protein response (Kimata et al. 2003). Accumulated P450 has been hypothesized to trigger the apoptotic response in mammalian cells (Szczesna-Skorupa and Kemper 2006) and overexpression of properly and mis-folded P450 induced ER stress and the UPR in HepG2 cells (Narjoz et al. 2009). However, most known foreign gene products that are toxic to plants include diphtheria toxin and ribonucleases (Agarwal et al. 2006; Czako and An 1991) and little supporting evidence of P450 overexpression being toxic to plants can be found in the literature. The use of CYP2K1 in chemically inducible promoter systems (Zuo and Chua 2000) would provide a mechanism of a plant scientist to confirm the toxicity of the gene product and determine the mechanism by which it occurs. It would be wise for scientists who wish to develop a plant with such aflatoxin metabolizing ability to utilize genes that produce proteins that not only have the ability to metabolize aflatoxin, but also have been
successfully expressed in a number of different systems. CYP1A2 has been
successfully expressed in a number of systems including *E. Coli* (Kranendonk et al. 1998), insect cells (Crofts et al. 1998), yeast (Bellamine et al. 1994), and tobacco (Shiota et al. 2000) where the CYP was catalytically active contributing to herbicide metabolism. Numerous e-mails to the authors went unanswered so the ability of the transformed CYP1A2 expressing plant to activate AFB₁ is unknown.

While AFB₁ has been shown to induce the G to T transversion mutation in human hepatocytes with rat microsomal AFB₁ activation (Aguilar et al. 1993), the lack of detection of an AFB₁ induced mutation in codon 249 of *p53* should not have been surprising. Given the preponderance of evidence behind the requirement of hepatitis B infection for hepatocellular carcinoma in high AFB₁ occurring regions (IARC 1993a, 2002), the lack of preference of codon 249 for mutation by AFB₁ (Sengstag et al. 1999), codon 249 mutation not being dependent upon the number or persistence of AFB₁ adducts (Denissenko et al. 1998), region specific frequencies of codon 249 mutation in hepatocellular carcinomas (Soini et al. 1996), and evidence of DNA repair polymorphisms possibly contributing to the mutational hotspot (Lunn et al. 1999) demonstrate there are more factors contributing to codon 249 mutation than mere AFB₁ activation.
References


Fig. A-1. Plant tissue transformed with *Agrobacterium tumefaciens* possessing a vector containing the *CYP2K1* gene sequence gave no viable transformants while tissue transformed with *Agrobacterium tumefaciens* possessing an empty vector had kanamycin resistant shoots.
Fig. A-2. Melting curve profile of p53 Exon-7 DNA from cells treated with vehicle (DMSO) and with Aflatoxin-B1 (AFB1). The melting curve indicates that there were differences in the DNA sequences between the vehicle and the AFB1 treated cells.
APPENDIX B

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Education:

Ph.D. Toxicology
Utah State University Logan, Utah.

Title: The effects of Cache Valley particulate air pollution on human lung cells.

Bachelor of Science The University of Utah Salt Lake City, Utah. Major: Health Promotion and Education. Minor: Chemistry

Bachelor of Arts The University of Utah Salt Lake City, Utah. Major: Behavioral Science and Health. Foreign Language: Portuguese.

Relevant Experience

Quansys Biosciences
Scientist

Jan. 2007- Present

Currently leading company’s technical support and technical sales efforts.

Authored and revamped the company’s MSDS forms.

Successfully managed the creation and implementation of an ISO-9001 compliant cell culture facility and wrote the SOPs. Cells grown include NIH-3T3, HELA, HEK-293, and Jurkat.
Successfully worked with a European company to manage a project which involved the development and validation of an antibody based, bovine tuberculosis diagnostic assay involving multiple markers. The development was at one point the company’s largest revenue stream. The manuscript outlining the assay has been published (Whelen et al Below).

Adapted the antibody microarray ELISA technology for diagnostic purposes such as the detection of drugs of abuse and neurodegeneration.

Spearheaded the modification of the company’s best selling products to enable their usage on an additional platform thereby expanding the marketability of the products.

Currently leading efforts to improve the company’s validation protocols to fulfill current ISO 9001 standards and to prepare to implement ICH guidelines.

Knowledge, Skills, and Abilities

*In vitro* toxicology

Demonstrated the ability to develop, implement, and interpret toxicological studies.

Including being very experienced in non-linear regression analysis suitable for the establishment of benchmark doses as well as measurement of standard curves.

  Experienced with working in a regulated environment. Currently serve as an ISO 9001 quality auditor. Familiar with ICH, TSCA, and REACH regulations.

Demonstrated p53 independent apoptosis induction by the known human carcinogen and potential biological warfare agent; Aflatoxin B1 in human lung cells. Confirmed p53 expression by a different lung cell type.

Utilized genomic technologies such as microarray and q-PCR as well as Western blotting to examine changes in gene expression caused by particulate air pollution on
human lung cells.

Determined cytotoxicity of locally collected particulate air pollution on human lung cells as well as determined its effects on the signal transduction pathways of the cytokines interleukin-1 (IL-1) and interleukin 6 (IL-6).

Determined particulate air pollution (PM) causes ER stress, activates calpain, and NF–κB and upregulates Akt in human lung cells. Established PM mediated Akt activation and cytokine release is apparently through a calpain mediated mechanism.

Capable of working with hazardous and carcinogenic materials.

Assisted major professor in reviewing papers submitted for publication in peer reviewed journals.

**Oral and Written Communication Skills**

Taught graduate level classes in toxicology as graduate teaching assistant.

Presented multiple Power Point presentations on current topics in toxicology.

Created, presented, and discussed scientific posters in national scientific conferences.

Using available literature assisted a local cosmetics manufacturer in determining the role of ceramides and cholesterol in dermal barrier repair and the role of pH and ceramide signaling in dermal healing.

Use communication skills daily in technical support and technical sales role including sales presentations to large groups. Experienced in communicating scientific concepts to non-scientists.

Fluent in English and Portuguese. Working knowledge of Spanish.

**Molecular biology and biotechnology techniques**

Using virology techniques, manipulated a virus genome (Baculovirus) for infection
and expression of the recombinant trout protein Cytochrome P450 2K1 (CYP2K1) in insect cells.

Additional experience in the manipulation of microorganisms includes cloning and recombinant gene expression of CYP2E1, CYP1A2, and CYP2K1 in *Escherichia coli*.

Obtained broad experience in mammalian tissue culture of Bio-safety level 2 (BSL-2) human lung cells BEAS-2B, BCMV-1A2, and B-3A4. Also experienced with the BSL-1 cells A549, NIH3T3, HEPG2, HEK, and bovine MDBK.

Extensive experience with *in vitro* toxicity assays such as MTT and neutral red. Possess extensive knowledge of polymerase chain reaction (PCR) experimental design and implementation. This includes mutagenesis, gene expression and quantification, gene detection, reverse transcriptase PCR (RT-PCR) and mutation detection. Proficient at working with RNA as well as DNA samples.

Plant transformation (tobacco and *Arabidopsis*) using the pathogenic bacterium *Agrobacterium tumefaciens*.

Facility with widely used computer software such as Microsoft Word, Excel, PowerPoint, Outlook, Adobe Acrobat, Illustrator, PhotoShop, SigmaPlot, ArrayAssist, Graphpad Prism, and SigmaStat. Familiar with the EPA benchmark dose software, SAS, and SPSS.

Extensive experience at regression analysis and statistical methods. Experienced in the handling of hazardous, carcinogenic, and cytotoxic chemicals.
Honors and Awards


Graduate Student Senate Travel Award Utah State University

February 2005 and 2006

Three time Waldron Biotechnology Award Utah State University.

Taylor and Francis Outstanding Graduate Student Award Society of Toxicology Food Safety Specialty Section New Orleans, LA March 2005.

Willard L. Eccles Research Fellowship Utah State University.

Health Promotion and Education Departmental Scholarship University of Utah May 1998

Eta Sigma Gamma Health Education Honor Society Chi Chapter Research Vice President University of Utah 1998-1999


Publications:


Watterson, T. L., Sorenson, J. Martin, R. S., Coulombe, R. A. (2006b). Effects of PM$_{2.5}$ collected from Cache Valley Utah in Human Bronchial Epithelial Cells. *In 45th Annual Meeting of the Society of Toxicology. Society of Toxicology, San Diego, California.*


**Training:**

Insect Cell Culture and Baculovirus Expression Vector Systems Utah State University.

**Other Experience**

Mountain West Society of Toxicology Student Advisor 2005-2007
Designed and implemented the website for the Mountain West section of the Society of toxicology.


Underground Storage Tank Division.
Assisted in database upgrade evaluations, assisted in reworking certification tests and utilized statewide database to generate compliance reports in a regulatory setting.

Volunteer: St, Mark’s Hospital, Senior Health Center. 1994 – 1999.
Created and updated a database involving frequently prescribed drugs, the drugs’ manufacturers, and the information needed to enroll patients in the manufacturers’ indigent patient assistance programs. Worked with medical director and social worker to disseminate the database to other senior care centers.