Published in final edited form as:

Toxicol Appl Pharmacol. 2013 January 1; 266(1): 48–55. doi:10.1016/j.taap.2012.10.017.

Cell-Specific Oxidative Stress and Cytotoxicity after Wildfire Coarse Particulate Matter Instillation into Mouse Lung

Keisha M. Williams*, Lisa M. Franzi, and Jerold A. Last

Pulmonary, Critical Care, and Sleep Medicine, School of Medicine, University of California, Davis, CA

Abstract

Our previous work has shown that coarse particulate matter (PM_{10-2.5}) from wildfire smoke is more toxic to lung macrophages on an equal dose (by mass) basis than coarse PM isolated from normal ambient air, as evidenced by decreased numbers of macrophages in lung lavage fluid 6 and 24 hours after PM instillation into mouse lungs in vivo and by cytotoxicity to a macrophage cell line observed directly in vitro. We hypothesized that pulmonary macrophages from mice instilled with wildfire coarse PM would undergo more cytotoxicity than macrophages from controls, and that there would be an increase in oxidative stress in their lungs. Cytotoxicity was quantified as decreased viable macrophages and increased percentages of dead macrophages in the bronchoalveolar lavage fluid (BALF) of mice instilled with wildfire coarse PM. At one hour after PM instillation, we observed both decreased numbers of viable macrophages and increased dead macrophage percentages as compared to controls. An increase in free isoprostanes, an indicator of oxidative stress, from control values of 28.1 ± 3.2 pg/mL to 83.9 ± 12.2 pg/mL was observed a half-hour after PM instillation. By one hour after PM instillation, isoprostane values had returned to 30.4 ± 7.6 pg/mL, not significantly different from control concentrations. Lung sections from mice instilled with wildfire coarse PM showed rapid Clara cell responses, with decreased intracellular staining for the Clara cell secretory protein CCSP 1 hour after wildfire PM instillation. In conclusion, very rapid cytotoxicity occurs in pulmonary macrophages and oxidative stress responses are seen 0.5–1 hour after wildfire coarse PM instillation. These results define early cellular and biochemical events occurring in vivo and support the hypothesis that oxidative stress-mediated macrophage toxicity plays a key role in the initial response of the mouse lung to wildfire PM exposure.

Keywords

PM_{10-2.5}; macrophage; TNF- α ; CCSP; isoprostanes; Clara cells

© 2012 Elsevier Inc. All rights reserved.

Correspondence: Jerold Last; Center for Comparative Respiratory Biology and Medicine, University of California; 451 Health Sciences Drive, Davis, CA, 95616; telephone: 530-752-6230; fax: 530-752-8632; jalast@ucdavis.edu.

*Present Address: Department of Pathology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, Lansing, MI 48824

Conflict of interest statement: The authors have no conflicts of interest to declare.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Thousands of wildfires occur annually in California (California Department of Forestry and Fire Protection, 2008), with increases predicted in the future due to global climate change (Spracklen et al., 2008). Among the deleterious impacts of wildfires are the health effects associated with acute smoke inhalation (Shusterman et al., 1993; Künzli et al., 2006; Sharip et al., 2008; Delfino et al., 2009; Weinhold, 2011). Shusterman et al. (1993) found smoke inhalation caused over 50% of the local emergency room visits related to the 1991 Oakland area wildfires, while only 1% of the emergency room visits were burn-related. Thus, pulmonary toxicity is the major health concern after acute exposure to wildfire smoke.

In late June of 2008, several wildfires burned throughout California's central and northern regions. During this time period, poor air quality from wildfire smoke was measured and recorded throughout the highly dispersed network of air monitoring stations in California. Average atmospheric concentrations of particulate matter less than 10 micrometers in diameter (PM_{10-2.5}) were reported to be more than 4 times normal concentrations. The daily average ambient PM_{10-2.5} concentrations recorded for the weeks immediately before and after the wildfire episode were approximately 24 $\mu\text{g}/\text{m}^3$, as compared to an average PM_{10-2.5} concentration of 99 $\mu\text{g}/\text{m}^3$, and peak hourly concentrations of 381 $\mu\text{g}/\text{m}^3$, during the time of the wildfires (Wegesser et al., 2009) (California EPA Air Resources Board, 2008; California Department of Forestry and Fire Protection, 2008).

Our research group collected PM samples from the smoke-laden air in the San Joaquin Valley during the major 2008 wildfire episode. We subsequently used these samples to study pulmonary effects of wildfire smoke PM with a mouse bioassay (Wegesser et al., 2009; Wegesser et al., 2010), finding an unexpected decrease in macrophages recovered from the lung via bronchoalveolar lavage after exposure to coarse and fine wildfire PM as compared to control animals. Furthermore, as compared to the effects of coarse PM collected from ambient air in the same region at times when the wildfires were not occurring, there was also a significant decrease in the number of macrophages recovered in the bronchoalveolar lavage fluid (BALF) after instillation of wildfire PM_{10-2.5}. We hypothesized in these early studies that a rapid killing of macrophages occurred after phagocytosis of wildfire PM, but recognized that we could not rule out the alternative hypothesis that macrophages that had ingested wildfire PM adhered more to the lung, and were less easily washed out from the lung by the lavage procedure.

Our early studies also demonstrated decreased total antioxidant concentrations in the lung lavage fluid from mice exposed to wildfire PM, as compared to control animals or to mice treated with the same dose and size of PM isolated from normal ambient air, suggesting a role for oxidant stress in the toxicity of these particles to lung macrophages (Wegesser et al., 2010). Subsequent mechanistic studies in vitro with the coarse fraction of wildfire PM demonstrated direct toxicity of wildfire PM to the RAW 264.7 macrophage cell line (Franzi et al., 2011). We found increased cell death and biomarkers of oxidative stress after wildfire PM incubation with RAW 264.7 cells as compared to coarse PM collected from the same region when wildfires were not occurring (Franzi et al., 2011). Using a strain of RAW 264.7 cells containing a reporter gene for NF- κ B activation, we were able to demonstrate increased NF- κ B expression in macrophages exposed to wildfire PM (Franzi et al., 2011) as compared to macrophages exposed to an equal dose of "normal" PM isolated from ambient air from the same geographical region.

Thus, our previous studies showed a decrease in numbers of macrophages recovered from BALF after wildfire coarse PM instillation in vivo, direct macrophage toxicity after exposure to wildfire coarse PM in vitro, greater toxicity of wildfire PM on an equal dose

basis as compared to both ambient regional coarse PM and vehicle controls, and increased NF- κ B activation in cultured macrophages exposed to wildfire PM as compared to in macrophages exposed to an equal dose of “normal” ambient air PM.

Our previous studies with PM collected from ambient air obtained from the same region from which we collected the wildfire PM examined time points between 3 and 72 hours after PM instillation, a time interval that allowed for relatively large numbers of neutrophils to participate in the lung’s inflammatory response to particles (Wegesser and Last, 2008). In the present study we examined the effects of wildfire PM instilled into mouse lungs at very early time points, 0.5 and 1 hour after administration, an interval when only macrophages are observed in the lung lavage fluid. This experimental design allowed us to examine macrophage cytotoxicity in vivo in lungs under experimental conditions where the role of neutrophil recruitment and neutrophilic inflammation was not yet a confounding variable. To the best of our knowledge, there are no previously published studies of the effects of wildfire PM in vivo at time points prior to six hours after exposure by inhalation or instillation. Most previous investigations of the effects of wildfire PM have typically focused on time points of 24 hours after instillation whether performed in vivo (Wegesser et al., 2009) or in cultured cells (Jalava et al., 2006). We are aware of only one published study in vivo (Wegesser et al., 2010) that examined the effects of wildfire PM exposure of mice as early as six hours after PM exposure and only one such study in vitro (Nakayama Wong et al., 2011) that has examined the effects of wildfire PM exposure upon cultured cells as early as three hours after PM exposure.

In addition, we directly addressed the previously unanswered question of whether the low numbers of macrophages recovered in the lung lavage fluid from mice instilled with wildfire PM was due to cytotoxicity to the macrophages in vivo or to “increased stickiness”, whereby macrophages that had ingested wildfire PM adhered more to the lung, and were less easily washed out from the lung by the lavage procedure.

MATERIALS AND METHODS

Animals

Male, 8-10 week old, Balb/C mice were purchased from Charles River Breeding Laboratories (Hollister, CA or Raleigh, NC), and were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility. The mice were allowed to acclimate for one week after arrival at the facility. A 12-hour light/12 hour dark cycle was used and the mice had free access to food and water. All experiments were performed under protocols using experimental procedures and anesthesia and euthanasia methods approved by the UC Davis Institutional Animal Care and Use Committee. N values, the number of animals in each group tested, are indicated in the various Figures.

PM sampling and instillation

Coarse PM with an aerodynamic diameter between 10.2 and 2.1 μ m (PM_{10-2.5}) was collected during the summer of 2008 in Escalon, CA using a high-volume sampler (Model GS2310; Anderson, Inc.) and a four-stage cascade impactor (Series 230; Anderson, Inc.); aluminum substrates were used on each stage (Wegesser et al., 2009). Samples were removed from the aluminum substrates, sealed in 2mL Eppendorf tubes, and stored at -80°C . This specific PM sample has previously been characterized for its polycyclic aromatic hydrocarbon (PAH) content (Wegesser et al., 2010), and also found to contain $<1\text{U}$ of endotoxin/50 μ g of coarse PM endotoxin by Limulus amoebocyte lysate (LAL) assay (Wegesser et al., 2009; Wegesser et al., 2010).

PM was suspended in phosphate buffered saline (PBS) thirty minutes before experimentation. The intratracheal (IT) instillation method used has been described previously (Wegesser and Last, 2008). Briefly, the mice were lightly anesthetized in an enclosed chamber via isoflurane inhalation from a cotton ball soaked in the anesthetic. While the mice were still anesthetized, 100 μ g (or less, as indicated in the specific figure legend) of suspended coarse PM in 50 μ L of PBS, or 50 μ L of PBS alone, was instilled into the animals.

Pulmonary sample collection

Necropsies were performed 0.5-1 hour after PM instillation. Each mouse was euthanized with a 0.3mL intraperitoneal injection of a pentobarbital and phenytoin drug formulation (Beuthanasia). The trachea was cannulated, and the lung was lavaged twice (each) with 1 mL volumes of sterile PBS to collect a total of 2 mL of BALF. BALF samples were centrifuged at 2500 rpm for 10 minutes and the supernatant was collected. The cell pellet was re-suspended in a red blood cell lysis solution, centrifuged again for 10 minutes at 2500 rpm, and finally re-suspended in 500 μ L of PBS. BALF samples that were not used immediately for cell counting were stored in an ultra-cold (-80°C) freezer for later use. The lung tissue was fixed by tracheal perfusion with 1% paraformaldehyde in PBS (PFA) under 25 cm of pressure. The lungs were subsequently embedded in paraffin, with care taken to embed the tissue with the large conducting airways parallel to the front of the tissue block (Kenyon and Last, 2005) and cut into 5- μ m thick sections. Paraffin-embedded lungs were stored at room temperature. In some experiments the lungs were fixed via the vasculature as described below using a method developed by Pino et al. (1992). Due to the extremely limited amount of wildfire PM available for these experiments, most of the reported results are from mice whose lungs were lavaged to determine inflammatory cell content. A small subset of mice was studied whose lungs were not lavaged to allow for accurate quantitation of macrophage cell numbers in the airways of the vascularly perfused lungs.

Vascular perfusion

After mice were euthanized, the chest cavity was opened to expose the heart and sutures were tied to the descending aorta and the inferior vena cava. A needle from an infusion set was inserted into the right ventricle, and a cut was made to both the inferior vena cava and descending aorta above the ligature to allow exit flow for solutions. A 60-mL syringe, holding 30 mL of PBS, was connected to plastic tubing fitted with a three-way stopcock. The stopcock was attached to the infusion set allowing the PBS to be slowly injected into the cardiopulmonary system. The stopcock lever was then switched to allow PFA to perfuse into the cardiopulmonary system. The PFA solution was allowed to flow via gravity under 25 cm of pressure for 10-15 minutes. The lungs and heart were then excised from the chest cavity, stored overnight at 4°C , then placed in 70% ethanol until they were embedded as described above.

Quantification of macrophage cells

Macrophages in BALF—Total cell numbers in the BALF were counted using a hemocytometer and viability was assessed using the Trypan blue exclusion technique. Trypan blue solution was purchased from Sigma (St. Louis, MO). Percentages of each inflammatory cell type in the BALF were determined from cytospin preparations that were differentially stained with the Hema 3 staining kit from Fisher Scientific (Kalamazoo, MI). The absolute macrophage content in the BALF was estimated from the total live cell count multiplied by the fraction of cells identified to be macrophages based upon the differential cell counts.

Macrophages in lung sections—For immunohistochemistry, a 5- μ m lung section from each animal was stained against the F4/80 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution of the antibody and visualized via DAB precipitation using the Cell and Tissue Staining Kit for the rat secondary antibody (R&D systems, Minneapolis, MN). All positive staining cells in each lung section were viewed and counted using light microscopy on a Zeiss AxioSkop 50 microscope. Axiovision Rel. 4.7 software was used for imaging.

Determination of oxidative stress

Free isoprostanes in BALF supernatant—The 8-Isoprostane Enzyme-Linked Immunosorbent Assay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI) was used to quantify free isoprostane concentrations in the BALF supernatant. The assay was performed as detailed in the kit manual, with a detection limit of approximately 2.7 pg/mL. Absorbance values were read at 405 nm using an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, California). Graphpad Prism 5 software (Graphpad software, San Diego, CA) was used to generate the standard curve from the standard concentrations and their corresponding absorbance value then used to interpolate sample concentrations based on the standard curve.

Intracellular Clara Cell Secretory Protein (CCSP) analysis—5- μ m thick lung sections were stained with an anti-CCSP antibody (Biovend, Candler, NC), a rabbit antiserum containing polyclonal IgGs raised against a peptide antigen from the CCSP sequence, at a 1:2000 or 1:2500 dilution of the antibody and visualized via DAB precipitation using the ABC immunoperoxidase kit for the rabbit secondary antibody (Vector Labs, Burlingame, CA). Controls were run with each assay, and included no primary antibody or no secondary antibody added. Five airways were selected for counting in each lung section by using a random number generator. General size and location of the conducting airways was noted. These airways were then imaged with Axiovision Rel. 4.7 software using light microscopy with the Zeiss AxioSkop 50 microscope (Carl Zeiss Inc, Jena, Germany). Staining intensity was scored on a semi-quantitative scale from 0-3, where 0 = no brown staining and 3 = dark brown staining. Each field was scored with consideration for the average intensity in the airway epithelium, and the average score for the five fields of view was then taken. Two different observers, each blinded to the lung sample source, scored each lung tissue sample. The average of these two scores was used for statistical analysis.

TNF- α analysis in BALF—We used a commercial ELISA kit (R and D systems, Minneapolis, MN) to measure TNF- α concentrations in the BALF. The limit of detection for mouse TNF- α ranged from 0.4-7.2 pg/mL, with a mean value of 1.9 pg/mL. Absorbance values were read at 450 nm with a correction wavelength set at 540 nm using an Emax Precision Microplate Reader and corresponding Softmax Pro 3.1.2 microplate analysis software (Molecular Devices, Sunnyvale, California).

Statistical Analysis

Graphpad Prism software was used for statistical analysis. Results are reported as mean $\pm$ standard error of the mean (SEM). Outliers in treatment groups (values greater than 2 standard deviations from the mean) were discarded from the analysis. When multiple comparisons were made, one-way ANOVA was used with Tukey's post hoc test. When comparisons were made between only two treatment groups, Student's t-test was used. Welch's correction for unequal variances between groups was used when significant differences between group variances were found. To evaluate differences between treatment groups that had been scored using a semi-quantitative rubric for CCSP staining experiments,

the Mann Whitney non-parametric test was used. Statistical significance was taken as a p value <0.05.

RESULTS

Effect of wildfire PM_{10-2.5} on Macrophages 0.5 and 1 hour after instillation

There was a significant decrease ($p < 0.01$) to less than half of the control values in the number of macrophages recovered in the BALF at 0.5 and one hour after wildfire PM instillation (Figure 1). At these relatively short time points with regard to the lung inflammatory response, the BALF cell population is comprised of more than 97% pulmonary macrophages, the same value that is observed in control animals (Figure 1). The macrophage-dominant cellular composition of the BALF at these short time points allows us to address macrophage-specific oxidative stress and cytotoxicity in these in vivo studies. At later time points (in our hands as early as 2 hours after PM instillation, data not shown) there is a massive influx of neutrophils into the lung lavage fluid (Wegesser and Last, 2008), which would complicate the interpretation of assays performed on the BALF cell population.

To rule out the possibility that the low recovery of macrophages in the BALF was the result of the activated macrophages being more difficult to lavage from the lungs under the protocol we used, we counted the number of pulmonary macrophages in the lungs of mice that had not been lavaged to recover cells from the airway lumen. In these specific experiments we fixed the lungs via the vasculature to avoid potential artifacts caused by washing the airways with fixative. There were significantly ($p = 0.0045$) lower numbers of macrophages in the lungs after PM instillation, about half of the number found in the control mice, as shown in Figure 2. The agreement between the relative macrophage counts in the fixed lungs and the number of macrophages recovered in the BALF is striking. Note also the relatively larger number of macrophages in a representative section of the airway lumen from a PBS-instilled mouse as compared to a mouse instilled with the wildfire PM (Figure 2, insets A versus B).

Cytotoxicity of wildfire PM

To further confirm that the decrease in macrophages in the BALF of mice instilled 0.5 or 1 hour previously with wildfire PM was a result of killing of the macrophages by the particles we examined the number of dead cells (determined by Trypan-Blue uptake) in the BALF. As shown in Figure 3, there was a significant increase in the percentage of dead cells, presumptively macrophages, in the lavage fluid obtained from the mice exposed to wildfire PM one hour previously as compared with PBS-instilled mice ($p < 0.01$). As dead cells and cell debris would be rapidly cleared from the lungs by mucociliary clearance and by decomposition of dead (and dying) cells to structures that would not be scored by Trypan Blue staining, this analysis almost certainly underestimates the total number of dead macrophages in the BALF.

Oxidative stress

We hypothesized that the cytotoxicity of the wildfire PM was related to its ability to cause oxidative stress in the lung. Therefore, we measured isoprostane concentrations in the BALF as a biomarker of lipid peroxidation in the lungs of mice instilled with wildfire PM. At the 0.5-hr time point, we observed a three-fold increase in free isoprostanes in the BALF supernatant of mice instilled with wildfire coarse PM as compared to controls, $p < 0.001$ (Figure 4). It is noteworthy that the concentration of free isoprostanes had returned to control values in the BALF from mice instilled 1 hour previously with wildfire PM (Figure 4), suggesting that the lipid peroxidation events that gives rise to the free isoprostanes in the

lavage fluid occurs very rapidly after PM instillation but either does not continue thereafter or is counteracted by one or more antioxidant defense mechanisms induced in the lung.

To test the hypothesis that the rapid decrease in isoprostanes in the BALF between 0.5 and 1 hour after PM instillation was at least partially due to their removal by antioxidant defenses elaborated by the lung epithelial cells, we examined specific antioxidant release from the airway epithelial Clara cell population by measuring whether there was decreased intracellular CCSP staining, an indirect measure of CCSP release. As shown in Figure 5, there was decreased staining intensity for CCSP in Clara cells one hour after wildfire coarse PM instillation as compared to the PBS vehicle control ($p = 0.0253$). Representative lung sections from a proximal airway from a control and PM-instilled mouse are shown in the insets (Figure 5, A and B). This observed decrease in staining was uniform throughout the conducting airways (i.e. proximal bronchial regions to terminal bronchiole regions).

To complement the results presented herein, we attempted to examine other potential biomarkers of oxidant stress, cytotoxicity, and cellular damage. Additional assays were performed to measure protein, total antioxidant capacity, TUNEL staining, and LDH content in the BALF to try to further quantify oxidative stress and cellular damage in the lungs of the mice instilled with wildfire PM10-2.5. No significant differences were observed with any of these assays (data not shown).

We also examined the concentration of TNF- α , a cytokine known to appear very early in lung injury in rodent models (Lee and Yang, 2012), in the BALF from our PM-instilled mice. We compared time-matched PBS-instilled control mice with wildfire PM-instilled animals at 0.5 and 1 hour after PM administration (Figure 6). The PM-instilled mice contained 321 ± 31 pg/mL of TNF- α in their BALF ($p < 0.001$ versus PBS controls = 0) after 0.5 hours, and 883 ± 96 pg/mL ($p < 0.001$ versus PBS controls = 132 ± 110) after 1 hour. We often see slightly elevated, but highly variable, increases in biomarkers of inflammation in BALF at the 1-hour time point in our experiments. The concentration of TNF- α in the BALF was significantly higher at the 1-hour time point than at 0.5 hour, $p < 0.01$.

DISCUSSION

Most of the experiments reported in this paper were performed several years after collection of the wildfire PM (in 2008) that we studied here. Only enough of the coarse fraction was still available to allow us to perform in vivo studies. Thus, comparative studies with fine or ultrafine PM fractions are not presented in this paper. To specifically examine the cytotoxic effects of wildfire PM10-2.5 on the pulmonary macrophage population in vivo, we focused our experiments on the 0.5- and 1-hour time intervals between PM instillation and necropsy, a time period prior to measurable neutrophilic influx. Previous studies by Van Oud Alblas and Van Furth (1979) have indicated that mouse monocytes do not develop into macrophages in this time span. Therefore, we can assume that the macrophage cell population we examined during this short time period after wildfire PM instillation is the resident pulmonary macrophage population that was present at the moment of PM instillation. The decrease in macrophages found at this time period (Figures 1 and 2) in the lavage fluid, as well as in the lungs, supports our previous findings at 24 hours after instillation of wildfire PM (Wegesser et al., 2009). Furthermore, these acute decreases in this particular cell population suggest that pulmonary macrophages may be a specific target of wildfire PM toxicity.

We have previously shown that wildfire coarse PM is more cytotoxic to macrophage cell cultures than urban coarse PM, collected with the same high-volume sampler under similar conditions, on an equal mass basis (Franzi et al., 2011). Here, we demonstrate that specific

cytotoxicity to lung macrophages is also evident in a whole animal instilled intratracheally with the same PM preparation (Figure 3). While specific cell types cannot be determined from the viable cell count using the Trypan Blue exclusion technique, at the 1-hour time point the macrophage population represents the vast majority (>97%) of the cells identified in the BALF. Other cell types present in both wildfire PM- and saline-instilled mice are seen at such low percentages that it is highly unlikely that they could contribute significantly to the increase in dead cells. We conclude that macrophage-specific toxicity is occurring at the 1-hour time point, with a trend towards an increased percentage of dead macrophages in the BALF at 0.5 hour.

In earlier studies we were not able to rule out the alternative explanation of similar findings of decreased recovery of macrophages in lung lavage fluid 24 hours after wildfire PM instillation as being due to enhanced adherence of macrophages to alveolar (and other airway epithelial cell) surfaces (Wegesser et al., 2009). In the present study we counted resident macrophages in the unlavaged lungs of vascularly perfused mice after exposure to wildfire PM using a macrophage-specific antibody to selectively visualize the macrophages in situ, and compared the total number of tissue macrophages present with comparable values from mice instilled with physiological saline. As shown in Figure 2, there were less macrophages in the lung tissue of the wildfire-exposed mice than their matched controls, proving that the decreased yield of macrophages in the lung lavage was not due to increased adherence of macrophages to airway surfaces after exposure to wildfire PM.

Macrophage cytotoxicity has previously been shown to occur in studies of cultured cells exposed to PM from other sources than wildfires (Chan et al., 2011; Gerlofs-Nijland et al., 2007; Kubatova et al., 2006; Li et al., 2002). Acute effects of particles prepared from various sources on cultured cells of various types have become a focus of study within the past decade, and measures of oxidative stress and toxicity have been shown to change within several hours after PM exposure (Ayes et al., 2008; Chan et al., 2011; Goto et al., 2004a; Goto et al., 2004b; Kaewamatawong et al., 2005; Mills et al., 2007; Nemmar et al., 2003). However, it is difficult to extrapolate results across studies that use different doses and types of particles of different sizes with different chemical compositions arising from different sources.

We have also previously shown that wildfire coarse PM is a much more potent inducer on an equal mass basis of expression of an NF- κ B-driven reporter gene in macrophage cell cultures than is coarse PM from the same region, collected with the same high-volume sampler under similar conditions (Franzi et al., 2011). Thus, there was a correlation between macrophage cytotoxicity and elicitation of oxidative stress by wildfire PM in these in vitro experiments. We looked for a corresponding biomarker of oxidative stress in the current studies to determine whether a similar correlation occurred in vivo in our mouse model exposed to wildfire PM.

The increased free isoprostanes measured in the BALF supernatant samples of mice instilled with wildfire PM is indicative of oxidative stress (Figure 4). More specifically, the increased concentration of this lipid peroxidation marker in the lung lavage fluid suggests that cell membrane integrity of the target cells may be affected. The temporal progression of oxidative stress, as indicated by the increased isoprostanes seen at the 0.5-hour time point, followed by cell death and decreased macrophages at the 1-hour time point, supports the hypothesis that oxidative stress in macrophages that have ingested wildfire PM is the mechanism of cytotoxicity, as the pulmonary macrophages would have been the first line of defense against the instilled wildfire particles. Our previous results using a RAW 264.7 macrophage cell line that was modified with a NF- κ B-induced reporter gene has shown increased NF- κ B activation after exposure to wildfire PM as compared to ambient air PM.

Taken together, the increased concentration of lipid peroxidation products shown in the present study identifies early wildfire PM-induced oxidative stress effects in vivo that would lead to NF- κ B activation.

How might oxidative stress be linked to lung inflammation in our experiments? In the present study we found that TNF- α levels were significantly elevated in mice instilled with 100 μ g of coarse wildfire PM 0.5 and 1 hour after PM instillation. Our results point to TNF- α as a significant cytokine involved in the mouse lung inflammatory response to wildfire PM. Jalava et al. (2006) reported slightly decreased levels of TNF- α in RAW264.7 macrophage cells exposed for 24 h to wildfire coarse or fine PM as compared to seasonal average PM; however, cytokine levels were increased significantly in macrophages exposed to a mixture of seasonal average and wildfire PM. The difference in TNF- α levels we report in Figure 5 as compared to the results reported by Jalava et al. (2006) for RAW264.7 macrophage cells may be due to in vivo versus in vitro differences, timing of measurements, or differences in the specific PM preparations evaluated. We should also note that the use of in vitro assays, although quite useful for mechanistic studies, do not account for other inflammatory cells and cytokine/chemokine influences that are almost certainly present in the lung during an inflammatory insult. Happonen et al. (2008) and Wegesser et al. (2010) previously showed elevated TNF- α levels in lung lavage fluid collected 4 hours or 6 hours, respectively, after instillation of coarse PM into mouse lungs.

TNF- α gene expression is regulated by NF- κ B (Shukla et al., 2000) in lungs of mice exposed to PM. TNF- α activates expression of the KC gene (KC is the mouse homologue of IL-8), which in turn results in neutrophil recruitment to the lung (Jimenez et al., 2002). We have previously reported increased concentrations of TNF- α and of KC in the lung lavage fluid supernatant 6 hours after wildfire PM instillation into mouse lungs (Wegesser et al., 2010). We have also observed elevated levels of other neutrophil chemokines in the lavage fluid in these earlier experiments, including MIP 1 α (Wegesser et al., 2010), a potent neutrophil chemoattractant. Thus we suggest that the very early pathogenetic sequence occurring in our mice instilled with coarse wildfire PM is phagocytosis of the PM by macrophages followed by activation of NF- κ B leading to transcription of genes that lead to rapid death of the macrophage and/or secretion of neutrophil-attracting chemokines that promote rapid (within 2 hours of exposure to PM) neutrophilic inflammation in the lungs.

One major advantage of the whole animal model compared to the in vitro system we used previously to study wildfire PM effects (Franzi et al., 2011) is that we can also begin to investigate the lung responses by other pulmonary cell types. Our present results show decreased immunohistochemical staining for intracellular CCSP after wildfire PM instillation. Since demonstrable epithelial sloughing is not occurring, we believe that this is an indirect measure of CCSP release into the airway lumen. CCSP has been shown to have antioxidant properties and is a recognized response of a biomarker to oxidative stress (Mango et al., 1998).

Clara cells are a particularly important cell group to study in regards to inhaled coarse PM effects on airway epithelial cells, because this cell population is located in an anatomical target region in humans for this size of PM (Lippmann and Schlesinger, 1984). Clara cells are also the major sites of xenobiotic metabolism in the lung epithelium. In vitro studies in human bronchial epithelial cell cultures exposed to the fine fraction of our preparation of wildfire PM have shown significant up-regulation of gene expression of CYP1B1, a Phase I metabolic enzyme (Nakayama Wong et al., 2011). Furthermore, there is significant cross talk between Clara cells and pulmonary macrophages, specifically with regard to CCSP release as an anti-inflammatory mediator after pulmonary insults (Synder et al., 2010). Our finding of increased CCSP secretion by Clara cells one hour after PM instillation suggests

that very rapid macrophage-Clara cell cross talk is occurring in the mice instilled with wildfire PM.

In summary, our studies reported here confirm and extend our previous in vitro and in vivo observations of wildfire PM-induced macrophage cytotoxicity. Wildfire PM-induced oxidative stress occurs very rapidly after lung exposure to the particles and may cause some or all of the observed toxicity to macrophages. Significant biological responses from the airway epithelium occur concurrently and may be part of the lung's defensive response to oxidative stress. Neutrophil-attracting chemokines also appear very rapidly in the lung lavage fluid, and are probably elaborated by macrophages and/or airway epithelial cells directly in response to PM-induced oxidative stress.

Acknowledgments

The authors thank Dr. Teresa Wegesser for particle sample collection and advice; Dr. Nicholas Kenyon for advice; Dr. Jennifer Bratt for her assistance during tissue sample collection; Dr. Laura Van Winkle, Don Anderson and Shannon Murphy for assistance with CCSP staining; and Trio for support. This study was partially supported by funding from the NIH training grant HL-07013, and from the University of California Toxic Substances Research and Teaching Program through the Atmospheric Aerosols and Health UC Davis Lead Campus Program.

REFERENCES

- Ayres JG, Borm P, Cassee FR, Castranova V, Donaldson K, Ghio A, Harrison RM, Hider R, Kelly F, Kooter IM, Marano F, Maynard RL, Mudway I, Nel A, Sioutas C, Smith S, Baeza-Squiban A, Cho A, Duggan S, Froines J. Evaluating the toxicity of airborne particulate matter and nanoparticles by measuring oxidative stress potential—A workshop report and consensus statement. *Inhalation Toxicol.* 2008; 20:75–99.
- California Environmental Protection Agency Air Resources Board. [Accessed 11 September 2012] Weekly Listing: National 24-Hour PM10 Averages. 2008. Available from <http://www.arb.ca.gov/adam/weekly/weeklydisplay.php>
- California Department of Forestry and Fire Protection. [accessed 11 September 2012] 2008 Fire summary. 2008. Available from <http://www.fire.ca.gov/downloads/redbooks/2008/02-wildland-statistic-all-agencies/11-2008-FIRE-SUMMARY.pdf>
- Chan JKW, Fanucchi MV, Anderson DS, Abid AD, Dickinson DA, Kumfer BM, Kennedy IM, Wexler AS, Van Winkle LS. Susceptibility to inhaled flame-generated ultrafine soot in neonatal and adult rat lungs. *Toxicol. Sci.* 2011; 124:472–486. [PubMed: 21914721]
- Delfino RJ, Brummel S, Wu J, Stern H, Ostro B, Lipsett M, Winer A, Street DH, Zhang L, Tjoal T, Gillen DL. The relationship of respiratory and cardiovascular hospital admissions to the southern California wildfires of 2003. *Occupat. Environ. Med.* 2009; 66:189–197.
- Franzi, L.M, Bratt, J.M.; Williams, K.M.; Last, J.A. Why is particulate matter produced by wildfires toxic to lung macrophages? *Toxicol. Appl. Pharmacol.* 2011; 257:182–188. [PubMed: 21945489]
- Gerlofs-Nijland ME, Dormans JAMA, Bloemen HJT, Leseman DLAC, Boere AJF, Kelly FJ, Mudway IS, Jienez AA, Donaldson K, Guastadisegni C, Janssen NAH, Brunekreef B, Sandstrom T, van Bree L, Cassee FR. Toxicity of coarse and fine particulate matter from sites with contrasting traffic profiles. *Inhalation Toxicol.* 2007; 19:1055–1069.
- Goto Y, Hogg JC, Shih C, Ishii H, Vincent R, van Eeden SF. Exposure to ambient particles accelerates monocyte release from bone marrow in atherosclerotic rabbits. *Am. J. Physiol: Lung, Cell Mol. Physiol.* 2004a; 287:L79–L85. [PubMed: 15003928]
- Goto Y, Ishii H, Hogg JC, Shih C, Yatera K, Vincent R, van Eeden SF. Particulate matter air pollution stimulates monocyte release from the bone marrow. *Am. J. Respirat. Crit. Care Med.* 2004b; 170:891–897. [PubMed: 15256391]
- Happo MS, Hirvonen MR, Halinen AI, Jalava PI, Pennanen AS, Sillanpaa M, Hillamo R, Salonen RO. Chemical compositions responsible for inflammation and tissue damage in the mouse lung by coarse and fine particulate samples from contrasting air pollution in Europe. *Inhal Toxicol.* 2008; 20:1215–1231. [PubMed: 18855153]

- Jalava PI, Salonen RO, Halinen AI, Penttinen P, Pennanen AS, Sillanpaa M, Sandell E, Hillamo R, Hirvonen M. In vitro inflammatory and cytotoxic effects of size-segregated particulate samples collected during long-range transport of wildfire smoke to Helsinki. *Toxicol. Appl. Pharmacol.* 2006; 215:341–353. [PubMed: 16678872]
- Jiménez LA, Drost EM, Gilmour PS, Rahman I, Antonicelli F, Ritchie H, MacNee W, Donaldson K. PM(10)-exposed macrophages stimulate a proinflammatory response in lung epithelial cells via TNF-alpha. *Am J Physiol Lung Cell Mol Physiol.* 2002; 282:L237–248. [PubMed: 11792628]
- Kaewamatawong T, Kawamura N, Okajima M, Sawada M, Morita T, Shimada A. Acute pulmonary toxicity caused by exposure to colloidal silica: Particle size dependent pathological changes in mice. *Toxicol. Pathol.* 2005; 33:745–751.
- Kenyon NJ, Last JA. Reversible and irreversible airway inflammation and fibrosis in mice exposed to inhaled ovalbumin. *Inflamm Res.* 2005; 54:57–65. [PubMed: 15750712]
- Kubatova A, Dronen LC, Picklo MJ, Hawthorne SB. Midpolarity and nonpolar wood smoke particulate matter fractions deplete glutathione in RAW 264.7 macrophages. *Chem. Res. Toxicol.* 2006; 19:255–261. [PubMed: 16485901]
- Kunzli N, Avol E, Wu J, Gauderman WJ, Rappaport E, Millstein J, Bennion J, McConnell R, Gilliland FD, Berhane K. Health effects of the 2003 Southern California wildfire on children. *American Journal of Respiratory and Critical Care Medicine.* 2006; 174:1221–1228. [PubMed: 16946126]
- Lee IT, Yang CM. Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced airway and pulmonary diseases. *Biochem Pharmacol.* 2012; 84:581–590. [PubMed: 22587816]
- Li N, Kim S, Wang M, Froines J, Sioutas C, Nel A. Use of stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. *Inhalation Toxicology.* 2002; 14:459–486. [PubMed: 12028803]
- Lippmann M, Schlesinger RB. Interspecies comparisons of particle deposition and mucociliary clearance in tracheobronchial airways. *Journal of Toxicology and Environmental Health.* 1984; 13:441–469. [PubMed: 6376822]
- Mango GW, Johnston CJ, Reynolds SD, Finkelstein JN, Plopper CG, Stripp BR. Clara cell secretory protein deficiency increases oxidant stress response in conducting airways. *Am. J. Physiol: Lung Cell Mol. Physiol.* 1998; 275:L348–L356.
- Mills NL, Törnqvist H, Gonzalez MC, Vink E, Robinson SD, Söderberg S, Boon NA, Donaldson K, Sandström T, Blomberg A, Newby DE. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *New Engl. J. Med.* 2007; 357:1075–1082. [PubMed: 17855668]
- Nakayama Wong LS, Aung HH, Lame MW, Wegesser TC, Wilson DW. Fine particulate matter from urban ambient and wildfire sources from California's San Joaquin Valley initiate differential inflammatory, oxidative stress, and xenobiotic responses in human bronchial epithelial cells. *Toxicol. in Vitro.* 2011; 25:1895–1905. [PubMed: 21703343]
- Nemmar A, Nemery B, Hoet PHM, Vermeylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: Role of histamine. *Am. J. Respirat. Crit. Care Med.* 2003; 168:1366–1372. [PubMed: 12969870]
- Pino MV, Stovall MY, Levin JR, Devlin RB, Koren HS, Hyde DM. Acute ozone-induced lung injury in neutrophil-depleted rats. *Toxicol Appl Pharmacol.* 1992; 114:268–276. [PubMed: 1609419]
- Sharip A, Kajita E, Jones M, Hwang B. Health effects of LA County wildfires in October 2007. *Adv. Dis. Surveillance.* 2008; 5:129.
- Shukla A, Timblin C, Berube K, Gordon T, McKinney W, Driscoll K, Vacek P, Mossman BT. Inhaled particulate matter causes expression of nuclear factor (NF)-kappaB-related genes and oxidant-dependent NF-kappaB activation in vitro. *Am J Respir Cell Mol Biol.* 2000; 23:182–187. [PubMed: 10919984]
- Shusterman D, Kaplan JZ, Canabarro C. Immediate health effects of an urban wildfire. *Western J. Med.* 1993; 158:133–138.
- Snyder J, Reynolds S, Hollingsworth J, Li Z, Kaminski N, Stripp B. Clara cells attenuate the inflammatory response through regulation of macrophage behavior. *Am. J. Respirat. Cell Mol. Biol.* 2010; 42:161–171. [PubMed: 19423773]

- Spracklen DV, Bonn B, Carslaw KS. Boreal forests, aerosols and the impacts on clouds and climate. *Philos Transact A Math Phys Eng Sci.* 2009; 366:4613–4626. [PubMed: 18826917]
- Van Oud Alblas A, Van Furth R. Origin, kinetics, and characteristics of pulmonary macrophages in the normal steady state. *J. Exp. Med.* 1979; 149:1504–1518. [PubMed: 448291]
- Verma V, Polidori A, Schauer JJ, Shafer MM, Cassee FR, Sioutas C. Physicochemical and toxicological profiles of particulate matter in Los Angeles during the October 2007 Southern California wildfires. *Env. Sci. Technol.* 2009; 43:954–960. [PubMed: 19245042]
- Wegesser TC, Pinkerton KE, Last JA. California wildfires of 2008: Coarse and fine particulate matter toxicity. *Environ Health Perspect.* 2009; 117:893–897. [PubMed: 19590679]
- Wegesser TC, Franzi LM, Mitloehner FM, Eiguren-Fernandez A, Last JA. Lung antioxidant and cytokine responses to coarse and fine particulate matter from the great California wildfires of 2008. *Inhalat. Toxicol.* 2010; 22:561–570.
- Wegesser TC, Last JA. Lung response to coarse PM: Bioassay in mice. *Toxicol Appl. Pharmacol.* 2008; 230:159–166. [PubMed: 18384828]
- Weinhold B. Fields and forests in flames. *Env. Health Perspect.* 2011; 6:925–931.

HIGHLIGHTS

1. We studied very early events (0.5-1 hour) after giving wildfire PM10-2.5 to mice
2. Wildfire PM10-2.5 rapidly kills lung macrophages in mice
3. Wildfire PM10-2.5 rapidly elicits oxidative stress in mice
4. Wildfire PM10-2.5 rapidly elicits Clara cell CCSP secretion in mice
5. Wildfire PM10-2.5 rapidly elicits TNF- α secretion into BALF in mice

Figure 1.

Cell content of BALF from mice intratracheally instilled with wildfire PM 0.5 or 1 hour before sacrifice. Macrophages/mL are represented with white bars and neutrophils/mL are represented with gray bars. Age-matched control mice were instilled with 50 μ L of PBS and sacrificed 0.5 and 1 hour after PBS instillation. Sample sizes (N) are indicated in parentheses. Values represent group means $\pm$ SEM. * $p < 0.01$ versus matched control group

Figure 2.

Macrophage cell count in lung sections. A) representative image from PBS-instilled animals that were vascularly perfused as described in the text, and B) representative image from PM-instilled animals. Sample sizes (N) are indicated in parentheses. Arrows indicate F4/80 positive-staining macrophages. Scale bar = 50 μm. C) Cell counts of macrophages that stained positive for F4/80 in lung sections. Values represent group means $\pm$ SEM. * $p = 0.0045$ versus matched control group

Figure 3.

Dead cells in BALF expressed as percentages of total cells recovered. Control mice were instilled with PBS and sacrificed 0.5 and 1 hour after instillation (the two PBS groups were pooled as there was no significant difference between them, $p = 0.39$, t-test). Mice were instilled with coarse wildfire PM and sacrificed 0.5 or 1 hour after instillation. Sample sizes (N) are indicated in parentheses. Values represent group means $\pm$ SEM. * $p < 0.01$ versus matched control group

Figure 4.

Free isoprostane concentrations measured in BALF. The control group is composed of samples from mice instilled with PBS and sacrificed 0.5 and 1 hour after instillation. Treatment groups were instilled with coarse wildfire PM and sacrificed 0.5 or 1 hour after instillation. Sample sizes (N) are indicated in parentheses. Values represent group means $\pm$ SEM. * $p < 0.001$ versus matched control group

Figure 5.

Representative images of CCSP-stained lung sections for each treatment group and the resulting score for staining intensity. A) CCSP staining in airway epithelium sampled from the PBS group and B) CCSP staining in airway epithelium sampled from the PM group. Sample sizes (N) are indicated in parentheses. Scale bar = 50 μm length. C) Semi-quantitative scoring for CCSP staining intensity in airway epithelium. Values represent group means ± SEM. * $p = 0.0253$ versus matched control group

Figure 6.

TNF-a content of BALF from mice instilled with either PM (100 ug) or PBS 0.5 or 1 hour previously. Values represent group means $\pm$ SEM. (N), the number of mice analyzed, is shown in parentheses. * $p < 0.001$ versus matched control groups; ** $p < 0.001$ versus 0.5 hour PM group