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ALLERGIC AIRWAY INFLAMMATION IS DIFFERENTIALLY EXACERBATED BY DAYTIME AND NIGHTTIME ULTRAFINE AND SUBMICRON FINE AMBIENT PARTICLES: HEME OXYGENASE-1 AS AN INDICATOR OF PM-MEDIATED ALLERGIC INFLAMMATION

Christopher M. Carosino[1], Keith J. Bein[1,2], Laurel E. Plummer[1], Alejandro R. Castañeda[1], YongJing Zhao[1,2], Anthony S. Wexler[1,2], Kent E. Pinkerton[1]

[1]Center for Health and the Environment, University of California, Davis, California, USA
[2]Air Quality Research Center, University of California, Davis, California, USA

Ambient particulate matter (PM) originates from a range of sources and differs in composition with respect to season, time of day, and particle size. In this study, ambient PM samples in the ultrafine and submicrometer fine range were tested for the potential to exacerbate a murine model of allergic airway inflammation when exposure occurs solely during allergic sensitization, but not during subsequent allergen challenge. Temporally resolved and size-segregated PM samples were used to understand how summer or winter, day or night, and ambient ultrafine and submicrometer fine particle size influence PM's ability to exacerbate allergic inflammation. PM was collected in urban Fresno, CA. BALB/c mice were exposed to PM and house dust mite allergen (HDM) via intranasal aspiration on d 1, 3, and 5. HDM challenge occurred on d 12–14, with inflammation assessed 24 h following final challenge. While season or particle size did not predict allergic inflammation, daytime ultrafine and submicrometer fine particles significantly increased total cellular inflammation, specifically lymphocyte and eosinophil infiltration, compared to allergic controls. Further studies examined PM-mediated changes within the lung during the period where allergen sensitization occurred by measuring direct effects of PM on pulmonary oxidative stress and inflammation. Pulmonary levels of heme oxygenase-1 (HO-1), a biomarker of oxidative stress, but not cellular inflammation, demonstrated a remarkable correlation with the degree of allergic inflammation in animals sensitized to allergen and PM concomitantly, suggesting acute PM-mediated HO-1 levels may serve as a predictive indicator of a particle's ability to exacerbate allergic airway inflammation.

Epidemiological studies demonstrate correlations between (a) episodes of high ambient particulate matter (PM) and increased asthma exacerbations and (b) proximity to major highways and air pollution levels with increased rates of allergy and asthma in addition to increased risk of cardiopulmonary mortality (Dockery et al., 1993; Kim and Bernstein, 2009; Peterson and Saxon, 1996; Samet et al., 2000; von Klot et al., 2002; Beckerman et al, 2012; Cheng et al, 2014; Tsai and Yang, 2014). Toxicological studies frequently focus on near-freeway conditions and the ultrafine (UF) fraction of PM in urban environments. UF particles collected near heavily trafficked roadways were found capable of eliciting exacerbated allergic airway inflammation (Li et al., 2010). In addition, other investigators utilizing concentrated ambient particles (CAP) to study ambient PM toxicity via inhalation exposure demonstrated that near-freeway particles (50 m) elicit an enhanced ability to exacerbate allergic airway inflammation compared to particles collected at a greater distance (150 m) (Kleinman et al., 2005, 2007).

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Address correspondence to Kent E. Pinkerton, PhD, Center for Health and the Environment, Old Davis Road, University of California, Davis, Davis, CA 95616, USA. E-mail: kepinkerton@ucdavis.edu

Ambient PM, in contrast to PM collected in close proximity to the point of source emissions, undergoes atmospheric transport, mixing, and processing, which greatly alter the chemical composition and size of PM (Brugge et al., 2007). The composition of ambient PM varies by season and time of day due to differences in source strength, activity patterns, atmospheric conditions, and particle chemistry (Bein et al., 2009). These factors make it difficult to identify and regulate the most potent sources and windows of PM exposure responsible for adverse public health effects.

To study how the submicrometer fraction of ambient PM contributes to allergic sensitization and exacerbation in the context of season, time of day, and particle size, a novel source-oriented sampling technique was used to collect and extract submicrometer fine (SM) and UF ambient particles associated with atmospheric processing, within seasonal and temporal sampling periods in the city of Fresno, CA (Bein et al., 2009). Located in the San Joaquin Valley (SJV) air shed, Fresno consistently has some of the highest levels of ambient PM concentrations and some of the highest asthma rates in the state (UCLA Center for Health Policty Research, 2007); one-fifth (20.2%) of all children and adolescents suffer from asthma in Fresno County, in which the city resides, and it is a consistent U.S. Environmental Protection Agency (EPA)-designated nonattainment area for $PM_{2.5}$ (U.S. Environmental Protection Agency, 2009). Since the SJV is one of the largest agricultural areas in California, residents of Fresno are exposed to a combination of urban, agricultural, and rural PM sources, as well as highly processed regional background PM. Due to the geography and topography of the SJV, it is frequently impacted by long-term stagnation events where pollutants are trapped and recirculated by an atmospheric temperature inversion, which tends to increase pollutant concentrations and atmospheric processing.

The aim of this study was to examine how allergic sensitization in a murine model of allergic airway inflammation is affected by exposure to SM or UF PM collected during winter or summer and day or night. BALB/*c* mice were exposed to PM plus house dust mite allergen (HDM) via intranasal aspiration on d 1, 3, and 5. HDM challenge occurred on d 12–14. Endpoints that constitute hallmarks of allergic airway inflammation were examined 24 h following the final allergen challenge (d 15). Current hypotheses of the mechanisms for PM-mediated adjuvant effects revolve around inductions of pulmonary reactive oxygen species (ROS) leading to a hyperpolarized adaptive response (Li et al., 2008). To understand whether PM-induced oxidative stress was present and correlated with the augmentation of subsequent allergen-induced inflammation, levels of heme oxygenase-1 (HO-1) protein, a previously established biomarker of pulmonary ROS (Choi and Alam, 1996), were assayed for the same seasonal and temporal PM samples following a single acute exposure to mimic oxidative stress generated during the period of allergic sensitization.

METHODS

Temporal and Seasonal PM Collection

PM was collected in Fresno, CA, during August 2008 (summer) and January 2009 (winter) for a source-oriented sampling study of SM and UF ambient PM (Bein et al., 2009). Of the samples collected, eight were employed in this study: four summer samples, representing the UF (particle diameter $D_p < 0.17$ μm) and SM ($0.17 < D_p < 1$ μm) fractions of daytime (9:00 a.m. to 5:00 p.m.) and nighttime (12:00 a.m. to 6:00 a.m.), and four winter samples, representing the same size fractions and times of day. Table 1 summarizes the different PM samples, as well as mean aerodynamic diameter sampled and mean diameter of particle distribution in suspension after being extracted from collection substrates and used for animal exposures.

PM was collected with a novel source-oriented sampling technique that directly samples specific sources, source combinations, or predetermined temporal windows from the

TABLE 1. Particle Characteristics

Particle	Airborne particulate size fraction collected	Resuspended extracted particle diameter (DLS)
Summer_Day UF	$D_p < 170$ nm	417 nm
Summer_Night_UF		395 nm
Winter_Day_UF		345 nm
Winter_Night_UF		469 nm
Summer_Day_SM	$170 < D_p < 1{,}000$ nm	417 nm
Summer_Night_SM		451 nm
Winter_Day_SM		270 nm
Winter_Night_SM		543 nm

Note. Particles were collected by ChemVol samplers in the ultrafine (UF; $D_p < 0.17$ μm) and submicrometer fine (SM; $0.17 < D_p < 1$ μm) fractions for daytime and nighttime, summer and winter. The mean diameter for each particle fraction once in suspension was determined by dynamic light scattering (DLS).

ambient mixture into ChemVol (CV) samplers, which are high-flow-rate, impaction-based samplers that collect relatively large amounts of size-segregated PM in short periods of time. SM was collected on polyurethane foam (PUF) substrates, and UF was collected on borosilicate glass microfiber after-filters. PM was extracted from the after-filters and PUF substrates using novel processes designed to maximize particle extraction efficiencies and minimize extraction artifacts, including any potential compositional biases associated with the extraction process. In theory, this technique could be applied to collect larger particulates (coarse); however, this sampling apparatus was designed to sample only SM and UF particulates. Details of the collection and extraction protocols and their application are described elsewhere (Bein et al., 2009).

PM utilized in this study was collected using temporally defined windows to compare day and night PM and to accommodate periods when the source-oriented sampling technique could not be executed. Daytime PM could only be sampled in a temporal manner, as pollutants tended to be so well mixed that it was not possible to sample specific sources with any degree of certainty. It was additionally not possible to consistently discern, or "unmix," the nighttime source mixture, so an additional CV (nighttime CV) was operated during those periods when the sampling algorithms did not recognize any of the predetermined CV source combinations. Since the sampling technique was only able to isolate sources and source combinations during a small fraction of the nocturnal sampling period, the nighttime CV was by far the most frequently operated CV. As a result, it captured the complete nighttime mixture minus a portion of the source combinations assigned to the source-oriented CV in conditions where independent CV sampling was successfully triggered. Therefore, nighttime CV nearly represented what would have been collected by continuously operating a CV during the same hours each night. The daytime mixed layer and nighttime CV, from both the summer 2008 and winter 2009 experiments, were used in the current study (eight distinct particle types).

House Dust Mite Preparation

House dust mite allergen (HDM, Greer Laboratories, Lenoir, NC) was purified to remove endotoxin contamination to levels less than 2 endotoxin units (EU)/dose from stock levels of >1000 EU/dose via multiple passes through Detoxi-Gel (Pierce, Rockford, IL), ReductEtox (Sterogene, Carlsbad, CA) columns, and high-capacity removal spin columns. Removal methods continued until endotoxin levels were sufficiently decreased as determined by kinetic chromogenic *Limulus* amebocyte lysate assay. Total protein was assayed via Lowry assay (Bio-Rad, Hercules, CA) to ensure consistent dosing of 25 μg/dose.

Animals

Eight- to 10-wk-old male BALB/*c* mice, weighing 22.3 ± 1.3 g, were obtained from Harlan Laboratories (Hayward, CA) and allowed to acclimate for 1 wk prior to the onset of the study. Animal housing and experiments were approved by the University of California, Davis, Institutional Animal Care and Use Committee.

Allergic Inflammation Study: Allergen Sensitization and Challenge

Mice were randomly divided into 11 exposure groups (Figure 1): (1) Vehicle/Vehicle, "sham" ($n = 4$); (2) 15 μg PM/Vehicle, "PM control" ($n = 3$); (3) 25 μg HDM/25 μg HDM ($n = 14$, HDM/HDM, "allergic control"); and (4–11) 25 μg HDM + 15 μg PM/25μg HDM ($n = 7$–8, HDM + PM/HDM). Vehicle was sterile Hanks buffered saline solution. The PM sample chosen to serve as a PM control (PM/Vehicle) in the absence of the HDM sensitization/challenge protocol was from the Winter Night UF collection, since it produced the highest pulmonary cellular inflammation of all 8 particle types, as measured by bronchoalveolar lavage (BAL), in the 24-h PM-only studies. These same studies were used to assess the ability of PM to increase the oxidative stress marker HO-1. Our PM dose was selected based upon dose-response pilot studies (unpublished data) to represent a biologically relevant dose approximately equal to an exposure for 1 wk to ambient PM at a concentration of 65 μg/m^3, levels documented over a period of 24 h in Fresno in 2009 (California Environmental Protection Agency Air Resources Board, iADAM, Air Quality Data Statistics, 2009).

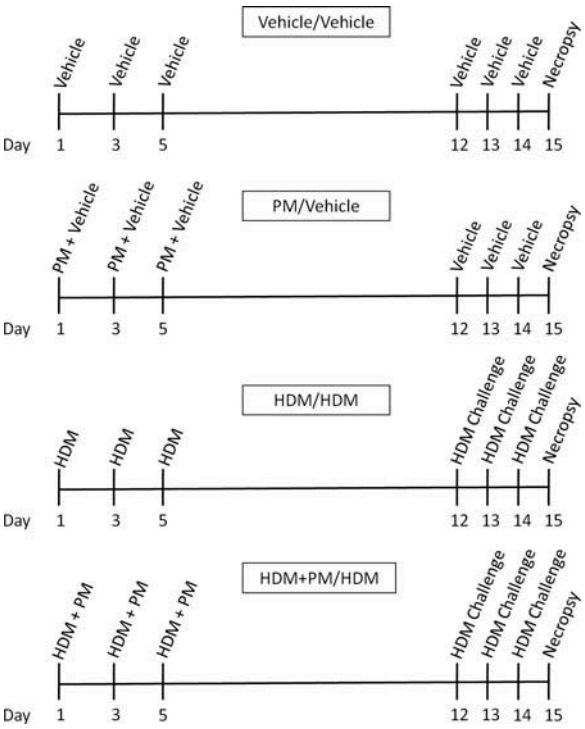

FIGURE 1. Allergen sensitization and challenge exposure regimen. Allergic sensitization to 25 μg house dust mite protein (HDM) occurred via intranasal instillation ± 15 μg PM on d 1, 3, and 5 (total 45 μg dose). Animals were challenged via intranasal instillation with HDM on d 12–14 with necropsy performed on d 15. Exposure scenarios: Vehicle/Vehicle (sham), PM/Vehicle (PM control), HDM/HDM (allergic control), and HDM + PM/HDM.

On d 1, 3, and 5, mice were exposed to vehicle, PM + vehicle, HDM, or HDM + PM via intranasal aspiration (Figure 1). The total PM dose for the 3 d was 45 μg. Mice then had a 1-wk recovery period from dosing. Mice were subsequently challenged on d 12, 13, and 14 to either vehicle or HDM. Each exposure was performed by first putting the animals under anesthesia with isoflurane (2% isoflurane, 1 L/min oxygen). Next, vehicle, PM + vehicle, HDM, or HDM + PM was administered via intranasal aspiration by suspending the solution above the nares and allowing animal inspiration to provide the motive force for aspiration. Animals were sacrificed with an overdose of Beuthanasia-D (Schering-Plough, Whitehouse Station, NJ) on d 15, 24 h post final exposure, and samples were collected.

Blood was collected via cardiac puncture with an ethylenediamine tetraacetic acid (EDTA) prerinsed syringe, put into EDTA-coated microtainer tubes, and centrifuged at 3000 × g for 10 min. Plasma was collected and frozen at −80°C until further use. In addition, all animals were lavaged with two identical volumes of Hanks buffered salt solution with Ca^{2+}/Mg^{2+} (HBSS, pH 7.3, Invitrogen, Carlsbad, CA) to determine cellular inflammation. The left lung was removed and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at 30 cm water pressure for histological analysis. Right lung lobes were collected and flash frozen for biochemical analysis.

Acute Inflammation Study: Assessment of PM-Induced Oxidative Stress

Mice were randomly divided into nine exposure groups corresponding to the eight temporally collected PM samples plus vehicle

control (n = 5–6/group) to assess acute pulmonary inflammation and ability of particles to induce pulmonary oxidative stress in vivo. Mice were exposed via oropharyngeal aspiration to 50 μg PM and sacrificed 24 h postexposure with an overdose of Beuthanasia-D (Schering-Plough). Mice were intratracheally cannulated, and lungs were lavaged as described earlier. Caudal lobe tissue from the right lung was weighed and homogenized with a cell lysis kit (Bio-Rad, Hercules, CA), and protein concentration was obtained via a modified Lowry protein assay (Bio-Rad DC). Protein levels were utilized to normalize HO-1 results found via enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Farmingdale, NY) in lung homogenate to determine pulmonary HO-1 protein concentration (pg/g).

Bronchoalveolar Lavage (BAL) Immune Cell Differentials

Total cell count and viability in BAL were determined from resuspended cells by trypan blue exclusion via hemocytometer (Sigma-Aldrich, St. Louis, MO). Aliquots of resuspended cells were centrifuged onto slides using a Shandon Cytospin (Thermo Shandon, Inc, Pittsburgh, PA) and stained with differential stain (DippKwik, American MasterTech, Lodi, CA) for counting of macrophages, neutrophils, eosinophils, and lymphocytes (300 cells/slide).

Analysis for Immunoglobulin E

The ELISA for HDM-specific immunoglobulin E (IgE) was made by coating plates with HDM in excess in coating buffer (Na_2CO_3, $NaHCO_3$, and NaN_3 in double-distilled H_2O) for 12 h at 4°C. Plates were washed with phosphate-buffered saline (PBS, pH 6.8) with 0.05% Tween 20 (Sigma-Aldrich) and then blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich). Plasma samples (50 μl/well in necessary dilutions) were added to the wells and left to incubate for 12 h at 4°C. Plates were washed and horseradish peroxidase (HRP)-conjugated anti-IgE-HRP (Southern Biotech, Birmingham, AL) was added to sample wells for 1 h at room temperature. After washing, 3,3′,5,5′-tetramethylbenzidine (TMB; Pierce-Thermo, Rockford, IL) was added to detect HRP activity and stopped with 2 N H_2SO_4 after 30 min. The plates were read with a Spectramax Microplate reader at 450 nm (Molecular Devices, Sunnyvale, CA) and reported as fold of the sham (Vehicle/Vehicle) absorbance values.

Histochemistry and Morphometric Analysis

Left lung lobes were inflation fixed at a hydrostatic pressure of 30 cm in 4% paraformaldehyde. Following fixation, the left lung lobe was divided into proximal, mid-level, and distal regions. Each region was embedded, sectioned, and stained with hematoxylin and eosin for pathological evaluation as well as identification of the main axial pathway. An additional serial section was stained with Alcian blue and periodic acid–Schiff (AB/PAS) stain for analysis of intraepithelial mucosubstance content. Four quadrants of the axial pathway at each level of the airway (proximal, mid-level, and distal) were captured for morphometric analysis in ImageJ (National Institutes of Health, Bethesda, MD) at 40×. A Mertz overlay (rolling cycloid arc grid) was utilized with 518 points per image. Intersections with positively stained mucosubstances, basal lamina, and epithelium were counted to determine mucosubstance volume, basal lamina surface area, and epithelium volume (Russ and Dehoff, 2000).

Statistical Analysis

All values are presented as mean ± SEM unless otherwise noted. Data were analyzed for normality via the Shapiro–Wilk test, and logarithmic transformations were applied as necessary to achieve a normal distribution. Factorial analysis of variance (ANOVA) was applied to test whether PM and particle characteristics (season, day/night, size) predicted responses. Differences against allergic control

animals were tested via ANOVA followed by Dunnett's post hoc testing to identify groups that were significantly different from controls. Significant difference from the control was considered to be at p values of < 0.05. Pearson's correlation coefficients were obtained to determine whether results from acute exposures in healthy BALB/*c* mice exposed to comparable levels of PM to those used during sensitization correlated with the exacerbated allergic inflammation observed upon allergen-only challenge, nearly 2 wk following PM exposure. This statistical approach was also applied to determine whether correlations existed between endotoxin levels within the PM samples and the resulting inflammation. Statistical analyses and graphing were performed using SAS JMP (Cary, NC) and GraphPad Prism computer software (San Diego, CA).

RESULTS

Bronchoalveolar Lavage Fluid (BALF) and Cell Differentials

The total number of cells recovered, as well as inflammatory cell differentials within bronchoalveolar lavage fluid (BALF), were assessed by cell counting on cytospin slides (Figure 2). To establish baseline cell number and types, two additional groups were utilized: (1) a Vehicle/Vehicle group, sham, consisting of a buffered saline solution at dosing volumes equivalent to those of all exposed and allergic groups, and (2) a PM/Vehicle group, PM control, consisting of equal doses of the same suspension of Winter/Night/UF delivered for acute HO-1 study and allergen studies but in the absence of allergen during sensitization. Winter/Night/UF was selected for incorporation as the negative PM control because it produced the highest levels of acute total cellular inflammation (Figure 3A). These two exposure conditions were found to not be significantly different from each other 15 d following the 3 vehicle or vehicle and PM doses delivered on d 1, 3, and 5 with vehicle challenges on d 12–14.

Compared to mice exposed only to allergen (HDM/HDM, allergic control), mice exposed to HDM plus daytime PM consistently trended toward increased allergic cellular inflammation following allergen challenges as compared to nighttime PM. Factorial ANOVA testing of a full cross of season, day/night, and particle size revealed that only daytime collection significantly predicted any changes in total cellular inflammation, lymphocytes and eosinophils, and neutrophils (Figure 2). When PM + HDM groups were compared with allergic control mice (HDM/HDM), only mice sensitized to HDM with Winter/Day/UF displayed significantly elevated total cells recovered from BALF (Figure 2A). Lymphocyte infiltration was significantly increased for all daytime particle exposures, Summer and Winter/UF and SM, compared to allergic control (Figure 2B). Eosinophils (Figure 2C) were significantly elevated for Winter/Day/UF as compared to allergic control. Neutrophils (Figure 2D), macrophages (Figure 2E), and cell viability (Figure 2F) were not significantly altered from that of allergic control.

Heme Oxygenase-1 (HO-1) Assay as a Measure of Oxidative Stress

Previous studies suggested that PM exacerbates allergic airway inflammation during allergic sensitization by increasing oxidative stress within lungs and immune system and that this rise in extra- and intracellular oxidative stress results in a hyperpolarization of the adaptive response (Li et al., 2009). To determine the effect of PM on the state of oxidative stress during allergen sensitization (d 1–5) when PM dosing occurred in our allergic model, cells recovered by BALF (Figure 3A) and HO-1 protein levels (Figure 3B) were assayed 24 h post PM exposure. Of the eight temporal PM samples, three daytime (Summer/Day/SM, Winter/Day/UF, Summer/Day/UF) showed significantly elevated HO-1 protein levels in lung compared to vehicle control (Figure 3B). However, while only three daytime particles yielded significant increases in the level of HO-1, six particles

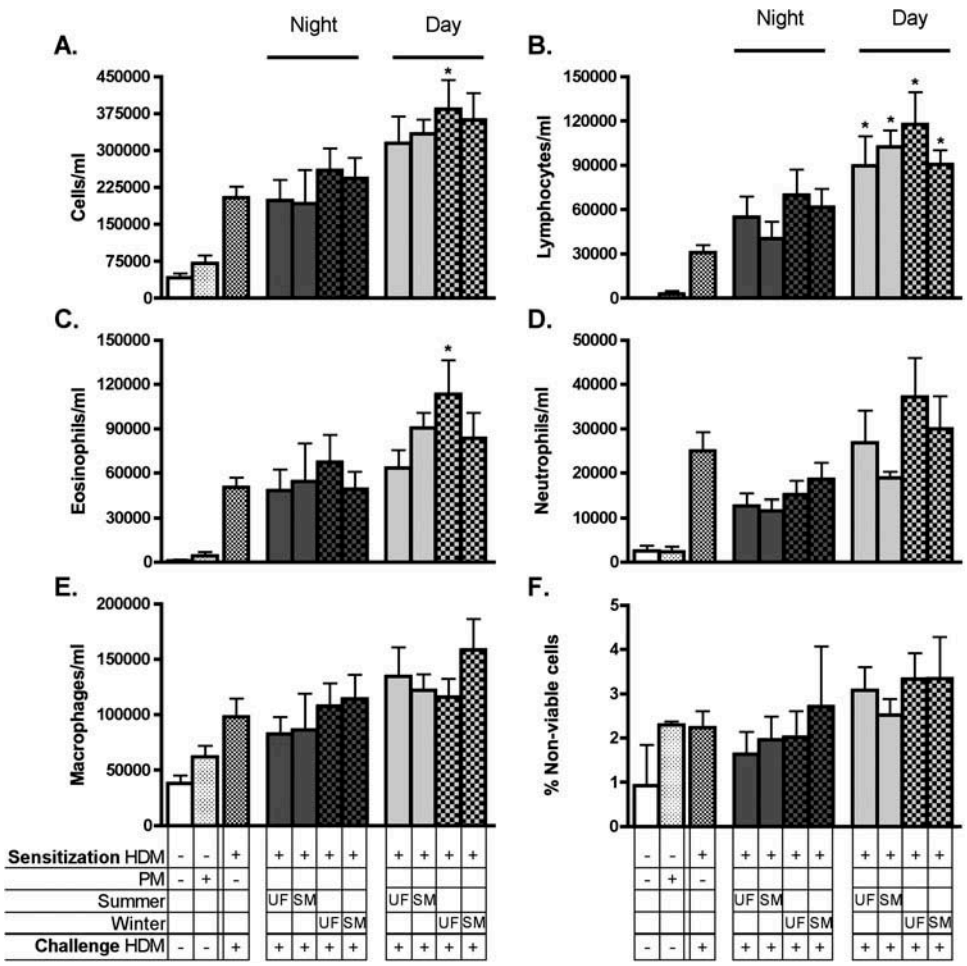

FIGURE 2. Allergic inflammation (bronchoalveolar lavage). To determine the ability of ambient PM exposure during sensitization to exacerbate allergic inflammation, BAL was performed. Shown are the lavage results of (A) total cells recovered, (B) lymphocytes, (C) eosinophils, (D) neutrophils, and (E) macrophages from lavage and (F) cellular viability at 24 h after final challenge with HDM from animals sensitized and challenged with HDM. Data is expressed as cells/ml of recovered lavage fluid, mean ± SEM. The group of three bars on left side of the *x*-axis represents the controls: sham, PM control (Winter/Night/UF), and allergic control, respectively. The center group of bars on the *x*-axis represents nighttime PM samples. The right group of bars on the *x*-axis represents daytime PM samples. Asterisk indicates significant difference from allergic control (third bar from left) at $p < .05$.

significantly elevated the number of cells recovered over vehicle levels: Summer/Night/UF, Winter/Night/UF and SM, Summer/Day/UF and SM, and Winter/Day/UF (Figure 3A).

Correlation Between HO-1 and Allergic Sensitization/Airway Inflammation

The correlation between PM-induced pulmonary levels of HO-1 and allergic inflammation was tested to determine whether subsequent allergic airway inflammation was possibly a manifestation of acute increases in pulmonary oxidative stress induced by PM exposure during sensitization. Significant positive correlations were found between pulmonary HO-1 protein levels observed acutely after PM exposure and allergic airway inflammation in the allergic model by Pearson's correlation coefficient (Table 2). Total allergen-induced inflammatory BALF cells and lymphocyte and eosinophil specific cellular inflammation significantly correlated with HO-1 protein levels, while neutrophils did not. No marked correlation was found between resuspended particle size and HO-1 protein levels. Nor was

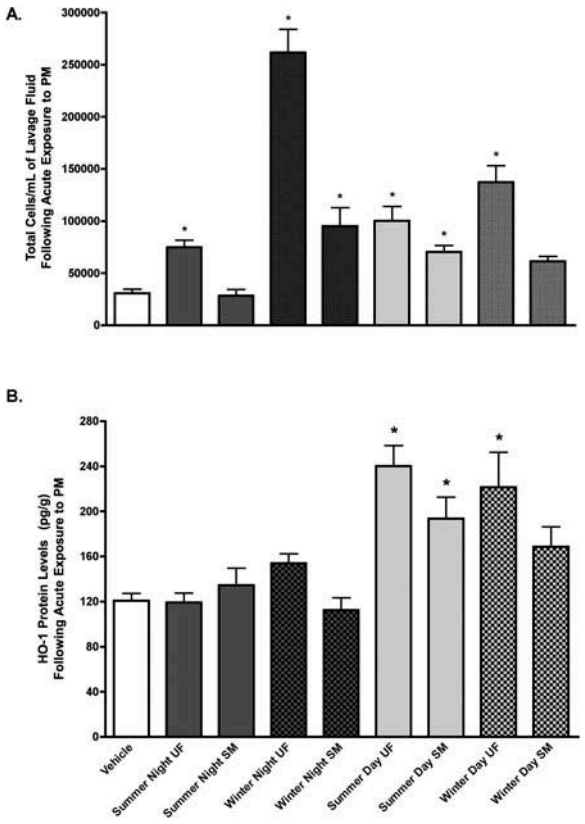

FIGURE 3. PM-induced acute inflammation and heme oxygenase-1 response. To determine the effect of PM exposure, which occurred concomitantly with allergen sensitization, on the oxidative state and inflammatory response in the lung, an acute PM study was performed and BAL and pulmonary tissues were collected. Total cells recovered (A) from BAL 24-h after an acute exposure to ambient PM show significant increases in cellular inflammation in 6 of 8 groups, with Winter/Night/UF yielding the strongest response. Determination by ELISA of HO-1 protein levels in homogenized lung tissue (B), however, demonstrated a markedly different trend in response to PM exposure in contrast to cellular inflammation. All daytime particles yielded the highest reported levels of HO-1, with Summer/Day/SM and UF and Winter/Day/UF reaching statistical significance. Asterisk indicates significant difference from vehicle control, $p < .05$.

a significant correlation found between HO-1 protein levels and PM-induced acute pulmonary inflammation, the more commonly measured endpoint in acute toxicity studies.

Intraepithelial Mucosubstances, HDM-Specific IgE, and Endotoxin

Stored intraepithelial mucosubstances of the airways were measured as an additional assessment of allergic pulmonary inflammation, since a non-allergen-challenged lung in mouse contains few intraepithelial mucosubstances. All animals exposed to allergen demonstrated significantly increased mucosubstance content in the proximal, mid-level, and distal axial pathways compared to Vehicle/Vehicle sham and PM control groups. The potent allergic response of enhanced mucosubstance production due to HDM sensitization and challenge was not further enhanced by exposure to PM by season, time, or size (Supplemental Figures 1A–1C). PM endotoxin levels did not significantly correlate with (1) HO-1 protein, (2) acute total cellular, or (3) allergic total cellular, lymphocyte, and eosinophil recruitment (Supplemental Table 1). Allergen-specific levels of IgE increased for all allergen-exposed groups compared to the vehicle-only (sham) and PM control groups. However, statistical significance between the allergic control (HDM/HDM) and PM plus allergen animal groups (HDM + PM/HDM) was not detected (Supplemental Figure 2). If an allergen dose that elicited no baseline allergic response were utilized, these endpoints might have been significantly increased; however, as humans experience HDM allergy without a necessary adjuvant, it was sought to mimic that baseline allergy, and as such our ability was diminished to detect small differences in these endpoints.

DISCUSSION

In this study, PM samples were tested for the potential to exacerbate a murine model of allergic airway inflammation. Temporally resolved and size-segregated PM samples were used to understand how season, time of day, and particle size influence the ability of PM to exacerbate allergic inflammation when exposure occurs solely during allergic sensitization. Our results indicated that both ultrafine (UF) and submicrometer fine (SM) PM composing the daytime mixed layer possess greater potential for increased pulmonary leukocyte recruitment than particles present at night beneath a nocturnal inversion layer. In addition, a single PM dose study was conducted to determine the degree of PM-induced pulmonary

TABLE 2. Heme oxygenase-1 as a biomarker of the ability of PM to exacerbate allergic airway inflammation. Correlations were performed between induction of pulmonary HO-1 protein levels in response to oxidative stress following an acute exposure to ambient PM and allergic total cellular and eosinophil, lymphocyte, and neutrophil specific inflammation, acute pulmonary inflammation, and resuspended ambient PM size. Strong positive correlations with HO-1 were found for total cellular inflammation and for lymphocyte and eosinophil specific airway infiltration. Neutrophil infiltration in the allergic model and acute cellular inflammation and particle size did not correlate significantly with pulmonary HO-1 levels. Pearson's correlation coefficients and resulting p-values are presented in the table.

Comparison vs. HO-1 Protein	Pearson correlation coefficient	*p-value*
Allergic Study- Total Inflammation (Cells/ml)	**0.797**	***p=0.018****
Allergic Study- Lymphocytes/ml	**0.799**	***p=0.017****
Allergic Study- Eosinophils/ml	**0.755**	***p=0.030****
Allergic Study- Neutrophils/ml	0.485	*p=0.224*
Acute Study[1]- Total Inflammation (Cells/ml)	-0.247	*p=0.234*
Re-suspended Particle Size	-0.139	*p=0.743*

*, Significant associations, $p<0.05$. [1]Acute pulmonary inflammation data obtained from the HO-1 study

oxidative stress that would be present in our allergic model from the concomitant exposure to PM during allergic sensitization (Figure 1). Through this single, acute PM study, it was possible to determine that acute elevations in pulmonary HO-1 protein levels, but not acute pulmonary inflammatory responses, correlate markedly with exacerbated allergic inflammatory response when animals sensitized to allergen during PM exposure were subsequently challenged with allergen alone. This correlation suggests that the differential ability of PM to induce oxidative stress from exposure during allergic sensitization can manifest itself as a subsequent, exacerbated allergic inflammatory response, even when the subsequent allergen challenge occurs in the absence of particle exposure as demonstrated here.

Factorial ANOVA of data revealed that PM collected from the daytime mixed layer significantly predicted increases in pulmonary inflammation. Specifically, in comparison to the allergic control group (HDM/HDM), Day/Winter/UF induced a significant rise in total number of cells recruited to the lung as well as a significant elevation in eosinophil recruitment. However, all daytime PM-sensitized groups demonstrated significant elevations of lymphocyte infiltration when compared to the allergic control, regardless of the particle size or season sampled, whereas no nighttime samples significantly varied from the allergic control.

Differences between source emissions and atmospheric processes driving daytime and nighttime mixing of the Fresno airshed may potentially account for the strongly diurnal nature of these results. For example, activity patterns, such as farming and heating/cooling and subsequent types of emission sources and their spatiotemporal distribution, differ between daytime and nighttime. Atmospheric photochemical processing occurs primarily during the daytime, and large diurnal temperature and relative humidity variations affect gas-to-particle partitioning, potentially contributing to increased particle toxicity.

Depending on meteorological conditions, boundary-layer conditions may be significantly different between day and night. Daytime boundary-layer conditions are characterized by large mixing depths and turbulent mixing that tend not only to dilute and homogeneously distribute atmospheric emissions, but also to increase their geospatial extent. Therefore, daytime Fresno airshed PM is not only influenced by local sources but also by the surrounding agricultural communities and highly photochemically processed regional background. In contrast, the nighttime boundary layer is characterized by low-lying nocturnal inversions that tend to trap and recirculate local

emissions, thereby isolating the sampled local nighttime PM from any regional or background influences and suppressing turbulent mixing. All of this likely leads to elevated pollutant concentrations and limits exposure to photochemically produced secondary compounds. Therefore, nighttime sampling is largely influenced by fresh, unprocessed emissions from local sources close to the site of sampling. Since our studies were performed using an equal mass dose, daytime–nighttime differences in atmospheric dilution are only relevant in that they determine the relative distribution of sources and chemical components constituting each PM sample. In general, the predominant nighttime PM sources captured include vehicular emissions, both gasoline- and diesel-powered engines, and residential and commercial emissions, including space heating, such as woodstoves and fireplaces, and cooking, such as meat broiling, barbequing, and pan-frying. Daytime captured PM, in addition to these sources, is also influenced by construction, landscaping, and agricultural emissions, which may persist for extended periods during stagnation events common to the SJV, as well as potential long-range sources, such as wildfires and trans-Pacific transport.

Daytime sources, especially mobile sources, emit more fine PM and organics, such as polycyclic aromatic hydrocarbons (PAH), than most nighttime sources (Guor-Chengfang et al., 1999). In addition, secondary organic aerosols may contribute significantly to the organic fraction of PM through photochemical oxidation of volatile organic compounds (VOC), further altering particle chemistry (Yan et al., 2009). Photochemical oxidation is a process that occurs primarily during the daytime and thus, may be less relevant for nighttime particles (Cox, 1979). Therefore, it is conceivable that the combination of sources and photochemical processing of daytime PM is responsible for the diurnal nature of the differences reported here.

Particles collected during the same season and time period did not elicit markedly different immunotoxicity with regard to size fraction (SM or UF). However, as the particles were collected by impaction, then extracted, lyophilized, and resuspended, particle size once in suspension increased over the originally collected particle size determined by the cut-points of the ChemVol samplers (K. J. Bein et al., personal communication). This postcollection particle aggregation may have resulted in a change in particle–lung interaction and region-specific deposition during aspiration, which is a limitation any noninhalation study encounters. Further aerosolization studies would be required to elucidate particle–lung deposition-specific changes to our reported toxicity. While particle aggregation, and consequent altered size distribution in suspension, was anticipated via extraction and resuspension, the focus of this study was to evaluate particle-mediated changes within two sampled size fractions relative to the extracted particles' chemical differences, which were conserved despite aggregation effects. Although no apparent size-specific differences existed within SM and UF fine size ranges, coarse PM ($2.5 < D_p < 10\ \mu m$), which is respirable in humans, would be an additionally interesting particulate fraction to study. Our sampling equipment was not designed to capture this size fraction, and therefore it was not possible to examine this size fraction in these studies.

Part of the study involved using pulmonary HO-1 as an in vivo oxidative stress marker (Choi and Alam, 1996) to predict the PM-mediated effect witnessed within the allergic study. In the allergic study, PM exposure occurred concomitant with allergic sensitization up until d 5; however, tissues were not collected until following allergen challenge to assess allergic inflammation at d 15. As indicated by our PM control group (PM/Vehicle), any PM inflammatory effect was not apparent by the measured endpoints at this time point. It was postulated that HO-1 levels would have resolved to baseline and determining lingering PM-specific effects from prior exposure during sensitization would be difficult due to the allergic challenge and robust response. In addition, visual identification of any macrophages on cytospins following allergic challenge containing particles was extremely rare. Only three

groups possessed any macrophages (<1%) witnessed with any apparent PM, Summer Night UF and Winter Day and Night SMF, and these groups did not markedly correlate to a specific response in either direction. Therefore, it is conceivable the main PM effect occurs following PM dosing concomitant with allergic sensitization. Thus, PM was utilized in the format of a common acute study of pulmonary inflammation where tissue collection occurred 24 h following PM exposure. Total cellular inflammation in BALF from this acute study did not significantly correlate with the ability of PM to exacerbate allergic airway inflammation; pulmonary HO-1 levels, however, demonstrated a strong positive correlation with total cellular inflammation, as well as lymphocyte and eosinophil specific recruitment in the allergic model.

This finding is in agreement with the hypotheses that PM adjuvant effects are mediated through PM-induced oxidative stress in the lung and, specifically, increased intracellular ROS in antigen presenting cells (Li et al., 2008). Pulmonary HO-1 levels were reported to be an effective marker of oxidative stress (Ayres et al., 2008; Li et al., 2009), and prior in vitro research indicated HO-1 as a biomarker of exposure to quantum dot nanoparticles, which elicit their acute toxicity via generation of reactive oxygen intermediates (McConnachie et al., 2013). Further, evidence from in vivo studies utilizing chronic UF ambient particle exposure demonstrated activation of the Nrf-2 signaling pathway, a regulator of HO-1 and a pathway subject to diminished upregulation with aging that might potentially explain additional susceptibility in the aging population to PM pollution (Zhang et al., 2012). To this point, Li et al. (2013) recently demonstrated that adoptive transfer of dendritic cells exposed to UF particles with allergen resulted in a more severe allergic response in Nrf-$2^{-/-}$ dendritic cells than for Nrf-$2^{+/+}$. Taken together with the findings from this study, the data suggest that further research into Nrf-2 mediated HO-1 activity and induction may be critical to understanding the ability of particles to worsen allergic airway inflammation. In our studies, daytime sources and photochemical processing appear to be significant contributors to the effects of SM and UF ambient PM in exacerbating allergic airway inflammation, as daytime particles were associated with higher levels of intrapulmonary HO-1 protein.

In summary, this study demonstrates that time of day, not sampled size fraction or season, most predominately influences the ability of PM to exacerbate subsequent allergic inflammation when exposure occurs during sensitization. Particles from daytime summer and daytime winter in both SM and UF size ranges worsen allergic airway inflammation. Evidence from an acute study of PM-induced toxicity indicated that HO-1 protein levels, and not acute cellular inflammation, more reliably predict the ability of PM to exacerbate allergic airway inflammation. Daytime sources and processing of PM appear to be most potent in enhancing this particle effect in the PM of the SJV.

CONCLUSIONS

Daytime SM and UF ambient PM acts to exacerbate allergic airway inflammation following allergen challenge, when exposure to ambient PM occurs during the phase of allergic sensitization. This PM-enhanced allergic response significantly increased pulmonary cellular recruitment due to exposure to daytime PM including both SM and UF size fractions of both winter and summer seasonal ambient PM in Fresno, CA. Pulmonary HO-1 protein was found to have strong positive correlations with PM-enhanced allergic response. These findings support that oxidative stress is a key element in PM-mediated effects on allergic inflammation during allergic sensitization and suggest that HO-1 protein may be a useful indicator of the ability of PM to exacerbate allergic inflammation even when data are obtained from acute toxicity studies.

SUPPLEMENTAL DATA

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/15287394.2014.959627

AUTHOR CONTRIBUTIONS

CMC, LEP, and AC performed the experiments and contributed to acquisition of data. CMC analyzed the data and interpreted the results. KJB and YZ performed the collection and extraction of the particle samples. The article was written by CMC and revised critically by KJB, ASW, and KEP. All authors read, corrected, and approved the article.

COMPETING INTERESTS

The authors do not have competing interests.

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