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Authors

Van Winkle, Laura S

Bein, Keith

Anderson, Donald

et al.

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Biological Dose Response to PM_{2.5}: Effect of Particle Extraction Method on Platelet and Lung Responses

Laura S. Van Winkle^{*,†,1}, Keith Bein^{†,‡}, Donald Anderson[†],
Kent E. Pinkerton^{*,†}, Fern Tablin^{*}, Dennis Wilson[§], and Anthony S. Wexler^{‡,¶}

^{*}Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, [†]Center for Health and the Environment, [‡]Air Quality Research Center, [§]Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine and [¶]Departments of Mechanical and Aerospace Engineering, Civil and Environmental Engineering, and Land, Air and Water Resources, University of California, Davis, California 95616-8732

¹To whom correspondence should be addressed at Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California at Davis, One Shields Avenue, Davis, CA 95616. Fax: 530-752-5300. E-mail: lsvanwinkle@ucdavis.edu.

ABSTRACT

Particulate matter (PM) exposure contributes to respiratory diseases and cardiopulmonary mortality. PM toxicity is related to sources and composition, such as abundance of polycyclic aromatic hydrocarbons (PAHs). We exposed adult male BALB/c mice, via oropharyngeal aspiration, to a range of doses of PM_{2.5} collected during the winter in downtown Sacramento near a major freeway interchange (SacPM). Two preparation methods (spin-down and multi-solvent extraction) were tested to remove particles from collection filters. Three doses were analyzed 24 h after treatment for (1) leukocytes and total protein in bronchoalveolar lavage fluid (BALF), (2) airway-specific and whole lobe expression of PAH-sensitive genes (CYP1B1 and CYP1A1) and *IL-1 b*, (3) lung histology, and (4) platelet function. Both extraction methods stimulated biological responses, but the spin-down method was more robust at producing *IL-1 b* and CYP1B1 gene responses and the multi-solvent extraction induced whole lung CYP1A1. Neutrophils in the BALF were increased 5- to 10-fold at the mid and high dose for both preparations. Histopathology scores indicated dose-dependent responses and increased pathology associated with spin-down-derived PM exposure. In microdissected airways, spin-down PM increased CYP1B1 gene expression significantly, but multi-solvent extracted PM did not. Platelet responses to the physiological agonist thrombin were approximately twice as potent in the spin-down preparation as in the multi-solvent extract. We conclude (1) the method of filter extraction can influence the degree of biological response, (2) for SacPM the minimal effective dose is 27.5–50 µg based on neutrophil recruitment, and (3) P450s are upregulated differently in airways and lung parenchyma in response to PAH-containing PM.

Key words: air pollution; cytochrome P450 monooxygenase; filter extraction

Particulate matter (PM) in the fine and ultrafine size fractions (PM_{2.5}) is known to contribute significantly to disease. Greater than 30% of the U.S. population is exposed to PM_{2.5} in excess of the National Ambient Air Quality Standards (NAAQS) (ALA, 2009). Numerous epidemiologic studies demonstrate a correlation between ambient PM concentrations and increased morbidity and mortality with lags of 1–4 days depending on endpoint (Larrieu *et al.*, 2009; Ostro *et al.*, 2007; Stieb *et al.*, 2009), yet the links between these health effects and PM composition

have only been partially identified. A number of recent studies have implicated the polycyclic aromatic hydrocarbon (PAH) content of PM as a potent mediator of health effects (Chan *et al.*, 2013; den Hartigh *et al.*, 2010; Gale *et al.*, 2012; Padula *et al.* (forthcoming); Val *et al.*, 2011) and this is further supported by epidemiologic data that indicate near roadway PM_{2.5} is especially potent (Gan *et al.*, 2013; Gauderman *et al.*, 2007; Jerrett *et al.*, 2013). Finally, recent studies have focused on effects on blood cells, including platelets, providing a link between inhaled fine

PM and cardiovascular risk (Frampton *et al.*, 2012; Poursafa and Kelishadi, 2010; Tablin *et al.*, 2012; Wu *et al.*, 2012; Zhang *et al.*, 2013).

A major difficulty with studying ambient PM is that it is a complex mixture that varies considerably in composition depending on the source, aging, and atmospheric processing, co-pollutants, and the season. Furthermore, air pollution regulations are improving our air, changing the amount and the type of PM to which we are exposed. California leads the country in particulate air pollutant regulation because many metropolitan areas are in violation of the PM NAAQS (ALA, 2009). Thus, the composition of PM in California urban areas may foretell compositions in other regions of the United States as similar regulations are more widely adopted. Collection of ambient PM to use in experimental studies of health effects also poses challenges in that typical collection involves concentration of the material onto filters from which it is subsequently extracted. In an effort to understand the role of filter extraction on health effects, we collected PM_{2.5} from an urban site near a major freeway in downtown Sacramento California in winter 2011.

Collected PM_{2.5} was extracted from the filters using 2 different methods. These PM_{2.5} extracts were used in an inflammation susceptible BALB/c mouse model to study the pulmonary and platelet inflammatory response and the effect of the PM on 2 P450s commonly involved in PAH metabolism in the lung: CYP1A1 and CYP1B1. The goal of the current study was to characterize the acute dose response to Sacramento urban PM_{2.5} (SacPM) and to compare acute health effects of the same PM after being subjected to 2 sample preparation methods. The first method (referred to as spin-down extraction) is a water-based approach using a combination of probe sonication, shredder homogenization, and filtration in a spin-down column. The second method (referred to as multi-solvent extraction) is more complex, using multiple solvents in bath style sonication and liquid-liquid extraction followed by micro-pore membrane filtration and solvent removal, but takes into account the strong likelihood that this PM will be high in PAHs and so uses several steps to optimize recovery of components less soluble in water. Since acute inflammation as indicated by recruitment of neutrophils is a convenient marker of potency of PM, we selected doses of each preparation that would give similar levels of neutrophil recruitment.

This project is a precursor to a larger study to examine the health effects of both urban and rural PM on the lung and cardiovascular system over time after exposure. The long-term goal of this research is to define whether PM with different compositions adversely affects health so that the most toxic sources of PM can be identified and controlled. The goals of the current study were 3-fold: (1) to test 2 methods of particle extraction for future use, (2) to pick an effective dose, and (3) to compare the biologic response to different doses of particles.

MATERIALS AND METHODS

PM_{2.5} sample acquisition. All PM samples used during these studies were collected from an urban sampling site located on the rooftop of a 2 story building at the northeast corner of T St and 13th St in downtown Sacramento, California, during the winter of 2011 using a PM_{2.5} high volume sampler system (Tisch Environmental Inc., TE-6070V-2.5-HVS) equipped with a PM₁₀ size selective head (Tisch Environmental Inc., TE-6001) and operating at a flow rate of 40 CFM. This site is within a quarter of a mile of a large freeway interchange. The coarse PM size fraction (PM_{2.5-10} = 2.5 < D_{p50} < 10 μm) was collected on

aluminum foil substrates and the fine fraction (PM_{2.5} = D_{p50} < 2.5 μm) was collected on Teflon-coated borosilicate glass microfiber filters (Pall Corporation, TX40H120WW-8X10). Only PM_{2.5} was used in the current study and 2 separate filter extraction techniques were employed to extract the particles from the filters prior to toxicological testing. PAHs were determined via Thermal Desorption Gas Chromatography Mass Spectrometry according to the protocols of the Desert Research Institute in Reno, Nevada (Chow and Watson, 2012).

Spin-down extraction. For both the treatment and control, a 1 in² filter piece is processed using a QIAshredder column in a total volume of 500 μl of Dulbecco's phosphate-buffered saline (PBS) solution. The top layer (Teflon-bound glass microfibers) of the 1 in² filter piece is removed, leaving the backing behind, and then cut into 3 equal strips. The strips are rolled up with the particle laden surface toward the inside of the cylinder and then stacked one on top of the other inside the top of a QIAshredder column (Qiagen, Cat. No. 79654), which is weighed to obtain the pre-extraction weight. Dulbecco's PBS (500 μl) without CaCl₂ or MgCl₂ is added and the membranes are sonicated. The collection tubes are attached and centrifuged at 7600 × g for 4 min. Particles settle at the bottom of the collection tube. The supernatant is collected and reapplied to the column containing the rolled membranes and probe sonicated for approximately 5 s. This process is repeated in triplicate and then the centrifuged particles are resuspended in the supernatant and filtered one final time through a clean Qiagen filter. Any fluid lost during this process is replaced with fresh PBS to give a final volume of 500 μl. The extracted membranes still retained in the Qiagen filter are washed with 500 μl of distilled water to remove salts and dried. After drying, the filters and shredded membranes are weighed to obtain the extraction post-weight. This is subtracted from the pre-weight to obtain the mass of extracted PM. Control mice were treated with suspensions from similarly treated clean filters. Treated mice were given suspensions from collection filters.

Multisolvent extraction. This is a novel technique designed to maximize the amount of PM extracted from the filter while minimizing potential extraction artifacts such as compositional biases in the extracted PM, volatilization losses and contamination by filter material, which was recently published in detail (Bein and Wexler, 2014). In brief, a multistep process was deployed that includes a combination of bath style sonication in different solvents of varying polarity (water, dichloromethane, and hexane), liquid-liquid extraction, micropore membrane filtration, solvent removal, and extensive gravimetric analyses. In brief, sonication provides the mechanical energy necessary to separate the particles from the filter, multiple solvents are used to increase the amount of extracted PM, liquid-liquid extraction reduces volatilization losses, micropore filtration removes potential contaminant filter material, and gravimetric analysis provides the accurate PM mass measurements required for comparative dose response studies. A detailed description of the various steps and results from the application of this extraction technique to a large number of filter substrates in terms of total and composition-specific extraction efficiencies are described elsewhere (Bein and Wexler, 2014; Chan *et al.*, 2013).

For oropharyngeal aspiration, PM was resuspended in PBS and sonicated for 5 min using a 10 s on and 10 s off cycling program. Control mice were treated with 50 μl suspensions from

clean filters. Treated mice were given 50 μ l suspensions from similarly treated collection filters.

Experimental design and animal exposure. Eight week reproductively capable adult male BALB/c mice (Harlan Laboratories, Hayward, California) were allowed to acclimate in filtered air for 7 days. Mice were provided with Laboratory Rodent Diet (Purina Mills, St Louis, Missouri) and water *ad libitum*. All animal procedures followed approved institutional animal care and use protocols. Animals were exposed to the same volume of particle extracts (50 μ l) via oropharyngeal aspiration under light 2.5% isoflurane anesthesia (De Vooght *et al.*, 2009). Six mice were used for each timepoint and exposure dose ($N=6$ per group). Different doses of the extract were used with the goal of identifying equivalent dosing regimens for the 2 extracts in relation to neutrophil recruitment to the bronchoalveolar lavage fluid (BALF). For comparison of sample preparation methods, doses were chosen to be 5.5, 27.5, and 55 μ g of the spin-down extract and 10, 50, and 100 μ g of the multisolvent extract to yield similar dose response profiles of percentage neutrophils as an indicator of acute inflammation.

BALF differentials and total protein. The right lobes were lavaged with approximately 0.6–0.7 ml of 0.9% sterile saline 2 times. The resultant BALF was collected into 5 ml round bottom tubes and kept on ice. BALF was centrifuged at 2000 rpm at 4°C for 10 min to pellet cells. The BALF supernatant was transferred to a 1.5-ml microfuge tube for total protein determination. The cell pellet was resuspended in 0.5 ml sterile 0.9% saline. Viable and total leukocyte counts were performed using Trypan Blue exclusion assay. A cytospin slide was prepared from the resuspended cell pellet. Slides were fixed with methanol and stained with DiffKwik Differential Stain kit (Mastertech, Lodi, California). BALF cell profile was determined by counting 500 cells per animal using a light microscope. Total protein in the BALF was determined using a Bradford protein assay (Biorad) performed in a standard 96-well plate using manufacturer's protocol. All samples were assayed in triplicate. The plate was read in a SpectroMax plate reader (Molecular Devices).

Quantitative RT-PCR. Lung compartmental RNA was isolated from microdissected intrapulmonary airways and surrounding parenchymal tissue from RNAlater (Ambion, Austin, Texas) stabilized lung tissue using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, California) as previously described (Baker *et al.*, 2004). RNA purity was confirmed through spectrophotometric absorbance at 260/280 nm. Quantification of CYP1A1 (Mm00487218_m1), CYP1B1 (Mm00487229_m1), IL1B (Mm00434228_m1), and the reference gene RPL13A (Mm01612987_g1) in the airway and parenchymal compartments were performed using inventoried Taqman probes and primers (Applied Biosystems, Foster City, California) as previously described (Baker *et al.*, 2004; Stelck *et al.*, 2005). Results were calculated using the comparative C_t method (Livak and Schmittgen, 2001). Results are expressed as a fold change in gene expression relative to sham (filter extract exposed) animals.

Immunohistochemistry. Paraffin sections from the left lung lobe of 3 mice per treatment group were immunostained for rabbit anti-CYP1A1 antibody (Xenotech) and rabbit anti-CYP1B1 as described (Chan *et al.*, 2013). An avidin-biotin peroxidase (Vectastain ABC, Vector Laboratories, Burlingame, California) kit

was used to detect primary antibody-binding sites. Nickel-enhanced 3',3'-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis, Missouri) was used as the chromagen. Controls included the substitution of primary antibody with PBS. A series of dilutions were used to determine optimal antibody concentration. Sections from all groups were run together to minimize run to run variability.

Histologic evaluation. Histopathologic evaluation of lung lesions was done on 2 hematoxylin and eosin-stained sections of lung representing short and long axial pathway of the left lung lobe from each animal in each treatment group. Slides were evaluated in random order without knowledge of treatment group assignment. All terminal bronchiole-alveolar duct junctions in each section were examined and a subjective severity score assigned relative to airway epithelial changes, inflammation in alveolar ducts and surrounding parenchyma and periarteriolar inflammation and medial hypertrophy in the terminal branches of the pulmonary artery. Similarly, an overall severity score was assigned to each specimen. Lesions were scored from 0 to 4 with 4 being the most severe changes. Results were tabulated and average group scores calculated with statistical analysis by the Kruskal-Wallis non-parametric ranking test with a $P < 0.05$ significance level.

Platelet alpha granule proteins and integrins. Mouse platelet activation was analyzed in whole blood by flow cytometry using the following antimouse antibodies, along with appropriate isotypes for each: a biotin-conjugated monoclonal antibody to the α_{2b} subunit for the major platelet integrin $\alpha_{2b}\beta_{3a}$ (CD41, BD Pharmingen) followed by Streptavidin Alexa 633 (in vitrogen), a FITC-conjugated monoclonal antibody to P-selectin for alpha granule secretion (CD62P, BD Pharmingen), and a PE-conjugated monoclonal antibody to LAMP-1 for lysosomal granule secretion (CD107a, eBioscience). A resting (unstimulated) sample and samples stimulated with either 10 mM ADP or 0.1 U/ml thrombin were examined for each animal. After stimulation of whole blood, platelets were labeled with the preceding antibodies for 1 h and fixed in 1% (final) paraformaldehyde prior to analysis by flow cytometry (FC500, Beckman-Coulter, Miami, Florida). Platelets were defined by forward, and side scatter characteristics and 10 000 events were collected within the platelet gate for each animal and each condition.

Statistics. All data are reported as mean \pm standard error of the mean unless otherwise stated. Statistical outliers were eliminated using the extreme studentized deviate method (Graphpad, La Jolla, California). Within treatment group, comparisons for continuous data were performed using a 1-way ANOVA followed by PLSD post hoc analysis using StatView (SAS, Cary, North Carolina). Lesion scoring data were analyzed using a Kruskal-Wallis 1-way ANOVA test. P -values of less than 0.05 were considered statistically significant.

RESULTS

As expected, the multisolvent extraction technique resulted in higher PAH content in the sample. The total PAH content of extracted PM was 330 ± 30 and 120 ± 10 ppm by mass for the multisolvent and spin-down techniques, respectively.

The spin-down extract caused an equal response to the multisolvent extract (at a lower dose) in terms of both induction of total cells into the BALF and increasing the neutrophil response (Figs. 1A and 1B) with the mid and high dose of both

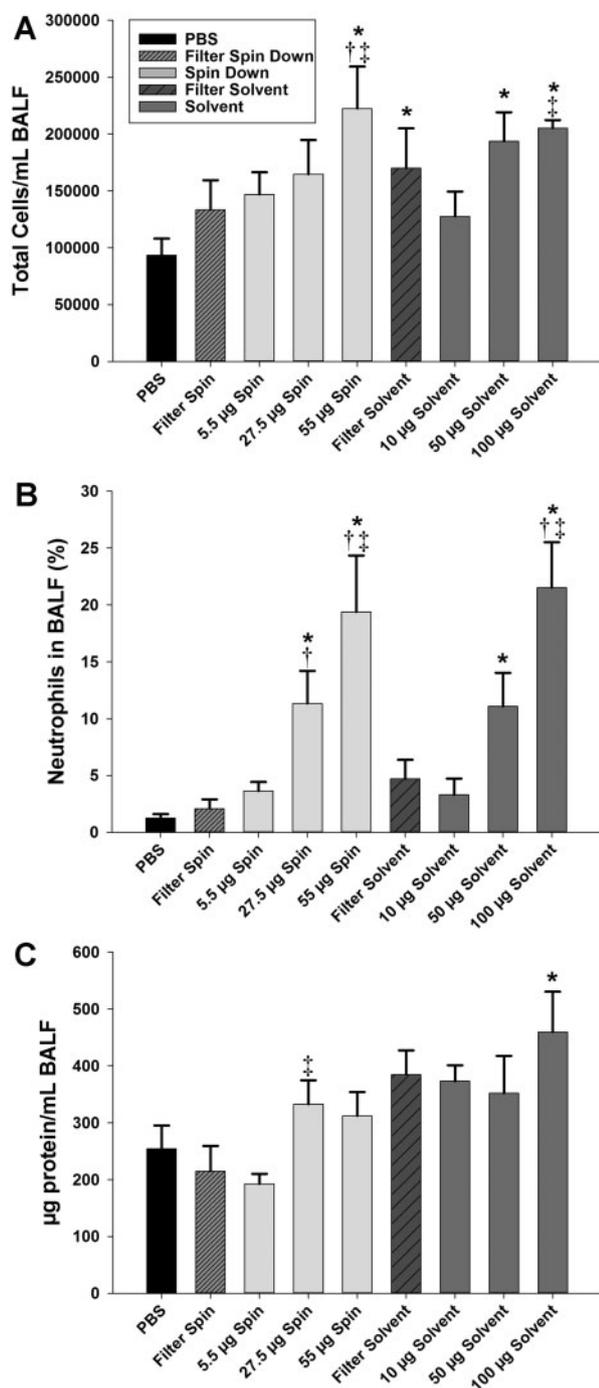


FIG. 1. Bronchoalveolar lavage fluid (BALF) was analyzed for total cellular infiltrates (A), percent of neutrophils (B), and total protein (C) as an indication of potential lung injury at 24 h following SacPM administration. Two preparations were compared (spin-down “spin” vs multisolvent “solvent”) and the dose response that yielded equivalent neutrophil infiltration is shown. To examine whether there was a possible effect of the filter blank preparations (filter son and filter extract) an additional group was administered PBS as a control. $N = 6/\text{group}$. (*) significantly different from PBS control. (†) significantly different from respective filter control. (‡) Significantly different from lowest dose group of same treatment. $P < 0.05$.

preparations increasing neutrophils in the BALF. Notably the multisolvent sham filter extract also significantly increased total cells in the BALF compared with the PBS control. Lung injury was modest as only one dose (mid dose) from each

preparation induced an increase of protein into the BALF (Fig. 1C). The response to high dose appeared similar despite not reaching statistical significance.

At 24 h after dosing, the spin-down preparation induced more apparent pathology at the terminal bronchiole alveolar duct junction at all doses tested (low, medium, and high) compared with the multisolvent extracted sample (Fig. 2). Spin-down extract-treated mice had bronchiolar epithelial hyperplasia, accumulation of neutrophils, and macrophages in alveolar ducts and periarteriolar inflammation. Multisolvent extract-treated mice had less inflammation and fewer neutrophils. Arteriolar inflammation was minimal in multisolvent extract-treated mice. To quantify these changes, pathology scoring was utilized (Fig. 3). Overall lesion scores indicated a dose responsive severity of injury in the spin-down extract exposed group. The lesion scores for the multisolvent extract exposed group peak at the mid dose and are approximately half as severe as the maximal response in the spin-down extract exposed group (Fig. 3A). Notably the responses in the high and mid dose spin-down extract exposed groups involved fairly equal inflammation in the vessels, the parenchyma, and the bronchioles, but the inflammation in the multisolvent extract exposed groups consisted primarily of inflammation in the vessels and parenchyma and not the bronchioles (Fig. 3B).

Since both preparations appear to affect the inflammation patterns at the conducting airway level based on pathology, an additional study was performed to separate conducting airway responses from those of the whole lung. RNAlater preserved lung tissue (Baker et al., 2004) was microdissected to isolate the conducting airways. The remainder of the lung lobe (whole lung lobe) was analyzed intact for mRNA expression of genes involved in detoxification and toxification of PAHs. CYP1A1 (Figs. 4A and 4B), CYP1B1 (Figs. 4C and 4D), and IL-1B (Figs. 4E and 4F) were examined. IL-1B, a gene associated with inflammation and macrophage activation, has been documented to be upregulated in previous studies of PAH-containing PM (den Hartigh et al., 2010). Airway CYP1A1 mRNA was significantly decreased by exposure to the spin-down sample in the high dose only (Fig. 4A). Within the lung lobe CYP1A1 was increased by the mid dose of the multisolvent extract sample, but not the high dose (Fig. 4B). CYP1B1 was increased in both the airways and the whole lung by the spin-down extract but only whole lung mRNA expression was increased by the multisolvent extract (Figs. 4C and 4D). Both sample preparations induced the expression of IL-1B (Figs. 4E and 4F) but the spin-down extract was more potent, inducing an increase at the mid dose in whole lung (Figs. 4F). Gene expression at a single timepoint gives only a partial picture of the temporal pattern of changes as well as the distribution and abundance of the corresponding proteins within the tissue itself. As a result, we also examined the abundance of 2 key P450s (CYP1A1 and CYP1B1) in the terminal bronchioles for the highest doses tested (Fig. 5). CYP1A1 protein was induced in both of the extract preparations with the multisolvent extract exposure exhibiting greater induction (Fig. 5D). Increased expression of CYP1A1 was noted in both the epithelium of the terminal bronchiole as well as in interstitial macrophages. Conversely, exposure to both extracts reduced CYP1B1 protein expression (Figs. 5G and 5H) at 24 h after dosing.

Due to the apparent correlation of (some) gene expression responses with tissue scores for inflammation as well as increased neutrophils in the BALF, Pearson linear correlations between these endpoints were determined. Positive correlations were only noted for the spin-down samples. Airway IL-1B gene

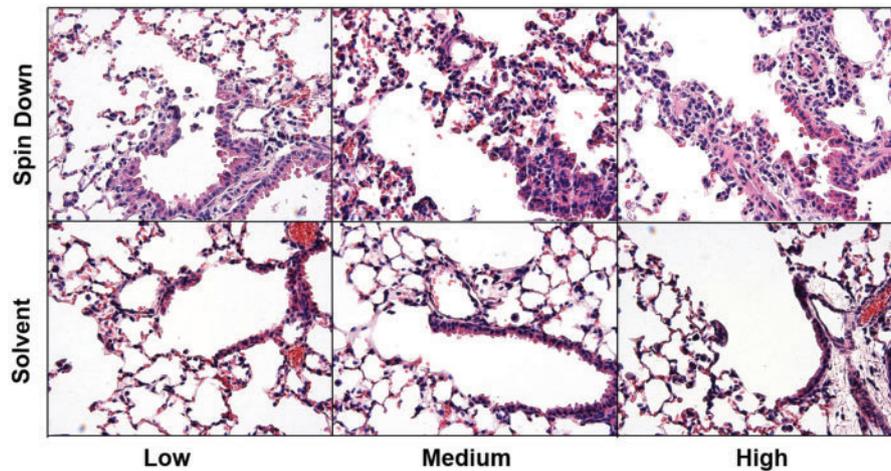


FIG. 2. Lung lesions in mice given collected ambient PM prepared by spin-down or multisolvent extraction. PM was administered by oropharyngeal aspiration at doses determined to be low, medium, or high based on induction of inflammatory cells in BALF. Multisolvent extract PM-treated mice were administered 25, 50, or 100 μg while spin-down-treated mice were given 5.5, 27.5, and 55 μg . Spin-down-treated mice have bronchiolar epithelial hyperplasia, accumulation of neutrophils and macrophages in alveolar ducts, and periarteriol inflammation. Mice treated with multisolvent extracted PM have less inflammation which has fewer neutrophils. Arteriol inflammation is minimal in extract-treated mice.

expression had a positive, and significant correlation with tissue inflammation (0.523165) and neutrophils in BALF (0.682310), but whole lung IL-1B gene expression only had significant correlation with neutrophils in the BALF (0.607316). Interestingly, CYP1B1 gene expression in both the airway (0.464732) and the whole lung (0.560052) also correlated with neutrophils in BALF. A stronger correlation was found though for CYP1B1 gene expression in the whole lung and the histology scores (0.689086).

The number of circulating platelets and blood hemoglobin content were measured (Supplementary Fig. 1). Hemoglobin content did not vary between groups and thus indicated no change in blood volume by group. The number of platelets in the blood was increased by exposure to the multisolvent extract at all doses, although all values were within normal limits. In contrast, when platelet response to the potent physiological agonist thrombin was studied, the spin-down preparation was approximately twice as potent as the multisolvent extract (Fig. 6). Platelet activation can be measured by the surface expression of alpha (P-selectin) and lysosomal (LAMP-1) granule proteins, both of which are secreted during activation. However, it should be noted that platelets may secrete more of one type of granule than another. Spin-down extracts showed a strong response to thrombin stimulation, as evidenced by both the number of cells that were positive for the platelet alpha granule protein P-selectin (Fig. 6A), and those positive for lysosomal granule membrane protein -1 (LAMP-1) (Fig. 6B). Additionally, unstimulated platelets from animals given the spin-down extract had high levels of the major platelet integrin CD41b, as would be expected in a normal healthy population of cells (Fig. 6E). The multisolvent extract resulted in an inability of platelets to respond to physiological stimuli such as thrombin, either by the expression of P-selectin (Fig. 6A) or LAMP-1 (Fig. 6B), but solvent-extracted PM restored this responsiveness.

DISCUSSION

In this study we have established that, for studies of $\text{PM}_{2.5}$ extracted from glass microfiber filters, the method of particle

removal from the filters has a substantial and significant effect on biological response. This may be due to modification of inherent, or bulk, particle properties during the filter extraction process. Further, we show that application of 2 different filter extraction protocols also results in particles with a difference in potency, as measured by neutrophil recruitment potential. We normalized the doses based on degree of neutrophil infiltration into the BALF. We set the high doses for each extraction method so that the same level of neutrophil recruitment was attained for each extract at the high dose. The extraction method that was optimized to retain particle bound and solvent extractable nonvolatile PAHs (multisolvent extraction), as well as particle mass from the filters, stimulated expression of CYP1A1 in the airways (at the mid dose) but did not increase CYP1A1 expression significantly in whole lung. On the other hand, inflammatory responses, including circulating platelet responses, which may be a key link between PM exposures and observed incidence of heart attacks and stroke, were greater in animals treated with the spin-down extract preparation.

CYP1A1 and CYP1B1 are 2 prototype CYPs that are abundant in lung tissue and can be induced by exposure to PAHs in the lung via activation of the Aryl hydrocarbon receptor AhR (Chan et al., 2013; Choudhary et al., 2003; Gebremichael et al., 1996). CYP1A1 is most abundant in alveolar type II cells and the endothelium (Forkert et al., 1996) and CYP1B1 is most abundant in airway epithelium (Chan et al., 2013). Exposure of mice by inhalation to concentrated ambient Fresno California PM was found to increase CYP1A1 gene expression in lung tissue including pulmonary vasculature, parenchyma, and airways (Tablin et al., 2012). A study of Milano winter PM_{10} and $\text{PM}_{2.5}$ removed from filters by sonication found that aspiration of either size fraction resulted in increased expression of CYP1B1 in the airways and alveoli of BALB/c mice (Farina et al., 2011). Our data indicate that the PAH content of the material is quite important for local lung responses, especially P450-mediated responses as the multisolvent extracted material had approximately 2.75-fold more total PAH than the spin-down preparation at the same dose. This may explain the large induction of CYP1A1 gene expression in the whole lung lobe exposed to the mid-level dose of the

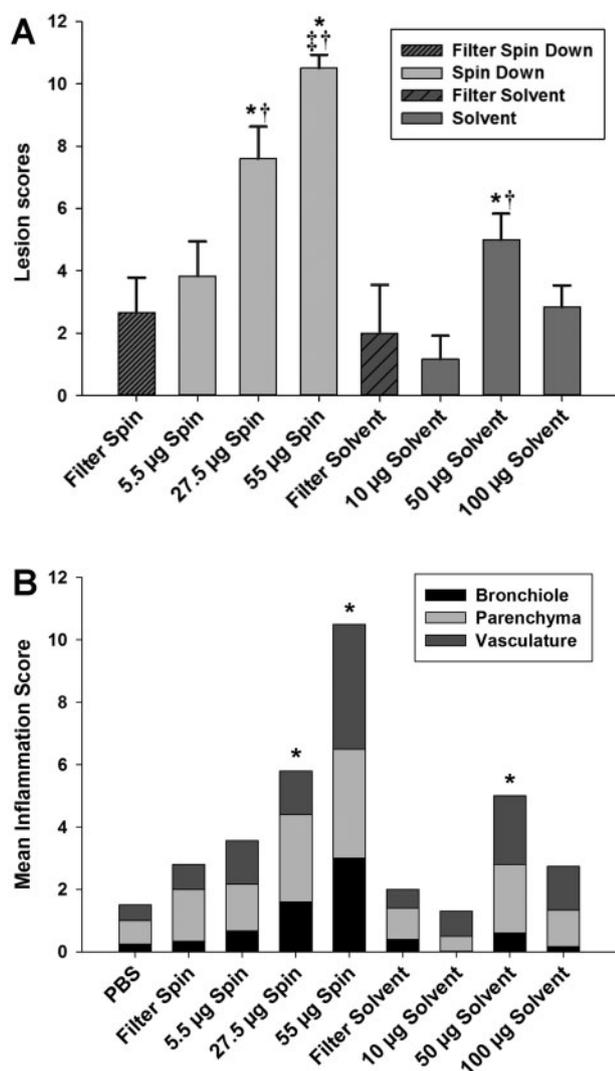


FIG. 3. All terminal bronchiole-alveolar duct junctions in each section were examined and an overall subjective severity score assigned relative to airway epithelial changes, inflammation in alveolar ducts and surrounding parenchyma, and periarterial inflammation and medial hypertrophy in the terminal branches of the pulmonary artery. Similarly, an overall severity score was assigned to each specimen. Lesions were scored from 0 to 4 with 4 being the most severe changes. Results were tabulated and average group scores calculated with statistical analysis by the Kruskal-Wallis non-parametric ranking test with a $P < 0.05$ significance level. $N = 6/\text{group}$. (*) significantly different from respective filter control. (†) significantly different from respective lowest dose. (‡) significantly different from respective mid dose.

multisolvent extracted particles. However, a higher dose of the same preparation did not result in an increase in gene expression for *CYP1A1* (but did increase protein expression). Further, the high dose of the spin-down preparation resulted in a significant decrease in *CYP1A1* gene expression. The explanation may be that gene expression is a rapid, and transient, phenomena and so the increase may have occurred at a different time point than 24 h after exposure. For instance, a gene may have been increased at 2 h after exposure but may appear unchanged at 24 h after exposure if a rapid response was needed to detoxify and excrete PAHs associated with a very high dose. We feel that this is a likely explanation for the high dose multisolvent extract data that show no change while the mid dose does show a change in response to PM exposure. *CYP1B1* was very responsive to the spin-down extract in conducting airways and alveoli,

and this correlated with tissue and BALF inflammation markers, but the multisolvent extract did not produce a response in the airways and produced only a small positive response at the highest dose in the whole lung.

It is curious that the 2 extract preparations differed substantially in their ability to stimulate P450 gene expression, even when derived from the same PM starting material and also acting through a similar mechanism of upregulation. Some of this may be due to different degrees of agglomeration of the material as it was delivered, but underscoring the importance of the extraction mechanism in determining the nature and the degree of the biological response. The extraction method likely alters interaction of components in the complex mixture ambient PM, thereby contributing to differences in biological responses. These interactions may involve both synergism and inhibition of toxicological effects. An illustration of this interaction has been shown for diesel exhaust where the sum of responses to individual components of the exhaust are significantly larger (by an order of magnitude) than the responses to the whole mixture, that is, the whole exhaust (DeMarini *et al.*, 2004; Hayakawa *et al.*, 1997), suggesting potential interference effects for complex multicomponent mixtures. Importantly, laboratory studies of mechanisms of PM-induced lung disease need to consider the composition of the sample and how it may be altered by processing, particularly of PAHs. Several recent human studies suggest a key role of PAHs, particularly the larger PAHs, in air-pollutant-associated childhood asthma (Gale *et al.*, 2012) as well as systemic inflammation (Delfino *et al.*, 2010). Because these responses may be time-dependent, future studies should consider evaluating CYP and other gene expression responses at additional timepoints. Further, the location of these responses in the lung tissue will be important as our data show that isolated conducting airways have different response profiles than whole lung.

Both preparations of SacPM were capable of inducing inflammation in the lung with the mid-level and high doses of both preparations causing a significant, dose-dependent increase in BALF neutrophils. However, since the high dose of the spin-down extract was approximately half that of the multisolvent extract, the spin-down preparation was more potent at inducing inflammation in the lung. This is also supported by a significant increase in *IL-1B* gene expression at the mid dose of the spin-down extract for whole lung (Fig. 4F) and by greater changes in the lung tissue pathology. *IL-1B* is an acute response cytokine that can be released by alveolar macrophages exposed to PM and can, in turn, stimulate lung epithelial cells to produce other proinflammatory mediators (Ishii *et al.*, 2004). The spin-down preparation induced *IL-1B* gene responses in the microdissected conducting airways and in the lung as a whole. Further, *IL-1B* does to a limited extent parallel BAL inflammation, suggesting a positive link. Involvement of the lung was greater on a histologic basis for the spin-down preparation versus the multisolvent preparation and included increased inflammation in 3 compartments (bronchioles, parenchyma, and vasculature). Notably the mid dose of the multisolvent extract also had a significant effect on these parameters, although in general the multisolvent extracted PM was half as potent that the spin-down extract.

This study provides additional evidence that inhaled $PM_{2.5}$ can have systemic effects. Although there have been numerous animal studies examining the effects of $PM_{2.5}$ exposure on cardiovascular endpoints (Tong *et al.*, 2010), few studies have evaluated the effect of PM on platelets (Tablin *et al.*, 2012). Platelets are key players in heart attacks and strokes which have been shown to be increased following exposure to PM. The evidence

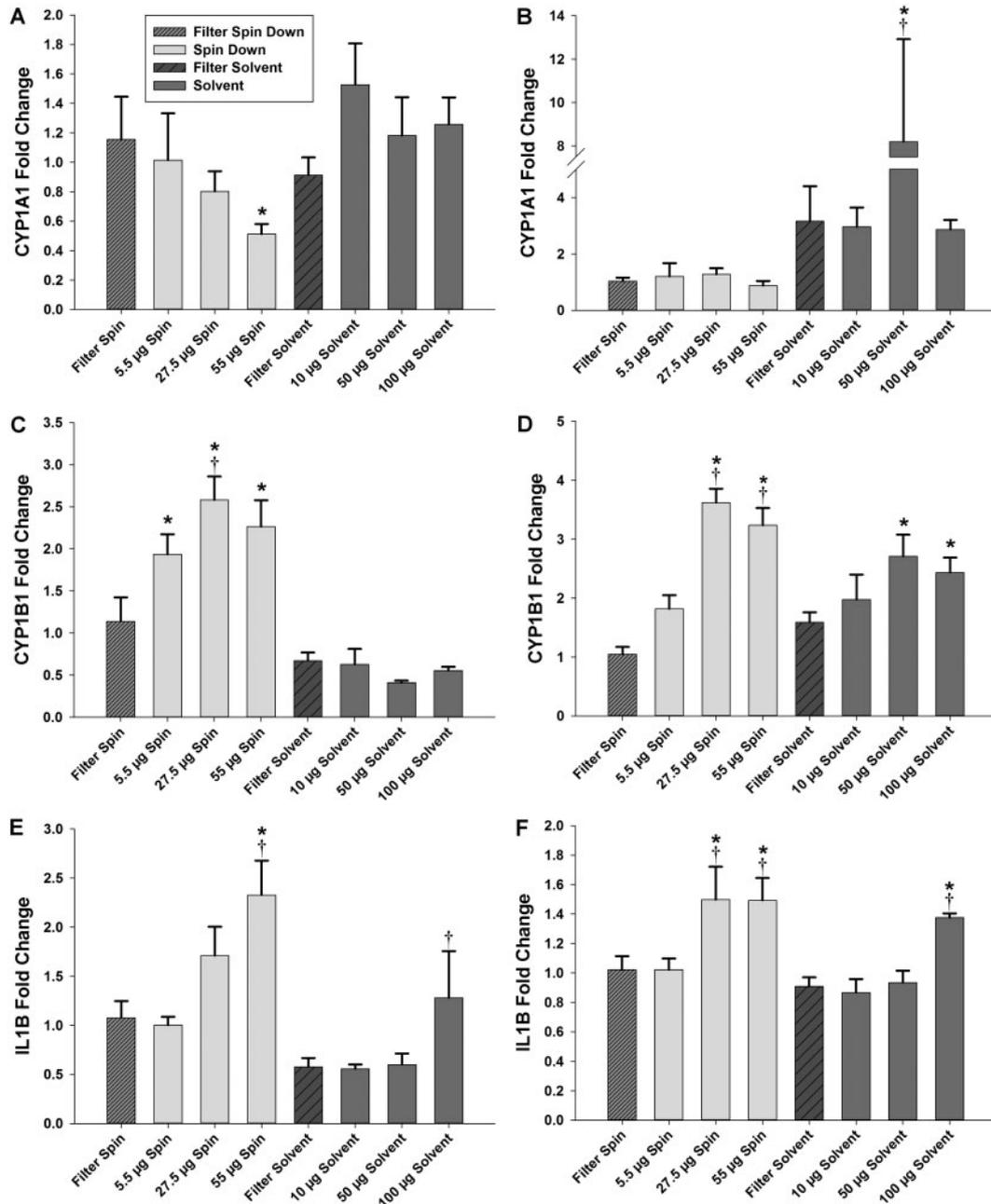


FIG. 4. Fold change in gene expression for CYP1A1 (A and B), CYP1B1 (C and D), and IL-1B (E and F) in the conducting airways (A, C, E) and whole lung (B, D, F) 24h after exposure to SacPM. All fold change was calculated relative to the spin-down filter control after normalizing to RPL13A reference gene. $N = 6/\text{group}$. (*) significantly different from respective filter control. (†) significantly different from lowest dose group of same treatment. $P < 0.05$.

from our study shows that platelet activation depends on how the test particles are prepared. The multisolvent extract exposed cells had significantly less CD41 in the resting population. CD41 is constitutively expressed on the platelet surface. When platelets are activated CD41 can be internalized and recycled or it may be shed in platelet microvesicles. Reduced CD41 suggests that the exposed cells were already activated, which was most likely the reason that they did not respond to thrombin. Further, the multisolvent extract (even from the filter sample) might have damaged and/or activated the platelets such that they were unable to secrete lysosomal granules and had only limited alpha granule release upon agonist stimulation. Previous studies have found that when mice were

exposed by inhalation to concentrated ambient particles over a 2-week period, particularly from rural sources, platelets are upregulated and show an even greater response to agonist stimulation, than in this study (Wilson et al., 2010). However, since most of the epidemiologic data on acute effects of urban PM_{2.5} indicates increases in mortality due to cardiovascular events (Dockery et al., 1993; Laden et al., 2006) and a lag time of 1 day or longer (Rosenthal et al., 2008), future studies should examine a time course of platelet response and the specific differences in urban and rural PM on platelet responses.

A key point to consider is why 2 extracts of the same PM give differences in response. The goal of this study is not necessarily to demonstrate that one extraction technique is better than

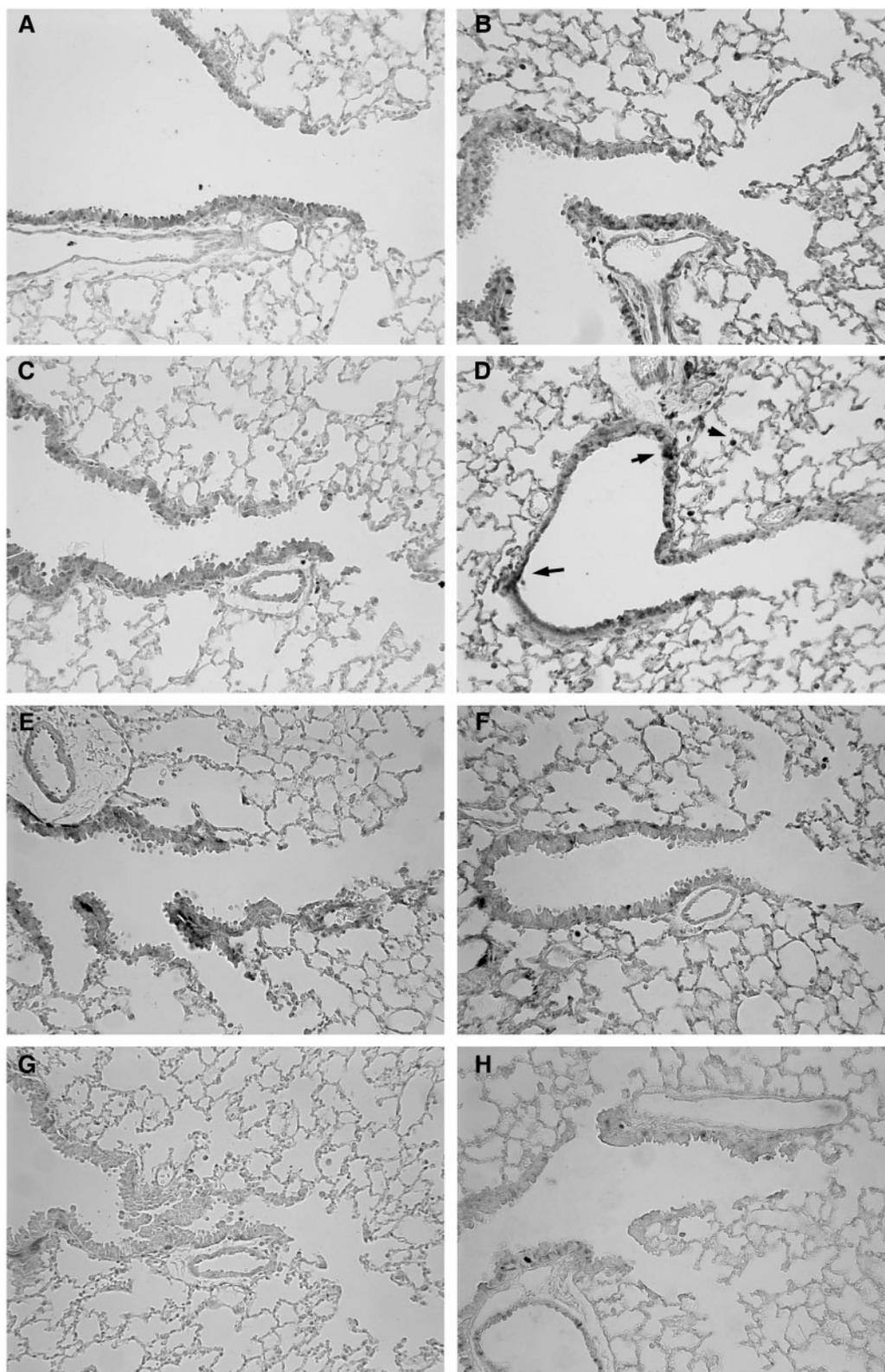


FIG. 5. Immunohistochemical localization of CYP1A1 and CYP1B1 protein in the lungs 24 h following treatment with the high dose of SacPM. CYP1A1 expression in control mice treated with the spin-down filter control preparation (A) or the multisolvent extract filter control (B). CYP1A1 expression in the airways treated with either the spin-down (C) or the multisolvent extract (D). Arrows indicate focal regions of the airways with high levels of expression (D). CYP1B1 expression in control mice treated with the spin-down filter control preparation (E) or the multisolvent extract filter control (F) or in the lungs of mice following treatment with either the spin-down (G) or the multisolvent extract (H). CYP1A1 protein is induced in both of the extract preparations containing SacPM with the multisolvent extract exposure exhibiting greater induction. Conversely, exposure to SacPM reduced CYP1B1 protein expression, regardless of extraction method. Three mice/group were examined for immunohistochemical staining.

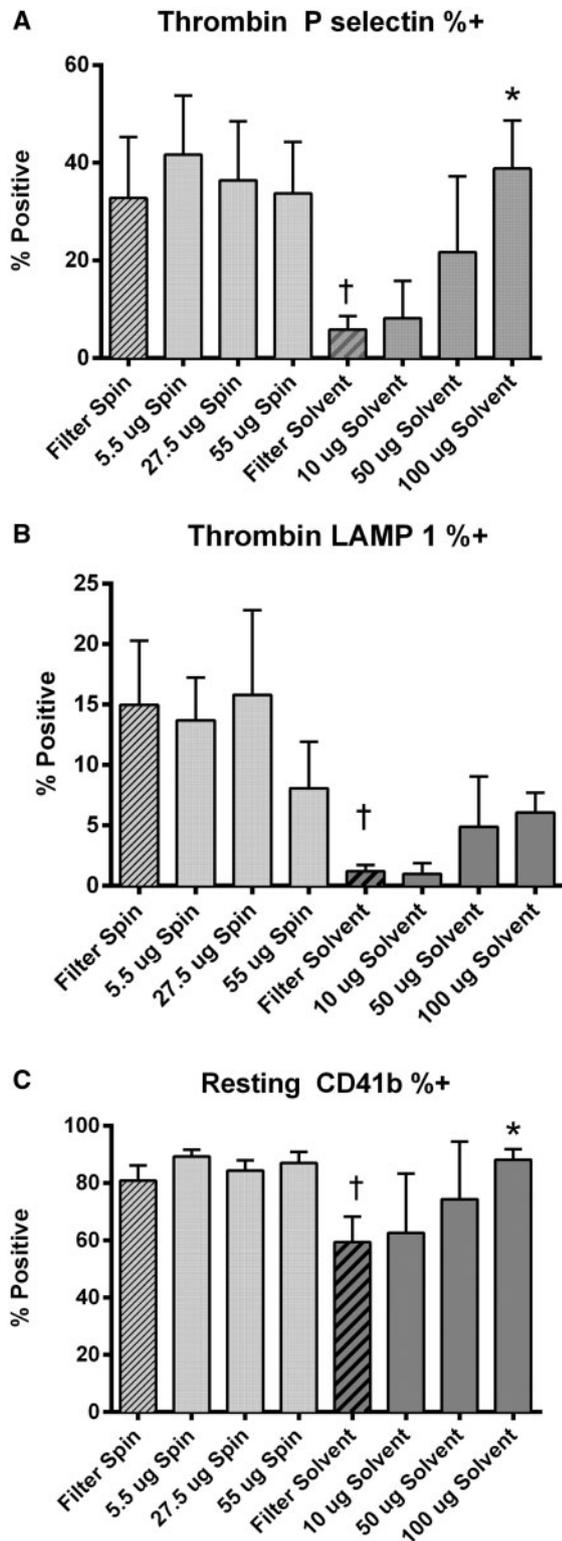


FIG. 6. Flow cytometric analysis of expression of platelet alpha granule membrane protein P-selectin (A) and lysosomal granule protein (LAMP-1) (B) in platelets stimulated by the potent physiological agonist thrombin. Platelets from animals exposed to the multisolvent extract are unable to respond to agonist stimulation by secretion of their lysosomal granules. There are high levels of expression of the integrin CD41b on the resting platelet surface from spin-down extracts, while it is significantly decreased in multisolvent extracts (C). (Dagger) significantly different from spin-down filter control group. (Asterisk) significantly different from filter control group same extraction method. $N = 6/\text{group}$.

another but rather that different extraction techniques designed with different sets of objectives can elicit differential toxicity and thus standardizing exposure objectives and extraction procedures to avoid introducing study bias is important. From the results of this work, it is perhaps reasonable to argue that the spin-down technique is “better” since it consistently elicits the largest toxicological response for almost all endpoints tested. However, a comparison of these 2 techniques based on the chemical composition of the extracted PM demonstrates that the multisolvent technique best conserves the original composition of the sampled PM (Bein and Wexler, under review). An alternate argument could be presented that the multisolvent technique is “better” since the primary objective of filter extraction is to conserve, as much as possible, the physical and chemical properties of the PM as it originally existed in the atmosphere. However, we cannot rule out that either extraction method may have also altered the properties of PM components that were removed from the filter and this may explain why control groups were different between the extracts. All PM samples used in this study were extracted from Teflon-coated borosilicate glass microfiber filters. Glass microfibers can be shed from the filter during the extraction process. These fibers can be retained in the extracted PM either as freely suspended microfibers or agglomerated with PM. The 2 extraction techniques include different methods to minimize the amount of microfibers retained in the extract. The multisolvent technique uses microporous membrane filtration while the spin-down technique uses a microcentrifuge-based cellular homogenization method. Based on direct gravimetric analysis of the extract controls, the spin-down technique does a better job of removing the microfibers than the multisolvent technique (Bein and Wexler, under review) and this is believed to account for the discrepancies between the toxicological responses to the controls. Overall the variance in the responses by particle extraction method presents a cautionary tale about how identical PM prepared using 2 different extraction methods can give different results.

This study is the precursor to a follow-up study to examine biological responses to PM from different locations. It is notable in the current work that all responses were observed just 24 h after dosing and so may continue to either increase (expected for some of the peripheral blood responses) or decrease (possibly some of the acute inflammation responses, especially for neutrophils) over time. One goal of this study was to set an optimal dose for the follow-up study. The optimal doses for the spin-down and multisolvent preparations were 27.5 and 50 μg , respectively. This is based on ability to recruit neutrophils at that dose as well as the lesion scores and gives room in any comparisons of different particle types to show an increase or a decrease over time. In conclusion, this study shows that extraction method for removal of particles from filters critically influences the biological responses observed following administration to the respiratory tract.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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