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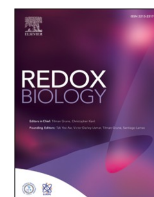

Lipid peroxidation index of particulate matter: Novel metric for quantifying intrinsic oxidative potential and predicting toxic responses

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ABSTRACT

Using particulate matter (PM) mass as exposure metric does not reveal the intrinsic PM chemical characteristics or toxic potential, which is crucial for monitoring the sources of emission causing adverse health effects and developing risk mitigating strategies. Oxidative stress and ensuing lipid peroxidation (LPO) in the lung are crucial underlying mechanisms of action by which PM drives cardiorespiratory disease. In the current study, we have postulated and demonstrated that the intrinsic potential of PM to elicit LPO, defined as "LPO index" as a novel approach for characterizing oxidative potential of PM (PM^{OP}) and predicting biological toxicity. First, we exposed unsaturated phosphatidylcholine (PC), an abundant phospholipid in the cell membrane, pulmonary surfactant, and lipoproteins to PM and analyzed the total burden of LPO byproducts generated as a measure of LPO index using a LPO reporter dye, BODIPY-C11. PM exposure resulted in a concentration-dependent increase in LPO. Second, we developed a novel method to expose the captured serum apoB100 lipoprotein particles to PM or its constituents and assessed the levels of specific oxidized-phospholipid on apoB100 particles by immunoassay using E06 monoclonal antibody (mab) that recognizes only PC containing oxidized-phospholipids (Ox-PCs). The immunoassay was highly sensitive to evaluate the PM LPO index and was modifiable by metal quenchers and exogenous antioxidant and radical quenchers. Third, to prove the pathophysiological relevance of Ox-PCs, we found that PM exposure generates Ox-PCs in mice lungs, pulmonary surfactant and lung cells. Fourth, we observed that treatment of macrophages with BAL fluid from PM exposed mice or PM-exposed pulmonary surfactant stimulated IL-6 production, which was abrogated by neutralization of Ox-PCs by mab E06 suggesting that Ox-PCs in lungs are proinflammatory. Overall, our study suggests that Ox-PCs as a probe of PM LPO index is a biologically relevant pathogenic biomarker and has a high value for evaluating PM^{OP}.

1. Introduction

Long-term exposure to ambient particulate matter (PM), mainly PM of aerodynamic diameter size less than 2.5 μm, is associated with the growing burden of chronic illnesses and premature deaths caused mainly by respiratory diseases (lower respiratory infection, obstructive pulmonary diseases, lung cancer) and cardiovascular diseases [1]. Major sources of PM in the atmosphere include biogenic emissions and anthropogenic emissions (such as automobiles, industries, power plants, agriculture, biomass burning). The global burden of disease (GBD) study [1] attributed 4 million deaths due to PM exposure in 2011, whereas *Burnett* et al. predicted 8.9 million deaths in the same year due to PM exposure using a new hazard-ratio model [2]. Because of ease of measurement, accuracy, and precision, most epidemiological studies, including GBD, have used the total mass of PM as a metric to correlate the exposure levels with adverse health outcomes [1]. However, PM mass metric fails to explain the differential health outcomes between the regions [3], seasons [4], or days with comparable levels of PM concentrations. Furthermore, PM mass metric assumes equal toxicity of PM derived from-i) combustion sources versus non-combustion sources (road dust, dessert dust, brake pad wearing); ii) primary emission versus secondary formation (generated by photochemical processing); iii)

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inorganic components (sulfates, nitrates) versus organic components (e.g., polycyclic aromatic hydrocarbons); iv) metal versus non-metal compounds. On the contrary, *Lelieveld* et al. [5], who developed a model to estimate the all-cause mortality from PM emitted by various point sources such as residential-commercial energy, industry, biomass burning, power plants, agriculture, and land use traffic, revealed that the mortality associated with PM exposure is markedly influenced by PM emission source and composition. In a clinical study on healthy subjects, instillation of PM collected from high and low polluted areas at equal mass showed differentially toxicity [6]. Experimental *in vitro* and *in vivo* model system found that PM collected from urban, rural, or industrial locations exhibit differential toxicities, including oxidative stress and inflammatory responses [7]. Therefore, an assumption of equal toxicity of PM per unit mass for modeling an exposure-response relationship in epidemiological studies could underestimate and mask the health risk posed by individual emission sources and also fails to reveal the intrinsic PM characteristics responsible for eliciting a toxic response in the lungs.

The knowledge of key chemical constituents of PM that exerts toxicity is essential for identifying and managing specific toxic emission sources as well as guiding environmental and health policies for mitigating adverse effects of PM. Numerous studies have attempted to establish a causal relationship between PM chemical constituents, PM exposure, and adverse health outcomes [3,8–11]. Despite increasing evidence, PM chemical composition has not been incorporated in epidemiological modeling studies for health risk assessment because of several challenges such as the highly heterogeneous chemical composition of PM, which varies with emission source, geographical location, meteorological conditions, space, and time; lack of toxicity profile of individual chemical species in PM; and analytical challenges to differentiate and quantify individual chemical constituents of PM between emission sources.

It is postulated that PM elicits pathogenic responses such as inflammation and cell death in the pulmonary and vascular system through oxidative stress-driven mechanisms [12]. Hence, there is a consensus that reactive oxygen species (ROS) generating capacity of PM referred to as “oxidative potential of PM” (PM^{OP}), could provide surrogate estimates of redox active organic and metal species in PM as well as toxic responses. Currently, the acellular and cellular methods to measure PM^{OP} features ROS generation or antioxidant depletion simulating one of the mechanisms of PM oxidative capacity. The most widely used acellular assay for measuring PM^{OP} is the dithiothreitol (DTT) assay (OP^{DTT}) and antioxidant depletion assay (glutathione assay (OP^{GSH}), and ascorbic acid (AA) assay (OP^{AA}). Employing these OP assays, a growing body of evidence has found a positive association between high PM^{OP} and cardiorespiratory diseases, especially in the regions of moderate PM concentrations [13–15]. However, the performance of these OP assays to evaluate PM^{OP} in epidemiological studies has been inconsistent and underpowered because of the variability in the sensitivity of individual OP assays to different redox-active organic or metal species in the PM sample [16, 17]. There is a need for an alternative robust bioassay for estimating PM^{OP}, which simulates other potential oxidative stress mechanisms and also pathophysiologically relevant in cardiorespiratory diseases.

ROS mediated lipid peroxidation (LPO) of phospholipids containing polyunsaturated fatty acids (PUFA) in pulmonary surfactant, and the cell membrane is a key pathological event in driving lung injury following oxidants exposure including PM [15,18]. The LPO process results in diverse reactive byproducts, including oxidized phospholipids, lipid peroxides, *trans*-4-hydroxy-2-nonenal, and malondialdehyde, which are shown to oxidize surfactant proteins [19], activate inflammatory responses [20] and elicit cell death signals [21,22] in lungs. We hypothesize that ‘LPO index’ defined as the intrinsic capacity of PM to oxidize phospholipids could serve as pathophysiologically relevant surrogate metric for evaluating PM oxidative potential and biological toxicity. In the current study, we have used 1-palmitoyl-2-arachidonoyl-*sn*-phosphatidylcholine (PAPC), a predominant phospholipid in the cell membrane, as a target substrate for oxidation to characterize the intrinsic potential of PM to induce LPO using BODIPY-C11 reporter dye. Next, using captured serum apoB-100 lipoprotein particles as a target substrate, we have developed a novel sensitive physiologically relevant immunoassay to assess LPO index of PM, which probes only Ox-PCs but not native phospholipids. Lastly, using PM exposed -mouse model, -pulmonary surfactant, and lung cell line, we demonstrate the physiological relevance of Ox-PCs and their role in mediating inflammatory responses in the lungs.

2. Materials and methods

2.1. Chemical and reagents

Dithiothreitol, Glutathione, N-acetyl L-cysteine, Disodium EDTA, Deferoxamine, DCFHDA, and 9,10 Phenanthrenequinone were purchased from Sigma Aldrich. The BODIPY C11 581/591 (Cat no #D3861) was purchased from Invitrogen-Thermo scientific. 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, antibody MB47 and monoclonal antibody E06 were purchased from Avanti Polar Lipids. Ascorbic acid was purchased from SRL chemical Pvt. Ltd. The IL-6 ELISA kit was purchased from Invitrogen-Thermo scientific. DL-Methionine and D-mannitol was purchased from S.D. Fine- Chem Limited, Mumbai, India. Clinical surfactant, Neosurf (Bovine Lipid Extract Surfactant Suspension) manufactured by Cipla Pharmaceutical company was purchased from local medical store. The secondary antibody goat anti-mouse IgM-HRP (Cat no #ab97230) was purchased from Abcam.

2.2. $PM_{2.5}$ extraction and preparation of PM fractions

$PM_{2.5}$ samples were collected on a pre-combusted quartz filter during the winter of 2014–15 (December–January) at Kanpur. The aerosol sampling was performed using high volume impaction-based PM sampler [23]. These aerosol filter samples were procured from Dr. Tarun Gupta laboratory at the Indian Institute of Technology, Kanpur and his laboratory has characterized the chemical composition of the study PM samples (details in supplementary methods). PM sample was extracted from the quartz filters in 100% HPLC grade methanol using an Ultra bath sonication for 10 min. After extraction, the PM samples were dried under nitrogen gas and stored at −80°C until further analysis. Blank filters were subjected to a similar extraction procedure. To prepare PM-whole fraction (PM-WF), a known amount of PM (4 mg/ml) was suspended in phosphate-buffered saline (PBS); sonicated for 5 min using probe sonicator, and the obtained fine suspension was immediately used for experimentation. To prepare PM-soluble fraction (PM-SF), the stock of PM-WF (4 mg/ml) was centrifuged at 2500 RPM for 5 min to remove insoluble components, and the supernatant was collected and used for experiments.

2.3. Preparation of oxidized-PAPC (Ox-PAPC)

A known amount of PAPC (10 mg/ml, Avanti Polar Lipids) prepared in chloroform was dried under nitrogen gas in an amber glass vial, exposed to air for 72 h at room temperature for oxidation, and then resuspended in chloroform and stored at −80°C until further analysis. For experimentation, Ox-PAPC stock solution was dried under nitrogen gas and then suspended in PBS.

2.4. DTT assay and ascorbic acid assay

DTT assay was performed as described previously [24] with modifications. PM-WF or PM-SF (50 or 100 μg/ml) was exposed to DTT (100 μM) in potassium phosphate buffer (0.1 M, pH7.4) at 37 °C in 100 μl reaction volume, and the reaction was stopped at designated time points (0, 30 and 60 min) by adding 100 μl of 10% trichloroacetic acid. To this reaction mixture, 25 μl of 10 mM 5,5′-dithiobis (2-nitrobenzoic acid and

then 75 μl of Tris-HCL buffer (0.4 M, pH 8.9) was added, and the formation of 5-mercapto-2-nitrobenzoic was monitored spectrophotometrically at 412 nm using a multimode plate reader (Molecular Devices). The 9,10-Phenanthrenequinone and blank filters were used as positive and negative controls respectively in every run (data not shown). For ascorbic depletion assay [25], PM samples (PM-WF or PM-SF) were incubated with 2 mM of ascorbic acid solution (prepared using HPLC grade water) in a 96-well UV plate, and the absorption was measured at 265 nm every 5 min for 1 h. The maximum rate of depletion of DTT or ascorbic acid was determined by linear regression of the linear section data, plotted as absorbance against time, and the result was expressed as nmol/min.

2.5. PM LPO index using BODIPY C11

Boron dipyrromethane difluoride (BODIPY)-C11 is a fluorescent reporter dye that is widely used to measure oxidized phospholipids as a marker of LPO inside the cells [26]. BODIPY-C11 is a fatty acid analog whose fluorescence characteristics shift from red to green range of visible spectrum upon oxidation of the polyunsaturated butadienyl component of the dye [26]. Twenty micrograms of PAPC in chloroform or equivalent volume of chloroform were transferred into amber glass vials and dried under nitrogen gas. To these vials, 500 μl of PM-WF of various concentrations (suspended in PBS) or PBS alone was added, vigorously mixed, and incubated for 1.5 h at 37 °C with shaking (100 RPM). After the incubation period, BODIPY-C11 solution (final concentration, 10 μM) was added to each tube and incubated for an additional 30 min with shaking. In the end, the reaction mixture was transferred to an opaque 96-well plate, and the levels of oxidized and unoxidized BODIPY-C11 were measured at ex/em 485/520 nm and ex/em 580/595 nm, respectively, using a multiplate reader (Molecular Device). The data was represented as a ratio of oxidized to unoxidized BODIPY-C11. To measure the levels of oxidized phospholipid content in PAPC and Ox-PAPC stock solution, equal amounts (20 μg) of PAPC and Ox-PAPC in chloroform was transferred to amber glass tubes; dried under nitrogen gas and then incubated with 500 μl of BODIPY C11 solution (final concentration 10 μM) for 1 h with shaking. The reaction mixture was transferred to an opaque 96-well plate, and fluorescence was measured.

2.6. PM LPO index using DCFH assay

As described by *Navab et al.* [27], the DCFH-DA solution (2 mg/ml) was prepared in an amber glass tube by dissolving in methanol and stored at room temperature for 30 min, which results in the conversion of DCFH-DA to DCFH. A 10 μl aliquot of DCFH stock solution was transferred to amber color polypropylene tubes, and the methanol was evaporated under nitrogen gas. To the same tubes, 20 μg of PAPC in chloroform or equivalent volume of chloroform was added and dried under nitrogen gas. Subsequently, 25 μl of whole fraction of PM sample of varying concentration in PBS was added, and the reaction volume was made up to 1 ml and mixed vigorously. The tubes were incubated for 1.5 h at 37 °C with shaking. In the end, the reaction mixture was transferred to an opaque 96-well plate, and fluorescence was read at Ex/Em 485/535 nm using a multiplate reader.

2.7. Immunoassay to assess PM LPO index using apoB100 particles

The schematic of the immunoassay procedure is represented in Fig. 3. The blood sample was collected from healthy volunteers for serum isolation under the protocol approved by the JSS Academy of Higher Education & Research Institutional Ethical Committee. Briefly, the microtiter plate wells were coated with capture antibody MB47 (100 μl of 2.5 μg/ml stock) and incubated overnight at 4ºC. After washing with PBS containing 0.1% tween-20 (PBST), the wells was blocked with 100 μl of 1% BSA (fatty acid-free) for 1 h at room temperature. After washing again with PBST, 100 μl of diluted normal serum (1:50) was added and incubated for 2 h. Joseph Witztum and colleagues [28] reported that 0.25 μg/well of MB47 is sufficient to capture a saturated and equal amount of apoB100 lipoprotein particles into each well from the serum. Post-incubation, the plates were washed thrice with PBST and incubated with 100 μl of PM-WF or PM-SF sample (10–100) μg/ml for 2 h at 37 °C with shaking in the dark. Fenton reagent ($FeCl_2$ (0.65 mM): H_2O_2 (30 mM)) was included as a positive control. Subsequently, plates were washed thrice with PBST, and 100 μl of mab E06 (1.25 μg/ml, Avanti Polar lipids) was added and incubated for 1.5 h at room temperature. Post-incubation, plates were rewashed, and 100 μl of anti-goat IgM-HRP (1:2000 dilution) was added and incubated for another 1.5 h. After washing, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the plates, and after 15 min of incubation, 50 μl of stop solution (2 N H_2SO_4) was added. The plate was read at 450 nm using a multimode plate reader. The data were represented as Ox-PCs normalized to the vehicle (serum without PM).

2.8. Antioxidant and metal chelators treatment

PM-WF or PM-SF samples of varying concentration was incubated with antioxidant (GSH, NAC or methionine), metal chelators (EDTA or deferoxamine) free radical quenchers (mannitol) or respective vehicle for 45 min at 37 °C with shaking. Subsequently, a desired volume of the reaction mixture was directly used to assess the PM LPO index.

2.9. Mice studies

C57BL/6 mice (male, 6–8 weeks) were procured from a commercial vendor (ADITA BIOSYS PVT. LTD, Tumkur, Karnataka) and housed at the Central Animal Facility of JSS Medical College maintained at 25ºC temperature and 12 h light and dark cycle. The mice had ad libitum access to a standard sterilized chow diet and water. Mice were administered intranasally with 10 μg/mouse of PM-WF in 25 μl volume of PBS every other day for 10 days. Control mice were administered with an equal volume of PBS alone. The JSS Academy of Higher Education & Research Institutional Animal Ethical Committee approved the animal work carried in this study.

2.10. Bronchoalveolar lavage (BAL) assay

Bronchoalveolar lavage assay was performed in mice as described elsewhere [29]. Briefly, mouse lungs were lavaged with 1 ml of PBS twice, and the pooled lavaged fluid was centrifuged for 8 min at 1500 RPM. The supernatant from the BAL fluid was stored at −80 °C until analysis.

2.11. Exposure of PM to bovine lipid extract surfactant (BLES) and evaluation of LPO by immunoassay

BLES (Neosurf, CiplaMed, 27 mg/ml), a clinical surfactant was diluted to 5 mg/ml working stock solution with sterile PBS. Fifty microliters of the working stock of BLES solution were incubated with PM samples of varying concentrations in amber color polypropylene tube, and the final reaction volume was made up to 200 μl. The reaction mixture was vortexed and incubated at 37 °C overnight in the dark. After incubation, the levels of Ox-PCs accumulated in BLES were measured by immunoassay. Briefly, 100 μl of diluted reaction mixture (1:5) was plated onto wells of a microtiter plate and incubated overnight at 4 °C in the dark. After washing, the plate was blocked with 1% BSA for 1 h, again washed, and then incubated with mab E06 for another 1.5 h. Post incubation, plates were washed, and 100 μl of secondary ab IgM-HRP (1:2000) was added, and the rest of the procedure was similar as described above (immunoassay procedure).

2.12. Cell culture

Normal human bronchial epithelial cells, Beas2B were cultured in DMEM: HAMF12 medium containing 10% FBS and 1% of penicillin and streptomycin solution at 37 °C in 5% CO_2 [30]. For experimentation, Beas2B cells were plated at a cell density of 25000/well in a 96-well plate, and after overnight culture, the cells were treated with PM-WF or PM-SF for 24 h. Post incubation, the culture media was collected,

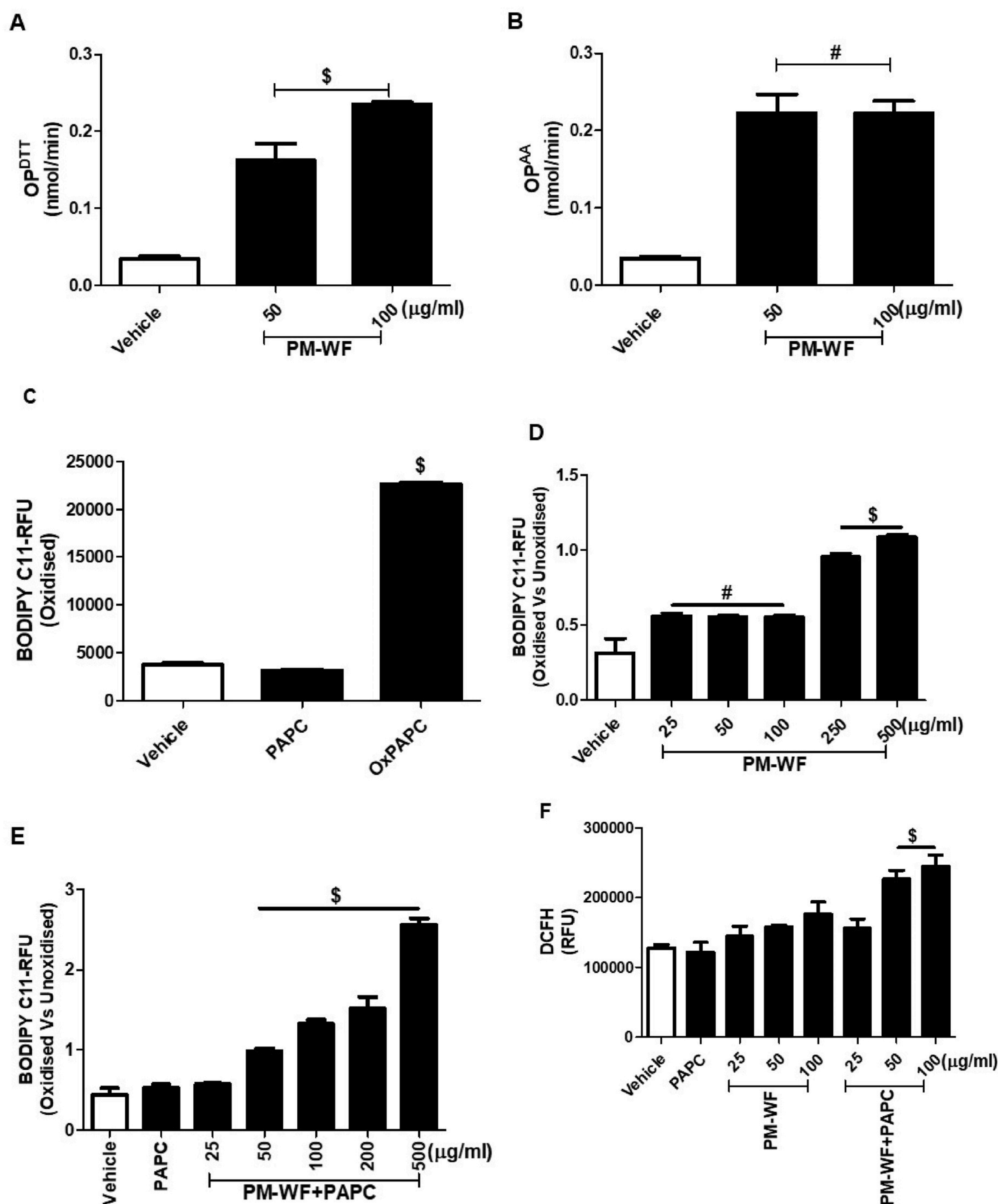

Fig. 1. Characterization of the oxidative potential of PM by conventional acellular assay and LPO index of PM using phospholipid, PAPC as substrate and BODIPY-C11 as LPO reporter dye. A-B. OP^{DTT} assay (A) and OP^{AA} assay (B) revealed the oxidative potential of the PM test sample. PM samples was incubated with DTT or AA, and the rate of depletion of DTT or AA was evaluated for 1 h, \$ $P < 0.0001$ and #$P < 0.05$ compared to vehicle. C. Validation of BODIPY-C11 probe to analyze lipid peroxide content in Ox-PAPC stock sample. Ox-PAPC sample was incubated with BODIPY-C11, and fluorescence was measured to monitor the levels oxidized and non-oxidized BODIPY-C11. \$ $P < 0.0001$, compared to vehicle D-F. Monitoring PM induced peroxidation of PAPC using BODIPY-C11 (D–E) or DCFH (F). Increasing concentrations of PM (25–500 μg/ml) was incubated in the absence or presence of PAPC for 2 h. Levels of lipid peroxide generated was monitored by BODIPY-C11 or DCFH probe. The data from BODIPY-C11 assay is represented as mean ± SD of the ratio of oxidized (ex/em, 485/520 nm) to reduced (ex/em, 580/595 nm) BODIPY-C11. The data from DCFH assay is represented as mean ± SD of relative fluorescence units. \$ $P < 0.0001$ compared to vehicle or PAPC alone; #$P < 0.05$ compared to vehicle.

centrifuged, and used for measuring Ox-PCs by immunoassay. Briefly, the 100 μl of culture media was coated onto a 96-well plate and incubated overnight at 4 °C. After washing with PBST and blocking with 1% BSA, 100 μl of mab E06 was added and incubated for 1.5 h. The rest of the procedure was similar as described above (immunoassay procedure).

J774.1 murine macrophage cell line was used to assess whether Ox-PCs drives the inflammation. J774.1 macrophages were cultured in a DMEM high glucose medium containing 10% FBS with 1% of penicillin and streptomycin solution [31]. Macrophages were plated at a cell density of 50,000 cells/well in a 96-well plate and treated with i) FBS free culture media diluted at 1:1 ratio with BAL fluid retrieved from PM or air-exposed mice and ii) diluted BLES reaction mixture treated with PM or vehicle. For neutralization of Ox-PCs, BAL fluid from PM exposed mice or oxidized BLES reaction mixture was incubated with mab E06 for 1 h prior to exposure to macrophages. Post-treatment, the culture media was collected, and IL-6 levels were measured using an IL-6 ELISA kit (Invitrogen Thermo scientific) by following the procedure recommended by the manufacturer.

2.13. Statistical analysis

Data presented for OP^{DTT} assay and OP^{AA} assay is the mean of three independent experiments performed in duplicates. All other cell-free and cell-based experiments were repeated at least two or three times and the data presented is representative of single experiment performed in triplicates. The data is presented as mean ± SEM, and the statistical analysis was performed by paired Student's T-test, one-way ANOVA and two-way ANOVA with Bonnferroni's post hoc test using Graph Pad Prism software version 5.0.

3. Results

3.1. Monitoring intrinsic potential of PM to oxidize phospholipid, PAPC using BODIPY-C11 LPO sensor

First, we characterized the PM^{OP} by commonly used OP assays, OP^{DTT} and OP^{AA}. $PM_{2.5}$ (henceforth referred to as PM) has been used in the entire study and unless otherwise stated, PM-whole fraction was used for the experiments (see details in methods). Both OP^{DTT} and OP^{AA} assay showed the intrinsic OP associated with the test PM samples. OP^{DTT} assay showed a concentration-dependent increase in PM^{OP} (Fig. 1A), whereas OP^{AA} assay indicated comparable levels of intrinsic OP at low and higher PM concentration (Fig. 1B). Next, we evaluated the OP of soluble fraction of PM (henceforth referred to as PM-SF) by OP^{DTT} and OP^{AA} assay. Although significant, OP of PM-SF was relatively moderate compared to PM-whole fraction (Supplemental Figs. S1A–B). Next, we evaluated the intrinsic potential of PM to oxidize phospholipid using BODIPY-C11. BODIPY-C11 is reactive to several oxy-radical species generated by oxidation of hydroperoxides but not to hydroperoxides per se [32]. Also, BODIPY-C11 is insensitive to superoxide and transition metals per se [32]. We first validated the utility of BODIPY-C11 to assess the levels of oxidized lipids in a cell-free model system by incubating an equal concentration of unoxidized PAPC or oxidized-PAPC (Ox-PAPC) with BODIPY-C11. Ox-PAPC was generated by air oxidation of PAPC (see methods). The relative fluorescence units of BODIPY-C11 were markedly increased (2-times) by Ox-PAPC as compared to unoxidized PAPC. The relative fluorescence units of unoxidized PAPC was comparable to the vehicle (only BODIPY-C11), indicating undetectable levels of oxidized lipids in the unoxidized PAPC stock (Fig. 1C).

Next, to assess the intrinsic capacity of PM to induce LPO, we incubated increasing concentration of PM with or without PAPC for 2 h and then measured the levels of oxidized lipids generated using BODIPY-C11. The oxidation of BODIPY-C11 by PM per se was minimal, although there was a modest but significant increase in oxidation of BODIPY-C11 at doses 250 and 500 μg/ml (Fig. 1D). In contrast, PM incubation with PAPC for 2 h resulted in a significant concentration-dependent increase in the generation of oxidized lipids as detected by an increase in oxidation of BODIPY-C11 (Fig. 1E).

Next, we confirmed the intrinsic potential of PM to oxidize PAPC using a non-specific ROS dye, 2′, 7' dichlorofluorescein (DCFH) assay that is a commonly used technique to assess the OP of PM per se [33]. We found that Ox-PAPC but not unoxidized-PAPC increased oxidation of DCF, indicating reactivity of oxidized lipids with DCFH (Supplementary Fig. S2). As expected, PM per se increased oxidation of the DCF. However, PM-induced oxidation of DCF was markedly enhanced in the presence of PAPC, indicating accumulation of oxidized lipids (Fig. 1F).

3.2. PM-induced oxidation of PAPC is modifiable by antioxidants

Next, we addressed if direct antioxidant molecules modulate PM-induced oxidation of PAPC. First, we investigated whether co-treatment of Ox-PAPC with antioxidant, GSH, and N-acetyl L-cysteine (NAC) neutralizes the reactivity of oxidized lipids to BODIPY-C11. Co-incubation of Ox-PAPC with GSH or NAC significantly decreased the oxidation of BODIPY-C11 (Fig. 2A and B), indicating that GSH and NAC neutralize the reactivity of oxidized lipids in Ox-PAPC.

Next, we investigated if GSH or NAC diminishes the intrinsic potential of PM to oxidize PAPC. We incubated PM samples of varying concentrations with GSH, NAC or PBS for 45 min and then added the reaction mixture to PAPC and incubated for another 2 h. We found that pre-treatment of PM with GSH failed to reduce intrinsic potential to oxidize PAPC. Conversely, GSH presence enhanced PM-mediated oxidation of BODIPY-C11 (Fig. 2C) in the absence or presence of PAPC. To confirm this observation, we used DCFH assay and found that GSH presence augments PM-induced oxidation of PAPC (Supplementary Fig. 3). To further validate this observation, we used a traditional OP^{DTT} assay. In line with the data obtained by BODIPY-C11 dye and DCF assay, OP^{DTT} assay also showed that GSH presence failed to reduce PM^{OP} (Supplementary Fig. 4). Next, we evaluated the effects of NAC on PM-induced oxidation of PAPC using BODIPY-C11 assay. Interestingly, at all doses tested, NAC inhibited PM-induced oxidation of PAPC as compared to the vehicle-treated group (Fig. 2D). Together our data suggest that NAC but not GSH is more effective in reducing LPO index of PM in the cell-free system.

3.3. Immunoassay for evaluation of LPO index of PM using ApoB100 lipoprotein particles

Circulating ApoB100 containing lipoproteins are LDL and very low-density lipoprotein (VLDL), of which LDL is the most abundant circulating apoB100 containing lipoprotein. Phosphatidycholine (PCs) phospholipids including PAPC are the predominant phospholipids on the surface of LDL particles [34], and oxidized-phosphatidylcholine (Ox-PCs) represents major proatherogenic oxidized phospholipids on Ox-LDL [28]. We used apoB100 lipoprotein particles as a target substrate for assessing the LPO index of PM. Serum apoB100 particles were captured on a microtiter plate using MB47 antibody and exposed to increasing concentrations of PM-whole fraction for 2 h (see schematics Fig. 3A). To detect the levels of Ox-PCs on the apoB100 lipoprotein particles, we designed an immunoassay using an E06 monoclonal antibody (mab) that recognizes the phosphorylcholine polar head group of only Ox-PCs but not of non-oxidized PCs [28]. We first validated the immunoassay assay using the Fenton reagent as a positive control. Fenton reagent markedly increased the accumulation of Ox-PCs on apoB100 particles (Fig. 3B). The specificity of E06 to detect Ox-PCs but not native PCs was evaluated using PAPC and Ox-PAPC (Supplemental Fig. 5). Next, we observed that exposure of apoB100 particles to PM (whole-fraction) caused a concentration-dependent increase in the accumulation of Ox-PCs (Fig. 3B). The immunoassay was sensitive to detect and quantify Ox-PCs on apoB100 particles induced by PM concentrations as low as 10 μg/ml. Exposure to PM-soluble fraction also elicited a dose-dependent increase in Ox-PCs on apoB100 particles. The

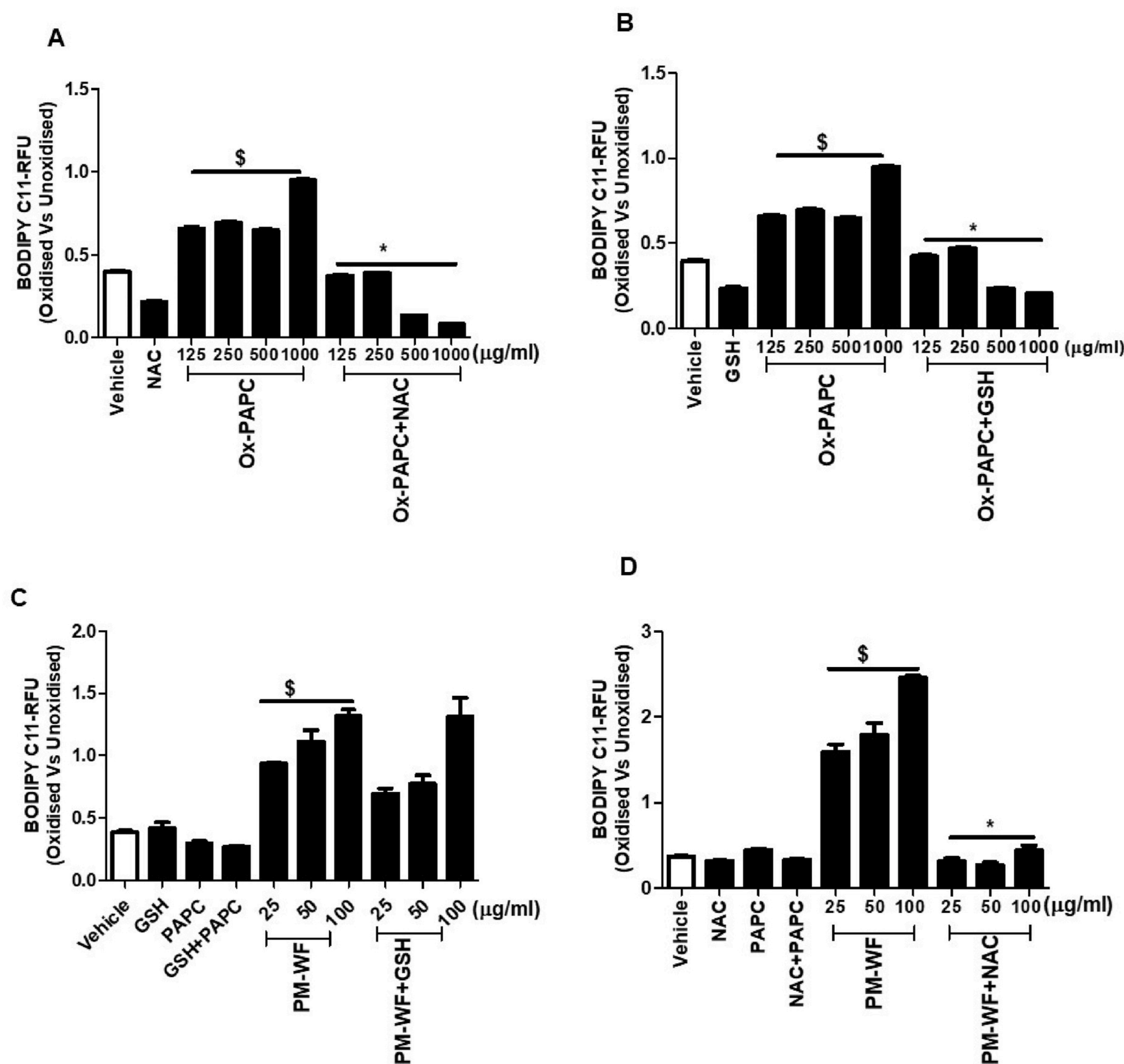

Fig. 2. Modulation of PM induced oxidation of PAPC by exogenous antioxidants, NAC or GSH. A-B. Treatment of Ox-PAPC stock sample by NAC (A) or GSH (B) neutralizes lipid hydroperoxide radical content as detected by oxidation of BODIPY-C11 dye. Ox-PAPC (125–1000 μg/ml) was incubated with NAC (0.5 mM) or GSH (1 mM) for 2 h, and subsequently, BODIPY-C11 dye was added. Fluorescence was measured to monitor the levels of oxidized and non-oxidized BODIPY-C11, $ P < 0.0001, compared to vehicle; *P < 0.0001, compared to respective concentration of Ox-PAPC alone. C-D. NAC (D) but not GSH (C) reduces the peroxidation of PAPC by PM. PM was pre-treated with vehicle (PBS), NAC (0.5 mM) or GSH (1 mm) for 1 h and then the mixture was incubated with PAPC for an additional 2 h. At the end of the reaction, BODIPY-C11 was added, and fluorescence was measured to monitor the levels of oxidized and non-oxidized BODIPY-C11. $ PM < 0.0001 compared to vehicle; *P < 0.0001 compared to PM-WF alone. The data is represented as mean ± SEM of the ratio of oxidized (ex/em, 485/520 nm) to reduced (ex/em, 580/595 nm) BODIPY-C11.

levels of Ox-PCs generated on apoB100 particles by PM soluble fraction was comparable to whole-PM at the highest dose (Fig. 3B). The metals and organic compounds in PM are main mediators of oxidative potential of PM [35] and chemical analysis revealed significant amounts of these components in our study PM sample (Supplementary Table S1–S2). To determine if the metals in our study PM sample are the key factors driving the oxidation of apoB100 particles, we treated the PM-whole fraction and PM-soluble fraction with metal chelators [36], EDTA and deferoxamine for 1 h before incubating the mixture with apoB100 particles. Both EDTA and deferoxamine significantly reduced the accumulation of Ox-PCs on apoB100 particles exposed to whole fraction of PM (Fig. 3C). Interestingly, both EDTA and deferoxamine completely abrogated the Ox-PCs generation on apoB100 particles suggesting that metals are the predominant components in the PM-soluble fraction responsible for PM LPO index (Fig. 3D). Quinones are the redox active organic compounds which are major contributors of PM^{OP}. To determine if quinones elicits oxidation of lipids in our assay system, we used 9–10, Phenanthrenequinone (PQ) is a major quinone in diesel exhaust particles and also present in PM [37]. PQ catalyzes ROS generation indefinitely by redox cycling and drives lung injury by inducing oxidative stress [37]. We found that PQ exposure resulted in a significant increase in oxidation of apoB100 particles (Fig. 3E) as compared to the vehicle as indicated by the levels of Ox-PCs. Next, we evaluated if exogenous antioxidant NAC modulates PM-induced oxidation of apoB100. In the presence of NAC, oxidation of apoB100 particles by whole PM or PM-soluble fraction was significantly reduced (Fig. 3F). We also investigated if radical quenchers, methionine (singlet-oxygen quencher) and mannitol (hydroxyl radical quencher) modulates apoB100 particles oxidation by PM. Methionine (10 mM) presence significantly reduced oxidation of apoB100 particles by PM-WF and PM-SF samples (Fig. 3G) indicating generation of singlet-oxygen reactive species by PM sample in our cell-free assay system. However, mannitol (10 mM) showed modest

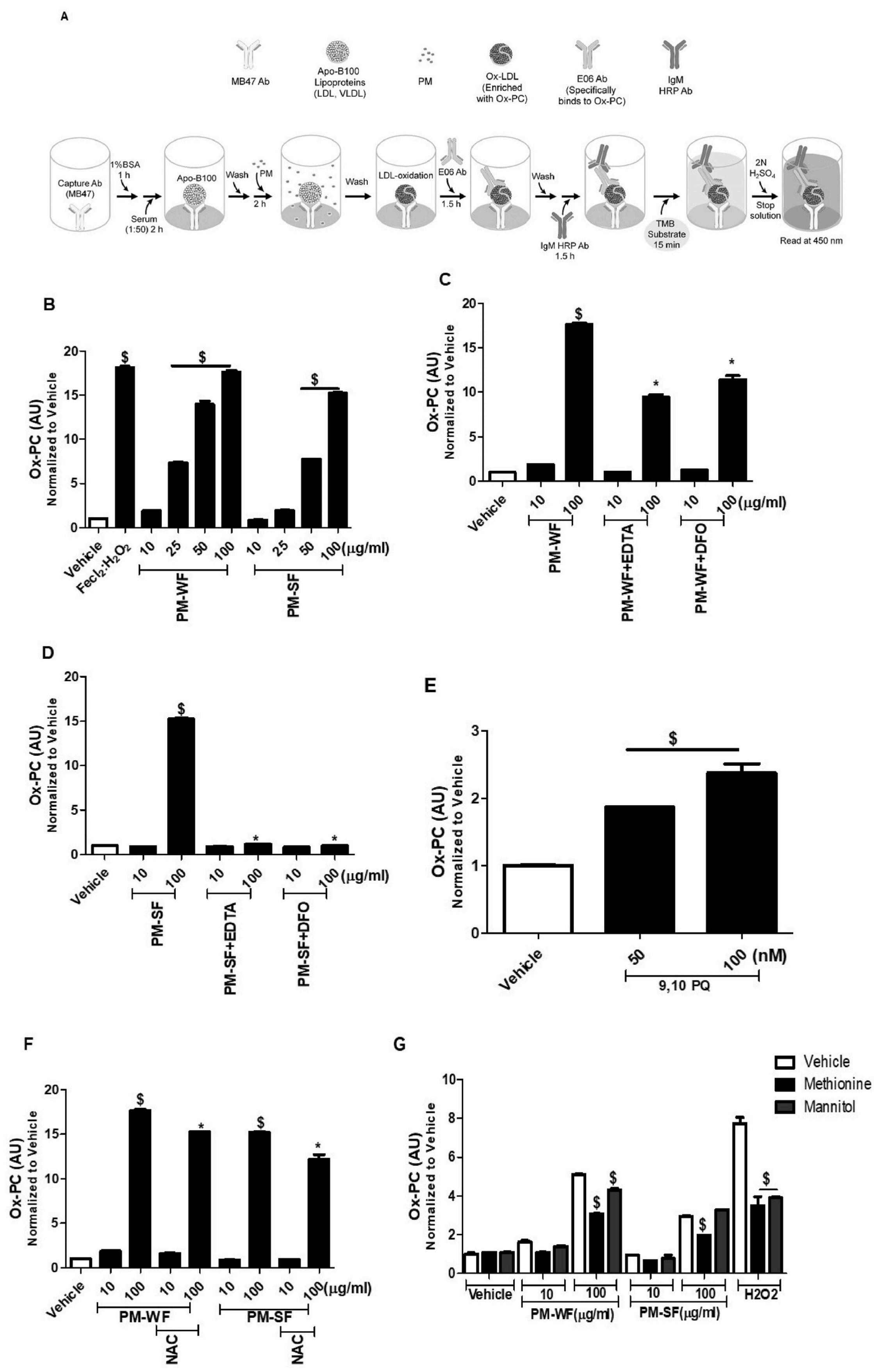

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Fig. 3. Monitoring PM-induced Ox-PCs levels on apoB100 particles by immunoassay as a measure of PM LPO index. A. Exposure of apoB100 particles to PM whole fraction (PM-WF) or PM soluble fraction (PM-SF) induces peroxidation of surface phospholipids as indicated by an increase in the levels of Ox-PCs. The captured apoB100 particles (see method) were exposed to Fenton reagent system ($FeCl_2$: H_2O_2), PM-WF or PM-SF for 2 h. The levels of Ox-PCs present on the surface of apoB100 particles was measured by immunoassay using E06 Mab. \$, $P < 0.0001$ compared to vehicle. B-D. PM induced peroxidation of apoB100 particles was reduced in the presence of metal chelators. PM-WF or PM-SF was pre-treated with vehicle (PBS), EDTA (1 mM) (C) or deferoxamine (DFO, 0.5 mM) (D) for 1 h and then the mixture was incubated with apoB100 particles for an additional 2 h. The levels of Ox-PCs generated on the surface of apoB100 particles were measured by immunoassay, \$$P < 0.0001$, compared to vehicle; *$P < 0.0001$, compared to PM alone E. Exposure of apoB100 particles to 9,10-phenanthrenequinone (PQ) induced peroxidation of apoB100 particles. The captured apoB100 particles were exposed to PQ (50 and 100 nM) for 2 h, and the levels of Ox-PCs on the surface of apoB100 particles were measured by immunoassay, \$ $P < 0.000$, compared to vehicle F-G. NAC and methionine mitigated the PM induced oxidation of ApoB100 particles. Captured apoB100 particles were pre-incubated with NAC, methionine or mannitol for 45 min and then exposed to PM-WF, PM-SF or H2O2 (1 mM) for additional 2 h. The levels of Ox-PCs generated on apoB100 particles were measured by immunoassay, \$ $P < 0.0001$ compared to vehicle; *$P < 0.05$, compared to PM alone (100 μg/ml).

effect in inhibiting oxidation of apoB100 by PM-WF whereas it failed to protect apoB100 oxidation by PM-SF (Fig. 3G). Both methionine and mannitol were markedly effective in blocking the oxidation of apoB100 by hydrogen peroxide (positive control) in our assay system (Fig. 3G). Taken together, these data suggest that intrinsic capacity of PM to elicit oxidation of apoB100 particles could be a surrogate measure of PM LPO index, and quantifying Ox-PCs by immunoassay is a robust method for evaluating PM LPO index, which is modifiable by metals and free radical content in PM.

3.4. *PM exposure generates Ox-PCs in mice lungs*

Next, we addressed the *in vivo* biological relevance of evaluating Ox-PCs for assessing PM LPO index. We exposed the mice to PM (10μg/mouse, every other day for 10 days) by intranasal instillation and evaluated Ox-PCs accumulation in BAL fluid by immunoassay using E06 Mab. We observed that PM exposure significantly increased the accumulation of Ox-PCs in BAL fluid (Fig. 4A) compared to the vehicle exposed group. The data demonstrated that PM exposure results in the generation of Ox-PCs in the lungs and therefore, Ox-PCs are physiologically relevant biomarker for evaluating intrinsic PM LPO index.

3.5. *Exposure of pulmonary surfactant and lung cells to PM generates Ox-PCs*

Next, we determined the source of Ox-PCs in lung exposed to PM. Pulmonary surfactant is composed of phospholipids (~80–90%), cholesterol (~2–10%), and ~5–10% proteins including surfactant proteins. A large proportion of phospholipids in surfactant is made of desaturated dipalmitoylphosphatidylcholine (30–45%) and the remaining is composed of unsaturated phospholipid, of which the unsaturated phosphatidylcholine is >30% [38]. Unsaturated phospholipids in the pulmonary surfactant are vulnerable to oxidation following exposure to environmental toxicants [39]. To test if PM exposure oxidizes unsaturated phospholipids in the pulmonary surfactant, we employed clinical surfactant, BLES that contains surfactant phospholipids and surfactant proteins (SP–B and SP-C) and used for the treatment of respiratory distress syndrome. We exposed BLES to Fenton reagent (as positive control) or PM and measured Ox-PCs accumulation by immunoassay. Both Fenton reagent and PM exposure significantly increased the accumulation of Ox-PCs in clinical surfactant (Fig. 4B and C).

After deposition of PM in the distal airways, soluble factors such as metals could cross the respiratory lining fluid and target the lung epithelium. Oxidized phospholipids generated within the cell membrane leaflets are shed by stressed cells and dying cells (apoptotic cells) [40]. To determine if exposure of lung epithelial cells to PM releases Ox-PCs, we exposed normal human bronchial epithelial cells, Beas2B, to whole fraction or soluble fraction of PM and measured Ox-PCs in the culture media by the immunoassay using E06 ab. We observed a significant accumulation of Ox-PCs in culture media of Beas2B cells exposed to PM as compared to vehicle (Fig. 4D). We also measured the accumulation of oxidized lipids in the culture media of Beas2B cells following exposure to whole fraction or soluble fraction of PM using BODIPY-C11 dye. In agreement with the immunoassay, BODIPY-C11 dye method also revealed a dose-dependent increase in the accumulation of oxidized lipids in the culture media of Beas2B cells following exposure to PM-WF or PM-SF (Supplementary Fig. 6). Because methionine was effective in ablating PM oxidative potential in our cell-free assay system (Fig. 3G), we also evaluated the effectiveness of methionine to inhibit LPO in Beas2B cells following exposure to PM. We found that methionine pre-treatment markedly reduced LPO in PM exposed Beas2B cells (Supplemental Fig. 7) suggesting that PM elicits LPO inside cells partly via generation of singlet-oxygen species. Taken together, the data suggest that oxidation of PC containing phospholipids in pulmonary surfactant and membrane of lungs cells are the potential source of Ox-PCs in the lungs of mice exposed to PM and therefore, Ox-PCs levels represent physiologically relevant biomarkers for probing LPO index of PM.

3.6. *PM-induced Ox-PCs elicit inflammation in macrophages*

Ox-PCs not only serve as a biomarker of oxidative lipid damage but also function as damage-associated molecular patterns [41]. We asked if Ox-PCs generated following exposure to PM elicits inflammation. J774 macrophages were exposed to BAL fluid retrieved from PM exposed mice in the presence and absence of Ox-PCs neutralizing mab E06 for 24 h and measured IL-6 levels in culture media. BAL fluid from PM exposed mice significantly increased secretion of IL-6 by macrophages (Fig. 5A) as compared to vehicle exposed mice, which was significantly reduced in the presence of E06 Mab. We also exposed macrophages to BLES treated with PM-whole fraction or PM-soluble fraction and measured IL-6 secretion. We noted a marked increase in IL-6 secretion by macrophages following exposure to BLES treated with whole-PM or PM-soluble fraction as compared to vehicle (Fig. 5B and C). However, in presence of mab E06, the capacity of BLES treated either with PM-whole fraction or PM-soluble fraction to stimulate IL-6 secretion by macrophages was markedly abrogated (Fig. 5B and C). Taken together, the data suggest that Ox-PCs accumulated in lungs following PM exposure elicits inflammation and therefore, are pathogenic.

4. Discussion

Although the mass of PM has been the conventional metric to derive the causal relationship between PM exposure and adverse health effects, it may not represent the best exposure metric for evaluation and prediction of toxic potential of ambient PM of a region or specific source. Because only a tiny fraction of metals and organic compounds in PM are toxic and most PM mass is made of biologically inactive or less toxic inorganic components such as ammonium sulfate, nitrates, NaCl, crust dust, and soil. The underlying mechanism of how PM exerts adverse health effects remains poorly understood. However, the consensus is that airborne PM generates oxidative stress in the lungs, which causes depletion of antioxidants, lipid peroxidation, cell death, and inflammation and ensues the pathogenesis of cardiorespiratory disease [42]. Hence, the measurement of the oxidative potential of PM is thought to be a better metric for evaluating PM toxicity in epidemiological studies.

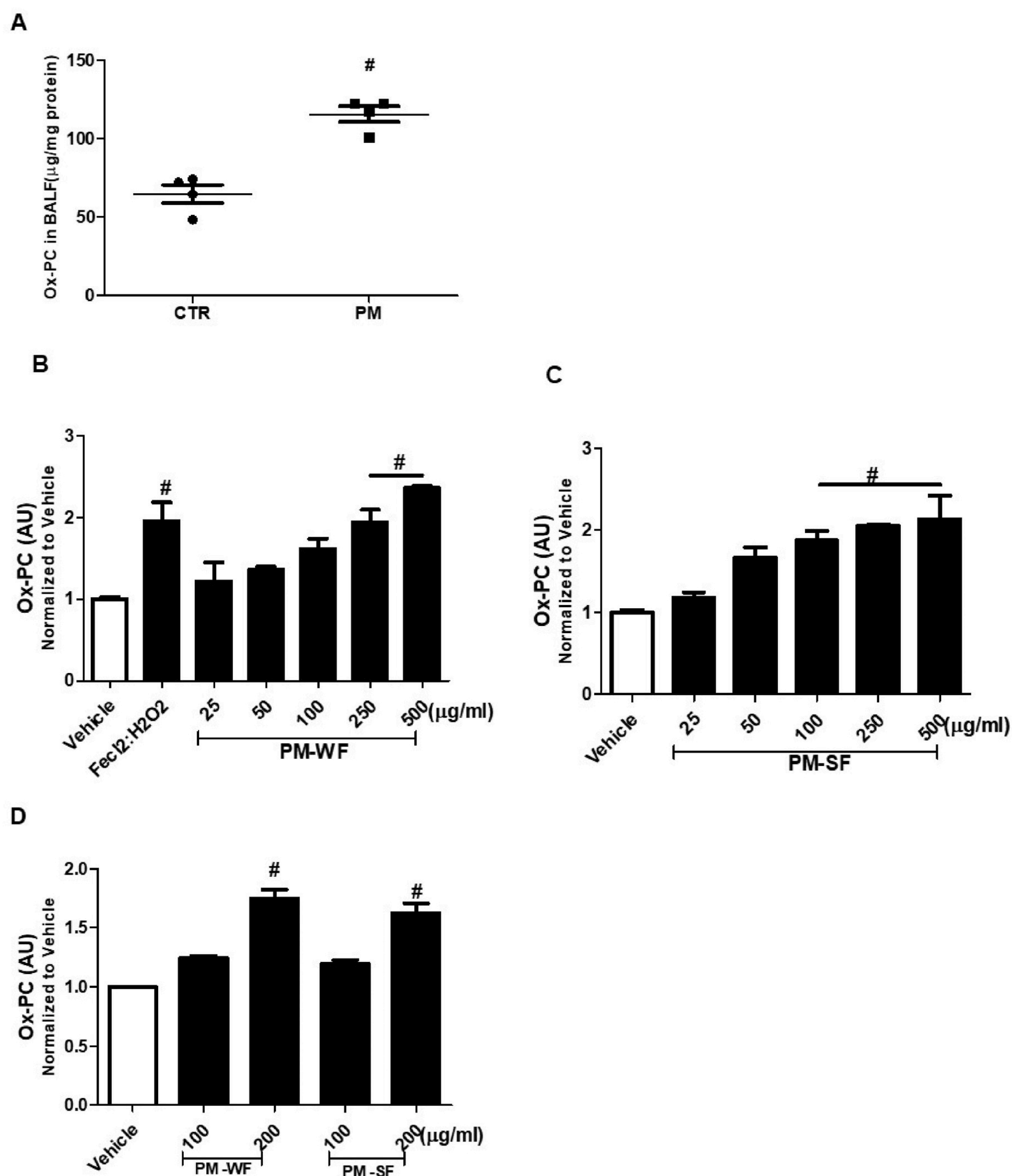

Fig. 4. PM exposure generates Ox-PCs in mice lungs, pulmonary surfactant and lung cells. A. Elevated levels of Ox-PCs in BAL fluid of mice exposed to PM. Mice (n = 4) were exposed to PM (10μg/mouse/day) or vehicle (saline) by intranasal instillation for 5 days. At the end of PM exposure, Ox-PCs levels were measured in the BAL fluid by immunoassay, $P < 0.05 p.m. compared to Control B–C) PM exposure oxidizes phospholipids in pulmonary surfactant and generates Ox-PCs. BLES was exposed to PM-WF (B) or PM-SF (C) at concentrations 25–500 μg/ml for 2 h. The Ox-PCs levels generated in BLES was measured by immunoassay. #P < 0.05, compared to vehicle D) PM exposure causes the release of Ox-PCs by Beas2B cells. Beas2B cells were treated with PM-WF and PM-SF at concentrations 100 and 200 μg/ml for 24 h. Ox-PCs levels were measured in the cell-free culture media by immunoassay, #P < 0.05 compared to vehicle alone.

Currently, OP^{DTT}, OP^{AA}, and OP^{GSH} assays are commonly used to assess PM's oxidative potential. These assays feature thiol depletion or consumption of antioxidants as a testing principle to measure PM^{OP} and simulate one of the deleterious responses (antioxidant depletion) of PM-induced oxidative stress in the lungs. In the current study, we have postulated and demonstrated that LPO index of PM as a method for assessing PM^{OP}. This approach simulates the potential of PM to induce lipid peroxidation in lungs. Further, our assay system features i) interaction of PM with physiological relevant phospholipid substrates ii) measurement of PC-containing oxidized phospholipids, which are proven to exert prooxidants and proinflammatory activity and are implicated in the pathogenesis of airway disease and cardiovascular diseases [43]. Hence, "PM LPO index" concept reflects and captures a newer mechanism of PM oxidative capacity and, therefore, could be valuable in predicting PM^{OP} and PM toxicity.

We employed natural phospholipid PAPC, which is abundantly present in pulmonary surfactant, cell membrane, and apoB100 particles, as the target substrate for assessing the LPO index of PM. Using BODIPY-

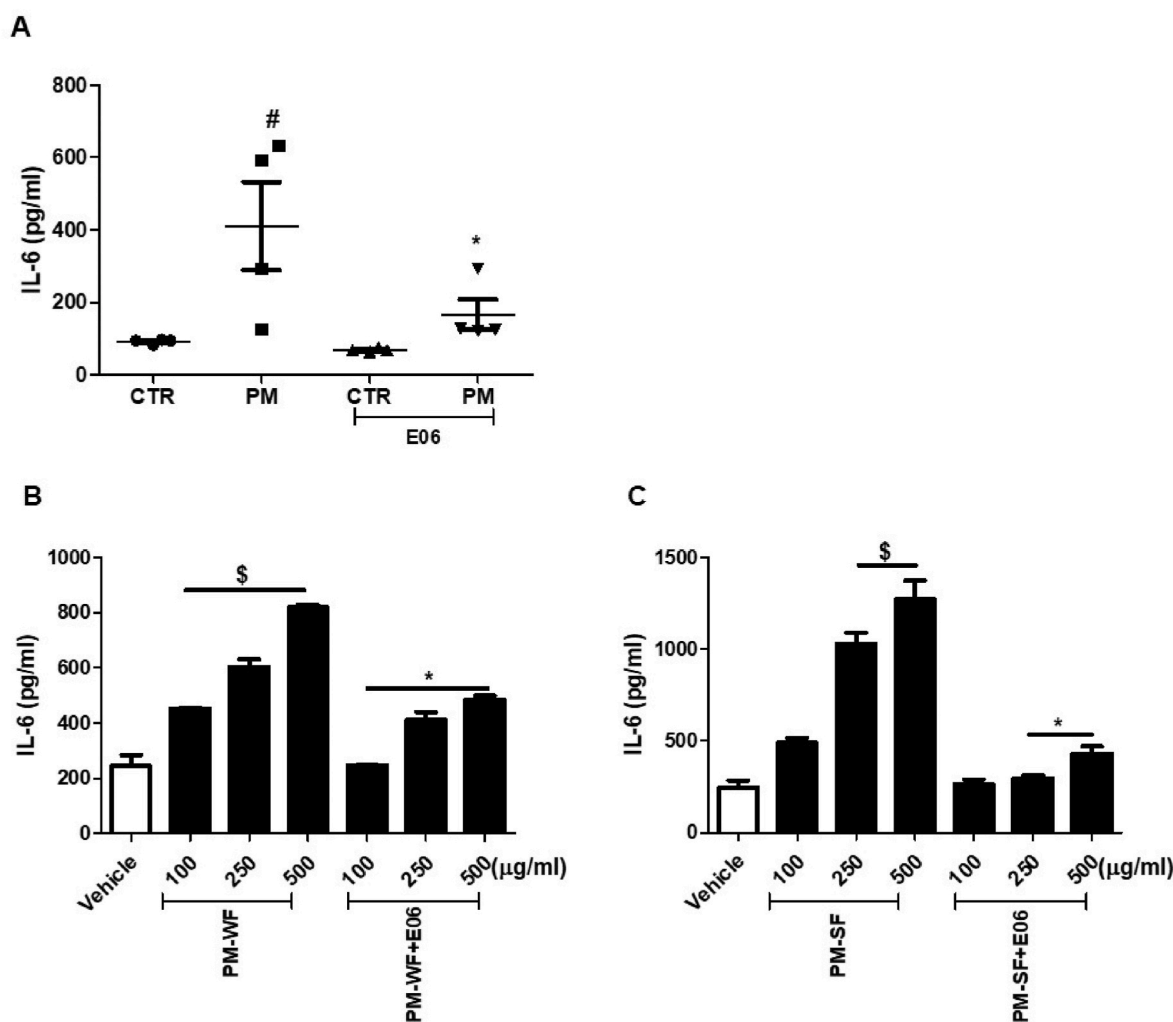

Fig. 5. Ox-PCs accumulated in mice lungs, and pulmonary surfactant following exposure to PM induces inflammatory responses in macrophages. A. BAL fluid from PM exposed mice induced greater IL-6 secretion in macrophages, which was attenuated in the presence of Ox-PCs neutralizing antibody E06. J774 macrophage cells were exposed to BAL fluid from mice exposed to vehicle or PM in the absence and presence of mab E06 for 24 h. IL-6 levels were measured in culture media by ELISA, #P < 0.05, compared to control; *P < 0.05 compared to PM alone. B–C. Exposure of macrophages to oxidized pulmonary surfactant caused by PM-WF (B) or PM-SF (C) stimulates IL-6 secretion, which was attenuated by mab E06. J774 macrophages were treated with BLES that was exposed to PM-WF or PM-SF. After 24 h of BLES treatment, IL-6 levels were measured in the culture media by ELISA, $ P < 0.0001, compared to vehicle; *P < 0.05, compared to respective concentration of PM, $ P < 0.0001 compared to vehicle, #P < 0.05 compared to respective concentration of PM alone.

C11 reporter dye, which selectively binds to lipid peroxyl radicals but not to superoxide [32], we demonstrated the intrinsic potential of PM to oxidize PAPC in a concentration-dependent manner. Intriguingly, antioxidant NAC suppressed PM-induced oxidation of PAPC but not GSH in the cell-free system. Although the precise mechanism why GSH failed to abrogate PM induced oxidation of PAPC is unclear, we believe it could be due to multiple mechanisms such as-i) GSH may form a stable complex with heavy metals [44] in PM and catalyzes the oxidation of PAPC; ii) oxidation of GSH by PM may result in significant accumulation of thiyl radicals [45] and glutathione disulfide (GSSG), which may promote oxidation of PAPC by redox cycling of the transition metals such as Fe(II) and Cu(II) present in PM [46]; iii) GSH may also catalyze superoxide dependent hydroxyl radicals generation in the presence of trace amounts of Fe(II) present in PM [47].

The advantage of using BODIPY-C11 as a probe for indexing LPO of PM is that it measures total lipid peroxyl radicals generated during oxidation of PAPC by PM. However, the loss of fluorescence signal with time due to photobleaching and quenching effects in cell-free assay systems could reduce the sensitivity and consistency of the BODIPY-C11 based method to quantify the PM LPO index. To address this limitation, we developed an immunoassay to assess the PM LPO index using the E06 antibody, which explicitly measures Ox-PCs [28]. For the immunoassay development, we could not use PAPC as a target substrate for assessing PM LPO index because of several technical challenges such as inefficient immobilization of PAPC onto the microplate wells; loss of coated PAPC while washing and blocking steps; and loss of oxidized phospholipids during the washing steps. Instead, we used apoB100 lipoprotein particles as a substrate for indexing LPO of PM because PAPC and other PC-containing phospholipids are abundantly present on the surface of apoB100 particles [48]. More importantly, apoB100 lipoprotein particles present in the human serum or plasma could be easily captured into microplate wells using MB47 antibody [28]. Joseph Witztum and colleagues have extensively characterized MB47 as apoB100 capturing antibody and E06 as Ox-PCs detecting antibody for measuring the levels of Ox-PCs content on apoB100 lipoproteins in patients with coronary artery disease [49,50]. We observed a concentration-dependent increase in Ox-PCs content on apoB100 particles following exposure to PM-whole fraction or PM-soluble fraction, indicating the sensitivity and suitability of the immunoassay for assessing PM LPO index.

Redox-active metals and organic compounds (such as quinones) absorbed onto the surface of PM are the main determinants of the oxidative potential of PM [16]. In our immunoassay system, apoB100

particles were sensitive to oxidation by PM associated redox-active chemical species including metals and quinone (9,10-Phenanthrenequinone). In the presence of metal chelators (EDTA and deferoxamine), PM potency to oxidize apoB100 lipoprotein was significantly abolished, indicating the sensitivity of the method to metal content in PM. The capacity of deferoxamine and EDTA to abolish the PM LPO index was much higher in PM-soluble fraction than PM-whole fraction, suggesting that metals are the predominant components in the soluble fraction of PM sample that drive the oxidation of apoB100 particles. Although significant, we found that the efficacy of NAC and methionine to reduce PM-induced peroxidation of apoB100 was significantly less compared to metal chelators. The data suggest that the chelators completely prevent metals' reaction with unsaturated fatty acids on the surface of apoB100 particles and, therefore, more efficacious than radical scavengers such as NAC and methionine in mitigating the oxidative capacity of PM. Both human and animal studies have found that Ox-PCs on LDL is proinflammatory and proatherogenic [28,51]. Furthermore, oxidized-LDL and oxidized-VLDL are shown to inhibit surfactant production by the alveolar type II cells [52]. Our findings reveal the plausible mechanism of how PM may mediate lung and vascular injury.

Studies in animal models suggest that PM-induced lung injury is characterized by an increase in LPO, proinflammatory cytokine levels, influx of inflammatory cells [53], cell death [21], and fibrosis [54]; however, the mediators that drive these pathogenic events are not entirely understood. We and others have shown that oxidative stress conditions caused by cigarette smoke exposure [31] or viral infection (H5N1 and SARS) [20] lead to the accumulation of Ox-PCs in the lungs. These Ox-PCs function as danger-associated molecular patterns [55] and induce acute lung inflammatory responses [20], impair pulmonary antibacterial defenses [31], and promote atherosclerosis [51]. We found that PM exposure also results in the accumulation of Ox-PCs in the lungs of mice and the peroxidation of PUFA in pulmonary surfactant and cellular membrane are potential sources of Ox-PCs in lungs. By using Ox-PCs neutralizing mab E06, we demonstrated that Ox-PCs accumulated in BAL fluid and BLES after PM exposure stimulates IL-6 production by macrophages suggesting that the Ox-PCs accumulation in lungs may exacerbate PM-induced lung injury.

In conclusion, we have demonstrated that the PM LPO index is a novel metric for characterizing PM^{OP}, and Ox-PCs levels are physiologically relevant surrogate markers for evaluating PM LPO index and predicting toxicity. The developed immunoassay is sensitive and quantitative for assessing the PM LPO index and is amenable for high-throughput assays for epidemiological studies. The limitation of the developed immunoassay involves using the human serum to capture apoB100 lipoprotein and the assay probes for only one specific oxidized phospholipid (i.e., Ox-PCs). However, in reality, an array of oxidized products are expected to be generated during PM-mediated oxidation of apoB100 particles [56]. Future studies will determine the utility value of the LPO index of PM as a method to assess the relationship between PM^{OP}, PM exposure, and health effects in epidemiological studies.

Declaration of competing interest

The authors have declared there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102189.

References

[1] A.J. Cohen, M. Brauer, R. Burnett, H.R. Anderson, J. Frostad, K. Estep, et al., Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015, Lancet 389 (10082) (2017) 1907–1918.
[2] R. Burnett, H. Chen, M. Szyszkowicz, N. Fann, B. Hubbell, C.A. Pope, et al., Global estimates of mortality associated with long-term exposure to outdoor fine particulate matter, Proc. Natl. Acad. Sci. U. S. A. 115 (38) (2018) 9592–9597.
[3] F. Dominici, R.D. Peng, M.L. Bell, L. Pham, A. McDermott, S.L. Zeger, et al., Fine particulate air pollution and hospital admission for cardiovascular and respiratory diseases, J. Am. Med. Assoc. 295 (10) (2006) 1127–1134.
[4] R.D. Peng, F. Dominici, R. Pastor-Barriuso, S.L. Zeger, J.M. Samet, Seasonal analyses of air pollution and mortality in 100 US cities, Am. J. Epidemiol. 161 (6) (2005) 585–594.
[5] J. Lelieveld, J.S. Evans, M. Fnais, D. Giannadaki, A. Pozzer, The contribution of outdoor air pollution sources to premature mortality on a global scale, Nature 525 (7569) (2015) 367–371.
[6] F.R. Cassee, M.E. Héroux, M.E. Gerlofs-Nijland, F.J. Kelly, Particulate matter beyond mass: recent health evidence on the role of fractions, chemical constituents and sources of emission, Inhal. Toxicol. 25 (14) (2013) 802–812.
[7] S. Kundu, E.A. Stone, Composition and sources of fine particulate matter across urban and rural sites in the Midwestern United States, Environ Sci Process Impacts 16 (6) (2014) 1360–1370.
[8] M.L. Bell, F. Dominici, K. Ebisu, S.L. Zeger, J.M. Samet, Spatial and temporal variation in PM(2.5) chemical composition in the United States for health effects studies, Environ. Health Perspect. 115 (7) (2007) 989–995.
[9] M.L. Bell, K. Ebisu, R.D. Peng, J.M. Samet, F. Dominici, Hospital admissions and chemical composition of fine particle air pollution, Am. J. Respir. Crit. Care Med. 179 (12) (2009) 1115–1120.
[10] R.D. Peng, M.L. Bell, A.S. Geyh, A. McDermott, S.L. Zeger, J.M. Samet, et al., Emergency admissions for cardiovascular and respiratory diseases and the chemical composition of fine particle air pollution, Environ. Health Perspect. 117 (6) (2009) 957–963.
[11] J.Y. Son, J.T. Lee, K.H. Kim, K. Jung, M.L. Bell, Characterization of fine particulate matter and associations between particulate chemical constituents and mortality in Seoul, Korea, Environ. Health Perspect. 120 (6) (2012) 872–878.
[12] F. Tao, B. Gonzalez-Flecha, L. Kobzik, Reactive oxygen species in pulmonary inflammation by ambient particulates, Free Radic. Biol. Med. 35 (4) (2003) 327–340.
[13] J.Y. Abrams, R.J. Weber, M. Klein, S.E. Sarnat, H.H. Chang, M.J. Strickland, et al., Associations between ambient fine particulate oxidative potential and cardiorespiratory emergency department visits, Environ. Health Perspect. 125 (10) (2017) 107008.
[14] S. Weichenthal, E. Lavigne, G. Evans, K. Pollitt, R.T. Burnett, Ambient PM2.5 and risk of emergency room visits for myocardial infarction: impact of regional PM2.5 oxidative potential: a case-crossover study, Environ. Health 15 (2016) 46.
[15] R. Thimmulappa, I. Chattopadhyay, S. Rajasekaran, Oxidative stress mechanisms in the pathogenesis of environmental lung diseaseS, in: S. Chakraborti, T. Chakraborti, R. Ghosh, N.K. Ganguly, N.L. Parinandni (Eds.), Oxidative Stress in Lung Diseases, vol. 2, Springer, Singapore, 2020, pp. 103–137.
[16] D. Gao, S. Ripley, S. Weichenthal, K.J. Godri Pollitt, Ambient particulate matter oxidative potential: chemical determinants, associated health effects, and strategies for risk management, Free Radic. Biol. Med. 151 (2020) 7–25.
[17] J.T. Bates, T. Fang, V. Verma, L. Zeng, R.J. Weber, P.E. Tolbert, et al., Review of acellular assays of ambient particulate matter oxidative potential: methods and relationships with composition, sources, and health effects, Environ. Sci. Technol. 53 (8) (2019) 4003–4019.
[18] R.S. Gangwar, G.H. Bevan, R. Palanivel, L. Das, S. Rajagopalan, Oxidative stress pathways of air pollution mediated toxicity: recent insights, Redox Biol 34 (2020) 101545.
[19] K. Rodríguez-Capote, D. Manzanares, T. Haines, F. Possmayer, Reactive oxygen species inactivation of surfactant involves structural and functional alterations to surfactant proteins SP-B and SP-C, Biophys. J. 90 (8) (2006) 2808–2821.
[20] Y. Imai, K. Kuba, G.G. Neely, R. Yaghubian-Malhami, T. Perkmann, G. van Loo, et al., Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury, Cell 133 (2) (2008) 235–249.
[21] Z. Dagher, G. Garçon, S. Billet, P. Gosset, F. Ledoux, D. Courcot, et al., Activation of different pathways of apoptosis by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture, Toxicology 225 (1) (2006) 12–24.
[22] M. Pardo, C. Li, Q. He, S. Levin-Zaidman, M. Tsoory, Q. Yu, et al., Mechanisms of lung toxicity induced by biomass burning aerosols, Part. Fibre Toxicol. 17 (1) (2020) 4.
[23] A. Kumar, T. Gupta, Development and Field Evaluation of a Multiple Slit Nozzle-Based High Volume PM2.5 Inertial Impactor Assembly (HVIA), vol. 15, 2015, pp. 1188–1200, 4.
[24] A.K. Cho, C. Sioutas, A.H. Miguel, Y. Kumagai, D.A. Schmitz, M. Singh, et al., Redox activity of airborne particulate matter at different sites in the Los Angeles Basin, Environ. Res. 99 (1) (2005) 40–47.
[25] I.S. Mudway, M.T. Krishna, A.J. Frew, D. MacLeod, T. Sandstrom, S.T. Holgate, et al., Compromised concentrations of ascorbate in fluid lining the respiratory tract in human subjects after exposure to ozone, Occup. Environ. Med. 56 (7) (1999) 473–481.
[26] E.H. Pap, G.P. Drummen, V.J. Winter, T.W. Kooij, P. Rijken, K.W. Wirtz, et al., Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY (581/591), FEBS Lett. 453 (3) (1999) 278–282.

[27] M. Navab, S.Y. Hama, G.P. Hough, G. Subbanagounder, S.T. Reddy, A. M. Fogelman, A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids, J. Lipid Res. 42 (8) (2001) 1308–1317.
[28] A. Taleb, J.L. Witztum, S. Tsimikas, Oxidized phospholipids on apoB-100-containing lipoproteins: a biomarker predicting cardiovascular disease and cardiovascular events, Biomarkers Med. 5 (5) (2011) 673–694.
[29] C.J. Harvey, R.K. Thimmulappa, S. Sethi, X. Kong, L. Yarmus, R.H. Brown, et al., Targeting Nrf2 signaling improves bacterial clearance by alveolar macrophages in patients with COPD and in a mouse model, Sci. Transl. Med. 3 (78) (2011), 78ra32.
[30] A. Sivanantham, D. Pattarayan, R. Bethunaickan, A. Kar, S.K. Mahapatra, R. K. Thimmulappa, et al., Tannic acid protects against experimental acute lung injury through downregulation of TLR4 and MAPK, J. Cell. Physiol. 234(5) (2018) 6463–6476.
[31] R.K. Thimmulappa, X. Gang, J.H. Kim, T.E. Sussan, J.L. Witztum, S. Biswal, Oxidized phospholipids impair pulmonary antibacterial defenses: evidence in mice exposed to cigarette smoke, Biochem. Biophys. Res. Commun. 426 (2) (2012) 253–259.
[32] G.P. Drummen, L.C. van Liebergen, J.A. Op den Kamp, J.A. Post, C11-BODIPY (581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro) spectroscopic characterization and validation of methodology, Free Radic. Biol. Med. 33 (4) (2002) 473–490.
[33] S. Dikalov, K.K. Griendling, D.G. Harrison, Measurement of reactive oxygen species in cardiovascular studies, Hypertension 49 (4) (2007) 717–727.
[34] M. Dashti, W. Kulik, F. Hoek, E.C. Veerman, M.P. Peppelenbosch, F. Rezaee, A phospholipidomic analysis of all defined human plasma lipoproteins, Sci. Rep. 1 (2011) 139.
[35] M. Lin, J.Z. Yu, Assessment of oxidative potential by hydrophilic and hydrophobic fractions of water-soluble PM, Environ. Pollut. 275 (2021) 116616.
[36] J. Aaseth, M.A. Skaug, Y. Cao, O. Andersen, Chelation in metal intoxication–Principles and paradigms, J. Trace Elem. Med. Biol. 31 (2015) 260–266.
[37] F. Zheng, F.M. Gonçalves, Y. Abiko, H. Li, Y. Kumagai, M. Aschner, Redox toxicology of environmental chemicals causing oxidative stress, Redox Biol 34 (2020) 101475.
[38] R. Veldhuizen, K. Nag, S. Orgeig, F. Possmayer, The role of lipids in pulmonary surfactant, Biochim. Biophys. Acta 1408 (2–3) (1998) 90–108.
[39] M. Al-Saiedy, R. Pratt, P. Lai, E. Kerek, H. Joyce, E. Prenner, et al., Dysfunction of pulmonary surfactant mediated by phospholipid oxidation is cholesterol-dependent, Biochim. Biophys. Acta Gen. Subj. 1862 (4) (2018) 1040–1049.
[40] M. Manček-Keber, M. Frank-Bertoncelj, I. Hafner-Bratkovič, A. Smole, M. Zorko, N. Pirher, et al., Toll-like receptor 4 senses oxidative stress mediated by the oxidation of phospholipids in extracellular vesicles, Sci. Signal. 8 (381) (2015) ra60.
[41] M. Di Gioia, R. Spreafico, J.R. Springstead, M.M. Mendelson, R. Joehanes, D. Levy, et al., Endogenous oxidized phospholipids reprogram cellular metabolism and boost hyperinflammation, Nat. Immunol. 21 (1) (2020) 42–53.
[42] S.G. Al-Kindi, R.D. Brook, S. Biswal, S. Rajagopalan, Environmental determinants of cardiovascular disease: lessons learned from air pollution, Nat. Rev. Cardiol. 17 (10) (2020) 656–672.
[43] G.O. Fruhwirth, A. Loidl, A. Hermetter, Oxidized phospholipids: from molecular properties to disease, Biochim. Biophys. Acta 1772 (7) (2007) 718–736.
[44] J. Liu, H. Liu, Y. Li, H. Wang, Probing the coordination properties of glutathione with transition metal ions (Cr2+, Mn2+, Fe2+, Co2+, Ni2+, Cu2+, Zn2+, Cd2+, Hg2+) by density functional theory, J. Biol. Phys. 40 (4) (2014) 313–323.
[45] M.L. Sagristá, A.E. García, M. Africa De Madariaga, M. Mora, Antioxidant and pro-oxidant effect of the thiolic compounds N-acetyl-L-cysteine and glutathione against free radical-induced lipid peroxidation, Free Radic. Res. 36 (3) (2002) 329–340.
[46] E.O.U. Eteshola, D.A. Haupt, S.I. Koos, L.A. Siemer, D.L. Morris, The role of metal ion binding in the antioxidant mechanisms of reduced and oxidized glutathione in metal-mediated oxidative DNA damage, Metall 12 (1) (2020) 79–91.
[47] D.A. Rowley, B. Halliwell, Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds, FEBS Lett. 138 (1) (1982) 33–36.
[48] H. Esterbauer, M. Dieber-Rotheneder, G. Waeg, G. Striegl, G. Jürgens, Biochemical, structural, and functional properties of oxidized low-density lipoprotein, Chem. Res. Toxicol. 3 (2) (1990) 77–92.
[49] S. Tsimikas, S. Kiechl, J. Willeit, M. Mayr, E.R. Miller, F. Kronenberg, et al., Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study, J. Am. Coll. Cardiol. 47 (11) (2006) 2219–2228.
[50] S. Tsimikas, E.S. Brilakis, E.R. Miller, J.P. McConnell, R.J. Lennon, K.S. Kornman, et al., Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease, N. Engl. J. Med. 353 (1) (2005) 46–57.
[51] X. Que, M.Y. Hung, C. Yeang, A. Gonen, T.A. Prohaska, X. Sun, et al., Oxidized phospholipids are proinflammatory and proatherogenic in hypercholesterolaemic mice, Nature 558 (7709) (2018) 301–306.
[52] J. Zhou, A.J. Ryan, J. Medh, R.K. Mallampalli, Oxidized lipoproteins inhibit surfactant phosphatidylcholine synthesis via calpain-mediated cleavage of CTP: phosphocholine cytidylyltransferase, J. Biol. Chem. 278 (39) (2003) 37032–37040.
[53] N. Gour, K. Sudini, S.M. Khalil, A.M. Rule, P. Lees, E. Gabrielson, et al., Unique pulmonary immunotoxicological effects of urban PM are not recapitulated solely by carbon black, diesel exhaust or coal fly ash, Environ. Res. 161 (2018) 304–313.
[54] H. Wang, X. Shen, J. Liu, C. Wu, J. Gao, Z. Zhang, et al., The effect of exposure time and concentration of airborne PM, Redox Biol 26 (2019) 101264.
[55] C.J. Binder, N. Papac-Milicevic, J.L. Witztum, Innate sensing of oxidation-specific epitopes in health and disease, Nat. Rev. Immunol. 16 (8) (2016) 485–497.
[56] S. Parthasarathy, A. Raghavamenon, M.O. Garelnabi, N. Santanam, Oxidized low-density lipoprotein, Methods Mol. Biol. 610 (2010) 403–417.