**Dose-dependent intracellular reactive oxygen and nitrogen species production from
particulate matter exposure: comparison to oxidative potential and chemical composition**

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Abstract

Elevated particulate matter (PM) concentrations have been associated with cardiopulmonary risks. In this study, alveolar macrophages and ventricular myocytes were exposed to PM extracts from 104 ambient filters collected in multiple rural and urban sites in the greater Atlanta area. PM-induced reactive oxygen/nitrogen species (ROS/RNS) were measured to investigate the effect of chemical composition and determine whether chemical assays are representative of cellular responses. For summer samples, the area under the ROS/RNS dose-response curve per volume of air (AUC_{volume}) was significantly correlated with dithiothreitol (DTT) activity, water-soluble organic carbon (WSOC), brown carbon, titanium, and iron, while a relatively flat response was observed for winter samples. EC_{50} was also correlated with max response for all filters investigated, which suggests that certain PM constituents may be involved in cellular protective pathways. Although few metal correlations were observed, exposure to laboratory-prepared metal solutions induced ROS/RNS production, indicating that a lack of correlation does not necessarily translate to a lack of response. Collectively, these results suggest that complex interactions may occur between PM species. Furthermore, the strong correlation between organic species and ROS/RNS response highlights a need to understand the contribution of organic aerosols, especially photochemically driven secondary organic aerosols (SOA), to PM-induced health effects.

Keywords: oxidative stress, alveolar macrophage, ventricular myocyte, particulate matter, chemical composition, oxidative potential

Highlights

- Cellular ROS/RNS production measured for 104 ambient PM samples.

- AUC found to be most robust dose-response parameter for characterizing samples.
- AUC correlated with DTT activity, WSOC, BrC, Ti, and Fe for summer samples.
- EC₅₀ correlated with max response for all filters.
- Correlation between organics and ROS/RNS highlight potential importance of SOA.

1. Introduction

Adverse health effects resulting from particulate matter (PM) exposure have received considerable attention with increasing epidemiological studies associating elevated PM concentrations with increases in cardiopulmonary hospitalizations and mortality, specifically increases in lung cancer, asthma, chronic obstructive pulmonary disease, arrhythmia, and ischemic heart disease.¹⁻⁷ Contributions from PM-associated deaths worldwide also rank among the top 10 global risks,⁸ and in 2013, the World Health Organization classified PM as a Group I carcinogen.⁹ Toxicology studies suggest that PM-induced oxidative stress may play a role in initiating these adverse effects.¹⁰⁻¹³ Specifically, PM-induced reactive oxygen/nitrogen species (ROS/RNS) production can initiate inflammatory cascades, and prolonged stimulation of these cascades can lead to oxidative stress and cellular damage, including lipid peroxidation, protein oxidation, and DNA/RNA alteration.^{14, 15} Additionally, there is a well-established link between chronic inflammation and cancer.¹⁶ A possible link between PM exposure and observed health endpoints therefore exists as PM can generate ROS/RNS via redox reactions and by inducing cellular pathways that produce ROS/RNS.^{17, 18}

Despite these associations, the specific mechanisms and components responsible for PM-induced oxidative stress have not been fully explored. Metals have received considerable attention in this regard as many are redox active and can produce ROS/RNS via Fenton-like reactions.^{19, 20} For instance, studies with standard reference materials and PM samples have found that oxidative

potential, cytotoxicity, and inflammatory responses were most significant in metal-containing samples.^{21, 22} Epidemiological studies have also found that specific metals (Fe, Ni, and Zn) were more strongly associated with mortality than fine particle (PM_{2.5}) mass alone.²³ Additionally, Huang et al.²⁴ showed that water-soluble metals in concentrated ambient particles were correlated with increased neutrophil influx and blood fibrinogen. Besides metals, organic carbon components were also found to be toxicologically relevant in inhalation and exposure studies,^{25, 26} and ROS have been linked to quinones, polycyclic aromatic hydrocarbons (PAHs), and other oxygenated aromatics, such as humic-like substances.²⁷⁻³⁰ Many of these studies, however, involve limited data sets and low-throughput methods.

Chemical assays have been developed and widely used to determine PM oxidative potential. In these assays, an anti-oxidant (e.g. DTT; ascorbic acid, AA; or other species present in the respiratory tract lining fluid) is used to simulate redox reactions that would occur in biological systems.^{31, 32} PM catalyzes electron transfer from the anti-oxidant to oxygen, and anti-oxidant decay provides a measure of redox-active species in the sample. These assays can be automated and high-throughput systems exist for DTT and AA.³³ Studies involving oxidative potential as measured by DTT have shown strong correlations with redox metals,³⁴⁻³⁶ water-soluble organic carbon (WSOC), and PAHs.³⁷⁻³⁹ However, the question remains as to whether chemical assays are representative of cellular responses.

In the present study, alveolar macrophages and ventricular myocytes were exposed to ambient PM extracts over a wide dilution series to fully capture dose-dependent reactive oxygen and nitrogen species (ROS/RNS) production and obtain dose-response parameters. The PM filters were collected at multiple rural and urban sites in the greater Atlanta area during the Southeastern Center for Air Pollution and Epidemiological (SCAPE) study. Response parameters were then

compared with chemical oxidative potential, measured by DTT,⁴⁰ to determine whether results from chemical assays represent cellular responses. Finally, water-soluble PM components were compared with cellular response parameters to elucidate components associated with ROS/RNS production. Spatial and seasonal trends were also investigated for each comparison.

2. Methods

2.1 Alveolar macrophage cell line. Alveolar macrophages are closely involved in foreign particle clearance and inflammatory signaling and were thus chosen as cells representing the first line of defense against environmental insults.^{41, 42} Immortalized murine alveolar macrophages (MH-S, ATCC[®]CRL-2019[™]) were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS, Quality Biological, Inc.), 1% penicillin-streptomycin, and 50 μ M β -mercaptoethanol (BME) at 37°C and 5% CO₂. Prior to sample exposure, MH-S cells were seeded at 2x10⁴ cells well⁻¹ onto 96-well plates pre-treated with 10% FBS in phosphate buffered saline (PBS, Cellgro). For seeding and thereon, all culture media was supplemented with 10% FBS without BME addition since BME is a reducing agent that may interfere with ROS/RNS measurement. Seeded plates were then incubated overnight to allow cells to adhere and acclimate.

2.2 Ventricular myocyte isolation and culture. Primary ventricular myocytes were chosen as an alternate active cell type to investigate, as cardiac health is known to be affected by pollution. Neonatal rat ventricular myocytes (NRVM) were harvested from 2-day-old neonatal Sprague-Dawley rats (Charles River Laboratories) based on pre-established protocol.⁴³ Isolated cells were resuspended in M199 culture medium supplemented with 10% FBS, 10mM HEPES, 0.1mM MEM non-essential amino acids, 3.5g L⁻¹ glucose, 2mM L-glutamine, 2mg L⁻¹ vitamin B12, and 50U mL⁻¹ penicillin. Prior to seeding, several iterations of pre-plating were performed to

increase the cardiomyocyte purity to 90-98%. Cells were then seeded onto 96-well plates pre-coated with polydimethylsiloxane (PDMS, Dow Corning Sylgard 184) and fibronectin (FN, Fisher Scientific) at 3.33×10^4 cells well⁻¹. Seeded plates were incubated at 37°C and 5% CO₂ and cultured for 2 days prior to exposure. Approximately 24hrs after seeding, cells were washed with PBS to remove dead cells and the media was replaced. From 48hrs on, 2% FBS was used for culturing and exposure.

2.3 PM collection and extraction. Ambient PM_{2.5} samples were collected from multiple urban (Jefferson Street, Roadside, Georgia Tech) and rural (Yorkville) sites around the greater Atlanta area (Fig. S1) between June 2012 and October 2013 as part of the Southeastern Center for Air Pollution and Epidemiology (SCAPE) study.^{40, 44-46} High-volume samplers (ThermoAndersen) were used to collect particles over 23hrs onto pre-baked quartz filters (Pallflex®Tissuquartz™). Collected samples were wrapped in pre-baked aluminum foil and stored at -20°C until extraction and analysis.³³ For this study, ambient samples were selected to span the range of extrinsic (per volume of air sampled) and intrinsic (per mass of PM) DTT activities observed in SCAPE.⁴⁰

The concentrations of various water-soluble species in the PM samples were determined and reported in previous studies.^{44, 47} Specifics on PM chemical characterization are described elsewhere.⁴⁴ Briefly, filter samples were sectioned and extracted in deionized (DI) water for chemical analysis. WSOC and brown carbon (BrC) absorption were then measured using an automated system comprised of an autosampler, a liquid waveguide capillary cell, a spectrophotometer, and a total organic carbon analyzer.⁴⁰ A Xact™ 625 automated multi-metals monitor was used to determine water-soluble metal concentrations using acidified water-soluble extracts.⁴⁴ Elemental and organic carbon were also determined using a thermal/optical transmittance analyzer on sections of filter samples.⁴⁰

In this study, collected particles were extracted following the procedure described in Fang et al.⁴⁴ with modifications for cellular exposure. Briefly, filter samples were sectioned into 1.45cm²-punches and extracted in cell culture media for measuring cellular ROS/RNS production or DI water for determining oxidative potential. Punches were submerged in extraction medium and sonicated for 30min using an Ultrasonic Cleanser (VWR International). Extracts were then filtered using 0.45µm PTFE syringe filters (Fisherbrand™) to remove quartz fibers, which are known to elicit inflammatory responses.^{48, 49} Prior to exposure, media extracts were supplemented with FBS and diluted over ten dilutions to obtain a dose-response curve from 1x to 0.00125x, with 1x being the undiluted extract.

2.4 Pure metal solutions. Concentrated pure metal solutions were prepared by dissolving metal salts [Cu(II)SO₄, ZnCl₂, C₆K₂O₁₂Ti, Cr(III)Cl₃, Fe(II)SO₄, Mn(II)SO₄] in DI water to a final concentration of 400mM (Cu, Zn), 200mM (Ti, Cr, Mn), and 1000mM (Fe). Prior to exposure, metal solutions were diluted 1000x in FBS-supplemented media to produce the “1x” solution. These concentrations were chosen to span the observed range for these metals in SCAPE.⁴⁰

2.5 Oxidative potential. PM oxidative potential was measured in previous studies using a semi-automated DTT assay system.³³ Details of this high-throughput system are described elsewhere,³³ and oxidative potential results from SCAPE have been reported and extensively discussed in the context of PM composition and source apportionment in prior publications.^{40, 44,}
⁵⁰ Briefly, the method consisted of three main steps: (1) oxidation of DTT by redox-active species, (2) reaction of residual DTT with DTNB to form 2-nitro-5-mercaptobenzoic acid (TNB), repeated at specific time intervals, and (3) measurement of TNB to determine DTT consumption.

2.6 Intracellular ROS/RNS measurement. ROS/RNS were detected using 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA, Molecular Probes C-400), a cell-permeable compound that becomes deacetylated intracellularly and is better retained than other commonly used probes due to its additional negative charges.^{51, 52} The deacetylated ROS/RNS probe (carboxy-DCFH) is subsequently oxidized by cellular ROS/RNS to produce a fluorescent compound (carboxy-DCF). The protocol established by Landreman et al.,⁵³ which measured ROS production from alveolar macrophages exposed to diesel exhaust particles, was used as a general reference for assay development and optimization in this study. The optimized ROS/RNS assay, shown schematically in Fig. 1, consisted of the following major steps: (1) pre-treatment of 96-well plates, (2) seeding of cells, (3) treatment of cells with ROS/RNS probe, (4) exposure of probe-treated cells to samples, and (5) detection of ROS/RNS.

Figure 1. Cellular assay optimized for measuring ROS/RNS production as a result of PM exposure. Major steps include: (1) pre-treatment of wells, (2) seeding of cells onto wells, (3) incubation of cells with carboxy-H₂DCFDA, (4) incubation of probe-treated cells with samples or controls, and (5) detection of carboxy-DCF (proportional to ROS/RNS) using microplate reader.

In the first step, 96-well plates were pre-treated to reduce cell aggregation near hydrophobic walls and ensure a uniform cell density throughout the well. Next, wells were seeded at a density optimized for ROS/RNS measurement (MH-S) or predetermined to form a confluent monolayer tissue (NRVM).⁴³ MH-S cell density was optimized with the following considerations: (1) too few cells could result in saturation where different PM samples yield the same ROS/RNS signal, and (2) too many cells could result in overcrowding stress and increased background ROS/RNS production.

Approximately 24hrs after seeding, the cell culture media was removed and cells were washed with PBS. 120 μ L of ROS/RNS probe diluted to a final concentration of 10 μ M was then added to each well and incubated for 40min at 37°C and 5% CO₂. After probe treatment, the probe solution was removed and replaced with 120 μ L of PM extract or control solution. All controls were dissolved or extracted in cell culture media supplemented with FBS. Positive controls included LPS (1 μ g mL⁻¹), H₂O₂ (100 μ M), and reference filter extract (10 filter punches mL⁻¹, 1 per filter sample, from various ambient filters collected at the Georgia Tech site). LPS and H₂O₂ have been shown to induce oxidative stress and inflammation in macrophages,^{54, 55} whereas reference filter extract contains ambient particles that should elicit similar cellular pathways as filter samples of interest. Negative controls include blank filter extract (2 punches mL⁻¹) and control cells (probe-treated cells exposed to media only, no stimulants). After sample/control addition, cells were incubated for 24hrs. This sample incubation time was chosen to maximize positive and negative response signal separation (Fig. S2). After 24hrs, samples and controls were removed and replaced with PBS. Fluorescence was then measured (ex/em: 485/525nm) using a microplate reader (BioTek Synergy H4).

Cellular metabolic activity was determined for selected samples using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Biotium). Briefly, samples were removed after exposure and replaced with media containing MTT. Cells were then incubated for 4hrs, during which the dye was reduced by cellular NAD(P)H to produce an insoluble purple salt. Finally, dimethyl sulfoxide was used to solubilize the salt and absorbance at 570nm was measured using a microplate reader.

2.7 Statistical analysis. Linear regressions between cellular response and oxidative potential or PM composition were evaluated using Pearson's correlation coefficient. Significance was determined using multiple imputation to calculate the total variance associated with the slope of each regression. Details of this method are given in Pan and Shimizu.⁵⁶ Briefly, fit parameters were assumed to be normally distributed and 10 parameter "estimates" were obtained for each parameter using the average and standard deviation determined from the fit. Estimates were then plotted against the component of interest to obtain 10 fits, each with a corresponding slope. The generated slopes and variances were then used to calculate the between and within variance to obtain the total variance. Finally, the student's *t*-test and a 95% confidence was used to calculate and evaluate the associated *p*-values.

3. Results

3.1 PM-induced dose-dependent ROS/RNS production. ROS/RNS production, expressed as fold increase in fluorescence over control cells, was measured over 10 dilutions for each PM sample. A representative dose-response curve with three distinguishable regions is shown in Fig. 2. In the first region, there exists a dose below which there is no observable difference in

response compared to control cells. Next, there is a region where response increases with dose. Finally, there exists a dose beyond which there is no further increase in response.

Figure 2. Representative dose-response curve of ROS/RNS produced as a result of ambient PM exposure (filter ID: YRK 07/10/12). ROS/RNS production is expressed as fold increase over control, defined as probe-treated cells incubated with stimulant-free media. Dose is expressed as either (a) mass in extract (μg) or (b) volume of air sampled (m^3). Data shown are means $\pm$ SE of exposure experiments performed in triplicate. Hill equation (HE) parameters shown include: max response, Hill slope (rate at which max response is attained), EC_{50} (dose at which 50% of max response is attained), threshold (dose at which response is 10% above baseline), and AUC (area under the dose-response curve).

To characterize each dose-response, the Hill equation, used extensively in pharmacology to determine drug dose-response relationships,^{57, 58} was applied to each data set to obtain response parameters. These parameters, labeled in Fig. 2a, include the max response attained as a result of PM exposure, the rate at which the max response is attained once there is response (Hill slope), the dose at which 50% of the max response is attained (EC_{50}), and the dose at which response is 10% above the fitted baseline (threshold).

In addition to the classical dose-response shown in Fig. 2, some samples exhibited behaviors that required adjustments to analysis methods (Fig. S3):

(1) **Maximum response not attained:** the Hill equation was applied and the maximum response was estimated from the fit. Fitted parameters in these cases were generally associated with large uncertainties.

(2) **Decreased response at higher doses:** doses after the initial decrease in response were excluded from the fit. MTT for selected filters showed a decrease in cell viability at the same dose where ROS/RNS production decreased.

(3) **No response above baseline at all doses:** a maximum response of 1 and a threshold equal to the highest dose investigated was assigned. EC_{50} and Hill slope were not calculated.

The area under the fitted dose-response curve (AUC), labeled in Fig. 2b, was also considered as a measure of the overall effect of PM exposure across the dose range investigated.⁵⁹ Doses (in μg) were converted to volume of air sampled to obtain dose-response curves across the same dose range. Uncertainties associated with AUC determination were approximated by averaging AUCs calculated by fitting dose-response data with each point removed systematically. Max response and Hill slope values did not change with dose conversion. Furthermore, the uncertainties associated with AUC determination were low for all samples investigated, including those with non-classical dose-response behaviors.

3.2 ROS/RNS production and oxidative potential. AUC per volume of air sampled (AUC_{volume}) and AUC per mass of PM (AUC_{mass}) were compared with extrinsic and intrinsic DTT activity, respectively. Results are shown in Fig. 3, colored by season for both cell types. It is noted that the DTT activity results have been reported previously in Verma et al.⁴⁰ For summer filters, AUC_{volume} was significantly correlated with DTT activity ($n = 47$, $R = 0.63$, $p < 0.05$), but a

relatively constant AUC_{volume} was observed for winter samples ($n = 32$). A statistically significant correlation between AUC_{mass} and DTT activity (Fig. 3b) was observed for summer data, while no correlation was observed for winter data. No other dose-response parameter was significantly correlated with DTT activity (Fig. S4) and no spatial trends were observed.

Figure 3. AUC per volume of air sampled (a) and per mass of PM (b) as determined by Hill equation fits to ROS/RNS dose-response data for ambient samples spanning a wide range of (a) extrinsic and (b) intrinsic DTT activity. Each data point represents a single ambient filter for which a dose-response was obtained (10 dilutions performed in triplicate). Data points are colored by season as determined by solstice and equinox dates between June 2012 and October 2013. Linear regressions and Pearson's correlation coefficients are shown for summer and winter filter samples. $n = 104$ ambient filters (10 spring, 47 summer, 15 autumn, and 32 winter); * indicates significance, $p < 0.05$

3.3 ROS/RNS production and PM composition. Absolute mass concentrations and

relative mass fractions of all water-soluble PM components⁴⁴ were compared with AUC_{volume} and AUC_{mass} , respectively. Seasonal trends for these correlations are shown in Figs. 4 and S5. For summer filters, AUC_{volume} was significantly correlated with WSOC ($R = 0.66$), brown carbon absorption (BrC, $R = 0.62$), titanium (Ti, $R = 0.71$), and iron (Fe, $R = 0.65$) (Fig. 4), while AUC_{mass} was significantly correlated with titanium fraction ($R = 0.66$) and chromium fraction (Cr, $R = 0.79$) (Fig. S5). For winter filters, a relatively constant AUC_{volume} was observed for WSOC, BrC, and Fe, while no statistically significant correlations were observed for AUC_{mass} and water-soluble PM

constituents. Other correlations, including metals grouped by source apportionment results,⁴⁴ were not statistically significant (Tables S1 and S2).

Figure 4. AUC per volume of air sampled for ambient samples spanning a wide range of (a) water-soluble organic carbon (WSOC), (b) brown carbon (BrC), (c) titanium (Ti), and (d) iron (Fe). Each data point represents a single ambient filter for which a dose-response was obtained (10 dilutions performed in triplicate) and fitted using the Hill equation. Data points are colored by season as determined by solstice and equinox dates between June 2012 and October 2013. Linear regressions and Pearson's correlation coefficients are shown for summer and winter filter samples. $n = 104$ ambient filters (10 spring, 47 summer, 15 autumn, and 32 winter); * indicates significance, $p < 0.05$. Removal of the sample with high WSOC introduces a slight slope (0.03) in the winter correlation, which is still relatively flat (panel A).

3.4 Comparison between MH-S and NRVM. AUC_{mass} data for ambient filters

investigated using both cell types are shown in Fig. 5. A total of 18 filters were analyzed with both cellular assays. AUC_{mass} was significantly correlated between the cell types ($R = 0.80$, $p < 0.05$). No seasonal or spatial trends were observed.

Figure 5. AUC per mass of PM for MH-S and NRVM. Each data point represents a single filter dose-response obtained from 10 dilutions (1x to 0.00125x) performed in triplicate. An orthogonal regression and the corresponding Pearson's coefficient is shown. A 1:1 line is shown for reference. $n = 18$ ambient filters; * indicates significance, $p < 0.05$.

4. Discussion

4.1 Cellular ROS/RNS assay optimization. In the present study, MH-S and NRVM were exposed to PM extracts containing water-soluble components in an assay optimized for measuring ROS/RNS production (Fig. 1). Previous cellular assays^{53, 60} were used as a general guideline for method optimization. For this study, the following parameters were optimized using MH-S and later adapted for NRVM: cell density, ROS/RNS probe concentration, and sample incubation time (Table S3). Optimization experiments with stimulant-free, probe-treated cells showed that 1×10^5 cells well^{-1} (commonly used in prior studies^{26, 53, 60}) produced a higher ROS/RNS baseline with greater signal variability. Likewise, studies have shown that cells experienced oxidative stress

under crowded conditions.^{61, 62} A lower cell density, 2×10^4 cells well⁻¹, produced a low baseline and was visually consistent with low-density reference images (ATCC). Further optimization experiments also showed that probe concentrations $\geq 30 \mu\text{M}$ resulted in visually enlarged cells and membrane irregularities similar to those observed under crowded conditions. These observations suggest that ROS/RNS productions observed using higher probe concentrations, such as $45 \mu\text{M}$,^{60, 63} may be due to basally stressed cells and may not accurately reflect PM exposure. Finally, a time series experiment (Fig. S2) showed that a significant fold increase in ROS/RNS production was not observed until 8-24hrs. This is much longer than exposure times used in previous cellular assays, typically ~ 2.5 hrs,^{60, 63, 64} but is in accordance with the LPS peak response time found in other inflammatory assays.⁶⁵ The final assay parameters are as follows: 2×10^4 cells well⁻¹ (MH-S), 3.33×10^4 cells well⁻¹ (NRVM), $10 \mu\text{M}$ ROS/RNS probe, and 24hrs sample incubation.

In addition to parameter optimization, a dose-response approach was used in this study. Prior studies using the macrophage assay involved normalizing raw fluorescence measurements to a positive control (e.g., zymosan or H_2O_2) in order to obtain a single value for comparison with DTT activity.^{60, 66} However, studies involving inflammatory stimulants and cellular endpoints, including IL-6, IL-8, NF- κ B, and TNF- α , showed that endpoints generally followed a sigmoidal dose-response curve.^{65, 67, 68} Here, we demonstrated clearly that ROS/RNS production from PM exposure was non-linear, highly dose-dependent, and could not be represented by a single concentration measurement. Furthermore, the specific dose-response region captured for each filter depended strongly on the dilution range used. A narrow dilution range could only capture the full response behavior for certain filters and may result in overestimates and underestimates for filters of lower and higher potency, respectively. Additionally, in this study, ROS/RNS production was reported as a fold increase over control cells to take into account variability between batches

of cells (basal cellular ROS production). This normalization scheme was chosen to reduce uncertainty in ROS/RNS production, since signals were observed to increase with increasing response and were especially variable with positive controls.

Another common scheme used in prior studies involved obtaining a linear slope for macrophage ROS production over a few dilutions.⁶⁹ However, as demonstrated in this study, the dose-response curve (Fig. 2) is highly non-linear and the slope varies significantly depending on the dose range chosen. Furthermore, large-scale pharmacology studies showed that different dose-response metrics were correlated with different subsets of drugs, highlighting the importance of considering information from multiple dose-response metrics.⁷⁰ For comparison studies where a single measure was required, the same study demonstrated that AUC was robust whereas other metrics varied in informativeness depending on dose. Another study involving simulation and experimental results also demonstrated that AUC was the most robust metric in classifying and ranking drugs.⁵⁹ Conversely, EC_{50} and Hill slope were found to be less reliable and more unstable due to their sensitivity to dose range, especially in cases where either the base or maximum response are not observed.⁷¹ In the current study, all dose-response metrics were considered to avoid missing important information. Still, AUC was found to be the most robust metric in this study.

4.2 Comparison between MH-S and NRVM. To determine whether PM-induced ROS/RNS production varied between cell types that participate in the first line of defense (macrophages) and other active cells that would be exposed to pollution less directly (cardiomyocytes), all response parameters were compared for both cell types. AUC_{mass} was significantly correlated between the cell types ($R = 0.80$, $p < 0.05$) (Fig. 5). Both macrophages, which participate in the immune response, and ventricular myocytes, which contract to pump

blood, are active cells. Thus, the strong correlation suggests that the ROS/RNS response may be similar for different active cell types. However, the response is likely to be different for structural cells that are largely inactive and warrants future studies.

4.3 Associations between ROS/RNS production and oxidative potential. To determine whether chemical oxidative potential (i.e. DTT activity) is representative of cellular responses, all response parameters (AUC_{mass} , AUC_{volume} , threshold, Hill slope, EC_{50} , max response) were compared with DTT activity (Figs. 3 and S4). Distinct seasonal trends were observed for correlations between AUC_{volume} and extrinsic DTT activity (Fig. 3a). A strong, statistically significant correlation was observed for summer filters. Conversely, with the same sample variability as measured by root-mean-squared error (Table S4), a relatively constant AUC_{volume} was observed regardless of DTT activity for winter filters. Spring and autumn samples in the present study (Fig. S7) do not span the observed DTT range and the sample size is too small ($n = 10$ and 15 , respectively) to determine statistical significance, however, the majority of spring and autumn responses fall within summer and winter prediction intervals. This suggests that spring and autumn samples may exhibit overall trends that fall somewhere between summer and winter trends. AUC_{mass} was also significantly correlated with intrinsic DTT activity for summer samples (Fig. 3b), however, no statistically significant correlation was observed for winter samples. The observed seasonal differences suggest that a simple correlation does not exist for oxidative potential as measured by DTT and cellular ROS/RNS responses and appear to be dependent on PM composition (section 4.4). However, due to its automation and high throughput, DTT may serve as a useful tool for screening samples for cellular analysis, as low DTT will most likely correspond to a low cellular response.

Other dose-response parameters were not significantly correlated with DTT activity. The lack of correlation with other parameters may be the result of large uncertainties associated with parameter determination for samples that did not attain a maximum response. Indeed, AUC was the most robust measurement with low associated uncertainties and did not require samples to attain maximum response. This is in agreement with other drug studies, where AUC was the most reliable measure for differentiating drug performance.^{59, 72} Collectively, these results suggest that DTT activity may not fully represent every aspect of the cellular ROS/RNS response, as each dose-response parameter serves as a different measure of toxicity. Since DTT was significantly correlated with AUC for summer samples, DTT may be more indicative of the overall effect of PM, which AUC represents.

Although neither EC_{50} nor max response was correlated with DTT activity or composition, a significant positive correlation was found between these parameters for all filters investigated (Fig. S8). This is in contrast to the expected anti-correlation, where a more potent sample (lower EC_{50}) would induce more ROS/RNS production (higher max). Previous studies investigating anti-oxidant activities found a significant correlation between EC_{50} and anti-oxidant concentration.⁷³ ⁷⁴ It is therefore possible that certain species in more potent samples are involved in anti-oxidant production pathways, resulting in neutralization of ROS/RNS species and thus a lower maximal ROS/RNS production. Alternatively, species may induce cellular protective pathways that prevent or reverse oxidative damage, thus reducing cellular damage and possibly enabling cells to produce more ROS/RNS. Studies with pro-inflammatory and anti-inflammatory molecules generally did not include correlations with Hill slope or threshold, however these parameters could potentially better capture other cellular pathways or simply include contributions from a combination of cellular events. Further studies are required to test these hypotheses.

4.4 PM constituents associated with AUC.

To assess whether a good predictor exists for cellular ROS/RNS production, all cellular response parameters were compared with water-soluble PM constituents, mass fractions of constituents, and constituents grouped by sources (Tables S1 and S2). Distinct seasonal patterns for AUC_{volume} correlations with composition were observed for WSOC, a surrogate of secondary organic aerosols (SOA), BrC, and to a lesser extent Ti and Fe (Fig. 4), where a statistically significant correlation with AUC_{volume} was found for summer samples and a relatively flat AUC_{volume} was found for winter samples.

The observed seasonal variability could potentially be explained by chemical composition, as PM collected during different seasons at the sampling sites differ in mass concentration and chemical composition.^{40, 44-46, 50} Similarly, seasonal variability has been observed for sources of organic species. Using positive matrix factorization (PMF) analysis of high-resolution aerosol mass spectrometry (HR-ToF-AMS) data, Xu et al. showed that organic aerosols at these sites are dominated by SOA, which can be further deconvolved into different subtypes.⁴⁶ With more intense photochemistry in summer, the correlation between AUC_{volume} and WSOC for summer samples may reflect contributions from photochemical SOA and/or aged SOA.⁴⁶ There may also be significant contributions from less-oxidized and more-oxidized oxygenated organic aerosol (LO-OOA and MO-OOA, respectively), which are present at higher concentrations in summer due to stronger photooxidation and emissions of biogenic volatile organic compounds.⁴⁶ This further highlights the importance of understanding the contribution of SOA to PM-induced health effects, especially those arising from photochemical oxidation reaction pathways.

Similarly, seasonal variability observed for AUC_{volume} vs. BrC may be the result of different sources of BrC inducing different cellular pathways. Although biomass burning is the predominant source of BrC, especially in winter, BrC may also have non-negligible contributions from aged

SOA.⁷⁵ Furthermore, while BrC was observed in both summer and winter during the filter collection period,⁴⁰ biomass burning organic aerosol (BBOA) was not resolved from PMF analysis of HR-ToF-AMS data in summer, suggesting an unknown source of BrC other than biomass burning.⁴⁶ The cellular ROS/RNS assay may only be sensitive to BrC from the unknown source, rather than BBOA. This is further supported by the low AUCs observed for winter samples even though BBOA was previously found to be highly redox active compared to ambient PM and other sources of organic aerosols.⁵⁰ Additionally, it is possible that some BrC species may not pass through the cell membrane or that transport through the membrane may be hindered once a certain intracellular concentration is attained, resulting in a consistent response regardless of concentration. This may be especially true for humic-like substances, a source of BrC from biomass burning that includes large molecular weight hydrophobic compounds.⁷⁶ Transport of large compounds across the cellular membrane could be impeded, which may result in lack of increased response. While size-dependent impedance on cellular transport has been observed for macromolecules⁷⁷ and nanoparticles,⁷⁸ further studies may be needed to establish this for atmospheric aerosols.

Among the metal species, only Fe and Ti were significantly correlated with AUC_{volume} for summer filters. Both are transition metals with positive oxidation states that have been correlated with DTT activity, a measure of the concentration of redox active species, and shown to participate in reactions that generate ROS/RNS.^{34, 38, 44, 79, 80} Furthermore, Fe (II) may produce ROS/RNS via Fenton-like reactions⁸¹, while Ti (IV) may interfere with protein structure by interacting with cysteines or forming other proteins complexes.^{82, 83} Titanium dioxide (TiO₂) is also known to induce pulmonary inflammation.⁸⁴ No other response parameter was correlated with any metals, and no spatial trends were observed for any species. This is in contrast with previous ROS studies

441 using filter samples collected in California where correlations with Fe, Cu, Cr, Pb, Mn, Zn, As,
442 Ni, and V were observed.^{60, 63, 64, 69} However, this is not surprising as previous studies used
443 different metrics (e.g. measurement at single PM dose, linear slope from few PM doses, etc.) to
444 represent ROS production. While Hill slope is similar to the linear slope method used in some of
445 these studies, we found this metric to be strongly influenced by dose range and previous
446 pharmacology studies also found this metric less reliable.⁷¹ Still, correlations between DTT
447 activity and K, Mn, Fe, Cu and Zn were also previously reported in SCAPE studies.⁴⁰ The lack of
448 correlation with other metals, however, does not reflect a lack of response in the present study.
449 Exposure to pure metal salt solutions at concentrations observed in ambient filters induced dose-
450 dependent ROS/RNS production (Fig. S6). These results demonstrate that complex interactions
451 may occur such that a simple sum of individual effects cannot recapitulate observed response. For
452 example, while metals induce ROS/RNS production, complex interactions between species may
453 hinder metal-cell interactions or individual organic species may have a greater contribution that
454 masks metal contributions. Verma et al.³⁷ previously showed that certain metals (V, Zn, and Fe)
455 were retained on a C-18 column, which largely removes hydrophobic compounds. This supports
456 the hypothesis that certain metals may be involved in metal-organic complexes, which may be too
457 large to freely diffuse across the cell membrane and interact with cellular components that promote
458 ROS/RNS production. Furthermore, the relative WSOC:metal ratio may mediate this effect, where
459 a higher WSOC:metal ratio may exhibit more metal-cell hindrance due to enhanced metal-organic
460 complex formation. In the present study, the WSOC:metal ratio averages from 18-23, whereas the
461 ratio ranges from 0.98-3 for ambient samples from California.^{60, 63, 64} This substantial difference
462 could explain the few correlations between cellular response and metal concentrations observed in
463 the present study, where a significant portion of metals may be complexed with organics. However,

while metal-organic complexes have been observed in aqueous solutions,^{85, 86} further studies are required to establish this for organics and metals present in PM extracts. Interactions with proteins in FBS, added to PM extracts to prevent cell starvation, may also be possible and could either promote or prevent species-cell interactions depending on whether the protein involved participates in cell interactions or not, respectively.^{87, 88} These interactions may be important considerations as proteins, sugars, and lipids are also present in the alveolar fluid, however, further studies are required to establish whether PM species participate in these interactions.

Implications. PM-induced intracellular reactive oxygen/nitrogen species (ROS/RNS) production from 104 filters collected from the greater Atlanta area was measured using an optimized alveolar macrophage and ventricular myocyte cellular assay. Results from this study demonstrate that a wide dose range (instead of a single point) should be considered to accurately compare cellular responses between different PM samples. Furthermore, multiple dose-response metrics (i.e. max response, EC₅₀, Hill slope, threshold, AUC) should be examined as each metric may serve as a different measure of toxicity. Overall, AUC was found to be the most robust metric, as its informativeness did not depend on dose range.

Seasonal differences in the correlation between water-soluble DTT activity, a measure of the concentration of redox-active species in the sample, and intracellular ROS/RNS produced as a result of interactions between PM species and cells were also observed in this study, which may be largely driven by PM composition. Nevertheless, DTT may serve as a useful tool to screen samples for cellular analysis. It should be noted that only the water-soluble DTT activity and water-soluble PM components were considered in this study. Results may differ for total DTT activity, where water-insoluble components would also be present.

Thus far, most aerosol health studies have focused on the effects of primary pollutants, though ambient studies have repeatedly shown that SOA dominates ambient fine PM, even in urban centers.⁸⁹ In this study, the observed correlations between cellular ROS/RNS production and organic species (WSOC, BrC) in summer samples highlight the importance of understanding the contribution of organic aerosols, especially photochemically-driven summertime SOA, to PM-induced health effects. Furthermore, results from the current study also show that PM species cannot be treated as individuals in water-soluble PM extracts. While few water-soluble metals were correlated with ROS/RNS response, exposure to individual metals induced measureable ROS/RNS production. These results suggest that complex interactions may occur between PM species, resulting in the involvement of multiple cellular pathways such that the overall cellular response cannot be approximated by a simple sum of the individual effects of components. Mixture effects may therefore be an important consideration in future studies. This study highlights the need to systematically investigate ROS/RNS production from different PM mixtures. In particular, generating well-characterized PM mixtures in laboratory experiments and measuring their cellular ROS/RNS response could provide fundamental data to interpret results from ambient mixtures.

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510 ABBREVIATIONS

511 PM: particulate matter; ROS/RNS: reactive oxygen and nitrogen species; DTT: dithiothreitol;
512 WSOC: water-soluble organic carbon; BrC: brown carbon; FBS: fetal bovine serum; PBS:
513 phosphate buffered saline; SE: standard error; AUC: area under the curve

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Graphical Abstract