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Model Combustion-Generated Particulate Matter Containing Persistent Free Radicals Redox Cycle to Produce Reactive Oxygen Species

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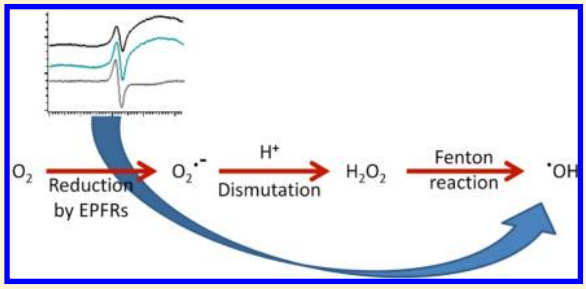
ABSTRACT: Particulate matter (PM) is emitted during thermal decomposition of waste. During this process, aromatic compounds chemisorb to the surface of metal-oxide-containing PM, forming a surface-stabilized environmentally persistent free radical (EPFR). We hypothesized that EPFR-containing PM redox cycle to produce ROS and that this redox cycle is maintained in biological environments. To test our hypothesis, we incubated model EPFRs with the fluorescent probe dihydrorhodamine (DHR). Marked increases in DHR fluorescence were observed. Using a more specific assay, hydroxyl radicals (•OH) were also detected, and their level was further increased by cotreatment with thiols or ascorbic acid (AA), known components of epithelial lining fluid. Next, we incubated our model EPFR in bronchoalveolar lavage fluid (BALF) or serum. Detection of EPFRs and •OH verified that PM generate ROS in biological fluids. Moreover, incubation of pulmonary epithelial cells with EPFR-containing PM increased •OH levels compared to those in PM lacking EPFRs. Finally, measurements of oxidant injury in neonatal rats exposed to EPFRs by inhalation suggested that EPFRs induce an oxidant injury within the lung lining fluid and that the lung responds by increasing antioxidant levels. In summary, our EPFR-containing PM redox cycle to produce ROS, and these ROS are maintained in biological fluids and environments. Moreover, these ROS may modulate toxic responses of PM in biological tissues such as the lung.



INTRODUCTION

Airborne pollutants, including fine (diameter <2.5 μm) and ultrafine (<0.1 μm) particulate matter (PM), are often generated through widely used thermal processes such as the combustion of fuels or the thermal decomposition of waste. In recent decades, increased environmental awareness has led to a growing number of epidemiologic studies examining the relationship between airborne emissions and public health. Not surprisingly, many of these studies have revealed associations between air pollution and adverse health effects, including increased daily mortality,[1] as well as increased mortality due to lung cancer and cardiopulmonary diseases.[2] Noteworthy is that these associations persisted even after adjustment for individual factors such as smoking status.[2] In support of these findings, data collected from 20 U.S. cities suggested that levels of PM_{10} (PM < 10 μm) were associated with increased mortality rates from cardiovascular or respiratory disorders.[3] Moreover, hospital admissions related to pulmonary and cardiovascular conditions are among the most prevalent associations reported for particulate air pollution exposures. For example, as much as 5% of cardiovascular disease-related hospital admissions are estimated to be attributable to air pollution.[4]

Although toxicological studies have thus far been unable to identify the exact mechanism of PM-related toxicity, the dominant hypothesis is that smaller particulates such as ultrafine particles (UFP) induce the generation of reactive oxygen species (ROS) on the particle surface.[5] Moreover, studies have shown that UFP often elicit a greater biologic effect than larger particles of the same substance.[6] UFP are able to penetrate both deeper and more effectively into the lungs compared to larger PM.[7] Greater surface area compared to larger PM may also aid UFP in the production of ROS through an increased opportunity for interaction with cell surfaces.[8]

Production of ROS associated with UFP may also be related to the generation of surface-associated environmentally persistent free radicals (EPFR) that form during combustion and incineration processes. Oberdorster et al.[9] asserted that pathogenic PM likely produce free radicals in cell-free systems

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and are thus capable of inducing oxidative stress. Donaldson et al.[10] proposed that surface-associated free radicals are capable of eliciting biologic damage such as lipid peroxidation, protein oxidation, DNA damage, and depletion of antioxidant defenses.

In support of this hypothesis, aromatic compounds often chemisorb to the surface of metal-oxide-containing PM under postflame, cool-zone conditions associated with thermal processes, resulting in the formation of surface-stabilized EPFRs.[11] Moreover, our colleagues reported the presence of semiquinone-like radicals in $PM_{2.5}$ generated from combustion processes and the fact that aqueous extracts of the $PM_{2.5}$ damaged DNA in human cells.[12] Finally, studies of the toxicological properties of cigarette smoke demonstrated that semiquinone radicals are capable of redox cycling, resulting in the production of sustained ROS upon reaction with biological systems.[12,13]

We hypothesize that chemisorption of organic EPFRs and the reduced form of metal oxides result in a catalytic cycle on the particle surface that generates ROS, while regenerating both the EPFR and the oxidized form of the metal oxide. Because of the variability associated with the collection of environmental samples, to test this hypothesis in a manner in which the metal oxide and organic content can be carefully controlled, we synthesized model particle systems that produce surface-associated EPFRs, as verified extensively by EPR analyses.[14−16]

We further hypothesized that this EPFR-mediated ROS production can be maintained in the biological milieu and can even interact with other biological mediators to promote a sustained ROS production. We thus utilized *in vitro* approaches for assessing ROS production in both cell-free systems, as well as other biological fluids, incubated together with our model EPFR-containing PM.

$$O_2 \xrightarrow[\text{by EPFRs}]{\text{reduction}} O_2{}^{\cdot -} \xrightarrow[H^+]{\text{dismutation}} H_2O_2 \xrightarrow[\text{reaction}]{\text{Fenton}} {}^{\bullet}OH \quad (1)$$

Our colleagues have proposed that the semiquinone radical formed on the surface of the particles redox cycle to produce first the superoxide and then, via Fenton reactions with Cu(I), the first hydrogen peroxide, and then finally the hydroxyl radicals (eq 1). To date, molecular probes such as dichlorodihydrofluorescein, dihydrorhodamine 123 (DHR), and dihydroethidium (DHE), which form fluorescent products in the presence of ROS including superoxide, hydrogen peroxide, and $^{\bullet}OH$, have been commonly used in *in vitro* and, in some cases, *in vivo* experimentation. These assays have also been used in cell-free particle solutions to detect ROS production from surface-associated free radicals.[9] However, in studies presented herein, we compared data obtained using this approach to that of more specific measurements of $^{\bullet}OH$ production using a scavenging assay, with detection using HPLC. Finally, using both laryngeal epithelial cells in culture and tissues obtained from animals exposed through inhalation, we tested whether ROS production from EPFR-containing PM contributes to cytotoxic responses. Studies presented here highlight that some PM may indeed redox cycle to produce ROS that can be maintained in biological environments but that the selection of assays for the measurement of ROS is critical to the interpretation of data obtained in these types of experiments.

MATERIALS AND METHODS

Materials. Chemicals, including catalase (CAT), superoxide dismutase (SOD), nicotinamide adenine dinucleotide phosphate (NADPH), *N*-acetyl cysteine (NAC), 4-hydroxybenzoic acid (4HBA), and 3,4-dihydroxybenzoic acid (DHBA) were purchased from Sigma-Aldrich. Ascorbate (AA) was purchased from JT Baker, and DHR and dihydroethidium (DHE) were purchased from Calbiochem.

Generation of Model Particle Systems. The generation and characterization of the particle systems tested in these studies have been discussed in detail elsewhere.[14−16] Briefly, particles of 5% CuO (w/w) supported on silica were prepared by the impregnation of ultrafine silica powder (Cabosil, particle size <200 nm) with copper nitrate hemipentahydrate through incipient wetness, followed by calcination. The particulate samples were then exposed to the vapors of the adsorbates (monochlorophenol or dichlorobenzene) using a custom-made vacuum exposure system to produce EPFRs and physisorbed monochlorophenol and dichlorobenzene.

Generation of Reactive Oxygen Species by EPFR. General ROS production was determined by incubation of the particle systems with the fluorescent probe DHR. It is generally well-known that in the presence of ROS, DHR is oxidized to the fluorescent product rhodamine; thus, increases in fluorescence can be indicative of increases in ROS production. The particle samples used for these experiments included silica (SiO_2), copper oxide silica (CuO/SiO_2), monochlorophenol (MCP) physisorbed to SiO_2 by exposure at 100 °C (MCP100), dichlorobenzene (DCB) physisorbed to SiO_2, (DCB100) monochlorophenol chemisorbed to SiO_2 via CuO by exposure at 230 °C (MCP230), and DCB chemisorbed to SiO_2 via CuO (DCB230). The particle samples were suspended at a concentration of 1 mg/mL in 0.9% saline (w/v) containing 0.02% Tween 20 (v/v). To ensure adequate dispersion, the samples were incubated for 30 min in a bath-type sonicator. After sonication, the particle samples were incubated in the dark at room temperature for 1 h with 5 μM DHR, and fluorescence was monitored at *Ex*/*Em* = 485/528 nm in 10 min intervals. Following the analysis of ROS generation associated with the basic particle systems, additional incubations of selected particle systems were performed to evaluate the generation of ROS in the presence of 1800 U/mL CAT, 3000 U/mL SOD, SOD + CAT, 100 μM NADPH, 100 μM AA, or 100 μM NAC.

Generation of the Hydroxyl Radical by Model Combustion-Generated Particulate Matter. Liu and others[17] reported a method for utilizing 4HBA to trap $^{\bullet}OH$. This method was selected because 4HBA hydroxylation produces only one metabolite, 3,4-DHBA.[17,18] Thus, stoichimetrically, one mole of hydroxyl radical should produce one mole of 3,4-DHBA. The particle samples, prepared as described above, were incubated with 0.05 mg/mL 4HBA at 37 °C for 1 h. In some experiments, incubations were conducted in the presence of antioxidants and potential redox mediators (e.g., SOD, CAT, NADPH, AA, NAC, and uric acid). Hydroxyl radical production was then determined by quantifying 3,4-DHBA formation by HPLC with electrochemical detection. As such, the samples were injected onto a Waters Alliance 2695 HPLC System. The separation was accomplished using with a Hamilton RPR-1 250 × 4.1 mm I.D., 7 μm reverse-phase polymeric column, and isocratic elution of 90% 20 mM acetate/20 mM citrate, pH 5.4/10% methanol (v/v), at a flow rate of 0.5 mL/min. Elution of 3,4-DHBA was monitored using a 4-channel ESA Coularray electrochemical detector, with the electrodes set at 150, 350, 600, and 750 mV. 3,4-DHBA was quantified on the 350, 600, and 750 mV channels combined for all samples tested, with one exception. Samples incubated in the presence of CAT were quantitated using the 350 mV channel only due to baseline interference on the 600 and 750 mV channels.

Generation of the Superoxide Radical by Model Combustion-Generated Particulate Matter. The production of the superoxide was evaluated by modification of a method described previously.[19] Zhou et al.[20] reported that DHE reacts with the superoxide radical anion, resulting in the formation of 2-hydroxyethidium (2-OH-E^+) as the major end product. Thus, the formation of 2-OH-E^+ can be quantified as a diagnostic marker for the reaction of HE with superoxide.[21] Synthesis of the 2-OH-E^+ standard was accomplished through reaction of HE with nitrosodisulfonate (NDS), as described by Zielonka et al.[19] The separation was accomplished using a Phalanx (Higgins Analytical,

PS-2546-C183) 250 × 4.6 mm I.D., 3 μm C18 column, and isocratic elution of 65% 50 mM potassium phosphate buffer, pH 2.6, and 35% acetonitrile (v/v) at 0.6 mL/min.[22] A 4-channel ESA Coularray electrochemical detector was used to monitor the elution of HE and 2-OH-E$^+$. HE was quantitated on the 0 and 200 mV channels combined, while 2-OH-E$^+$ was measured on the 750 mV channel.

In other experiments, the incubations were carried out in the dimethyl sulfoxide, and the concentration of HE utilized for trapping superoxide was increased to 150 μM. Moreover, we utilized an alternate HPLC method for assessing 2-OH-E$^+$. In brief, the separation was accomplished using the same Phalanx column but with a gradient elution of 90% A [0.1% trifluoracetic acid (TFA)]/10% B [0.1% TFA in acetonitrile] to 35%A/65% B over 15 min, then to 20% A/80% B over 3 min, and then finally, a gradient to 100% B over 6 min. This time, detection was achieved using a Shimadzu RF-20A fluorescence detector with excitation/emission wavelengths of 510 and 595 nm.

Maintenance of EPFR and EPFR-Mediated Hydroxyl Radical Production in Biological Fluids. To determine the half-life of EPFR in biological matrices, samples containing 25 mg of MCP230 or DCB230 were placed in a microcentrifuge vial. Then, 500 μL of bronchoalveolar lavage fluid (BALF) or normal saline was added to each sample. Note that the BALF was obtained by one of our colleagues from the lungs of adult mice using a standard protocol and that the collections were approved in advance by the institution's animal care and use committee. The suspensions were shaken continuously for 0.5−12 h using a vortex mixer. A similar procedure was repeated with mouse serum. Samples of 25 mg of MCP230 or DCB230 were placed in centrifugal vials, and 150 μL of untreated mouse serum was added and allowed to interact with the particles for 0.25−12 h under vortex mixing. Note that any difference in sample dilution was simply a matter of the availability of the biological sample. After vortex mixing, all samples were then centrifuged for 5 min, and the liquid fraction was discarded. The particles were frozen at −25 °C and dried in a vacuum for 12 h at room temperature. The dried particles were then subjected to EPR analyses using a Bruker EMX-20/2.7 EPR spectrometer at a microwave power of 1 mW, 9 GHz frequency, 4 G amplitude, and 100 kHz frequency. EPFR concentration was expressed as a percent of the initial radical concentration, assumed to be 100%.

Next, to determine the ability of the particles to produce a hydroxyl radical in similar biological matrices, MCP230 or DCB230 was suspended at 1 mg/mL in BALF or normal saline containing 0.05 mg/mL 4HBA at 37 °C for 1 h. Hydroxyl radical production was then determined by quantifying 3,4-DHBA formation by HPLC with electrochemical detection, as described above.

Generation of ROS in Cells Following Exposure to Model Combustion Generated Particulate Matter. Human laryngeal epithelial (HEp-2) cells were provided by Dr. Stephania A. Cormier and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; v/v). The cells were trypsinized and were transferred into 96-well plates prior to experimentation. The effect of particle exposure on cellular ROS production was determined by first preloading with 5 μM DHR for 40 min, washing the cells with PBS, and then incubating the cells with particles for an additional 40 min. The particle suspension method was modified slightly for these cell studies. First, we utilized pulsed sonication rather than bath sonication. We postulated that due to EPFR decay, ROS production decreases with the increased amount of time taken for bath sonication. Thus, utilization of a brief pulsed sonication ensured that the cells were exposed to a maximal level of ROS production from the very beginning of the dosing period. In addition, since Tween 20 is somewhat toxic to cells, we utilized Tween 80 to aid in particle suspension. Note that Tween 80 is a nontoxic surfactant typically utilized in food products such as ice cream. Thus, prior to dosing, the particles were suspended in PBS containing 0.02% Tween 80 (v/v) and were pulse sonicated (10 s on, 10 s off) for 2 min using an ultrasonifier. Particles were then applied to cells at a concentration of 400−800 μg/mL. Given a plate surface of 2 cm^2/well and assuming that cells were confluent at the time of treatment, this provided an equivalent dose of 100−200 μg/cm^2. Following particle incubation, the PM-containing medium was aspirated, and the cells were washed twice with PBS. Finally, 100 μL of PBS was applied to the cells, and fluorescence was measured at *Ex*/*Em* = 485/528 nm. To confirm that increases in fluorescence were due to elevated oxidant production, some cell samples were preloaded with the antioxidant Trolox (100 μM).

To test for particle-induced changes in cell viability, the cells were treated with 100−200 μg/cm^2 particles. After 24 h of incubation, the medium was removed, the cells were washed with PBS, and ATP levels were assessed in the cells using the CellTiter-Glo Luminescent Cell Viability Assay kit from Promega. ATP concentrations in the cells were normalized to levels of cell protein, assessed using a Pierce BCA (bicinchoninic acid) Protein Assay kit (Thermo Scientific).

Animal Exposure. Using the InExpose nose-only inhalation system (SciReq, Montreal, QC, Canada), Brown-Norway neonatal rats, 7 days of age, were exposed to DCB230 particles for 7 consecutive days at a dose of 0.2 mg/mL. At 15 days of age, the animals were sacrificed. BALF and blood were then collected. Note that all animal procedures were approved in advance by the Institutional Animal Care and Use Committee at the LSU Health Sciences Center (New Orleans, LA), and all methodologies were in accordance with the recommendations of the Office of Laboratory Animal Welfare at the National Institutes of Health.

Ascorbate Measurement in Plasma and BALF from Exposed Animals. The method reported by Vislisel et al.[23] was utilized for the measurement of ascorbate levels in plasma. Through centrifugation, plasma was isolated from whole blood of exposed neonatal rats. To precipitate the protein, plasma or BALF samples were then diluted 1:5 with MeOH/H_2O (90:10, v/v) containing 250 μM DTPA and were incubated on ice for 10 min. Samples were then subjected to centrifugation of 12,000*g* at 4 °C for 10 min, and the supernatants were stored at −80 °C until analysis.

Glutathione and Glutathione Disulfide Measurements in BALF. BALF samples were rapidly thawed in 1:1 volumes of ice-cold 0.4 N perchloric acid containing 1 mg/mL EDTA. The samples were centrifuged, and 100 μL of each supernatant was injected immediately onto HPLC. The separation was accomplished on a Waters 2695 HPLC with a Supelco LC-8, 3 μm, 25 cm × 4.6 mm reversed phase column and isocratic elution of 10 mM NaH_2PO_4 containing 0.03 mM 1-octanesulfonic acid and 3% acetonitrile, pH 2.7, run at 0.35 mL/min. The elution of products was monitored using a Coularray electrochemical detector interfaced to a boron-doped diamond electrode maintained at 1500 mV. Quantitation of GSH and GSSG was achieved by comparison to authentic standards of each.

Statistics. All data are expressed as the means ± SEM. Statistical analyses were performed using either GraphPad Prism 5.0 or SPSS for Window software, as appropriate. For experiments where effects of treatment were assessed (across more than two groups), a one-way ANOVA followed by a Dunnett's multiple comparison or Fisher's LSD posthoc test was conducted. In other studies, where effects of both treatment and time were evaluated, a two-way ANOVA with either a Bonferroni or Student−Neuman−Keul's posthoc test was performed. Finally, in experiments where two treatment groups were compared at a single time point, an unpaired Student's *t* test was utilized. In all cases, $p < 0.05$ was accepted as statistical significance.

RESULTS AND DISCUSSION

Generation of Reactive Oxygen Species by EPFRs. To assess the redox potential of our particle systems in the absence of a biological environment, the production of reactive oxygen species (ROS) was monitored for 1 h after suspension of particles in solution. As assessed by DHR fluorescence, ROS production associated with SiO_2, DCB100, CuO/SiO_2, and MCP100 was generally quite low, while the EPFR-containing particle systems (DCB230 and MCP230) typically fluoresced at 5- to 70-fold higher levels (Figure 1A). Note that for the purposes of suspending the particles, we utilized a small concentration of Tween 20 (0.02% v/v). However, pilot

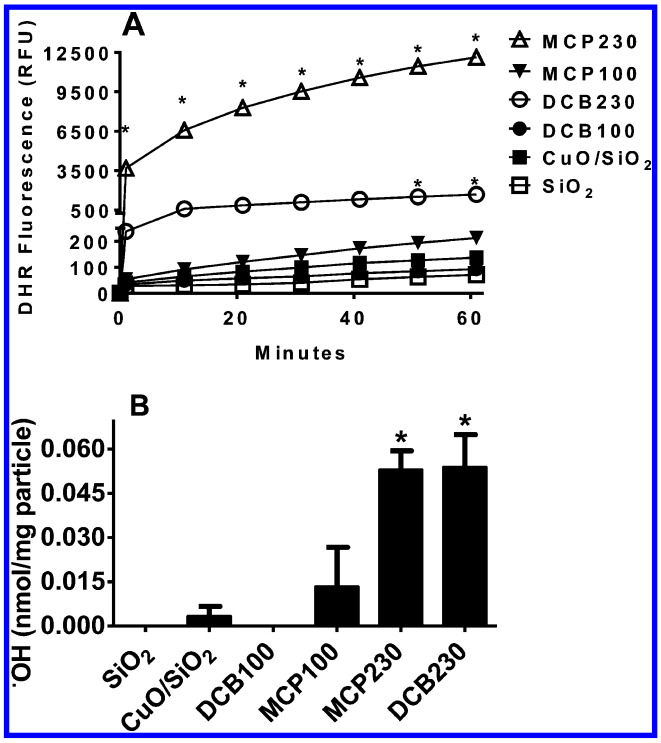

Figure 1. Reactive oxygen species (ROS) production measured in 1 mg/mL suspensions of model particle systems, including silica (SiO_2), copper oxide silica (CuO/SiO_2), monochlorophenol physisorbed to silica (MCP100), monochlorophenol chemisorbed to silica via CuO (MCP230), dichlorobenzene physisorbed to silica (DCB100), or dichlorobenzene chemisorbed to silica via CuO (DCB230). Increases in ROS levels were indicated by an increase in fluorescence of dihydrorhodamine 123 (DHR) (A), and hydroxyl radical levels were measured by scavenging with 4-hydroxybenzoic acid (4-HBA) over 60 min and then measuring the 3,4-dihydroxybenzoic acid product using HPLC with electrochemical detection (B). * $p < 0.05$ compared to SiO_2.

experiments comparing ROS production with and without Tween 20 showed no effect of the surfactant on ROS levels.

Although these initial studies suggested an ROS production mediated by suspension of particles in aqueous solution, we could not be certain which specific ROS was being detected. This is because fluorescent dyes like DHR and dichlorofluorescein are known to be fairly nonspecific, reacting with an array of species, including superoxide, hydrogen peroxide, hydroxyl radical, singlet oxygen, and even peroxynitrite.[24−27] We expected that a number of these species might be produced in our particle systems. Our hypothesis was that during the redox cycle of the surface-associated EPFR, oxygen is reduced to superoxide. The superoxide is then dismutated in solution to form hydrogen peroxide, which in the presence of Fe(II) or Cu(I), forms the highly reactive •OH via Fenton chemistry (eq 1). Thus, in order to examine particle-associated production of a specific ROS, we began by measuring •OH using an HPLC-based scavenging assay. The hydroxyl radical was not detected in suspensions of SiO_2 or DCB100 samples, but low levels were observed for CuO/SiO_2 and MCP100 (Figure 1B). However, •OH production associated with DCB230 and MCP230 was much greater, with levels as much as 15-fold higher than other particle systems ($p < 0.05$). Production of a superoxide radical was also measured by incubation of the particle systems with DHE, followed by measurement of 2-hydroxyethidium by HPLC. However, the superoxide radical was not detected in any of the particle suspensions (not shown).

Attenuation of EPFR-Induced ROS by Cotreatment with Antioxidant Enzymes. To verify that what was measured in the DHR fluorescence and hydroxyl radical assays was indeed ROS, experiments were conducted utilizing particle systems cotreated with and without the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), or the two enzymes in combination. SOD converts superoxide into hydrogen peroxide, and catalase metabolizes hydrogen peroxide to water. Thus, if the two assays we employed were indeed detecting ROS, the addition of either should decrease ROS production, presumably resulting in a lower level of detectable ROS. Co-treatment with either SOD or CAT to suspensions of MCP230 resulted in partial, but statistically significant, attenuation of DHR fluorescence (Figure 2A). However, treatment with SOD + CAT virtually abolished MCP230-associated ROS production (Figure 2A). With respect to •OH production, MCP230-associated •OH production followed a similar pattern compared to DHR fluorescence (Figure 2B), in that cotreatment with SOD, CAT, or a combination of the two resulted in significantly lower mean levels compared to that of EPFR-containing PM alone. However, in this case, SOD + CAT treatment produced similar results compared to those

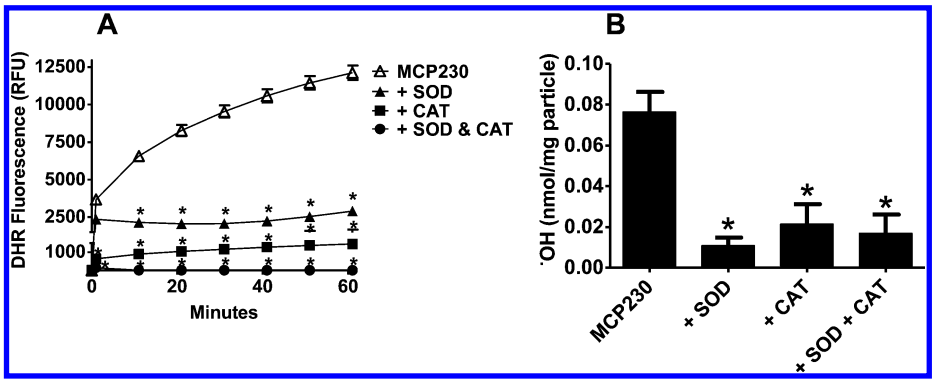

Figure 2. Effect of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), on MCP230-mediated ROS production. MCP230 was suspended at 1 mg/mL in 0.9% saline (w/v) containing 0.02% Tween (v/v), along with either 1800 U/mL CAT, 3000 U/mL SOD, or SOD + CAT. ROS levels were determined by following the fluorescence of dihydrorhodamine 123 (DHR) (A), and hydroxyl radical levels were measured by scavenging with 4-hydroxybenzoic acid (4-HBA) over 60 min and then measuring the 3,4-dihydroxybenzoic acid product using HPLC with electrochemical detection (B). *Denotes $p < 0.05$ compared to MCP230 alone.

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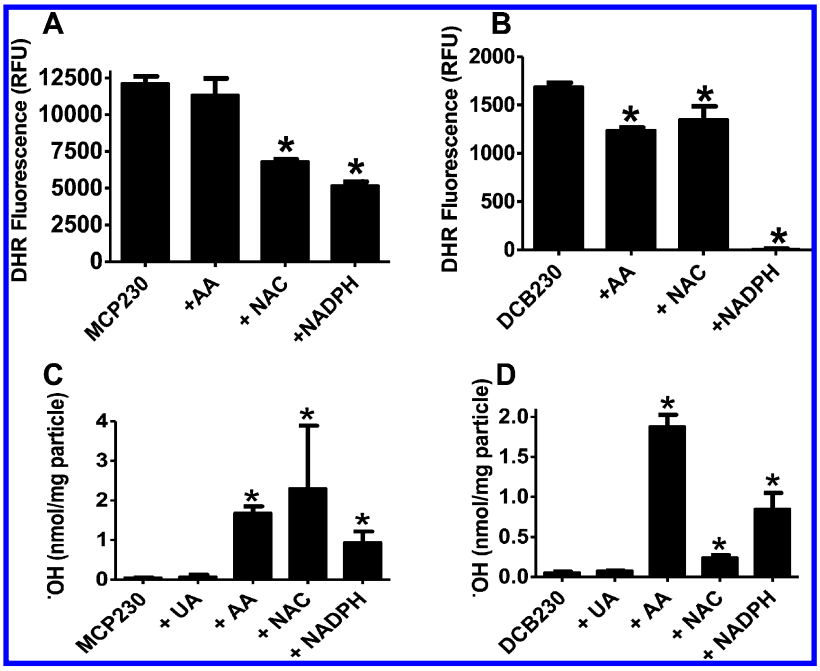

Figure 3. Effect of cotreatment with epithelial lining fluid constituents, including ascorbate (AA), urate, (UA), *N*-acetylcysteine (NAC, a model thiol), and NADPH, on MCP230-mediated ROS production. Particles were suspended at 1 mg/mL in 0.9% saline (w/v) containing 0.02% Tween (v/v) and then incubated for 1 h together with 100 μM NADPH, 100 μM AA, 100 μM urate, or 100 μM NAC. (A,B) ROS production was indicated by an increase in DHR fluorescence. (C,D) Hydroxyl radical production was measured by scavenging with 4-HBA and then measuring products using HPLC. *Indicates $p < 0.05$ compared to MCP230 or DCB230 alone.

from the treatment with SOD or catalase alone. This may have been due to the fact that treatment with either enzyme alone elicited such a dramatic reduction that detecting significantly greater effects of the combination treatment was simply not feasible.

One caveat is that in all of our experiments examining ROS production from particles in suspension, we were not initially able to detect superoxide production, even using the specific and sensitive HPLC-electrochemical detection assay reported by Zielonka et al.[19] However, (1) we were able to detect an SOD-inhibitable production of $^{\bullet}$OH (Figure 2B), suggesting that superoxide must have been produced and was a prerequisite for $^{\bullet}$OH formation (as in eq 1), and (2) using EPR methods and aprotic solvents, our colleagues have demonstrated its production in similar particle systems.[15] Thus, in follow-up experiments, we incubated our particle systems in DMSO rather than saline and increased our HE concentration 30-fold. We then measured 2-OH-E$^+$ using HPLC with fluorescence detection, as we found that its detection by electrochemical detection was highly sensitive to pH fluctuations. As such, we were able to detect increased superoxide levels in particles containing an EPFR or transition metal. Specifically, levels for 2-OH-E$^+$ increased from 0.40 μM $\pm$ 0.02 for SiO_2 to 0.60 $\pm$ 0.03, 0.60 $\pm$ 0.05, and 0.56 $\pm$ 0.02 for suspensions of MCP230, DCB230, and CuO/SiO_2, respectively. Thus, it is reasonable to assume that superoxide was produced for our incubations in saline but that perhaps in aqueous solution it dismutated too rapidly for adequate detection.

Interaction of Particle-Derived ROS with Other Biological Mediators. To further examine the redox capability of EPFR-containing particle systems, ROS production associated with MCP230 and DCB230 suspensions alone, or in the presence of biological agents commonly found in epithelial lining fluid (ELF) was assessed.[28] Included among these cotreatments were AA, NAC (as a representative thiol), and NADPH. All cotreatments resulted in significantly lower levels of DHR fluorescence compared to those of MCP230 and DCB230 alone (Figure 3A,B).

To determine the effect of ELF constituents on the production of a specific ROS, MCP230- and DCB230-mediated $^{\bullet}$OH production was measured alone and in the presence of uric acid (UA), NADPH, AA, and NAC. In contrast to results obtained using the DHR fluorescence assay, the $^{\bullet}$OH assay revealed that cotreatment with all agents tested resulted in significant 1.5−48-fold higher levels of $^{\bullet}$OH compared to EPFR-containing PM alone (i.e., MCP230 or DCB230, Figure 3C and D).

To further confirm the interaction between ELF constituents and ROS/$^{\bullet}$OH production, similar experiments were conducted utilizing a particle system that lacks the EPFR (CuO/SiO_2). Again, DHR fluorescence was significantly reduced 14−22% by all cotreatments (Figure 4A). In contrast, CuO/SiO_2-associated hydroxyl radical production was increased by cotreatment with NAC and AA, although these increases did not achieve statistical significance ($p = 0.076$, Figure 4B).

A thiol-, NADPH- or ascorbate-induced increase in $^{\bullet}$OH production resulting from surface-associated EPFR was not entirely unexpected. Each of these agents is known to participate in free radical chemistry. For example, although ascorbate is generally considered an antioxidant, it can also act as a reductant of ferric ion (or Cu^{2+}) to facilitate Fenton chemistry. This property of this otherwise water-soluble antioxidant is referred to as ascorbate-driven Fenton chemistry.[29] Other reductants, such as uric acid, can also facilitate the reduction of ferric ion (or Cu^{2+}), so presumably could also promote the redox cycle of our copper oxide-containing particles.[30]

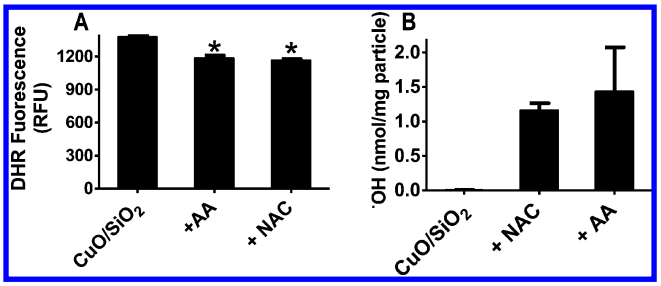

Figure 4. Effect of cotreatment with epithelial lining fluid constituents, including ascorbate (AA) and *N*-acetylcysteine (NAC), on CuO/SiO_2-mediated ROS production. Particles were suspended at 1 mg/mL in 0.9% saline (w/v) containing 0.02% Tween (v/v) and then incubated for 1 h together with 100 μM AA or 100 μM NAC. (A) ROS production was indicated by an increase in DHR fluorescence. (B) Hydroxyl radical production was measured by scavenging with 4-HBA, then measuring products using HPLC. * $p < 0.05$ compared to CuO/SiO_2 alone.

ELF typically contains a vast quantity of thiols and glutathione. Thiols also facilitate Fenton chemistry and promote superoxide production but through a more complex set of reactions.[31] First, the thiol anion reacts with Cu^{2+} to form a thiyl radical and Cu^+ (eq 2), which can in turn promote Fenton reactions and thus •OH production. However, the thiyl radical can also react with another molecule of thiol anion that, through a two-step reaction, results in thiol disulfide and superoxide (eqs 3 and 4). The dismutation of the superoxide to form hydrogen peroxide and its subsequent interaction with the metal oxide could ultimately produce another molecule of •OH. Thus, it is likely that similar chemistry occurred between the thiol, in this case *N*-acetylcysteine, and the metal oxide chemisorbed to the surface of the particle to promote the production of excess •OH.

$$RS:^- + Cu^{2+}(\text{or } Fe^{3+}) \rightarrow RS\cdot + Cu^{+}(\text{or } Fe^{+2+}) \quad (2)$$

$$RS\cdot + RS:^- \rightarrow RSSR\cdot^- \quad (3)$$

$$RSSR\cdot^- + O_2 \rightarrow RSSR + O_2^- \quad (4)$$

Inconsistencies between the DHR Fluorescence and Hydroxyl Radical Assay Data. Our data demonstrating opposing results for the coincubation experiments including ELF constituents, i.e., decreases in DHR fluorescence but increases in •OH formation in the presence of thiols or ascorbic acid (Figure 3), prompted our realization that the DHR assay may not precisely reflect a particle-mediated production of ROS. Several explanations for these findings are conceivable. First, the DHR may possibly form adducts with the EPFR to produce a fluorescent product. Second, and perhaps a more likely explanation is that the thiol or ascorbic acid indeed promotes hydroxyl radical production in a simple system of EPFRs. However, in the presence of DHR, these compounds may participate in the many side reactions that can occur upon DHR oxidation[26] so as to generate data that are difficult to interpret. Thus, we argue that more selective assays, such as the HPLC assays utilized here, may be more useful for mechanistic studies of EPFR-induced ROS production.

Maintenance of Particle-Associated ROS Production in Biological Matrices and Systems. Our final objective was to determine whether particle-associated ROS production was maintained in biological systems and whether this EPFR-mediated ROS production induced potentially cytotoxic responses. To answer this question, we began by incubating the particles with laryngeal epithelial cells. In these experiments,

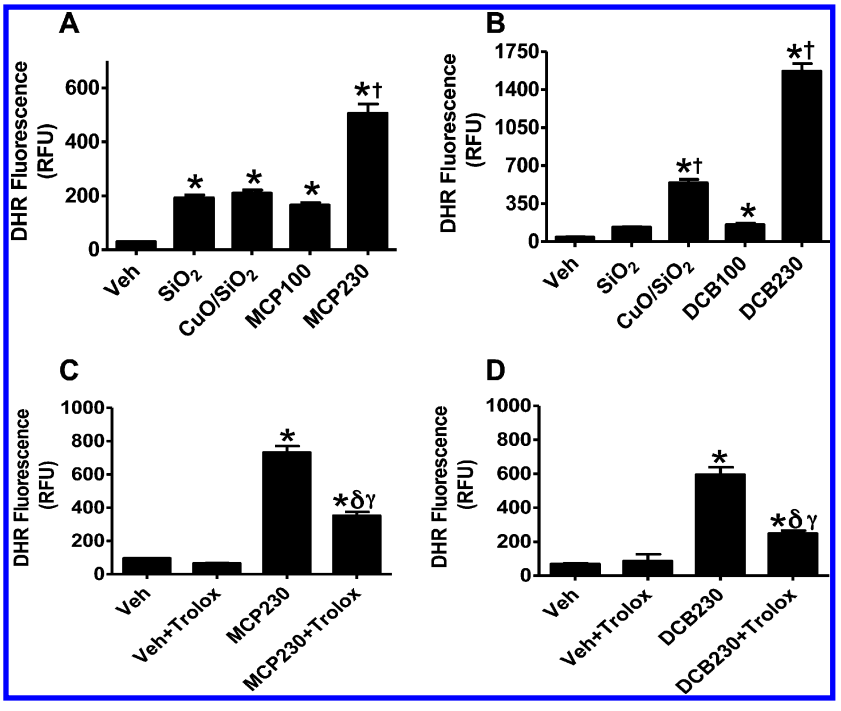

Figure 5. ROS production associated with EPFR in a human epithelial cell line and the effect of antioxidant cotreatment. HEp-2 cells were preloaded with 5 μM DHR and were treated with ±200 $\mu g/cm^2$ of the model particle systems suspended in medium containing 0.02% Tween 80 for 40 min. Cellular ROS production was assessed as DHR fluorescence, in the presence of (A,C) MCP-based or (B,D) DCB-containing particle systems, with (C,D) and without (A,B) cotreatment with the antioxidant Trolox. *$p < 0.05$ compared to cells treated with vehicle (veh). †, $p < 0.05$ compared to SiO_2. δ, $p < 0.05$ compared to MCP230 or DCB230. γ,$p < 0.05$ compared to veh + Trolox.

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incubation with most of the particle systems induced an increase in cellular DHR fluorescence (Figure 5A,B). However, incubation with MCP230 or DCB230 induced greater increases in fluorescence compared to that in the other particle systems, and these increases in ROS were inhibitable by cotreatment with antioxidant (Figure 6C,D). These data suggest that

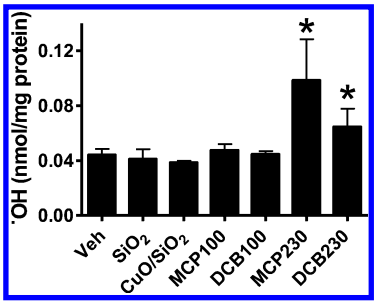

Figure 6. Hydroxyl radical production measured in Hep-2 cells treated with MCP- or DCB-containing particle systems. HEp-2 cells were incubated for 40 min with 200 $\mu g/cm^2$ of the model particle systems suspended in medium containing 0.02% Tween 80 and 0.05 mg/mL 4-HBA. Hydroxyl radical production was determined as the level of 3,4-dihydroxybenzoic acid detected using HPLC with electrochemical detection. *Indicates $p < 0.05$ compared to cells treated with vehicle only.

exposure to particles perturbs the cell environment in some way, so as to elicit at least a mild cytotoxic response, regardless of the type of particle. However, this response was exacerbated in the presence of an EPFR.

We then repeated the experiment, but this time, we measured •OH production. Compared to cells treated with vehicle only, significant increases in •OH production were detected for only those particle systems containing an EPFR (e.g., MCP230 or DCB230, Figure 6). These findings suggest that •OH production in cells is increased only by particles containing an EPFR–metal oxide system capable of promoting Fenton-related chemistry.

As a surrogate marker for cytotoxicity, we compared these findings to levels of ATP produced in treated cells. Note that preliminary experiments suggested that assaying for ATP was a sensitive method for detecting a particle-associated cytotoxic response. In these studies, ATP levels were significantly reduced for cells incubated with most of the particle systems, with the exception of SiO_2, but ATP was reduced at a lower dose of CuO/SiO_2 and MCP230 (Figure 7). Our findings of

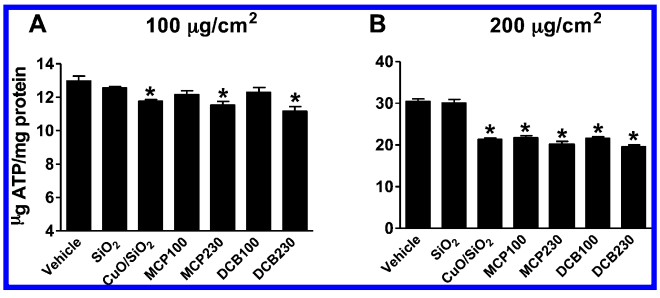

Figure 7. Effects of model PM exposure on levels of ATP in Hep-2 cells. Cells were treated with 100 (A) or 200 (B) $\mu g/cm^2$ particles for 24 h. After treatment, cellular ATP levels were determined using a luminescence assay, and concentrations were normalized to cell protein. *Denotes $p < 0.05$ compared to cells treated with vehicle only.

greater sensitivity of cells to MCP230 supported the hypothesis that EPFR-containing PM was more toxic. However, our finding of an increased toxicity of CuO/SiO_2 may suggest that although EPFR-mediated ROS production is important in determining toxic outcomes of certain PM exposures, other factors and mechanisms may contribute for some particle types. In these studies, we utilized the MCP100 and DCB100 particle systems as controls for PM lacking an EPFR and used CuO/SiO_2 as a control system for PM containing a redox-active transition metal but no EPFR, per se. Cu^{2+} is known to oxidize biomolecules, as it is utilized in standard methodologies for experimentally oxidizing LDL. Thus, it is not surprising that it, too, can reduce cell viability, presumably by oxidizing cell membrane lipids and/or proteins.

In order to further test the biological persistence of the particle-associated EPFR and its potential to produce ROS, EPFR levels and their half-lives in serum and BALF were measured and compared using EPR. As shown in Figure 8A and B, the DCB230-associated radical did not decay in saline but exhibited a half-life of 0.4 and 0.2 h in serum and BALF, respectively. However, MCP230 displayed significant decay in saline, with a half-life of 4.2 h. However, its half-life in BALF and serum was reduced to a proportionally smaller extent (i.e., to 3.5 h and 1.5 h, respectively). Thus, although both model EPFRs were maintained in the biological fluids tested, they exhibited differing behaviors compared to that of saline.

In other experiments, •OH was measured in saline compared to BALF, incubated together with MCP230. The experiment verified that MCP230 is capable of •OH generation in BALF, as approximately 0.07 nmol of •OH was detected per mg of MCP230 (Figure 8C). Of note, however, was that the levels of •OH produced in BALF were markedly lower than measurements taken in saline. Nevertheless, our ability to detect these radicals, as well as our ability to detect them in treated cells (Figure 6), confirms the ability of EPFR-containing particles to maintain some level of ROS production in a biological system or matrix.

Another caveat to this particular experiment worth noting is that the mean level of •OH production for incubations in saline (Figure 8C) was up to 10-fold greater than that measured in other experiments (Figures 1B, 2B, and 3C and D). This is because for these studies, we suspended particles using a brief 1 min pulse rather than a 30 min bath sonication. This was done to ensure that critical BALF antioxidants and other biological mediators were not depleted by the somewhat elevated temperature of the water bath prior to incubation with 4HBA. These observations prompted us to calculate the cycle length (i.e., the number of times the particle systems cycle through reductions and oxidations) of our particles systems suspended by pulse compared to bath type sonication. The length of the redox cycle was extrapolated from our measurements of spins/g, determined using EPR, and nmol •OH/mg particle, assessed by HPLC. Our findings suggest that for pulse sonication, both MCP230 and DCB230 redox cycle ~6 or 7 times (Table 1). However, for bath-type sonication, we detected cycle lengths that were significantly smaller (<1). Our findings of significantly higher levels of •OH determined for pulse sonication may suggest that maximal •OH production occurs within the first 30 min after suspension in solution and likely decreases thereafter due to EPFR decay. Thus, although these data support that our model EPFR are capable of forming a redox cycle, they may also suggest that in future experiments, more rapid suspension techniques are warranted.

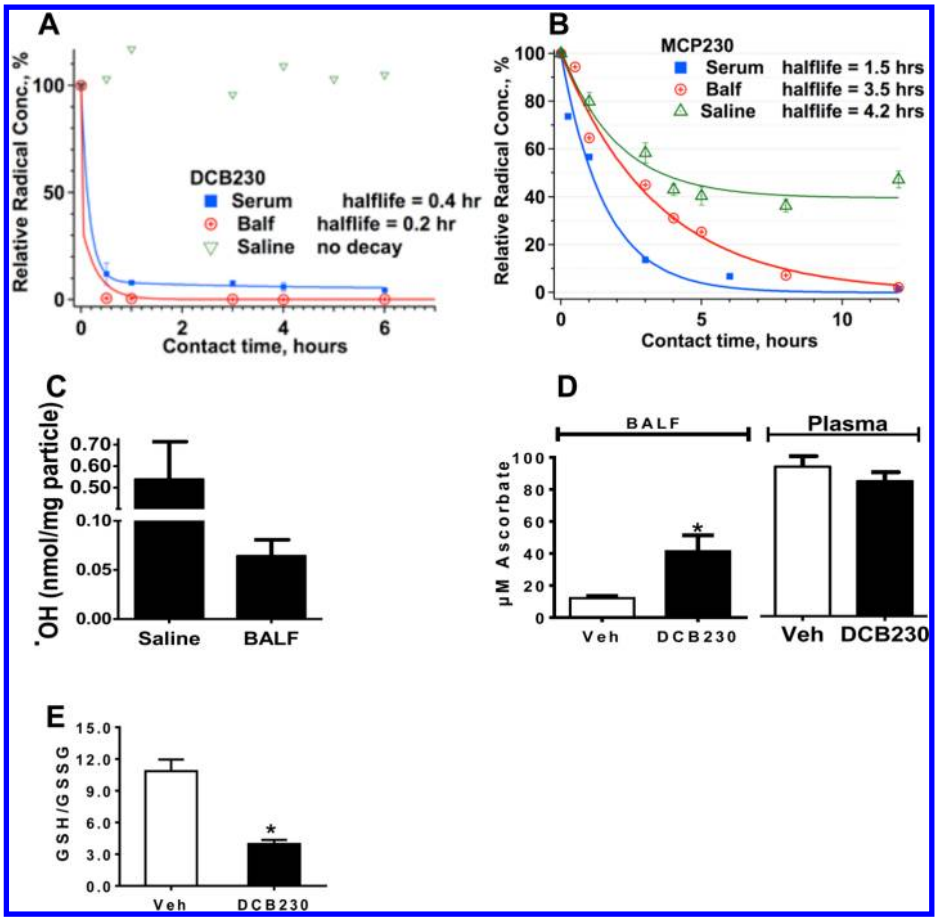

Figure 8. EPFR and EPFR-associated ROS production is maintained in biological environments. Using electron paramagnetic resonance, EPFR half-life was measured for DCB230 (A) or MCP230 (B) suspended in saline, bronchoalveolar lavage fluid (BALF), or serum of untreated adult mice. Calculated half-lives are shown. All decay data were fit to exponential function $y = y_o - A\exp(-kx)$ to obtain a best fit, where y is a percent drop in the radical concentration, and x is time. (C) Hydroxyl radical production was measured after a 1 h incubation of 1 mg/mL MCP230 in saline compared to BALF obtained from untreated adult mice. (D) Ascorbate levels were determined in plasma and BALF, and (E) the glutathione (GSH)/glutathione disulfide (GSSG) ratio was assessed in the BALF of neonatal rats following nose-only inhalation of DCB230 for 7 consecutive days. *Denotes $p < 0.05$ compared to the vehicle treatment.

Table 1. Length of the Redox Cycle for Suspensions of EPFR-Containing PM[a]

particle type	sonication method	•OH/EPFR (mean ± SEM)
MCP230	pulse	6.2 ± 4.5
	water bath	0.3 ± 0.1
DCB230	pulse	7.4 ± 11
	water bath	0.4 ± 0.1

[a]Using the spins/g determined by electron paramagnetic resonance (EPR) for each batch of particles produced and the level of hydroxyl radical produced determined by *in vitro* incubation in saline, we calculated the cycle length or the number of hydroxyl radicals (•OH) produced per environmentally persistent free radical (EPFR). Note that depending on the type of experiment, either pulse or bath type sonication was utilized, and the differences in cycle length for each are shown.

Finally, we hypothesized that if particles inhaled onto the surface of the lung redox-cycled to produce ROS, then antioxidant levels in that environment should be depleted. To test this hypothesis, neonatal rats were treated by inhalation with vehicle or EPFR-containing PM (e.g., DCB230), and both BALF and plasma were obtained. We chose to measure AA levels since it is an antioxidant known to be abundant in epithelial lining fluid.[28] Note that levels in the plasma were also measured, as we further hypothesized that a perturbation of AA in the plasma might suggest that the particles were capable of transit into the circulation. In brief, AA levels in plasma were comparable among the two treatment groups (Figure 8D), suggesting either that the particles remained sequestered within lung tissue or that particles escaping the lung did not maintain their ROS production. However, AA levels in BALF were significantly elevated in DCB230 treated rats compared to that in those treated with only vehicle (Figure 8D).

The increased level of ascorbate in BALF among EPFR-treated neonatal rats was contrary to our hypothesis of antioxidant depletion after particle treatment. However, we rationalized that these findings may reflect a compensatory response of the lung to the EPFR exposure, and literature reports support this assertion. First, Shannahan et al.[32] examined baseline levels of markers for cardiac and pulmonary disease among normotensive Wistar Kyoto (WKY), spontaneously hypertensive (SH), and spontaneously hypertensive heart failure (SHHF) rats. They reported that SHHF rats were associated with increased baseline levels of oxidative stress and inflammation. Moreover, SHHF rats were found to have significantly elevated levels of ascorbate in BALF and the heart but decreased ascorbate levels in the lung. In another relevant study,[33] exposure of WKY and SH rats to filtered air or residual

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oil fly ash resulted in increased levels of ascorbate, glutathione, and uric acid in BALF in both strains. These prior reports thus suggest that animals undergoing oxidative stress may exhibit a compensatory increase in AA and other antioxidant levels in lungs and BALF.

Thus, in an effort to explain our findings, we measured the ratio of reduced-to-oxidized glutathione (GSH/GSSG), a well-known marker for oxidative stress, in the BALF of exposed animals. Indeed, the GSH/GSSG ratio was dramatically reduced at 7 d of exposure (Figure 8E), suggesting that EPFR exposure induced an oxidative stress response in the BALF and lung. These findings thus strengthen our argument for an oxidative stress-induced, compensatory increase in ascorbate levels.

Environmental Health Implications of These Studies. The results of the studies presented here suggest that PM containing EPFRs are capable of redox cycling on the particle surface, resulting in sustained ROS production. This provides valuable insight into the potential role for free radicals in mechanisms of toxicity for combustion-generated PM, although particle size is also an important consideration.[34] For instance, based on the measurement of leukocytes and cytokine levels in BALF, Fahmy et al.[34] noted that fine PM containing EPFRs (MCP230) resulted in reduced cell viability but failed to induce pulmonary inflammation in chronically exposed neonatal rats. In contrast, Balakrishna et al.[14] noted that chronic exposure of neonatal rats to ultrafine PM containing EPFRs (MCP230), resulted in increased levels of lipid peroxidation. The study also demonstrated that ultrafine MCP230 demonstrated cytotoxicity. In another study, Balakrishna et al.[14] exposed neonatal rats to PM containing EPFRs (DCB230) and found significant pulmonary inflammation, including increases in airway hyperreactivity, increases in total leukocytes, as well as oxidative stress markers and various cytokines in BALF. Finally, in one of our other studies, adult rats exposed to DCB230 by intratracheal instillation exhibited elevated cytokine levels in BALF and oxidative stress markers in plasma.[35]

Repine et al.[36] also conducted animal experiments utilizing combustion-generated, free-radical-containing PM. The study exposed mice to PM that contained either high or low concentrations of free radicals for 6 h per day on 4 consecutive days. Results indicated that inhalation of PM containing the higher concentration of free radicals resulted in mitochondrial abnormalities, as well as induction of lung inflammation and oxidative stress.

Evidence in support for our findings of particle-associated $^{\bullet}$OH and for the environmental relevance of our model PM systems is provided by studies conducted by Alaghmand and Blough.[37] These studies examined $^{\bullet}$OH production associated with several standard reference materials (SRMs) that were purchased from the National Institute of Standards and Technology (NIST). The authors employed an HPLC-based fluorescence method to determine $^{\bullet}$OH.[37] Results indicated that although little or no $^{\bullet}$OH production was detected in the absence of electron donors, addition of NADPH resulted in substantial increases in $^{\bullet}$OH levels for several of the SRMs studied. This is consistent with increases we report here for cotreatment with AA, NAC, and NADPH. An additional area of consistency was that $^{\bullet}$OH production was attenuated in the presence of catalase.

In summary, these studies demonstrate that EPFR-containing PM redox cycle to produce the highly reactive $^{\bullet}$OH. This $^{\bullet}$OH production is maintained in biological milieu and may even be exacerbated in the presence of some ELF constituents. The EPFR-containing PM induces an oxidative stress in the lungs that promotes a compensatory increase in AA levels. These findings suggest that EPFR-mediated ROS production may indeed be maintained in the lung and further, may be a mechanism modulating toxic outcomes from PM exposures. However, our studies also highlight the important need for utilizing clearly defined assays for measuring ROS in studies elucidating mechanisms of PM toxicity.

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Notes

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ABBREVIATIONS

AA, ascorbic acid; BALF, bronchoalveolar lavage fluid; CAT, catalase; CuO/SiO_2, copper oxide silica; DCB100, dichlorobenzene physisorbed to silica; DCB230, dichlorobenzene chemisorbed to silica via CuO; DHE, dihydroethidium; DHBA, 3,4-dihydroxybenzoic acid; DHR, dihydrorhodamine 123; ELF, epithelial lining fluid; EPR, electron paramagnetic resonance; EPFR, environmentally persistent free radical; 4-HBA, 4-hydroxybenzoic acid; $^{\bullet}$OH, hydroxyl radical; MCP100, monochlorophenol physisorbed to silica; MCP230, monochlorophenol chemisorbed to silica via CuO; NAC, *N*-acetylcysteine; PM, particulate matter; ROS, reactive oxygen species; SiO_2, silica; SOD, superoxide dismutase; UFP, ultrafine particles; UA, urate

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