



In vitro and in vivo assessment of pulmonary risk associated with exposure to combustion generated fine particles

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ABSTRACT

Strong correlations exist between exposure to PM_{2.5} and adverse pulmonary effects. PM_{2.5} consists of fine ($\leq 2.5 \mu\text{m}$) and ultrafine ($\leq 0.1 \mu\text{m}$) particles with ultrafine particles accounting for >70% of the total particles. Environmentally persistent free radicals (EPFRs) have recently been identified in airborne PM_{2.5}. To determine the adverse pulmonary effects of EPFRs associated with exposure to elevated levels of PM_{2.5}, we engineered 2.5 μm surrogate EPFR-particle systems. We demonstrated that EPFRs generated greater oxidative stress in vitro, which was partly responsible for the enhanced cytotoxicity following exposure. In vivo studies using rats exposed to EPFRs containing particles demonstrated minimal adverse pulmonary effects. Additional studies revealed that fine particles failed to reach the alveolar region. Overall, our study implies qualitative differences between the health effects of PM size fractions.

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1. Introduction

Epidemiological studies demonstrate a clear correlation between exposure to particulate matter (PM) and adverse pulmonary effects such as asthma exacerbation (Delfino et al., 2004). Airborne PM is generated by a variety of sources including industrial processes and combustion of biomass and fossil fuels (Zheng et al., 2002). Recently, we reported the presence of environmentally persistent free radicals (EPFRs) associated with ambient PM_{2.5} samples collected from different locations across the United States (Dellinger et al., 2001). In combustion processes, high-temperatures initiate a cascade of chemical reactions which form phenoxy- and semiquinone-type radicals depending on the precursors present (Lomnicki et al., 2008a) that are stabilized and resistant to oxidation when associated with metal oxide containing particles and thus persist in the environment (i.e. EPFR). EPFRs are of significant interest due to their redox potential and ability to pro-

duce reactive oxygen species (ROS) in biological systems (Lomnicki et al., 2008b).

Although the adverse pulmonary effects of ambient PM have been extensively studied by epidemiologists, many of these studies were performed with a mixture of particles, collected from ambient air and were of various sizes and chemical compositions. Thus, the biological impact related to a specific chemical or particle size has remained an enigma. We have developed a surrogate model for EPFRs using 2-monochlorophenol (MCP) and particles having a median diameter of 2.4 μm . The process of EPFR synthesis is initiated by simple physisorption of the molecular precursors on the surface, followed by chemisorption at a metal oxide (e.g. Fe₂O₃ or CuO) surface site at high-temperatures (230 °C) like those encountered in the post-flame cool-zone of combustors where pollutants like dioxins are known to form. The adsorbate then reduces the metal oxide to form a EPFR (Lomnicki et al., 2008b).

We chose CuO as the metal oxide in our model system because it is present in relatively high concentrations in biomass such as woody wastes and debris as well as cigarette smoke (Wasson et al., 2005; Neuberger et al., 2009). 2-MCP was selected because it is typically produced from thermal treatment of wastes and is able to chemically react with CuO under combustion-like conditions. A sample of CuO/silica was exposed to 2-MCP at 230 °C which resulted in the formation of the persistent 2-MCP radical on the surface of the CuO/silica. CuO/silica and silica/MCP serve as control particles to understand the roles of CuO and MCP, in the biological responses following exposure. Our hypothesis was that an EPFR

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(e.g. silica/CuO/MCP), which undergoes redox cycling and produces ROS, is able to generate more oxidative stress and induce greater damage to human epithelial cells than the silica particles containing CuO or 2-MCP alone.

Excluding particle dose, time points, and end points for assessing toxicity; there are many other inherent limitations to *in vitro* systems. First, epithelial cells represent only a fraction of all of the cells present in the respiratory system. Second, epithelial cells lack many of the defensive capabilities of a lung *in vivo* including a blood supply, immune system, endogenous buffering system, and other biologically relevant factors. Third, the ability of the surrogate pollutant/particle systems to reach the alveolar region of the lungs remains controversial (Ferin et al., 1992; Kreyling et al., 2006). Thus, we further hypothesized that fine combustion generated particle surrogates ($\leq 2.5 \mu\text{m}$ in spherical diameter) would produce minimal adverse effects on pulmonary function.

To investigate the intrinsic ability of fine particles containing EPFR to decrease cell viability and generate oxidative stress in respiratory epithelial cells, an *in vitro* model was employed. Human laryngeal epithelial cells (HEp-2) were chosen, since they are used in many pulmonary toxicological assays (Rudolf et al., 2001; Kvolik et al., 2005) and represent target cells which are usually subjected to significant amounts of airborne particles. For the evaluation of the *in vivo* effects of an EPFR, we employed neonatal rats, which unlike adults, have immature lungs characterized by sacculus structure, thick alveolar walls, inadequate pulmonary capillary network and limited gas-exchange capabilities and are very sensitive to air pollutants (Hsia et al., 2004). The development of airway inflammation and oxidative stress in neonatal pulmonary tissue as result of exposure to air pollutants such as $\text{PM}_{2.5}$ can lead to the induction of airway remodeling and alter pulmonary function (Jeffery, 2001; Henson et al., 2006). Accordingly, we have assessed the impact of EPFRs in both an *in vitro* culture system and an *in vivo* system following inhalation exposure in neonatal rats.

2. Materials and methods

2.1. Reagents

2,7-Dichlorofluorescein-diacetate (H_2DCFDA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), hydrogen peroxide, oxidized glutathione (GSSG), reduced glutathione (GSH), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), methacholine (MeCh), SOD determination kit, glutathione assay kit all were obtained from Sigma (St Louis, MO). Resveratrol was purchased from Axxora (San Diego, CA) and Alamar Blue was obtained from Invitrogen (Carlsbad, USA). All organic solvents were of Fisher optima grade (Fisher Scientific, Hampton, NH).

2.2. Synthesis of surrogate combustion generated coarse particles

Silica/CuO was prepared by using the method of incipient wetness followed by calcination. Briefly, the silica gel powder (Sigma-Aldrich, grade 923, 100–200 mesh size) was introduced into a 0.1 M solution of the copper nitrate (Sigma-Aldrich, copper (II) nitrate hemipentahydrate, 99%) in the amount for incipient wetness to occur. The samples were allowed to gel for 24 h at room temperature and dried at 120°C for 12 h before calcination in air for 5 h at 450°C . The resulting powder was ground and sieved to a particle diameter of less than $6 \mu\text{m}$.

Silica/MCP was generated by physisorption of 2-monochlorophenol (2-MCP) on the surface of silica particles at 50°C while silica/CuO/MCP was generated at 230°C by chemisorption of 2-MCP on CuO through the hydroxy-substituent of 2-MCP which formed primarily a 2-chlorophenoxy radical and reduced Cu^{2+} to Cu^{+1} at the site of chemisorption (Lomnicki et al., 2008b). To prepare silica/MCP or silica/CuO/MCP particles, silica or silica/CuO were exposed to the vapors of 2-MCP using a custom made vacuum exposure system that consisted of a vacuum gauge, dosing vial port, equilibration chamber and 2 reactors. Each of the outlets from the equilibration chamber was equipped with a teflon vacuum valve to control the adsorbate flow and vacuum. The system contained two bulb-shaped Pyrex reactors and a suprasil quartz electron paramagnetic resonance (EPR) tube as a side arm for EPR spectral measurements. The bottoms of the reactors, containing the particles to be dosed, were placed in a small tube furnace in a vertical orientation. This furnace controlled the temperature of sample pre-treatment (surface cleaning and adsorbed water removal) and adsorption conditions. The transfer lines were maintained at 80°C to prevent condensation of the vapor.

Each sample was *in situ* re-oxidized in air at 450°C and evacuated to leave the surface clean and ready for the adsorption. The reactor containing the particles was evacuated to 10^{-1} Torr. Vapors of the adsorbates were introduced into the equilibration chamber at 10 Torr at the desired temperature of 50°C or 230°C for 5 min to prepare silica/MCP and silica/CuO/MCP respectively. The high-temperature (i.e. 230°C) mimics temperatures observed in the post-flame cool-zone of combustors. The port and dosing tube were evacuated for 1 h at the dosing temperature and 10^{-2} Torr pressure to remove any residual physisorbed dosant. The reactor was then sealed under vacuum with a vacuum tight PFE stop-cock and allowed to cool to room temperature. Surface (silica) induced stabilization of SQ-type radicals prolong their environmental and biological lifetimes which appear to be infinite under vacuum but days or hours in aerobic or aqueous conditions, respectively (Lomnicki et al., 2008b). All EPR measurements were performed 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. The size of the particles was confirmed prior to experiments using flow cytometry as previously described (Balakrishna et al., 2009).

2.3. Cell culture and treatment

Human laryngeal epithelial cells (HEp-2 cells) were purchased from ATCC (Manassas, VA) and were cultured in 75 cm^2 flask at the density of 2×10^4 cell/ cm^2 in Dulbecco's Modified Eagle's Medium-Reduced Serum (DMEM-RS) supplemented with 2% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO_2 . At 90% confluency, cells were harvested using 0.25% trypsin and were sub-cultured into 75 cm^2 flask, 6-well plates or 96 well plates. Cells were then allowed to recover for 1–2 days prior to treatment. Particles were suspended in cell culture medium by sonication for 4 min using 50% amplitude and a cycle of 30 s on and 30 s off (Sonics and Materials Inc., CT, USA). All experiments were performed using HEp-2 cells at passage 10–20 and were replicated with at least two independent cell passages.

2.4. Cell viability assay

The cytotoxic effect of particles on HEp-2 cells was determined by the Alamar Blue assay as previously described (Baudouin et al., 2007). Briefly, HEp-2 cells were cultured in 96 well plates and then, incubated with a suspension of particles (100–400 $\mu\text{g}/\text{ml}$) for 5 h to establish a dose response curve. Cell viability was estimated by measuring the emitted fluorescence of the reduced Alamar Blue using a plate reader (ex/em: 530/590) and was normalized to medium only treated cells (100% viability) and 0.1% saponin (0% viability). To investigate the influence of resveratrol on the cytotoxic effect of the particles, cells were pre-treated for 1 h and co-treated with 100 μM of resveratrol. Data were compared to medium containing 100 μM resveratrol (100% viability) and 0.1% saponin (0% viability).

2.5. Measurement of cellular reactive oxygen species (ROS)

The production of reactive oxygen species in HEp-2 cells was measured by pre-loading the cells with 10 μM 2,7-dichlorofluorescein diacetate (H_2DCFDA) at 37°C for 40 min in the dark. The cells were then washed and incubated with particle suspension (400 $\mu\text{g}/\text{ml}$) for 30 min at 37°C . After the treatment, cells were washed, scraped, lysed, sonicated for 15 s using 50% amplitude and centrifuged at $12,000 \times g$ for 15 min at 4°C . The intensity of DCF fluorescence in the cell lysate was measured using a plate reader (ex/em: 485/530) and was normalized to protein content measured by BCA protein assay (Thermo Fisher Scientific Inc., Waltham, MA).

2.6. Antioxidant enzyme activity

After treating the cells for 4 h with particle suspension, cells were washed with PBS, scraped, lysed, sonicated for 15 s on ice and centrifuged at $12,000 \times g$ for 15 min at 4°C . The protein concentration of cell lysates was measured by BCA assay method and data were compared to a standard curve.

The activities of different antioxidant enzymes were then measured in the cell lysates:

The activity of superoxide dismutase (SOD) was measured using an SOD determination kit (Sigma, MO). Briefly, 15 μg protein of cell lysates were incubated with xanthine oxidase enzyme and tetrazolium salt for 20 min at 37°C . The absorbance of the formazan salt resulting from the oxidation of tetrazolium salt was detected at 450 nm. The activity of SOD, expressed as % inhibition of the formation of formazan, was then calculated by using an equation provided by the manufacturer.

The activity of catalase enzyme was measured as previously described (Aebi, 1984). An appropriate volume of cell lysate containing 40–50 μg protein was mixed with 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H_2O_2 in 1 ml quartz cuvette. The decrease in absorbance of H_2O_2 was followed at 240 nm for 4 min. Catalase activity was calculated from the slope of the H_2O_2 absorbance curve and normalized to protein concentration.

The activity of glutathione reductase (GR) was directly measured as previously described (Guthenberg et al., 1985) by mixing 40–50 μg protein of cell lysate with 1 ml of 0.1 M phosphate buffer supplied with 2 mM EDTA containing 20 mM NADPH and 20 mM GSSG. The activity of GR was calculated from the slope of NADPH absorbance curve and was normalized to protein content.

The activity of GPx was measured using glutathione peroxidase cellular activity assay kit (Sigma, MO). Using 1 ml quartz cuvette, 40–50 µg protein of the cell lysates. The cell lysates were mixed with tert-butyl hydroperoxide (30 mM), reduced glutathione (2 mM), GR (0.5 unit/ml) and NADPH (0.25 mM) in 50 mM Tris-HCl (pH 8) at 25 °C. The decrease in NADPH absorbance was followed for 3 min at 340 nm. The activity of GPx was calculated from the slope of NADPH absorbance curve and was normalized to protein content.

2.7. Measurement of intracellular glutathione

HEp-2 cells grown to 85% confluence were exposed to surrogate pollutant/particle systems (400 µg/ml) for 4 h. After the treatment, the amount of total and oxidized glutathione was measured by the recycling method (Rahman et al., 2007). Particle-exposed cells were washed, scraped and lysed using 0.1 M potassium phosphate buffer supplemented with 5 mM EDTA, 0.1% triton X-100 and 0.6% sulfosalicylic acid. Total glutathione was measured by reducing oxidized glutathione content using GR enzyme (3 unit/ml) and NADPH (0.8 mM). The assay is based on the chemical reaction between GSH and DTNB to form TNB. The change in TNB absorbance was measured at 412 nm using a plate reader. To measure the amount of GSSG, 100 µl of cell lysate was incubated with 2-vinyl pyridine which covalently reacts with GSH but not GSSG, then treated the cell lysate with GR, NADPH and DTNB. Data were compared to GSH and GSSG standard curves and were normalized to protein content and the ratio of GSSG to total glutathione was calculated.

2.8. Detection of 8-isoprostane as a biomarker of lipid peroxidation

The supernatant of cells exposed to surrogate pollutant/particle systems for 4 h was collected, centrifuged at 12,000 × g for 10 min to remove particles and cell debris and was used immediately to measure 8-isoprostane levels. 8-Isoprostane, a biomarker of lipid peroxidation, was measured by a competitive enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI) using 50 µl cell supernatant. The concentration of 8-isoprostane in samples were calculated from an established standard curve.

2.9. In vivo experimental model

Neonatal Brown Norway rats were purchased from Harlan (Indianapolis, IN) and were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Animals received food and water ad libitum and all procedures were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

2.10. Animal exposure

At 7 days of age, rats were randomly divided into three groups (6 animals per group) and were exposed to vehicle, silica/CuO or silica/CuO/MCP using a nose-only exposure system (InExpose, Scireq, Montreal, Canada). Particles were suspended in saline containing 0.02% tween 80 by sonication for 4 min using 50% amplitude and oscillating cycles of sonication and pause of 30 second each (Sonic and Materials Inc., CT, USA) to form a particle suspension (0.2 mg/ml). The particle suspension was aerosolized at a constant rate of 0.5 ml/min and diluted with dry room air using an external pump that delivers 5 l/min into 12 nose-only exposure channels. Each animal was held in a soft restrainer that was connected to one channel and was allowed to inhale the aerosol through their nose only. Rats were exposed to aerosol for 20 min/day for 7 successive days.

In order to identify the exact amount of particles delivered to the neonatal rats, a gravimetric sampling method was employed. The weight of the particles collected was determined by weight the substrate (glass fiber filters; 0.7 µm pore size; Millipore, MA) both before and after sampling. All substrates were dried at 30 °C and conditioned (i.e. placed in an individual, labeled, bin and equilibrated in the weighing environment) overnight before each weighing. Substrates exposed to vehicle under the same experimental conditions were used as blanks to correct for weight changes due to delivery vehicle. Consistently, the amount of particle deposited on the membrane was approximately 50 µg; and this value did not vary significantly between the different particles. Thus, every neonatal Brown Norway rat was exposed to surrogate pollutant/particle systems equivalent to (2 mg/kg); consistently used in other animal studies evaluating the pulmonary responses to PM (Antonini et al., 2002; Roberts et al., 2003).

2.11. Identification of particle deposition profile in the lung

In order to determine the deposition pattern of fine and ultrafine particles in the lungs of neonatal rats following aerosol delivery, we exposed rats to a suspension of ultrafine (0.19 µm, dragon green) and fine (1.59 µm, flash red) fluorescent spheres (Bangs laboratories Inc., IN). Ten min post-exposure, rats were euthanized and the lungs were inflated by gentle infusion with 1 ml of 30% Tissue-Tek OCT compound (Sakura, CA) in PBS. Lungs were then isolated, embedded in 100% Tissue-Tek OCT compound and snap frozen on a mixture of ethanol and dry ice. Lung sections

(25 µm) from apical, middle and lateral regions of the lungs were obtained. Fluorescent spheres were visualized using a deconvolution system which consists of a LEICA DMRXA microscope, and SLIDEBOOK software (Meyer Instruments, Houston, TX).

2.12. Assessment of pulmonary function

Twenty-four hours after the last exposure, lung resistance to increasing doses of methacholine (MeCh, Sigma; 0, 12.5, and 25 mg/ml in saline) was assessed using the forced oscillation technique (FlexiVent, Scireq, Montreal, Canada) as previously described (Becnel et al., 2005). Rats were anesthetized by intraperitoneal injection of ketamine/xylazine (70/5 mg/kg), tracheostomized, and a polyethylene cannula was inserted into the trachea. Rats were mechanically ventilated using a computer-controlled ventilator that delivers a tidal volume of 10 ml/kg at frequency of 120 breath/min. A positive end expiratory pressure (PEEP) of (3 cm H₂O) was established and airway resistance data was collected. Baseline airway resistance (MeCh, 0 mg/ml) was subtracted from pulmonary tissue resistance to calculate the normalized resistance which was plotted as a function of MeCh dose. A cohort of rats was exposed to 1 mg/kg of lipopolysaccharide (LPS; *E. coli* 055:B5, Sigma, MO) 24 h prior to the pulmonary function assessment and served as a positive control for pulmonary dysfunction and inflammation.

2.13. Measurement of lung inflammatory response

Bronchoalveolar lavage fluid (BALF) was isolated by inserting and recollecting 1 ml of PBS containing 2% heat-inactivated FBS in the lung. Total BALF cellularity was determined using a hemocytometer. Cytospin slides were fixed and stained using the Diff-Quick kit (IMEB, Chicago, IL), and differential cell counts by two unbiased observers were obtained by counting 200–300 cells per cytospin preparation. The protein concentration of BALF was measured by the BCA assay method.

2.14. Measurement of oxidative stress biomarker in pulmonary tissue

After BALF isolation, lungs were immediately isolated, flash frozen in liquid nitrogen and stored at –80 °C until use. Lung lysates from vehicle and particle treated lungs were prepared by homogenization in 0.1 M phosphate buffer (pH 7.4) supplemented with protease inhibitor cocktail (Sigma, MO) and sonicated on ice for 15–20 s. Samples were centrifuged at 12,000 × g for 15 min at 4 °C; supernatant was then removed and stored in –80 °C to preserve for future analysis. Protein concentrations in the supernatant were assessed using BCA assay. Appropriate volume of lung homogenates containing 15–50 µg protein were used to measure the activity of SOD, catalase, GR and GPx in the lung lysate as previously described, data were then normalized to protein concentration. The ratio of oxidized to total glutathione was estimated by mixing an equal volume of lung lysate with 0.1 M potassium phosphate buffer supplied with 1.2% sulfosalicylic acid to inhibit γ-glutamyl transferase (γ-GT) to prevent the loss of glutathione, then, the recycling method was employed to measure glutathione (Rahman et al., 2007).

2.15. Statistics

All data were reported as mean ± SEM and analyzed using Prism (GraphPad Software Inc., Version 5.0.0). One-way ANOVA followed by Bonferroni or Tukey's post-test was used to test for differences among the groups. Two-way ANOVA was conducted for the cytotoxicity test. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Silica/CuO/MCP uniquely generates free radical signals

EPR spectra of chemical compounds which have a paramagnetic center depend on the interaction between the unpaired spin and the environment such as the external magnetic field, other unpaired spin and the nuclear spin. EPR spectrum is described numerically as g -values, which is usually close to 2 (Banerjee, 2007). An EPR spectrum of the surrogate pollutant/particle systems demonstrated a distinct radical signal with a g -value of 2.003 and 2.006 for silica/CuO/MCP indicating the presence of radicals on the surface of these particles (Fig. 1). Deconvolution of this radical signal indicates that the dominant form of the radical present is chemisorbed *o*-chlorophenoxy species similar to that observed in collected PM samples (Lomnicki et al., 2008b). In contrast, radical signals were not observed with either silica/MCP or silica/CuO. To confirm the size of the particles prior to experiments, a size distribution curve was generated. The analyses of the size dis-

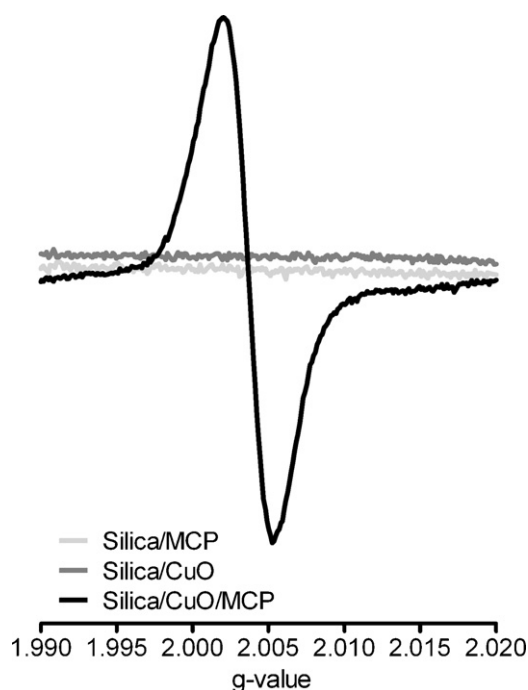


Fig. 1. EPR spectra of pollutant particle systems: silica/MCP, silica/CuO and silica/CuO/MCP.

tribution demonstrate that the diameter of the majority of the particles (>85%) was in the range of (1–3 μm) with a median size of 2.4 μm .

3.2. Cytotoxic effect of combustion generated pollutant/particle systems on human laryngeal epithelial cells

The presence of a radical signal with silica/CuO/MCP suggested that this surrogate pollutant/particle system maybe toxic to airway epithelial cells. To assess the toxicity of these particles, HEP-2 cells were treated with surrogate combustion pollutant/particle systems at different concentrations (100, 200, and 400 $\mu\text{g}/\text{ml}$) and viability after 5 h of exposure was determined. Cell viability decreased in a dose-dependent manner following exposure to each of the different surrogate particles (Fig. 2A). Silica/MCP was relatively non-toxic at the 100 $\mu\text{g}/\text{ml}$ exposure dose; however it significantly decreased cell viability by 14% and 20% when cells were incubated with 200 and 400 $\mu\text{g}/\text{ml}$, respectively. Both silica/CuO and silica/CuO/MCP induced significantly greater reductions in cell viability than control non-exposed cells at all doses. In addition, silica/CuO/MCP decreased cell viability substantially more than silica/MCP or silica/CuO at doses of 200 and 400 $\mu\text{g}/\text{ml}$. The cytotoxic effect associated with silica/CuO/MCP was partially reversed by treatment with the antioxidant resveratrol (Fig. 2B). Cells co-treated with silica/CuO/MCP and resveratrol exhibited significantly reduced viability (40%) when compared to control non-exposed cells but were not different from silica/MCP or silica/CuO treated cells. Resveratrol significantly increased the viability of silica/CuO/MCP treated cells suggesting that oxidative stress, at least in part mediated the cytotoxic effect of silica/CuO/MCP. Surprisingly, resveratrol did not alter the cytotoxic effect of either silica/MCP or silica/CuO indicating that these particles decrease cell viability through different mechanisms. The cytotoxic effect associated with exposure to silica/CuO/MCP (400 $\mu\text{g}/\text{ml}$) agrees with previously published data demonstrating the cytotoxicity of other airborne fine particles (Choi et al., 2004). Thus, the concentration of (400 $\mu\text{g}/\text{ml}$) was used in all subsequent in vitro experiments.

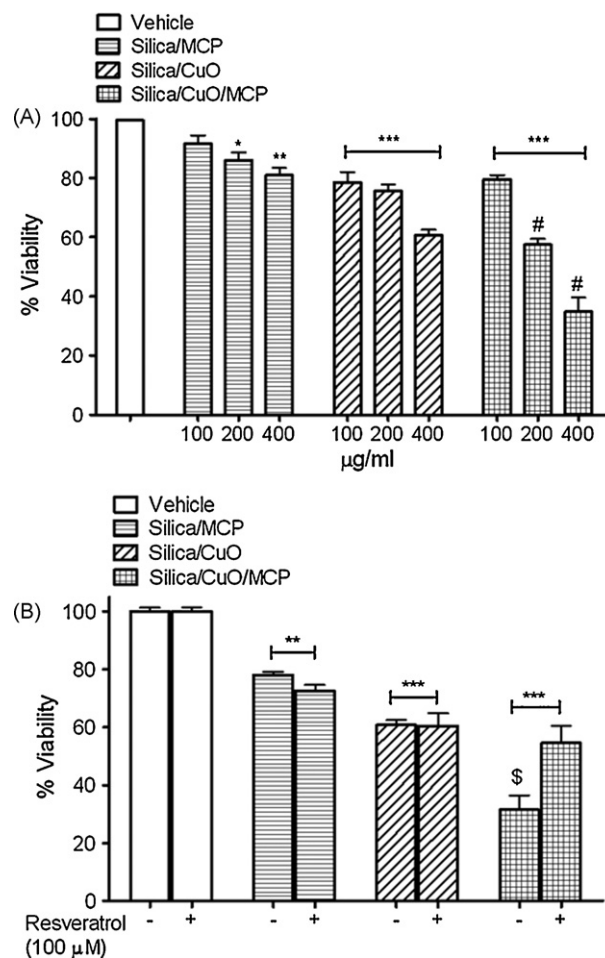


Fig. 2. Pollutant particle systems reduce human epithelial cell viability. HEP-2 cells were incubated with silica/MCP, silica/CuO and silica/CuO/MCP prior to assessment of cell viability. (A) Dose-dependent decrease in the cell viability associated with the exposure to increasing dose of pollutant/particle systems. (B) Resveratrol alters the cytotoxic effect of silica/CuO/MCP in epithelial cells. Cells were treated with particles (400 $\mu\text{g}/\text{ml}$) with or without 100 μM resveratrol. Data were normalized to cell viability of medium-only or medium containing 100 μM resveratrol treated cells (100% viable) and saponin treated cells (0% viable). Results are expressed as mean \pm SE of three replicates ($n=4$). (A) Two-way ANOVA, and (B) * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs vehicle, # $p < 0.01$ vs equivalent dose of silica/CuO or silica/MCP, \$ $p < 0.05$ vs all other groups.

3.3. Pollutant/particle systems generate reactive oxygen species (ROS) in vitro

The presence of a radical signal with silica/CuO/MCP suggested that this surrogate pollutant/particle system was able to redox cycle and that it was capable of inducing oxidative stress in a cellular system. To investigate this possibility, we exposed epithelial cells to these surrogate particle systems and measured their ability to generate ROS as detected by DCF fluorescence in cell lysates (Fig. 3). Exposure of epithelial cells to silica/CuO and silica/CuO/MCP resulted in significant increases in DCF fluorescence (240% and 280%, respectively), when compared to control non-exposed cells. The levels of DCF fluorescence in the lysates of silica/MCP treated cells, on the other hand, were similar to controls.

3.4. Pollutant/particle systems alter the activity of antioxidant enzymes and the level of oxidized glutathione in cultured epithelial cells

When the production of ROS exceeds the ability of the cell to neutralize the effects of the radicals, accumulation of pro-oxidants occurs in the cell leading to a state of oxidative stress (Gilmour et al.,

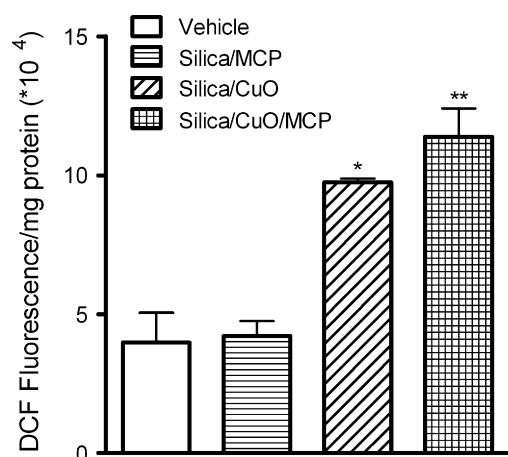


Fig. 3. ROS generated in epithelial exposed to pollutant particle systems. Cells were pre-loaded with H₂DCFDA for 40 min and then treated with silica/MCP, silica/CuO, silica/CuO/MCP (400 µg/ml) or vehicle. The intensity of DCF fluorescence was measured in cell lysates and normalized to protein concentration. Results are expressed as mean ± SE of two replicates (n = 4). *p < 0.05, **p < 0.01 vs vehicle.

2006). As the level of oxidative stress in the cell increases, different biological outcomes such as change in the activity of antioxidant enzymes and the depletion of glutathione occur (Li et al., 2003). To investigate the ability of surrogate combustion particle systems to alter the activity of cellular antioxidants, epithelial cells were exposed to each of the particles and the activity of antioxidant enzymes was assessed.

There was no difference in the activity of SOD in any of the exposed cell populations. Epithelial cells exposed to silica/CuO/MCP showed significant inhibition (27%) of catalase and GR (61%) activity as compared to control non-exposed cells (Table 1). Neither silica/MCP nor silica/CuO affected catalase or GR activities. The activity of GPx was significantly increased in silica/CuO and silica/CuO/MCP exposed cells (50% and 64%; respectively) as compared to control non-exposed cells. Since silica/MCP was not able to produce ROS in HEP-2 cells (Fig. 3), silica/MCP exposed cells were not expected to, nor did they show changes in the levels of antioxidant enzyme activity compared to non-exposed cells (Table 1).

The ratio of oxidized to total glutathione increased upon exposure to silica/CuO/MCP by 38%, but not upon exposure to silica/MCP or silica/CuO (Fig. 4). The oxidation of GSH indicates the inability of epithelial cells to scavenge ROS generated by silica/CuO/MCP and the development of oxidative stress in these cells.

3.5. Silica/CuO/MCP increased 8-isoprostane production in HEP-2 cells

Isoprostanes are produced by the non-enzymatic random oxidation of cellular phospholipids by oxygen radicals and are considered ideal markers of oxidative stress. Analysis of culture supernatant from cells exposed to combustion generated pollutant/particle systems indicated increased levels of 8-isoprostane associated with

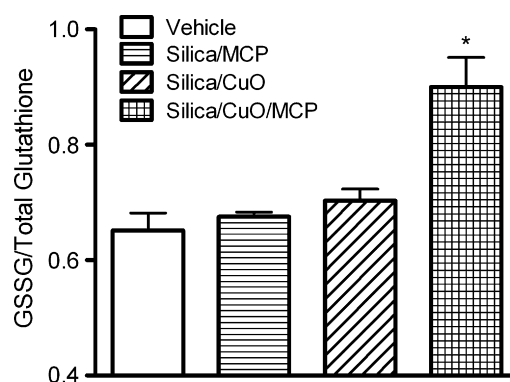


Fig. 4. Ratio of oxidized to total glutathione in epithelial cells exposed to pollutant particle systems. A significant increase in the ratio of oxidized to total glutathione was observed in HEP-2 cells treated with silica/CuO/MCP. Ratio is expressed as mean ± SE of 2 replicates (n = 3). *p < 0.05 vs vehicle.

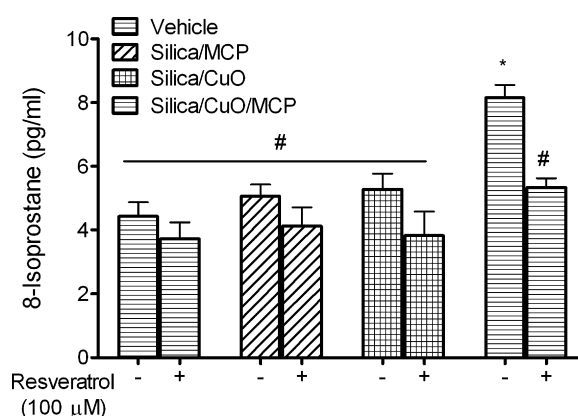


Fig. 5. Levels of 8-isoprostane in the supernatant of cells exposed to pollutant particles systems. Cells were exposed to vehicle, silica/MCP, silica/CuO, and silica/CuO/MCP with or without 100 µM resveratrol. Results are presented as mean ± SE of two replicates (n = 3). *p < 0.05 vs vehicle, #p < 0.05 vs silica/CuO/MCP.

the exposure to silica/CuO/MCP (240%) compared to vehicle treated cells (Fig. 5). In contrast to silica/CuO/MCP neither silica/MCP nor silica/CuO altered the level of 8-isoprostanes. Resveratrol significantly reduced the production of 8-isoprostane in silica/CuO/MCP exposed cells.

3.6. Deposition profile of fine and ultrafine fluorescent microspheres in the lungs of neonatal rats

Fluorescent fine and ultrafine spheres were similarly detected in apical, middle and lateral region of the lungs demonstrating that our nose-only exposure system provides relevant and reliable exposures. The site of deposition of the particles varied depending on the size of the spheres. Fine spheres (1.6 µm) were mostly localized in the terminal bronchioles and alveolar duct, while ultrafine

Table 1

Cellular antioxidant enzyme activities following exposure to pollutant particle systems (400 µg/ml). The activity of superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase were measured and normalized to protein concentration. Data are expressed as mean ± SE of two replicates (n = 3).

Enzyme activity	Vehicle	Silica/MCP	Silica/CuO	Silica/CuO/MCP
Total SOD (% inhibition rate)	85.10 ± 0.2357	85.53 ± 0.4703	87.46 ± 1.0010	86.74 ± 1.4210
Catalase (unit/mg protein)	9.881 ± 0.6408	9.392 ± 0.2546	9.352 ± 0.4626	7.153 ± 0.4302* [#]
Glutathione reductase (unit/mg protein)	0.0214 ± 0.0033	0.0230 ± 0.0010	0.0217 ± 0.0007	0.0347 ± 0.0053*
Glutathione peroxidase (unit/mg protein)	0.0140 ± 0.0008	0.0135 ± 0.0004	0.0213 ± 0.0007**	0.0230 ± 0.0018**

* One-way ANOVA: p < 0.05.

** One-way ANOVA: p < 0.01 vs vehicle.

One-way ANOVA: p < 0.05 vs silica/CuO.

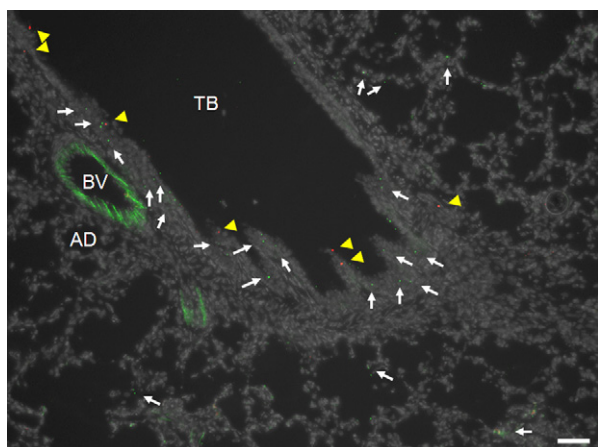


Fig. 6. Distribution of fluorescent microspheres in the lung. Rats were exposed to an aerosolized suspension of fluorescent microspheres. White arrows demonstrate the penetration of ultrafine spheres (0.19 μm , green) into the alveoli while yellow arrow heads display the deposition of fine spheres (1.59 μm , red) in the terminal bronchioles (TB) and alveolar ducts (AD). Scale bar = 50 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(0.19 μm) spheres mostly localized in the alveoli (Fig. 6). This particle deposition profile is consistent with the deposition profile of airborne particles in human lung after normal exposure to ambient air (Stuart, 1984). This suggests that ultrafine particles may elicit greater adverse pulmonary effects than fine particles.

3.7. Pollutant/particle systems failed to alter the redox balance in the lungs of exposed rats

To investigate the ability of the particles to generate oxidative stress *in vivo*, neonatal rats were exposed to the surrogate pollutant/particle systems via a nose-only inhalation system for 20 min/day for a period of 7 days. Pulmonary tissue was harvested and the activities of representative antioxidant enzymes were evaluated in lung homogenates (Table 2). Silica/MCP was excluded from this assay since they failed to generate ROS or induce oxidative stress *in vitro*.

A small but significant increase in the activity of SOD was observed in lung homogenates from rats exposed to silica/CuO and silica/CuO/MCP as compared to vehicle. The activity of catalase was significantly decreased in lungs exposed to silica/CuO/MCP compared to vehicle or silica/CuO by 41% and 31%; respectively. This data solidify the possible role of the phenoxyl ion associated with silica/CuO/MCP in the generation of H_2O_2 in lungs of exposed animals. No significant differences were observed in the activity of either GR or GPx among any of the groups. To study the ability of these particles to deplete GSH *in vivo*, the concentration of GSSG and GSH was measured in lung homogenates and the ratio of oxidized to total glutathione was calculated (Fig. 7). Neither exposure

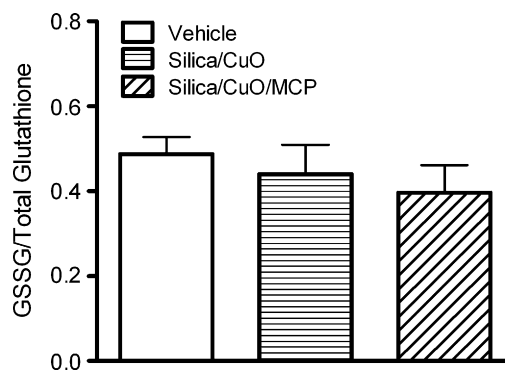


Fig. 7. Ratio of cellular oxidized to total glutathione in lung tissue of rats exposed to pollutant particle systems. Rats were exposed to vehicle or (0.2 mg/ml) of silica/CuO and silica/CuO/MCP for 20 min/day for 7 successive days. The cellular GSH and GSSG were measured and normalized to protein concentration. Ratio is expressed as mean \pm SE of two replicates ($n = 6$).

to silica/CuO nor silica/CuO/MCP was able to alter the *in vivo* glutathione ratio suggesting the inability of those particles to induce oxidative stress in lungs of exposed neonatal rats.

3.8. Neonatal exposure to particles failed to induce pulmonary inflammation

To determine if inhalation resulted in alveolar inflammation in neonatal rats, bronchoalveolar lavage fluid (BALF) was isolated. Total leukocytes in the BALF were counted and differentiated using morphological criteria by two unbiased investigators. The total number of cells recovered from the BALF of silica/CuO and silica/CuO/MCP exposed rats ($6.56 \times 10^5 \pm 0.52$ and $6.27 \times 10^5 \pm 1.45$; respectively) was slightly greater than vehicle only treated rats ($4.96 \times 10^5 \pm 0.78$). No significant differences in the percentage of macrophages, lymphocytes, neutrophils or eosinophils were observed among any of the groups (Fig. 8). In addition, no significant difference in the amount of protein in the BALF was observed between the lungs of rats exposed to silica/CuO or silica/CuO/MCP (1.10 ± 0.054 and 0.93 ± 0.037 g/l, respectively) and those exposed to vehicle (1.026 ± 0.12 g/l) as measured by the BCA assay. These data indicate that the surrogate combustion-fine pollutant/particle systems used in these studies do not induce pulmonary inflammation.

3.9. Rats exposed to surrogate pollutant/particle systems retain normal lung function

To determine if inhalation of surrogate pollutant/particle systems could alter pulmonary function, we exposed neonatal rats to silica/CuO, silica/CuO/MCP or vehicle for 7 successive days. A cohort of littermates were exposed to lipopolysaccharide (LPS) 24 h before assessing pulmonary function and served as a control demonstrating that we could detect changes in airway resistance

Table 2

Cellular antioxidant enzyme activity in the lungs of rats exposed to pollutant particle systems. Rats were exposed to vehicle or (0.2 mg/ml) of silica/CuO and silica/CuO/MCP for 20 min/day for 7 successive days. The cellular activity of superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase in lung homogenates was measured and normalized to protein concentration. Data are expressed as mean \pm SE of two replicates ($n = 6$).

Enzyme activity	Vehicle	Silica/CuO	Silica/CuO/MCP
Total SOD (% inhibition rate)	82.99 \pm 0.8105	85.44 \pm 0.2285*	84.89 \pm 0.2079*
Catalase (unit/mg protein)	30.06 \pm 3.2140	26.06 \pm 1.3810	17.74 \pm 1.4000** #
Glutathione reductase (unit/mg protein)	0.0308 \pm 0.0032	0.0344 \pm 0.0049	0.0317 \pm 0.0034
Glutathione peroxidase (unit/mg protein)	1.430 \pm 0.1773	1.104 \pm 0.1638	1.202 \pm 0.0681

* One-way ANOVA: $p < 0.05$.

** One-way ANOVA: $p < 0.01$ vs vehicle.

One-way ANOVA: $p < 0.05$ vs silica/CuO.

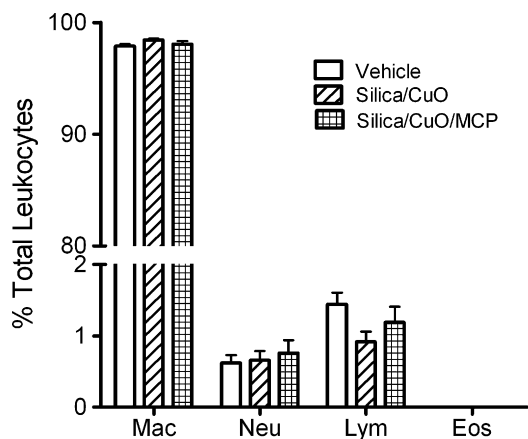


Fig. 8. BALF cellularity in neonatal rats following exposure to pollutant particle systems. The leukocyte population including macrophage (Mac), neutrophil (Neu), lymphocyte (Lym) and eosinophil (Eos) was determined and expressed as percent of total leukocytes recovered. Data are expressed as mean \pm SE of two replicates ($n = 6$).

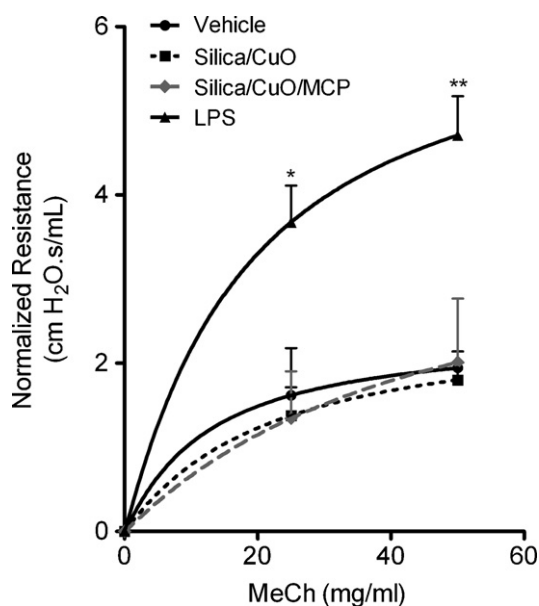


Fig. 9. Airway resistance in rats exposed to pollutant particle systems. Airway resistance was assessed in rats 24 h after the final exposure to fine particles. Airway resistance was measured after challenging the lungs with increasing doses of methacholine (MeCh) and normalized to basal airway resistance (0 mg/ml MeCh). As a positive control rats were exposed to (1 mg/kg) of lipopolysaccharide (LPS) 24 h prior to assessment of airway resistance. Data are expressed as mean \pm SE of two replicates ($n = 6$). * $p < 0.01$, ** $p < 0.005$ vs vehicle.

in these young rats. Lung resistance in response to 25 and 50 mg/ml of inhaled MeCh was significantly greater in LPS exposed rats than in any other group. Although a dose-mediated increase in airways resistance was observed with MeCh in all groups, the observed increases in resistance were no different among the vehicle, silica/CuO, or silica/CuO/MCP exposed rats in response to 25 or 50 mg/ml of MeCh suggesting that exposure to these particles did not alter pulmonary function (Fig. 9).

4. Discussion

We have previously reported that semiquinone-type (SQ) radicals, generated during combustion processes (Pryor, 1992; Lomnicki et al., 2008a), are able to generate ROS (Cosgrove et al., 1985; Crisostomo et al., 2007) and produce many deleterious effects in biological systems (Squadrito et al., 2001; Chung et al., 2007).

We have also reported the presence of semiquinone-type radicals in ambient PM_{2.5} samples collected from different locations across the United States (Dellinger et al., 2001). Since a substantial fraction of PM_{2.5} is generated from combustion sources and mainly consists of particles that are ultrafine ($\leq 0.1 \mu\text{m}$) in size (Zheng et al., 2002), we have studied the mechanism by which substituted phenoxy and semiquinone-type radicals are formed during the combustion process (Lomnicki et al., 2008b). In the present study we have synthesized surrogate pollutant/particle systems that resemble those generated during the combustion process and found in ambient air. Our surrogate particles are of a uniform size ($\sim 2.5 \mu\text{m}$) and chemical composition and we have begun to address the role of EPFRs in pulmonary effects associated with exposure to elevated levels of PM_{2.5}. We assessed the ability of the PFR-containing particles and the precursor particles to produce ROS, induce cytotoxicity, and induce oxidative stress in vitro using human laryngeal epithelial cells. The in vitro findings prompted us to then examine the ability of these particles to induce pulmonary inflammation, oxidative stress, and lung dysfunction in an in vivo setting. In particular, we looked at the effect of our surrogates on the developing pulmonary system using neonatal rats.

In the present study, all surrogate pollutant/particle systems elicited a dose-dependent decrease in epithelial cell viability (Fig. 2A) significantly different from non-exposed cells. Silica/MCP showed very limited cytotoxicity even at the highest concentration (400 $\mu\text{g/ml}$). In contrast, silica/CuO/MCP significantly decreased epithelial cell viability as compared to both silica/CuO and silica/MCP at higher concentrations (200 and 400 $\mu\text{g/ml}$). The weight of CuO and 2-MCP are relatively negligible as compared to the weight of the silica substrate and since they are all fairly uniform in size (1 μm in diameter) and shape; and therefore, the number of particles per mg weight and the surface area should be constant among all surrogate combustion particle systems. Thus, we believe that the significant decrease in cell viability associated with the exposure to silica/CuO/MCP is due to the presence of the EPFR in these particles. Co-treatment with resveratrol mitigated the cytotoxic effect of silica/CuO/MCP suggesting that oxidative stress is responsible, at least in part, for the decreased viability (Fig. 2B). Interestingly, resveratrol failed to alleviate the limited cytotoxic effect associated with exposure to silica/MCP or silica/CuO in these cells. Collectively, these data suggest that the EPFR is the cause of the enhanced cytotoxic effect associated with silica/CuO/MCP and that the limited cytotoxicity observed with silica/CuO and silica/MCP is due to something besides oxidative stress.

Exactly what is responsible for the limited cytotoxicity observed with silica/CuO and silica/MCP remains a mystery. It is possible that the low cytotoxicity profiles (20% non-viable) are due to the altered proliferation, morphological structure and/or protein synthesis machinery as has been documented with other in vitro particle exposures (Pernodet et al., 2006). However, cells receiving either of the surrogate pollutant/particle systems appeared identical at the light microscope level. Furthermore, average protein concentrations were not statistically different among the different exposures. Another alternative, not tested here, is that elevated secretion of tumor necrosis factor- α (TNF- α) by the epithelial cells is responsible for the reduced cell viability (Berkova et al., 1999; Fang et al., 2001).

Both silica/CuO and silica/CuO/MCP particles were able to generate ROS in the epithelial cells. The ability of a transition metal, such as copper to generate ROS in vitro has been previously reported (Cervantes-Cervantes et al., 2005). However, silica/CuO/MCP but not silica/CuO was able to inhibit the activity of catalase and suggesting that the EPFR-containing particles were able to continuously generate H₂O₂ in the exposed cells; whereas silica/CuO did not (Table 1). Silica/CuO/MCP also increased the activity of glutathione reductase and glutathione peroxidase and the ratio of

oxidized to total GSH (Fig. 4). These data indicate that the EPFR is the main player in the generation of oxidative stress rather than the copper or the 2-MCP. Although semiquinones are able to generate ROS including H_2O_2 and $\text{O}_2^{\bullet-}$, our data show significant modification in the activities of antioxidant enzymes associated with the neutralization of H_2O_2 (catalase, GPx); however, we did not observe significant changes in the activity of SOD indicating that $\text{O}_2^{\bullet-}$ was not generated in silica/CuO/MCP treated cells. Since H_2O_2 can diffuse across the cell membrane as previously reported (Schubert and Wilmer, 1991), but not $\text{O}_2^{\bullet-}$ which is negatively charged, we propose that ROS (H_2O_2 , $\text{O}_2^{\bullet-}$) was generated by the EPFR outside the cells and, then diffused across the cell membrane. Alternatively, ROS may have oxidized cell membrane lipid to produce 8-isoprostane, which can diffuse inside the cell and initiate the production of ROS in the mitochondria (Landar et al., 2006).

Usually cells respond to oxidative burden by fortifying their antioxidant defense mechanisms in order to protect themselves from any oxidative damage; however, if the defense mechanisms fail to neutralize the oxidative burden protein oxidation (Ramirez-Prieto et al., 2006), lipid peroxidation (Gutteridge, 1995), DNA damage, mitochondrial perturbation and apoptosis occur (Li et al., 2003). One of the eicosanoids produced by the oxidation of phospholipids by ROS is 8-isoprostane which is a stable and water-soluble compound. Therefore, 8-isoprostane has been proposed as an ideal marker of oxidative stress and lipid peroxidation (Morrow et al., 1995; Morrow and Roberts, 1997). The levels of 8-isoprostane in the supernatant of cells exposed to silica/CuO/MCP was significantly elevated (Fig. 5) demonstrating that silica/CuO/MCP was able to induce oxidative damage in epithelial cells. Resveratrol was able to partially reverse the cytotoxicity associated with silica/CuO/MCP exposure. This reduction in cytotoxicity was associated with a decrease in the level of 8-isoprostane in the supernatant of these cells; and overall it suggests that oxidative stress is responsible for the reduced viability induced by exposure to silica/CuO/MCP (Fig. 5). By contrast, silica/MCP failed to alter the cellular redox balance and produced limited cytotoxicity (20% non-viable) even at the highest concentration tested. Although silica/CuO was able to generate ROS in epithelial cells, the antioxidant defense system remained intact and limited cytotoxicity was observed. In summary, silica/CuO/MCP showed greater cytotoxic effect associated with the generation of oxidative stress in human laryngeal epithelial cells.

Our *in vitro* data indicated that EPFR-containing silica/CuO/MCP particles and to a lesser extent, silica/CuO were able to alter the redox balance in human epithelial cells. Although extrapolating conclusions from *in vitro* studies to humans will always be uncertain, we have investigated the ability of these particles to generate oxidative stress and pulmonary inflammation *in vivo*. Generation of oxidative stress and pulmonary inflammation in the developing neonatal lung was expected to induce airway remodeling and lead to persistent lung dysfunction (Jeffery, 2001; Henson et al., 2006). Using a dose that is equivalent to human exposure (Brown et al., 2005) and that has been used by other investigators (Antonini et al., 2002; Roberts et al., 2003), neonatal rats (7-day of age) were exposed to the particles for 7 successive days. We used a computer-controlled nose-only exposure system to deliver the aerosolized particle suspension to rats in a uniform and consistent pattern. Fluorescent spheres delivered to neonatal rats using this same system were subsequently localized at the bronchioles and small respiratory bronchioles of these rats (Fig. 6) resembling the deposition of ambient fine particles in the human lung under normal breathing conditions (Stuart, 1984).

In our study, all of the particles produced limited effects on the redox balance in the lungs of the exposed animals. Silica/CuO did not alter the activity of antioxidant enzymes except for SOD, whereas, silica/CuO/MCP increased the activity of SOD and inhibited

that of catalase as compared to vehicle exposed animals. This indicated that silica/CuO/MCP was able to generate H_2O_2 in the lungs of exposed rats. The release and dissolution of CuO and MCP in the lung lining fluid is least likely to happen, thus we believe that EPFR structure is the main source of this H_2O_2 . When the glutathione system is not overloaded with excessive oxidants, the contribution of catalase in the detoxification of H_2O_2 is very limited as compared to glutathione peroxidase (Antunes et al., 2002).

Surprisingly, no change in enzyme activity was observed with GPx or the ratio of oxidized to total glutathione. It is possible that the pulmonary antioxidant defense system exhibited site specific activity. For example, GPx has been considered the main antioxidant enzyme involved in the detoxification of oxidants in the alveolar region (Avisar et al., 1996), while lactoperoxidase (El-Chemaly et al., 2003) and catalase (Cohn et al., 1994; Baeza-Squiban et al., 2000) were essential in the neutralization of ROS generated in the epithelial cells of the trachea and bronchi. The inhibition of catalase but not GPx associated with the exposure to silica/CuO/MCP suggests that the impact effect of these particles is limited to the upper respiratory tract including the trachea and bronchi.

We further analyzed different pulmonary inflammatory biomarkers and measured lung function in the rats exposed to surrogate pollutant/particle systems. Neither silica/CuO nor silica/CuO/MCP increased the total number of leukocytes in the BALF of exposed rats as compared to vehicle treated rats. In addition, no significant differences were observed in any of the subcellular BALF populations among all groups. Analysis of BALF cytokine levels also failed to show increase in the level of any cytokine essential in pulmonary inflammation (i.e. IL-1b, IL-6, IL-10, IL-18, IFN- γ , KC, TNF α , MCP1, MIP1a, VEGF) (data not shown). These data strongly indicate that acute exposure to the EPFR-containing particles that are 2.5 μm in size did not produce pulmonary inflammation. Although the immune system of neonates is still developing and exhibiting limited capabilities as compared to adult immune system (Grigg and Riedler, 2000), our lab (You et al., 2006) and other investigators (Martinez-Burnes et al., 2001) have reported a pulmonary inflammatory response associated with various exposures. While particles are removed from the bronchioles by the mucociliary system, the clearance of particles from the alveolar region is mostly mediated by macrophages (Stuart, 1984). The inability of the 2.5 μm particle systems to activate macrophages and induce pulmonary inflammation suggests that they were cleared from the lungs by the mucociliary system and were not able to penetrate deep into the alveolar region. As expected, the limited ability of the particles to generate oxidative stress or inflammation in pulmonary tissue was associated with normal airway resistance in response to MeCh provocation as compared to vehicle exposed rats.

Our data reflect few but conclusive studies demonstrating the limited adverse pulmonary effects associated with exposure to $\text{PM}_{2.5}$. To study the acute effect of ambient $\text{PM}_{2.5}$, investigators usually expose animals to "concentrated ambient particles" (CAP). Goldsmith et al. demonstrated that exposing ovalbumin-sensitized mice to 787 $\mu\text{g}/\text{m}^3$ of CAP for three daily exposures of 5 h did not increase the airway inflammation or worsen pulmonary function (Goldsmith et al., 1999). Although asthmatic animals are susceptible and exhibit hyper-responsiveness to allergens, exposure to a high concentration of fine particles did not exacerbate the airway inflammation. Kodavanti et al. (2000) exposed bronchitic rats, which are believed to be susceptible to inflammation, to increasing doses of CAP but no significant inflammatory response was observed until the dose of CAP was increased to (640 $\mu\text{g}/\text{m}^3$). This was consistent with Holgate et al. (2003) who found no changes in any of the markers of inflammation in healthy volunteers associated with the exposure to CAP. The limited health impact associated with the exposure to these particles can be partially attributed to their limited capabilities to penetrate into the alveolar region,

which compose 90% (by surface area) of the lung. Controversially, some investigators have reported a link between the exposure to fine particles and cardio-pulmonary adverse effects (Koenig et al., 1993; Lee et al., 2006; Brauer et al., 2007; Brugge et al., 2007). It is unclear why these discrepancies exist; however, the variability in the chemical composition, size, etc. of these airborne samples maybe responsible for falsely positive/negative correlations between the exposure to fine particles and pulmonary dysfunction (Green and Armstrong, 2003). In conjunction with other data from our lab using EPFR-containing particles that are ultrafine in size (0.2 μm), these data clearly demonstrate that when delivered using a relevant exposure system (nose-only inhalation) particle size is critical in determining adverse pulmonary effects (manuscript in preparation).

In summary, the *in vitro* study demonstrated that the EPFR-containing particles generate greater oxidative stress and greater reductions in cell viability as compared to control particles containing the organic precursor and no EPFR. These data indicate that the chemical composition and the variety of compounds associated with particles may be responsible, at least partially, in the biological effect associated with the exposure to PM. In addition, the limited pulmonary inflammation and oxidative stress associated with the exposure to surrogate combustion-fine pollutant particles systems *in vivo* suggests that the size of the particle is as important as the chemical composition in assessing pulmonary toxicity. These data cumulatively suggest that there are distinct qualitative differences between the health effects of EPFR-containing PM due to size of the particle.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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