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Increased cytotoxicity of oxidized flame soot

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ABSTRACT

Combustion–generated particles released into the atmosphere undergo reactions with oxidants, which can change the particles' physiochemical characteristics. In this work, we compare the physical and chemical properties and cellular response of particles fresh from a flame with those oxidized by ozone and nitrogen dioxide. The reaction with ozone and nitrogen dioxide does not significantly modify the physical characteristics of the particles (primary particle size, fractal dimension, and surface area). However, oxidation affects the chemical characteristics of the particles, creating more oxygen and nitrogen containing functional groups, and increases their hydrophilicity. In addition, oxidized soot generates more reactive oxygen species, as measured by the dithiothreitol (DTT) assay. Furthermore, oxidized soot is 1.5 – 2 times more toxic than soot that was not reacted with ozone, but the inflammatory response, measured by interleukin–8 (IL–8) secretion, is unchanged. These results imply that combustion–generated particles released into the atmosphere will have an increased toxicity on or after high ozone days.

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1. Introduction

Carbonaceous particles (soot) from the combustion of hydrocarbon fuels can react in the atmosphere with oxidants such as ozone and nitrogen dioxide, modifying the soot surface characteristics. Zielinska (2005) speculated that this atmospheric oxidative aging may change the hazard posed to human health by soot. Additionally, Squadrito et al. (2001) proposed that long–lived free radical compounds associated with oxygenated species on ambient particles play a key role in particle induced adverse health effects. Our objective was to assess some of the physical, chemical, and cytotoxic characteristics of soot that has undergone oxidative aging.

The reaction of ozone and nitrogen dioxide with carbonaceous material has been extensively studied as it applies to ozone depletion (Kamm et al., 1999) and cloud condensation (Chughtai et al., 1991; Kotzick et al., 1997; Weingartner et al., 1997). In general, it was observed that oxygen and nitrogen containing functional groups are incorporated onto the soot surface, resulting in an increase of hydrophilicity. Evidence of atmospheric soot oxidation is seen in a direct correlation between ambient ozone concen–trations and the concentration of oxygenated hydrocarbons on ambient particulate matter (Wilson and McCordy, 1995). It should also be noted that, depending upon conditions in the flame, soot can contain as much as 10% oxygen (Stanmore et al., 2001).

Possible health effects associated with oxidative aging were observed in rats that had increased adverse effects when exposed to mixtures of ozone and different particle types (Adamson et al., 1999; Elder et al., 2000). However, it could not be determined if the increased toxicity of the mixture was due to oxidative aging of the particles or to biological interactions (i.e., ozone induced lung injury may cause increased susceptibility to particles). Sequential exposures to ozone followed by exposure to diesel particles have shown that ozone does increase susceptibility to particle induced injury (Kafoury and Kelley, 2005), but those results do not eliminate the possibility of increased toxicity of oxidized particles. Evidence of increased adverse effects due to oxidative aging was observed with diesel particles oxidized by ozone. The oxidized diesel particles had a greater ability to generate oxidants (Li et al., 2009) and increase the inflammatory response in human bronchial epithelial cells and rats compared to untreated diesel particles (Madden et al., 2000).

Diesel particles are typically coated with organic compounds, including polycyclic aromatic hydrocarbons (PAH). The surface molecules can be oxidized to polar species such as quinones, which have been shown to be the most potent fraction of diesel exhaust in causing oxidative stress and toxic effects on cells (Xia et al., 2004). However, there are indications that oxidation causes similar changes to the toxicity of carbonaceous particles without high concentrations of organic molecules. For example, the lung surfactants SP–D and dipalmitoyl phosphatidylcholine had differing binding patterns for carbon black and oxidized carbon black particles (Kendall et al., 2004). Oxidant production, as measured by the oxidation of dithiothreitol (DTT), was greater for an oxidized activated carbon as opposed to an untreated activated carbon (Sauvain et al., 2008). However, neither of these studies compared the effects between untreated and oxidized particles on cellular toxicity.

In this study, we used a human bronchial epithelial cell culture system to examine changes associated with atmospheric trans–formation of carbonaceous particles. We used soot generated from a methane–air flame, as it is primarily elemental carbon without

the sulfates, transition metals, and organic carbon found in diesel particles. The oxidative aging process in the atmosphere was simulated by reacting soot with ozone. The effects of oxidation on soot physicochemical characteristics were assessed and changes in viability of bronchial cells and release of the chemokine interleukin–8 (IL–8), a widely used biomarker of an inflammatory response, were determined.

2. Experimental

2.1. Particle generation and characterization

A downward flow methane–air diffusion flame described by Stipe et al. (2005) was used as a source of carbonaceous particles (Figure 1). Particles generated from this flame are composed almost entirely of elemental carbon and have no detectable organic carbon in thermo–optical analysis (Kirchstetter and Novakov, 2007). The methane and air flow rates were maintained with mass flow controllers (Teledyne Hastings) at 1.3 and 18.7 Lpm, respectively. In the post flame region, the exhaust was diluted with air at a flow rate of 14.7 Lpm. A sample was drawn 132 cm downstream from the dilution section and dried with a silica gel diffusion dryer (TSI model 3062).

Two conditions were investigated: soot (untreated with ozone) and oxidized soot. Soot from the post flame region was dried before being collected on a glass fiber filter (Pallflex 37 mm). Oxidized soot was generated by mixing dried flame exhaust at 0.4 Lpm with 55 ppm ozone in oxygen at a flow rate of 0.4 Lpm to achieve a final concentration of 28 ppm of ozone. Ozone was generated from the photolysis of oxygen (99.6% purity) with a Hg pen lamp (UVP Pen–Ray). Ozone was measured with a Dasibi 1003–AH ozone analyzer, with samples brought into range by dilution with nitrogen. Soot and ozone reacted in a flow tube (11.1 L volume) with a residence time of 14 minutes at ambient temperature. Nitric oxide produced from the flame (~4 ppm) also reacts with ozone to generate nitrogen dioxide; consequently, soot can react with both ozone and nitrogen dioxide in the flow tube. The reaction was stopped by removing the ozone in an activated carbon parallel plate denuder at ambient temperature (Tang et al., 1994), after which the oxidized soot was collected on a glass fiber filter.

A summary of all the measurements on particles, the source of the particles, and the instruments used is in Table 1. Particles were collected for transmission electron microscopy (TEM) on a carbon–coated grid placed on top of the glass fiber filter. Sufficient particle coverage on the grid was achieved with an exposure time of about five minutes. Measurements of soot TEM samples were made on an FEI Tecnai 12 transmission electron microscope. Soot particles had a fractal morphology consisting of agglomerates of spherical primary particles. The fractal dimension was calculated following the analysis of Park et al. (2004), which correlates the number of primary particles to the maximum length of the agglomerate. The number of primary particles in an agglomerate was calculated from the area of the particle and the area of a primary particle as described by Koylu et al. (1995).

Soot size distributions were measured with a TSI Scanning Mobility Particle Sizer (model 3071A Differential Mobility Analyzer, model 3025A Ultrafine Condensation Particle Counter) down–stream of the denuder. Soot size distributions were obtained with the mercury lamp off (i.e., only oxygen in the system) and oxidized soot size distributions were obtained with the mercury lamp on (i.e., ozone in the system) as shown in Figure 1. The BET surface area of the soot was measured with a gas adsorption analyzer (Micromeritics Tristar 3000). The change in surface composition of the soot was measured with an FTIR spectrometer (Nicolet Magna–IR 760) using a flow–through attenuated total reflectance (ATR) cell. Soot was collected on a Teflon filter (Pall), brushed off, and pressed onto the zinc selenide crystal surface in the ATR cell. The soot spectrum was recorded during exposure to ozone (0.33%) generated in air with a corona ozone generator (Yanco OL80W) at a flow rate of 40 cm^3 min^{-1}. Nitrogen oxides were also produced by secondary reactions in the generator, resulting in a nitric oxide concentration of 5.4 ppm and a nitrogen dioxide concentration of 2.3 ppm. The larger concentration of ozone was used when obtaining FTIR spectra to facilitate the detection of changes in the soot surface chemistry.

2.2. Particle suspension

Particles collected on filters were stored at –8 °C before the suspensions were generated. Suspensions for cell exposures were made with LHC basal medium (exposure medium for bronchial epithelial cells) with 0.004% dipalmitoyl phosphatidylcholine. Particles were gently brushed off the filter, weighed, and emptied into sterile tubes. The suspension was stirred for fifteen seconds on a vortex stirrer (Vortex Genie) then sonicated for 45 seconds in a bath sonicator (Branson, B–22–4 125 W); this process was repeated three times. The mass concentration in suspension was determined from the absorption at 640 nm, measured on a UV–visible spectrometer (Perkin–Elmer, Lambda 2 or Ocean Optics, HR4000). The absorption was calibrated by measuring the absor–bance of soot suspended in distilled water, heating to evaporate the water, and weighing the remaining soot.

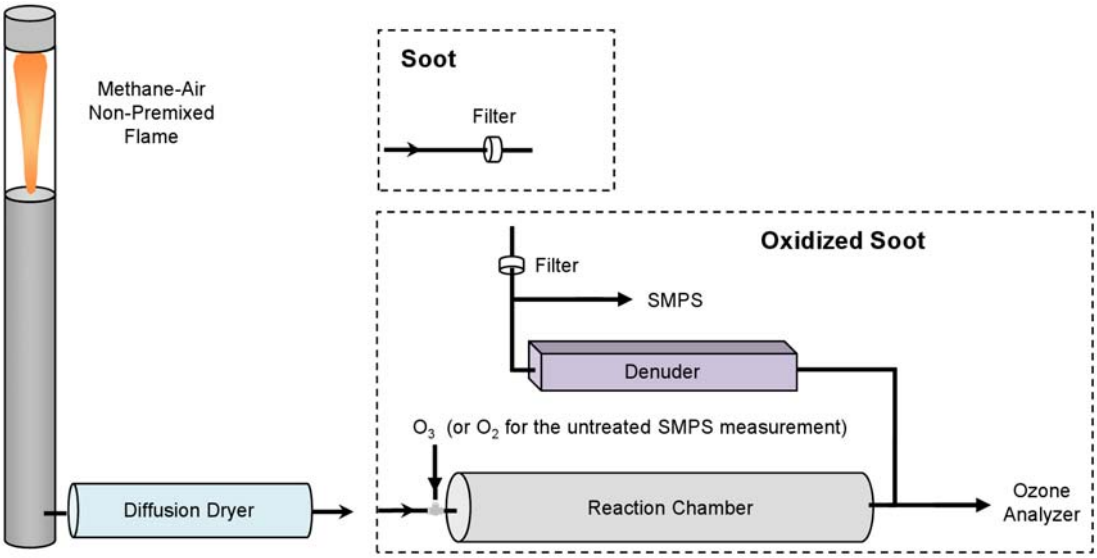

Figure 1. *Schematic of the experimental set–up. Post flame exhaust either can be collected as untreated soot or oxidized soot.*

Table 1. *Summary of methods, particle sample source or location in experimental set–up, and the instrument used to make the measurements*

Measurement	Sample Source/location	Instrument
Primary particle diameter, fractal dimension	TEM grid collected on filter	TEM
Size distribution	Downstream of denuder	SMPS
BET surface area	Filter	Gas adsorption analyzer
Surface composition	Filter, oxidized during FTIR measurement	FTIR Spectrometer
Suspension concentration	Filter	UV–Visible Spectrometer
(DTT) assay	Filter	UV–Visible Spectrometer
Toxicity – XTT assay	Filter	UV–Visible Spectrometer
Inflammation – IL–8 assay	Filter	SpectraMax Plate Reader

2.3. DTT Assay

Oxidant ability of the particles was measured with the DTT assay as described by Cho et al. (2005) and is briefly described here. All chemicals used in the assay were purchased from Sigma. Soot and oxidized soot suspensions (5, 25, and 50 μg mL^{-1}) were incubated at 37 °C with 100 μM DTT in a 0.1 M phosphate buffer (pH = 7.4) for 0, 10, 20, 30, 45, and 60 minutes. At the designated incubation time, a 0.5 mL aliquot of the mixture was mixed with 0.5 mL of 10% trichloroacetic acid, and a 0.5 mL aliquot was then mixed with 1.0 mL Tris HCl and 25 μl of 10 mM 5,5'–dithiobis–2–nitrobenzoic acid (DTNB). The DTNB reacts with the remaining DTT to produce 2–nitro–5–thiobenzoic acid, which was measured by its absorption at 412 nm with the UV–visible spectrometer.

2.4. Cell culture

The immortalized human bronchial epithelial cell line, 16HBE14o (provided by Dieter Gruenert, California Pacific Medical Research Center) was used to assess the cytotoxicity of soot suspensions. This cell line exhibits many features of normal human epithelial cells (Steimer et al., 2005). Cells were maintained in logarithmic phase of growth in collagen–coated flasks in minimum essential medium (MEM), supplemented with 10% fetal calf serum, 2 mM L–glutamine, 10 mM HEPES buffer, and antibiotics.

For experiments, cells were seeded into four collagen–coated 12 well plates at 10^5 cells per well two days before. On the day of the experiment, the media was removed, cells were rinsed with phosphate buffered saline (PBS), and then dosed with suspensions of particles in LHC basal medium with 0.004% dipalmitoyl phosphatidylcholine for four hours. Each dose was tested in triplicate wells. The control group consisted of nine wells distrib–uted over three plates that were exposed to LHC media with 0.004% dipalmitoyl phosphatidylcholine. Cells were dosed with 10, 25, 50, 100, 150, and 200 μg mL^{-1} of either soot or oxidized soot particles in suspension (2.6, 6.6, 13.2, 26.3, 39.5, and 52.6 μg cm^{-2}). A 200 μg mL^{-1} suspension of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1650 diesel particles was made following the same suspension protocol used to suspend the soot and oxidized soot. SRM1650 diesel particles were obtained from 4–cylinder direct injection diesel engines operated under a variety of conditions and are considered to be representative of heavy duty diesel exhaust particles (NIST, 1991). The diesel particles were used as an environmentally relevant control to provide context for the cellular response from flame soot. After exposure, cells were rinsed with PBS, and MEM was added for another four–hour incubation. The cell culture medium was collected and stored at –8 °C until used to measure (IL–8) release with an enzyme–linked immune-sorbent assay (ELISA) kit (Invitrogen) following the manufacturer's instructions. Cell viability was assessed using an XTT (2,3–bis–(2–methoxy–4–nitro–5–sulfophenyl)–2H–tetrazolium–5–carboxanilide, disodium salt, kit from Sigma) following the manufacturer's instructions. This widely used assay determines the number of viable cells in culture through the formation of a colored product in a mitochondria–based reaction. As all wells contain the same number of cells at the beginning of the exposure, a reduced absorbance in treated cells relative to untreated controls indicates cell killing, so results are expressed as percent absorbance of control value.

2.5. Statistical analysis

A paired student's t–test was applied between different exposure conditions to determine statistical significance. A difference between conditions is considered statistically significant at the $p < 0.05$ level.

3. Results

3.1. Particle characteristics

The size distributions of soot exposed to ozone or oxygen is shown in Figure 2. The oxidized soot has a slightly decreased number concentration and slightly increased mean diameter compared to the soot mixed with oxygen only (Table 2). There is also an increase in the number of small particles (below 40 nm) when ozone is present; the number concentration of particles smaller than 40 nm is approximately an order of magnitude larger when ozone is present.

The physical characteristics of the soot and oxidized soot are given in Table 2. The oxidized primary particles have an increase of about 2 nm compared to the soot primary particles, but the difference is not statistically significant. The fractal dimension of the soot particles is close to the generally accepted value of 1.78 for flame soot; the oxidized particles have a somewhat smaller fractal dimension. There is no significant change in surface area with oxidation.

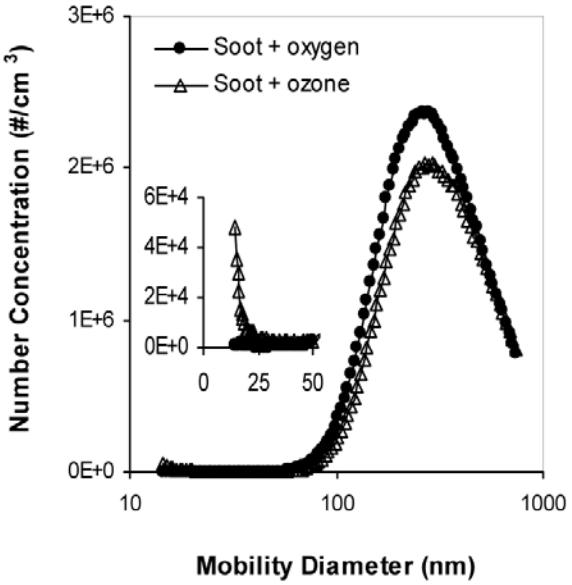

Figure 2. *Size distribution of soot mixed with oxygen and soot mixed with 28 ppm ozone (oxidized soot) at a residence time of 14 minutes. The inset shows the number concentration at diameters smaller than 50 nm.*

Table 2. *Measurements of physical characteristics of soot and oxidized soot, N is the number of measurements used to calculate the average value*

Sample	Soot	N	Oxidized Soot	N
Median Diameter (nm)	278	5	291	6
Number Concentration (# cm^{-3})	1.4×10^6	5	1.2×10^6	6
Surface Area ($m^2\ g^{-1}$)	124	3	123	3
Primary Particle Diameter (nm)	33.5 ± 7.3	54	35.1 ± 12.8	169
Fractal Dimension	1.73	10	1.57	28

The soot FTIR spectrum displays peaks at 1 260 and 1 350 cm^{-1}, indicating the presence of oxygen containing carbonyl groups, and nitrogen containing functional groups with peaks at 1 390 and 1 580 cm^{-1} before any reaction with ozone has occurred (Figure 3). When soot is exposed to ozone (1.3%) and nitrogen oxides (7.7 ppm) the absorbance increases at 1 310 and 1 500 cm^{-1}, attributed to the nitrogen–oxygen stretching frequencies in nitro functional groups. In addition, the absorbance is increased at 1 720 cm^{-1}, assigned to the carbonyl stretching frequency. At a lower ozone concentration (9 ppm) without nitrogen oxides, a similar, but smaller increase in absorbance due to carbonyl groups was observed.

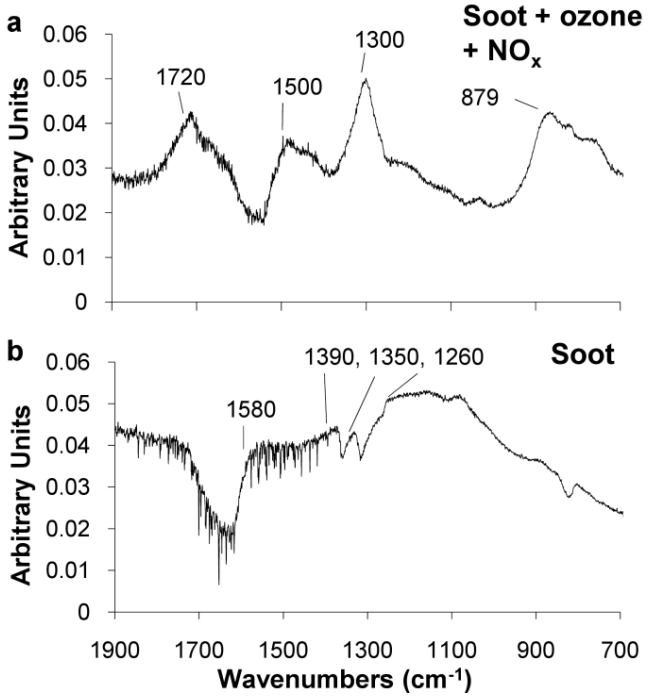

Figure 3. (a) *FTIR difference spectrum of the soot reacted with ozone and nitrogen dioxide,* ***(b)*** *FTIR spectrum of soot collected directly from the flame.*

3.2. Cellular response

Oxidized soot has a greater oxidative ability than soot, as assessed with the DTT assay (Figure 4). Both particle types display increasing DTT consumption rates with increasing particle mass.

The viability of cells, measured as the XTT absorbance, exposed to soot and oxidized soot decreased with increasing particle concentration up to 100 μg mL^{-1} (Figure 5). At concentrations above 100 μg mL^{-1}, the viability remained roughly constant at 40% and 25% of the control viability (0 μg mL^{-1}) for the soot and oxidized soot, respectively. At all concentrations tested, the oxidized soot was more toxic than soot. Both soot and oxidized soot were more toxic than the diesel particles at a dose of 200 μg mL^{-1}; however, due to the large variation in the diesel viability, the differences were not statistically significant.

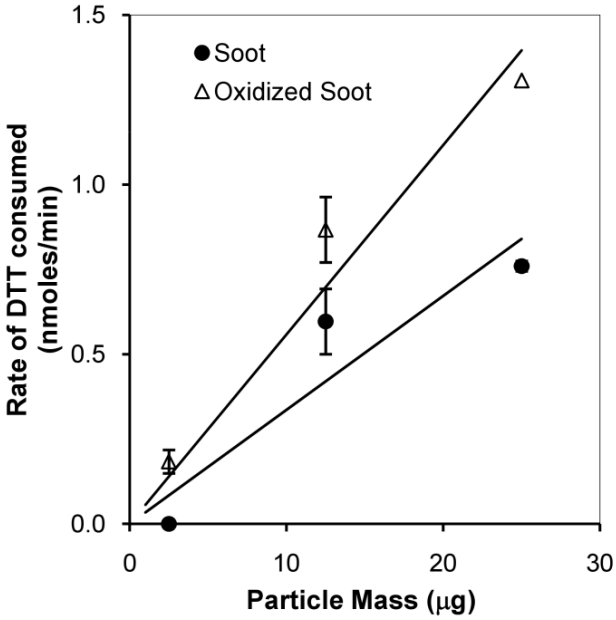

Figure 4. *DTT consumption rate compared between soot and oxidized soot.*

The IL–8 release during the four hours after exposure, corrected for reduction in viability, is displayed in Figure 6. Cells treated at concentrations above 10 μg mL^{-1} had a statistically significant increase of IL–8 release over the control group except at the highest dose. The largest increase of IL–8 secretion for both soot and oxidized soot was at the 50 μg mL^{-1} dose. The SRM 1650 diesel particles elicited a strong inflammatory response with an IL–8 secretion 3.6 times greater than the control level and larger than that from cells exposed to any dose of the soot or oxidized soot. The IL–8 secretion from cells exposed to soot was approx–imately the same as for cells exposed to oxidized soot, despite the increased cytotoxicity of oxidized soot.

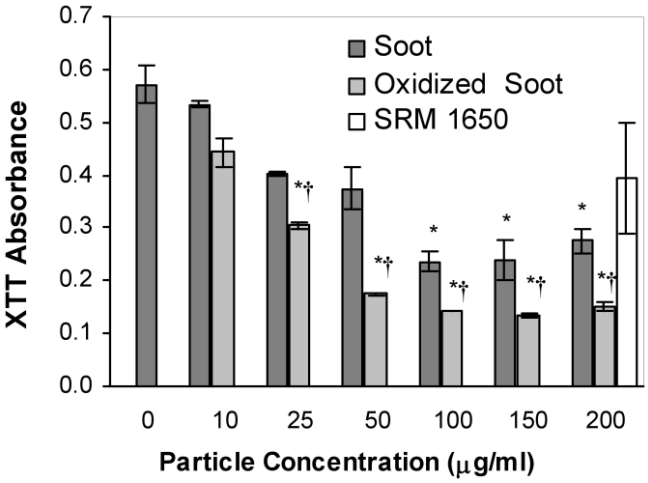

Figure 5. *XTT absorbance for cells exposed to suspensions of soot, oxidized soot, and diesel particles. Error bars represent the standard error of the mean. *Statistically significant compared to control at $p \leq 0.05$. †Statis–tically significant compared to soot at the same dose at $p \leq 0.01$.*

4. Discussion

In this study, reaction with ozone has a minimal effect on the physical characteristics of the soot particles, except for an increased number of particles less than 40 nm in diameter. These small particles are most likely due to reactions of ozone with gas phase hydrocarbons, producing compounds that condense onto existing particles or nucleate to form new particles (Seinfeld and Pandis, 1998). Additionally, there is a change in the fractal dimension, with particles exposed to ozone having a more open

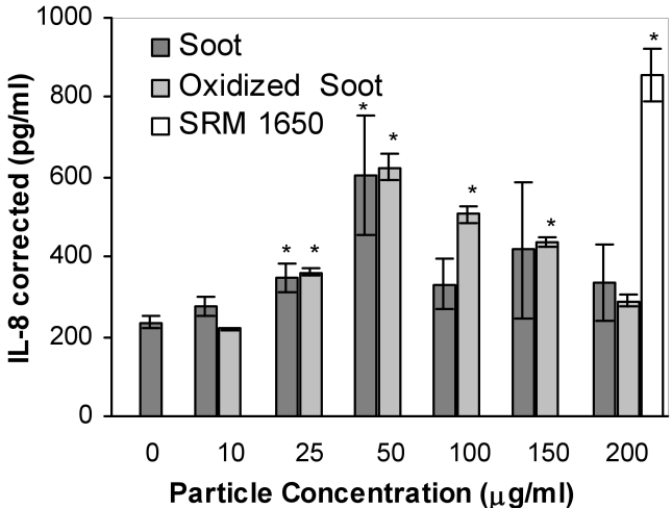

Figure 6. *IL–8 release from cells exposed to soot, oxidized soot, and SRM 1650 diesel particles. IL–8 values are corrected for the reduction in viability measured by the XTT assay. Error bars represent the standard error of the mean. *Statistically significant increased IL–8 compared to control at $p \leq 0.01$.*

shape. For the larger particles, a decreased number concentration and increased median diameter indicate that aggregation increases when ozone is present, but the effect is not large. The volume concentration remains approximately the same. This suggests no significant mass loss occurs from the oxidation reactions. Furthermore, preserved particle mass is suggested from the TEM images, where the oxidized soot has about the same primary particle diameter as soot. The conversion of soot to carbon dioxide by ozone is very slow at room temperature, even at high concentrations (Kamm et al., 2004); we do not expect any measurable mass loss from the soot at the residence time and ozone concentration used in this work (14 minutes, 28 ppm).

The reaction with ozone noticeably altered the chemical characteristics of the soot particles. We exposed fresh exhaust from the flame (both particles and combustion product gases) to ozone and nitrogen dioxide and observed an increase in nitrogen–containing and carbonyl functional groups. Moreover, we observed evidence of secondary particle formation. This oxidation resulted in an increased capability to generate reactive oxygen species (measured by the DTT assay) and increased cytotoxicity in lung cells. The oxidized soot is 30% to 240% more potent than soot for doses from 10 to 50 μg mL^{-1}, but the increased cytotoxicity does not translate to an increased inflammatory response. The inflammatory response increases with increasing dose of soot up to 50 μg mL^{-1}, but the oxidized soot causes virtually the same response as soot.

We used diesel particles to provide context to the cytotoxic and inflammatory responses of soot and oxidized soot. The soot and oxidized soot both caused a greater cytotoxic response than diesel soot, but did not cause as large an inflammatory response. The observed low cytotoxicity of diesel particles may be attributable to interference in the viability assay. We previously observed that diesel particles reacted with the tetrazolium compound in the MTT (3–(4,5–Dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide) assay to generate the colored formazan under cell free conditions (unpublished data). As the XTT assay is based on the same reaction, we expect a similar interference and view the viability results for the diesel particles as suspect. The greater inflammatory response of the diesel particles is likely due to the organic content of these particles, shown to be the primary source of a pro–inflammatory response in 16HBE14o cells to SRM 1650 diesel particles (Bonvallot et al., 2001). The soot particles have almost no organic content. It is expected that the oxidized soot has a thin coating of organic material generated during the reaction with ozone, but at a much lower concentration than the 17.5% extractable organic mass of the diesel particles (NIST, 1991). Therefore, it is likely that the observed inflammatory response from the soot is due to the physical nature of the particles rather than the chemical composition, and is thus not affected by the reaction with ozone. For example, Vogel et al. (2005) observed that diesel particles and ambient ultrafine particles stripped of their organic material still exhibit increased IL–8 expression.

Several other groups observed a similar increased potency of soot from oxidation with ozone. Koike and Kobayashi (2006) have shown that the increased reactivity towards DTT by oxidized carbon black is associated with an increased oxidative response in lung cells. This suggests that the increased cytotoxicity of the oxidized soot is due to its ability to increase the production of reactive oxygen species. This increase, however, does not increase the inflammatory response. Madden et al. (2000) also observed this effect; carbon black particles oxidized by ozone did not increase the inflammatory response in human lung cells and rats, but diesel particles oxidized by ozone did. They attributed the increased inflammation to the organic content of the diesel particles, which was not present in the carbon black.

Our results show oxidation of soot with negligible initial organic content increases cytotoxicity and the oxidant generation capability without increasing a pro–inflammatory response. These results are intriguing in that they suggest that even soot from a candle will have an enhanced ability to generate oxidative stress and cause greater cytotoxicity once oxidized by ozone in the environment.

We cannot attribute the difference in cytotoxicity entirely to the modified surface reactivity, since the particles undergo significant aggregation when dispersed in cell growth medium, and reach sizes on the order of 10 microns and in some cases sizes larger than the bronchial epithelial cells. Great care was taken to create suspensions with high mass concentrations of dispersed particles. However, despite the addition of surfactant, vortex stirring, and sonication, by the end of the exposure large aggregates had formed, with the aggregation rate strongly dependent on surface chemistry. Oxidized soot particles were more readily suspended in water and display a hydrophilic nature, unlike the soot that has not been reacted with ozone, which was difficult to suspend. The enhanced wetting of oxidized soot is expected, as the reaction between ozone and soot is known to create water–soluble polycarboxylic acids, even on particles with minimal organic carbon content, as is the case for the flame soot used in this work (Decesari et al., 2002). In addition, soot suspensions settled at a faster rate compared to the oxidized soot.

The assessment of particle toxicity with in vitro systems has always been plagued by particle aggregation in cell culture medium. The effect of particle size or degree of aggregation on toxicity is currently a very active area of research. Obtaining stable suspensions of dispersed particles to compare with their aggregated counterparts has proven difficult (Schulze et al., 2008) and initial studies produced mixed results. Wick et al. (2007) observed increased toxicity from aggregates compared to dispersed carbon nanotubes. However, Shvedova et al. (2007) observed that dispersed ultrafine carbon black caused the same inflammatory response at a dose 16 times less for agglomerated carbon black.

The greater aggregation at larger doses may explain why the inflammatory response to soot and oxidized soot has a maximum IL–8 release at a 50 μg mL^{-1} dose, rather than at a higher dose. Another explanation is that the particles bind IL–8, preventing its measurement. Despite rinsing of the cells after exposure, a substantial number of particles remain attached to the cells. Particles have been shown to bind IL–8 in a manner dependent on

their surface chemistry (Seagrave et al., 2004; Veranth et al., 2007; Kocbach et al., 2008). However, the presence of the surfactant in the particle preparation and serum in the incubation medium decreases the binding of IL–8 to particles (Seagrave et al., 2008; Kocbach et al., 2008).

Comparison of results obtained in these complex systems has to be done with caution, and drawing firm conclusions with this exposure format may not be possible. We suggest that using cells exposed at the air–liquid interface may provide better results, as this type of exposure has been shown to preserve the size distribution of the aerosol and is not affected by the extensive aggregation that particles undergo in cell culture medium (Holder et al., 2008).

5. Conclusions

Soot oxidized by ozone and nitrogen dioxide has different chemical properties than soot that was not reacted with ozone, while the physical properties are largely unchanged. Compared to soot, oxidized soot can generate more reactive oxygen species, is more toxic to lung cells, but the same inflammatory response was observed. Differences in the soot properties, especially aggregation and hydrophilicity, make it difficult to compare different types of soot and generate meaningful dose–response data.

Acknowledgements

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