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Evaluating the Toxicity of Airborne Particulate Matter and Nanoparticles by Measuring Oxidative Stress Potential—A Workshop Report and Consensus Statement

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Background: **There is a strong need for laboratory *in vitro* test systems for the toxicity of airborne particulate matter and nanoparticles. The measurement of oxidative stress potential offers a promising way forward.** ***Objectives:*** **A workshop was convened involving leading workers from the field in order to review the available test methods and to generate a Consensus Statement.** ***Discussions:*** **Workshop participants summarised their own research activities as well as discussion the relative merits of different test methods.** ***Conclusions: In vitro*** **test methods have an important role to play in the screening of toxicity in airborne particulate matter and nanoparticles.** ***In vitro*** **cell challenges were preferable to *in vitro* acellular systems but both have a potential major role to play and offer large cost advantages relative to human or animal inhalation studies and animal *in vivo* installation experiments. There remains a need to compare tests one with another on standardised samples and also to establish a correlation with the results of population-based epidemiology.**

PREAMBLE

There is extensive epidemiological evidence associating ambient particulate pollution with adverse health effects in humans (Schwartz et al., 2002). Nevertheless, fundamental uncertainty and disagreement persist regarding what physical and chemical properties of particles (or unidentified confounding environmental influences) can impact health risks, what pathophysiological mechanisms are operative, and what air quality regulations should be adopted to deal with the health risks. The mechanisms of PM related health effects are still incompletely understood, but a hypothesis under investigation is that many of the adverse health effects may derive from oxidative stress, initiated by the formation of reactive oxygen species (ROS) at the surface of and within target cells. There is a growing literature on specific health effects in association with cellular oxidative stress including the ability of PM to induce pro-inflammatory effects in the nose, lung and cardiovascular system. High levels of ROS cause a change in the redox status of the cell and its surrounding environment, thereby triggering a cascade of events associated

with inflammation and, at higher concentrations, apoptosis (Xiao et al., 2003; Li et al., 2002a, b; Squadrito et al., 2001; Schafer et al., 2003). Consequently, tests designed to quantify the potential of particles to exert oxidative stress have been developed, and are being used in a comparative manner to evaluate those particle properties most influential in particle toxicity. In the field of nanotechnology, there is an urgent need to develop test methods capable of predicting the risks associated with exposure to engineered nanoparticles. Tests developed for airborne particulate matter should be well suited to this purpose.

This paper summarises the talks and discussion that took place at a meeting held in London in November 2006 to address oxidative potential tests for airborne particles. The meeting took the form of short lectures from leading workers in the field describing their own research activities, followed by discussion, structured so as to produce a consensus statement.

INTRODUCTION

Ken Donaldson

Measuring the levels of pollution in the air provides a measure of exposure that is used as a surrogate for risk. Such air quality data are related to adverse health endpoints in numerous studies. It is also used to provide advice to susceptible groups on how to manage their risk. It follows that the closer the metric is to the actual harmful component of the exposure, the better the risk management and the relationship to adverse health effects in epidemiological studies are likely to be.

When exposure is transmitted into internal dose, mechanistic toxicologists, whose job is to address biological mechanisms, identify the true harmful entity in the dose as the *biologically effective dose* (BED). The BED is the entity that drives the adverse effect(s). The gap between the BED and the total dose, as derived from the exposure metric, can be considerable, especially in the case of ambient particles. Particles are heterogeneous in size and composition, undergo dynamic clearance and can be physicochemically complex. Dosimetry models in combination with knowledge of clearance mechanisms allow estimation of dose and dose rate (Schlesinger and Cassee, 2003), but these require knowledge of size distribution and chemical speciation. In recent decades, the mass of particulate matter measured by the PM_{10} or $PM_{2.5}$ conventions has been the metric of choice for ambient particles and has proved useful in demonstrating associations with a wide range of health outcomes, including mortality and morbidity among patients with cardiovascular and/or respiratory diseases (Brunekreef and Holgate, 2002; Pope and Dockery, 1999).

It can, however, be argued that PM_{10} and $PM_{2.5}$ mass are not ideal but represent some surrogate for the BED. This argument is based on the fact that much of the ambient particle mass consists of low toxicity components such as ammonium sulphates and nitrates, sea salt (sodium chloride), crustal dust and soil (Harrison et al., 2003). These contribute substantially to the mass metric but, except in rare circumstances, do not contribute substantially to the BED. In contrast, relatively tiny masses of transition metals and organic species may make a major contribution to the BED (Donaldson et al., 2005; Xia et al., 2006b).

Our understanding of the BED for PM remains hypothetical but oxidative stress has gained importance since the seventies as a central mechanism for the harmful effects of a range of particles at the cellular level (Table 1). Oxidative stress links the physicochemical activities of particles and the pathophysiological mechanisms underlying the common diseases that particles influence.

Put simply, components of particles have the potential to generate free radicals in the lung environment and thereby cause oxidative stress; oxidative stress is an important mechanism leading to inflammation (Donaldson et al., 2003) and inflammation plays a key role in airways disease and coronary heart disease (CHD), the diseases found in the main populations susceptible to the effects of PM. Inflammation is a well-documented feature of asthma (Li et al., 2003b; Walsh, 2006), COPD (O'Donnell et al., 2006) and coronary heart disease (Lucas et al., 2006) being central to their development and oxidative stress is made worse by inflammation through the oxidative activities of inflammatory leukocytes. Oxidative stress is also readily measurable in airways disease (MacNee, 2001) and CHD (Chen and Mehta, 2004). Lung cancer, another endpoint related to increased PM levels in chronic studies can have oxidative stress as an important factor in its causation, especially when caused by particles (Figure 1) (Knaapen et al., 2004).

TABLE 1
Examples of mechanisms by which particles generate oxidative stress

Exemplar particle	Mechanism of generation oxidative stress	Reference
Quartz	Chemical groups on fracture surfaces	Fubini, 1998
Welding fume, PM_{10}, asbestos	Fenton chemistry	McNeilly et al., 2005; Gilmour et al., 1996; Lund and Aust, 1991
DEP, PM_{10}	Organic chemical redox cycling e.g. quinones	Squadrito et al., 2001; Aust et al., 2002|; Nel et al., 2006; Xia et al., 2006b; Li et al., 2003a

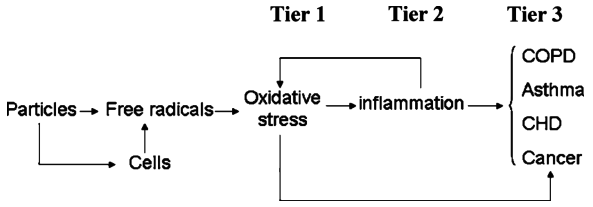

FIG. 1. Schematic of disease induction pathway from particle exposure.

For some time oxidative stress has been considered the dominant hypothesis of the BED of ambient particles supported by good toxicological and clinical evidence (Nel, 2005). The measurement of the oxidative potential of ambient particles would represent a more refined metric, bringing it closer to the BED with anticipated improvements in risk management and better associations with adverse health effects in epidemiological studies.

PARTICLE SAMPLING FOR *IN VITRO* TESTING OF PM OXIDATIVE PROPERTIES

Constantinos Sioutas

The ambient atmosphere is a dynamic system, in which the mixture of air pollutants changes over time. The fluctuation of important atmospheric parameters influencing the ambient PM concentrations (hence human exposure), including emission strengths of particle sources, temperature, relative humidity wind direction and speed, and mixing height, in time scales that are substantially less than a few hours. Ideally, PM collection for measurement of their oxidative potential should be conducted using direct and on line methods. Nevertheless, technologies allowing these on-line measurements are currently not available, and therefore particulate matter must first be collected before it can be assayed for oxidative potential.

In vitro assays, which are used for measuring the ROS content of PM generally, require high quantities (order of several mg of PM) for chemical and biological analyses. Often, high volume filter samplers are used, frequently preceded by a preselective PM inlet that removes particles larger than 10 or 2.5 μm in aerodynamic diameter, thereby allowing size fractionated sampling on surfaces during a long period. PM is typically collected on substrates or filters such as quartz, Teflon, and polyurethane foams (PUF). Once PM has been collected, vigorous methods are needed to remove the PM from the collection substrate (filters, foams) into suspension. Despite its simplicity and widespread use for PM sampling, filtration suffers from several drawbacks when used for *in vitro* studies. The first issue relates to the choice of the extraction solvent. If deionized, ultra pure water is used (a very common approach), insoluble PM bound species, which may be toxicologically important, will very likely not be extracted. To overcome this problem, organic solvents such as dichloromethane have been used. Removal of the organic solvent is necessary prior to the in vitro bioassay, given that the solvent itself may be toxic to cell cultures or elicit significant biological responses. This is normally done by means of lyophilization. This process will undoubtedly remove potentially toxic PM-bound labile species, such as semi-volatile organics. The sonication process itself may introduce sampling biases, including incomplete particle removal, physical changes (agglomeration, possibly de-aggregation) as well as altering the chemical or biological properties of PM. Furthermore, quartz filters tend to break up into fibers that need to be removed and separated from the PM suspension. The use of PUFs has the disadvantages of inadvertent trapping of some vapor phase organics as well as incomplete ultrafine particle collection when used as filters. An extensive literature of over 100 publications discusses sampling artifacts associated with the use of filters as PM collectors (e.g., Schauer et al., 2003; Eatough et al., 2003). These include loss of labile species, such as ammonium nitrate and more importantly organics from PM on the filter during prolonged sampling periods; adsorption of vapor phase organics (for quartz filters); reactions between particle and incoming gases (for all filters), for example reduction and transformation of PAH with O_3 to oxy-PAH (Tsaparakis et al., 2003).

To overcome some of the disadvantages associated with filtration, novel approaches have been developed, collecting particles in a fluid using a combination of particle concentration, followed by impaction and centrifugation as physical principles (Kim et al., 2001a, b). These particle concentrators are portable and have been demonstrated to increase ambient particle levels by enrichment factors up to 40 without significantly affecting particle properties such as size (Misra et al., 2004), bulk chemistry (Kim et al., 2001b; Khlystov et al., 2005) or single particle chemistry (Zhao et al., 2005) and morphology (Kim et al., 2001b). These concentrators can be used to provide elevated ambient PM exposures to animal or human subjects, as well as to collect a large amount of PM material in aqueous solution suitable for subsequent toxicological assays. Highly concentrated liquid suspensions of these particle modes are obtained by connecting the concentrated output flow from each concentrator to a liquid impinger (BioSampler^{TM}, SKC West Inc., Fullerton, CA). Particles are injected into the BioSampler^{TM} in a swirling flow pattern so that they can be collected by a combination of inertial and centrifugal forces. This inertia-based collection mechanism, coupled with the short residence time (i.e., order of 0.2 seconds) of particles and gases in the Biosampler precludes any inadvertent trapping of gaseous co-pollutants in the particulate layer (Khlystov et al., 1995). The main advantage of these technologies over filtration is that PM collection resembles a system closer to real world exposure and deposition onto human cells in respiratory system. Detailed studies have revealed very few or no artifacts during PM collection. Moreover, the concentration enrichment process minimizes volatilization losses in conventional particle collectors, such as impactors and filters, from $\sim$ 50–70% to less than 10%, as demonstrated by Chang et al. (2000). Disadvantages of these technologies include the fact that their operation is quite complicated, thus requiring fairly skilled

personnel. Moreover these systems have not been designed for automated use, therefore they cannot currently be used for continuous, unattended sampling over several days.

An alternative method for PM collection for *in vitro* toxicity studies is particle collection by impaction (Chang et al., 2001). This method has the advantage of a much smaller collection surface area (10–50 fold) over filtration, which in turn makes extraction easier, as particles are collected on top of a flat Teflon or aluminum surface, and not inside the fibers of a filter. Additionally, since the air flows around the collected PM and not through them (as in filtration) there is no adsorption of vapors onto the particle layer. The formation of the stagnation diffusion boundary layer around the particle collection area also appears to reduce losses of labile species by volatilization (Wang and John, 1988) by hindering mass transfer from the collected PM layer to the surrounding air stream. In conventional impactors, the lowest cutpoint is about 0.15 μm, which limits particle collection to the accumulation mode range of atmospheric PM. However, newer technologies such as the NanoMOUDI Cascade Impactor (MSP Corp) have a lower cutpoint of 10 nm, effectively capturing almost the entire ultrafine mode. The disadvantage of the NanoMOUDI is that it only samples at 10 L/min, which limits its ability to collect sizable amounts of PM within a reasonable time frame. Geller et al. (2002) overcame this problem by using the NanoMOUDI in conjunction with the particle concentrator noted earlier. By concentrating ambient particles in the 0.018–2.5 μm range by a factor of 20–22, Geller et al. measured size fractionated chemical speciation of ultrafine PM in the Los Angeles in sampling periods of 2–3 hours.

An interesting approach for measuring ambient bioaerosols using a modified electrostatic precipitator (ESP) was developed by Mainelis et al. (2002). In this sampler, an ionizer charges the incoming particles, which are then subjected to a precipitating electric field and are collected onto small square agar plates positioned along the flow axis. The original system, designed for sampling of microorganisms, collects >90% of PM at a flow of 4 L/min. This configuration may allow direct collection of particles onto cell cultures for *in vitro* testing of their redox properties. Because particle-laden air flows over the cell cultures at quasi-ambient relative humidity, particle collection using this method would be limited to at most 1 hr in order not to compromise the cell viability. In this case, the nominal sampling flow rate of this device may be insufficient for collecting an adequate PM mass for in vitro studies. However, similar to the case of the NanoMOUDI, using this ESP in conjunction with a particle concentrator could increase the sampling flow to 200–300 L/min and collect PM into the same small surface area designed for cell cultures, thereby making it possible to conduct these tests in short time periods.

In conclusion, despite its simplicity, particle collection by means of filtration remains problematic for use in toxicological testing. In general, biological outcomes for PM collected by filtration methods may not always agree with the use of other methods, including the concentrator-BioSampler tandem and could be bioassay specific. For instance, if the bioactivity is derived from water-soluble PM species, the two methods could yield similar results, but the outcome could be very different for compounds that are not water-soluble. In any other case, they will probably not. Given the nature of the discrepancies between the two methods, possibly related to sampling artifacts, it will be very difficult, if not impossible, to obtain even a modest correlation between the two. Other PM collection methodologies, including concentration enriched impactors and ESP, should be considered and pursued.

TESTS FOR OXIDATIVE STRESS POTENTIAL ANTIOXIDANT DEPLETION AS A MEASURE OF PARTICLE OXIDATIVE ACTIVITY

Frank J. Kelly, Sean Duggan and Ian S. Mudway

Ambient air contains a range of pollutants, the exact combination of which varies from one microenvironment to the next. Many of the individual pollutants that make up this ambient mix are free radicals, for example, nitrogen dioxide, or have the ability to drive free radical reactions, such as, ozone and ambient particulates. As a consequence, exposure to a wide range of air pollutants has the potential to give rise to oxidative stress within the lung. Inhaled particles generate oxidative stress through three inter-related pathways: firstly, by direct introduction of oxidising species into the lung, such as redox active transition metals (Mudway et al., 2004) or quinones (Squadrito, 2001; Li et al., 2003b; Xia et al., 2006b) absorbed on the particle surface. The second is by introducing surface adsorbed PAHs that can undergo bio-transformation *in vivo* into reactive electrophiles and quinones through the action of the cytochrome P450, epoxide hydrolase and dihydrodial dehydrogenase detoxification pathway (Bonvallot, 2001; Li et al., 2003a), and the third by stimulating inflammatory cells to undergo oxidative burst activity or upregulate inducible nitric oxide synthase and cause nitric oxide production (Porter et al., 2007). In healthy individuals the potential of inhaled particles to induce oxidative injury is constrained by endogenous extra- and intra-cellular antioxidant defenses, many of which are induced as an adaptive response to subtle changes in cellular redox status (Li et al., 2003b; Xia et al., 2006b). Hence, the capacity of ambient PM to elicit injury represents a function both of their inherent pro-oxidant and pro-inflammatory properties, but also the robustness of an individual's antioxidant defenses. This may in part explain the enhanced sensitivity of asthmatics to air pollutants, due to their impaired antioxidant defences at the air-lung interface (Kelly et al., 1999; Li et al., 2003b). Furthermore, numerous trials have shown that increased antioxidant intakes reduce air pollution related symptoms and lung function decrements (Romieu et al., 1998, 2002; Grievink et al., 1999; Samet, 2001) consistent with the view that oxidative stress is involved in these health impacts.

To quantify the oxidative potential of ambient PM, as well as to address the components driving the observed activity, our laboratory has established an *in vitro* screening system, which

involves the incubation of PM samples within a synthetic respiratory tract lining fluid (Zielinski et al., 1999; Mudway et al., 2004). The respiratory tract lining fluid (RTLF), represents the first physical interface encountered by inhaled materials and has been shown to contain high concentrations of the antioxidants ascorbate (vitamin C) (Willis and Kratzing, 1974; Skoza et al., 1983; van der Vleit et al., 1999), urate (Peden et al., 1990), and reduced glutathione (GSH) (Cantin et al., 1987; Jenkinson et al., 1988). Hence, examining the extent to which PM depletes antioxidants from this model with time (37°C, pH7.4) provides not only a quantitative output of activity, but also reflects reactions likely to occur *in vivo* at the air-lung interface. Following extraction of PM from a variety of filter matrices, particle suspensions are added to the synthetic RTLF, containing the equimolar concentrations of the antioxidants urate, ascorbate and glutathione (200 μM), at PM concentrations ranging from 10–150 μg/ml. The capacity of the particles to deplete ascorbate and reduced glutathione from this model is then monitored over a 4 h period, with the final concentrations of these antioxidants quantified by reverse phase high-pressure liquid chromatography with electro-chemical detection (Iriyama, 1984) and the enzyme recycling method of Tietze (Baker, 1990) respectively. Further characterization of the oxidative activity can be achieved by performing co-incubations with metal ion chelators such as ethylenediaminetetraacetic acid (EDTA), desferral (DES) and diethylene triamine pentaacetic acid (DTPA), as well oxidant depletion enzymes superoxide dismutase (SOD) and catalase (CAT) and the hydroxyl radical scavenger dimethyl sulfoxide (DMSO). Given that any measure of particle oxidative capacity needs to be robust over time it is important to ensure intra-assay standardization between experiments. To achieve this, we routinely run a number of particle-free and particle controls, the later consisting of residual oil fly ash (ROFA), as a positive control and an inert carbon black as a negative control (Zielinski, 1999). Blank filters or foams are also routinely extracted and run through the assay system. The results of a typical screening experiment using an ambient fine PM sample ($PM_{0.1-2.5}$) collected using a high volume impactor are illustrated in Figure 2.

These data demonstrate significant losses of both ascorbate and glutathione from the synthetic RTLF at the lowest dose examined (50 μg/mL), with the losses significantly greater than those observed with an equal dose of the positive control particle ROFA. Co-incubation of the particles with EDTA provided full protection against GSH losses, but no protection against ascorbate depletion over the 4h incubation was observed. Deferral, in contrast, provided only slight protection against PM-induced ascorbate losses, and none against GSH induced oxidation, whilst DTPA conferred full protection against the losses of both antioxidants from this system. Due to difference in the stability constants between these chelators and a variety of metal ions, allied to their capacity to redox inactivate metals, the pattern of protection seen with these chelators can be used to establish which metals are likely to be driving the observed oxidative activity. For example, whilst EDTA will complex both Fe and Cu, it will only redox inactivate the latter, whilst DTPA will complex both these metal ions and prevent their participation in the catalytic oxidation of ascorbate and glutathione (Buettner and Jurkiewicz, 1996). Hence the profile of responses illustrated in Figure 2 implicates Cu as a key driver of the oxidative losses of both antioxidants.

In addition to the partial discriminatory role of these chelators different antioxidants within the synthetic RTLF appear sensitive to oxidation by different metals. For example, using a variety of Fe, Cu and Zn salts we were able to show that Fe had little effect on glutathione, whilst causing a dose dependent loss of ascorbate. Cu in contrast depleted both ascorbate and glutathione to similar extents, whilst redox inactive Zn salts had no effect on either antioxidant over a 4h incubation period (Figure 3). Co-incubations with SOD, plus CAT with the ambient $PM_{0.1-2.5}$ samples in Figure 2 resulted in 57.7 and 60.8% inhibition of glutathione and ascorbate losses respectively relative to the 4h particle-free control. This reflects the fact that these antioxidants are consumed both by their reduction of metal ions in solution, but also by the superoxide subsequently formed during the reoxidation of these metals in the aerobic environment. Hence fully chelating the metals effectively prevents all metal-dependant oxidation, whilst superoxide, hydrogen peroxide scavengers only provide approximately 50% protection, assuming a minimal involvement from organic radicals. Interestingly in this model we have found no evidence that the hydroxyl radical scavenger DMSO prevents particle-induced antioxidant oxidation.

In addition to this screening approach a more simplified ascorbate-only model can be employed to determine the rate of ascorbate depletion by particle suspensions with time by following the decrease in absorbance at 265 nm. This ascorbate-only model provides an alternative high throughput method to the use of synthetic RTLF utilizing 96 well UV plates and two-hour incubation periods. We have used this method to rapidly screen ambient PM samples for their total, metal-dependent and metal-independent oxidative activities, employing DTPA to isolate the metal signature. Figure 4 illustrates that whilst DTPA will fully inhibit the catalytic oxidation of ascorbate by Fe and Cu salts, it does not inhibit quinone dependent oxidation, as long as the pH of the incubation medium is carefully controlled.

Determination of antioxidant depletion using the two models outlined above provides a robust, rapid and highly repeatable acellular screening method for obtaining quantitative measures of PM oxidative potential on an equal mass basis. To date we have utilised these methods to screen ambient PM_{10}, $PM_{2.5}$, $PM_{0.1-2.5}$ and $PM_{2.5-10}$ samples (Kunzli et al., 2006; Mudway et al., 2004; Mudway et al., 2005; Sandstrom et al., 2005), but the models are equally applicable to ambient ultrafine particles, or for the assessment of novel engineered nanoparticles. It should be noted however, that whilst these methods quantify inherent oxidative potential, i.e. that attributable to their content of pro-oxidant moieties, it does not reflect the total oxidative activity that requires the PM interaction with the cellular/tissue matrix to be considered. Despite this caveat, we believe that the depletion

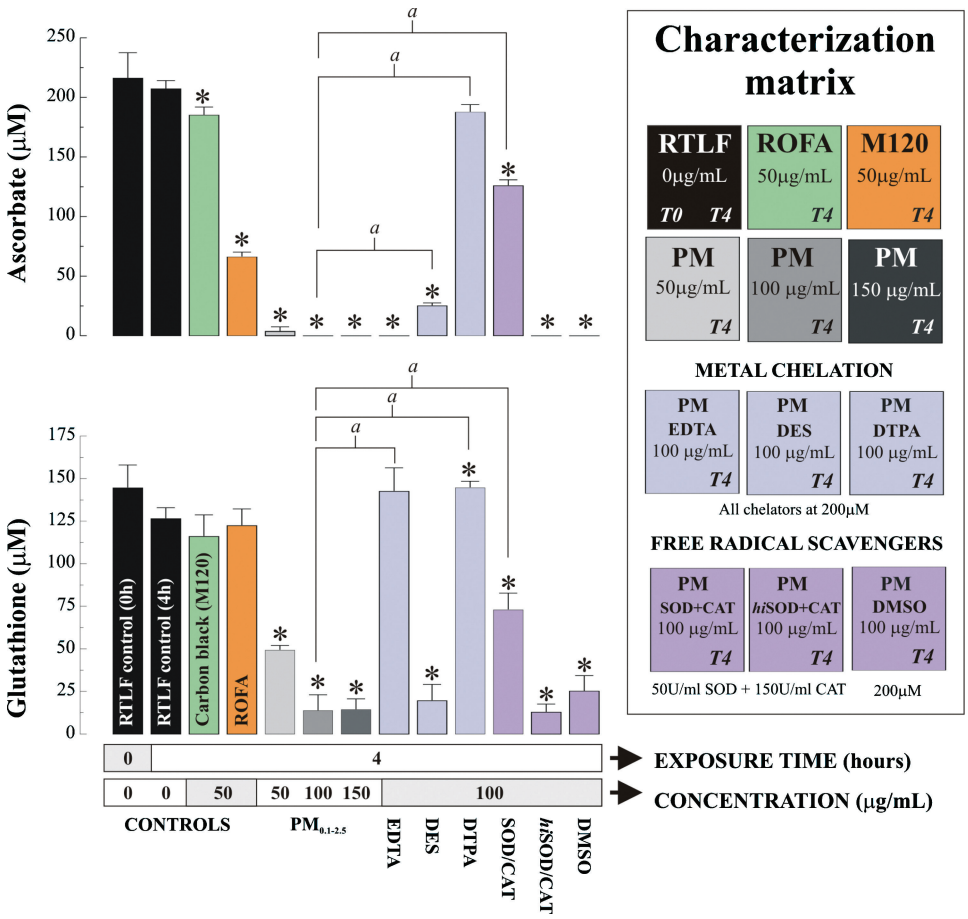

FIG. 2. Ascorbate and glutathione concentrations in a synthetic RTLF following a 4-h incubation with various concentrations of ambient $PM_{0.1-25}$ (50–150 μg/mL). Where PM–RTLF mixtures were co-incubated with metal chelators or free-radical scavengers this is illustrated, with additional details given in the sample characterisation matrix in the right hand panel. All data are represented as means ± SD of triplicate incubations. Comparison of concentrations in the treatment groups relative to the 4-h particle free RTLF control were performed using a two-way ANOVA with factors of concentrations and treatment. Post hoc comparison of groups was performed using the Student-Newman-Kuels test. '*' Indicates that ascorbate or glutathione concentrations in the treatment groups were significantly different ($P < 0.05$) than the 4h particle free control value; 'a' illustrates that the concentrations of antioxidants following chelator or free radical scavenger treatment were significantly different than those following incubation with 100 μg/mL $PM_{0.1-2.5}$ only. **KEY**: *hi* SOD/CAT — heat inactivated antioxidant enzyme control (95°C for 30 minutes).

of physiological antioxidants is a useful, and biologically meaningful measure of oxidative potential and would provide a useful component for future screening protocols aimed at identifying the toxic components of respirable ambient PM and potentially hazardous nanoscale materials.

OXIDANT GENERATION OF PARTICULATE MATTER BY MEASURING HYDROXYL RADICAL GENERATION IN OXIDANT CONDITIONS

Paul J.A. Borm

In the context of both toxicological and epidemiological research, it is well accepted that PM_{10} mass is not an ideal metric but represents some surrogate for the real causative components in PM. Relatively tiny masses of transition metals and organic species may redox-cycle and make a major contribution to the effects of PM (Kelly, 2003; Li et al., 2003b; Xia et al., 2006b). So, although our current and future PM standards are set on mass, we know that it is a surrogate of the biologically effective dose, at best, as most of the mass is actually biologically inactive. In fact, studies have shown that the particle number, which is not necessarily related to mass, can be a better descriptor of some health effects (Donaldson et al., 2005; Peters et al., 1997). This can be explained by the fact that combustion-derived nanoparticles (CDNP), the dominant particle type by number in urban air, represent a key component of the PM mix because they contain a

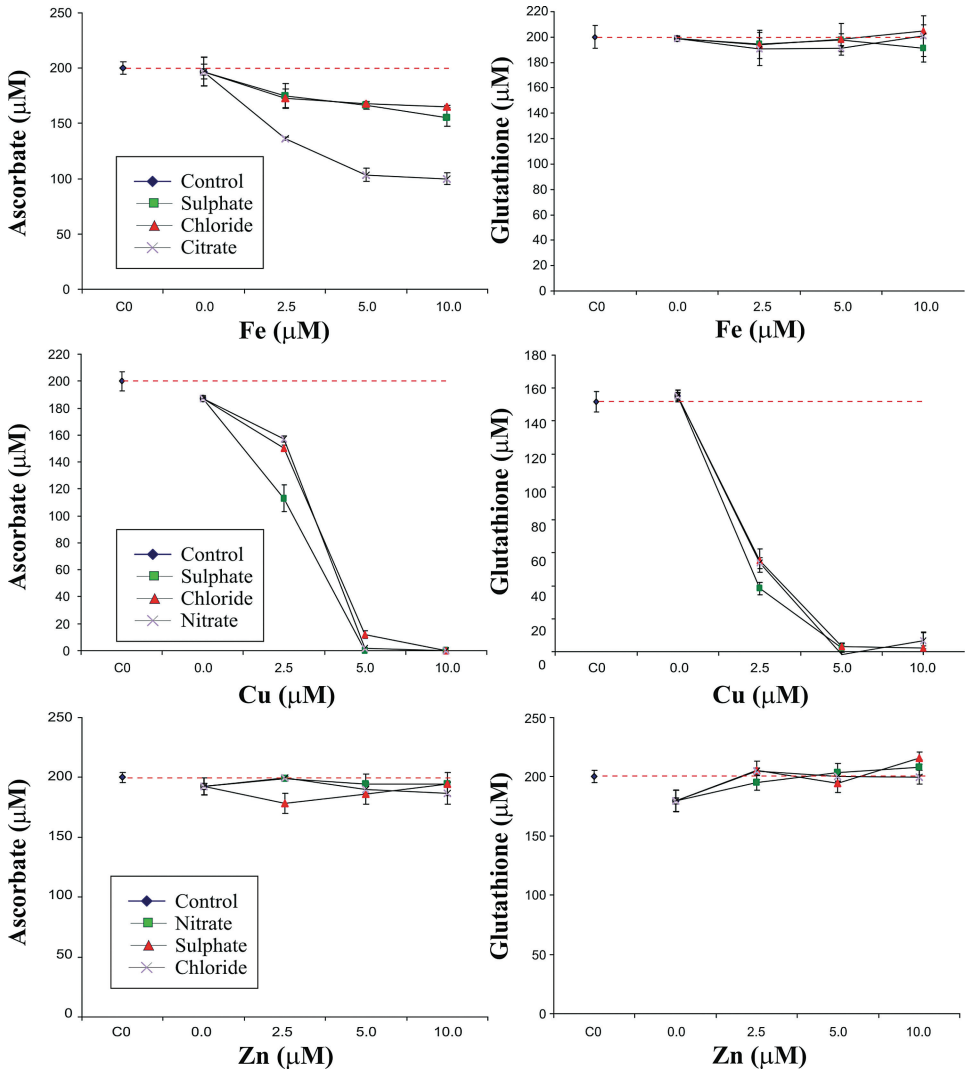

FIG. 3. Concentration of ascorbate and glutathione remaining in a synthetic RTLF following a 4 h incubation in the presence of a variety of Fe (III), Cu (II) and Zn (II) salts (37°C, pH7.4). All data are represented as means ± SD of triplicate incubations.

large *surface area*, *transition metals and organics*. Experimental studies have demonstrated that these components play a role in the pro-inflammatory effects of PM and model particles in animal and in vitro models, (reviewed in Donaldson et al., 2005). A common mechanism linking these parameters is their ability to generate oxidative stress both by direct generation of reactive oxygen species (ROS) and indirectly through induction of inflammatory responses in the lung. In fact, ROS production has been suggested as a unifying factor in the biological activity of pathogenic particles and ambient air pollutants in general (Donaldson et al., 1996; Nel et al., 2006). The measurement of oxygen radical generation as an indicator of PM's intrinsic toxicological hazard has features that make it highly advantageous as it integrates a number of aspects, including (i) redox activity of bound and soluble transition metals, (ii) the bioavailability of these metals for reaction, (iii) interactions between different metals in the reaction, (iv) redox cycling by complex organic contaminants, and (v) oxidative stress delivered by surfaces.

To measure the oxidative potential of particles on filters we have developed and validated over the past 5–7 years a method which recovers PM from filters by sonication in water, addition of hydrogen peroxide to the resulting suspensions to produce reducing conditions similar to those that pertain in the lungs and detection of very-reactive OH-radicals by a specific spin-trap (DMPO) and electron paramagnetic resonance (Shi et al., 2003). Although this system is highly artificial, it was recently shown that this method of measuring OH-generation is strongly correlated to depletion of antioxidants such as ascorbate and GSH in a reducing environment (Kunzli et al., 2006), and to the induction of oxidative DNA damage in lung epithelial cells *in vitro* (Shi et al., 2006). The significance of this method was also shown to be relevant in several field studies in Germany, Netherlands

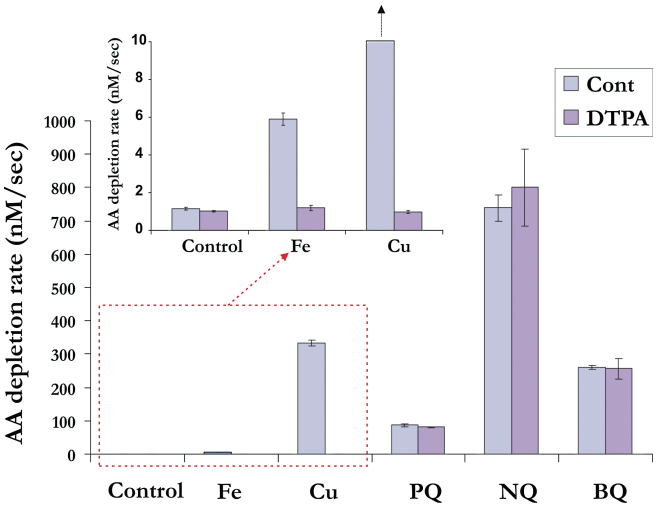

FIG. 4. Inhibition of ascorbate depletion by the redox active metals Fe(10 μM $Fe(NH_4)_2(SO_4).6H_2O$) and Cu (10 μM $CuSO_4.5H_2O$), as well as the redox-cycling quinones 9,10-phenatroquione (PQ–1 μM), 1,4 naphthoquinone (NQ–1 μM) and 1,4-benzoquione (BQ 1-μM) with the addition of the metal chelator DTPA (final concentration 200 μM). All data represent the mean (SD) of triplicate incubations.

and Europe. In the latter study oxidant generation was measured in 716 samples of $PM_{2.5}$ sampled over a two-year period in 20 European cities (Kunzli et al., 2006). The ultimate proof was given by a volunteer study in which 12 normal individuals were instilled with 100 μg of $PM_{2.5}$ from either a polluted or a non-polluted city in two different bronchial segments (Schaumann et al., 2004). Although all samples were delivered at equal mass, the oxidant activity of the samples was different, and pulmonary inflammatory response reflected this difference in a higher cell-count and cytokines in the segment instilled with the PM with higher oxidant activity (Schaumann et al., 2004).

We do not believe that this measure should replace the PM_{10} or $PM_{2.5}$ metrics at present but we do believe that a complementary metric that more closely approaches the BED should have intriguing scientific merit in testing the 'oxidative stress hypothesis' more specifically, both in the total population as well as in subgroups particularly susceptible to oxidative stress.

ACELLULAR AND CELLULAR ASSAYS FOR DETERMINING THE OXIDATIVE POTENTIAL OF AMBIENT PM

Andre Nel, Art Cho, John Froines and Costas Sioutas

Background

The Southern California Particle Center (SCPC) has developed a number of *in vitro* assays to determine the oxidant potential of ambient PM under abiotic and biotic conditions. The ability of ambient PM to generate reactive oxygen species (ROS) under these conditions constitutes one of the important predictors of ambient particle toxicity. Other physicochemical characteristics that are predictive of adverse biological effects in an urban environment such as Los Angeles include a small particle size and a large surface that is coated with bioavailable redox cycling organic chemical compounds and transition metals (Xia et al., 2006b; Li et al., 2003b).

Acellular Assays

In order to establish abiotic assays that provide a rapid readout of the oxidant potential of ambient particulate matter, the SCPC has developed a number of quantitative assays that reflect the chemical properties of ambient particles that are responsible for their ability to induce ROS production and oxidative stress under biological conditions. Oxidative stress refers to the cellular response as a result of the change in the redox status of the target cell. A key indicator of the redox equilibrium in the cell is the ratio of oxidized to reduced thiol antioxidants (Schafer and Buettner, 2001). Of particular importance is the ratio of reduced to oxidized glutathione (GSH/GSSG); this redox couple constitutes one of the most important homeostatic regulators of the redox balance in the cell (Schafer and Buettner, 2001). Changes in this ratio occur through the oxidation of GSH to GSSG by reactive oxygen and reactive nitrogen species as well as through GSH conjugation with electrophilic agents. PM-catalyzed electron transfers from cellular reductants such as NADPH to molecular dioxygen (O_2) lead to the formation of ROS. Initially, this consists mostly of the superoxide radical that subsequently comproportionates to hydrogen peroxide (Xia et al., 2006b). Hydrogen peroxide can be further reduced to the highly reactive hydroxyl radical by reduced transition metal ions such as Cu^{I} or Fe^{II} in the Fenton reaction. Conjugation of electrophilic species in PM occurs through the reaction of thiolate species with the electrophile.

To develop assays for the capacity of a PM sample to induce oxidative stress, three reactions have been utilized, namely: (i) PM-catalyzed DTT consumption, (ii) PM-catalyzed dihydroxybenzoate formation, and (iii) inactivation of glyceraldehyde-3-phosphate dehydrogenase.

The DTT Assay

This assay is based on the ability of redox active compounds associated with PM to transfer electrons from the dithiol, dithiothreitol (DTT), to oxygen (Figure 5). This generates superoxide that subsequently comproportionates to hydrogen peroxide and oxygen (Li et al., 2003a; Cho et al., 2005). The rate of this reaction is monitored by DTT consumption, determined by measuring the non-reacted DTT with the thiol reagent, 5,5′dithiobis-2-nitrobenzoic acid (DTNB) (Figure 5). Under the conditions of the assay, the reaction is proportional to the concentration of the redox active species. The sensitivity of the assay is due to the catalytic nature of the process. DTT consumption over time

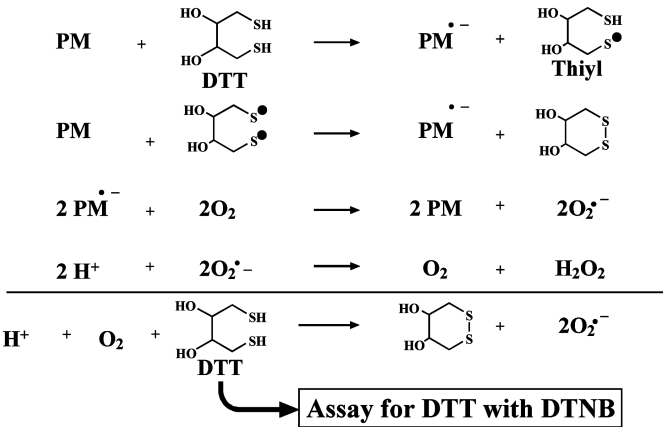

FIG. 5. Chemical basis of the DTT (dithiothreitol) assay. The DTT assay quantitatively measures the formation fo ROS by redox cycling chemicals such as quinines. The loss of DTT is followed by its reaction with 5,5′-dethiobis-(2-nitrobenzoic acid), (DTNB), which is converted to 5-mercapto-2-nitrobenzoic acid (Kumagai et al., 2002). The PM sample (5–50 μg/ml) is incubated with 10 μM DTT in a Tris buffer at pH 8.9 for 10–90 minutes. Aliquots of the incubation mixture are transferred to the DNTB solution and the optical density read at 412 nm.

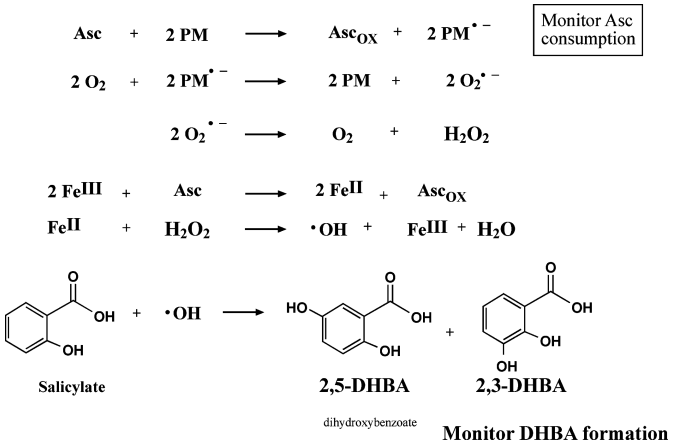

FIG. 6. Chemical basis of the ascorbate-dihydroxybenzoate (DHBA) assay.

constitutes the rate of the reaction. However, the rate is also a limitation of the assay, since incubation times of up to 45 minutes are needed to achieve significant consumption compared to the rate observed in the absence of sample. The reaction is associated with organic components, since it is unaffected by addition of the metal chelator, diethylenetriaminepentaacetic acid (DTPA). Catalase, which removes hydrogen peroxide, also does not affect the measured activity, indicating that hydrogen peroxide does not contribute significantly to DTT consumption. A methanol extract of diesel exhaust particles is used as a standard to monitor the consistency of each assay (Li et al., 2003a; Cho et al., 2005).

Ascorbate-Dihydroxybenzoate Based Redox Activity

This assay is based on the reaction between reduced transition metals such as Cu^{I} and Fe^{II} and hydrogen peroxide to generate the highly reactive hydroxyl radical. Hydroxyl will react rapidly with a substrate such as salicylic acid to form several dihydroxy benzoate isomers, mostly the 2,3- and 2,5 dihydroxybenzoates (DHBAs) (Coudray and Favier, 2000; Themann et al., 2001). The quantities of DHBAs at a given time are assayed by HPLC with electrochemical detection (Figure 6). As most metal ions in PM are likely to be oxidized, ascorbate is added to reduce them and to generate hydrogen peroxide by the reduction of oxygen. Redox active organic compounds such as quinones will consume ascorbate but do not generate DHBA. In our studies, we have found the consumption of ascorbate to be highly variable whereas DHBA formation is consistent and reproducible with Fe^{II} at 2 μM, which is used as a standard. DHBA formation is blocked by metal chelation with DTPA and by catalase, which consumes the peroxide. A detailed protocol for the procedure has not yet been published. The reaction and the detection of DHBA have been used by other atmospheric investigators (Donaldson et al., 1997; Liu et al., 2003) to assess redox capacity.

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Inactivation

This assay is based on the reaction between an electrophile and a reactive thiolate in GAPDH. The thiolate in the enzyme forms a covalent bond with electrophiles such as acrylonitrile (Campian et al., 2002) and certain quinones (Rodriguez et al., 2005). The reaction is direct and does not require oxygen, so the procedure is performed under anaerobic conditions to avoid complications from a second reaction, in which hydrogen peroxide reacts with the thiolate to form a sulfenic acid. This assay is still in the development stage; we are trying to establish conditions for a high throughput protocol. A series of controls are needed to monitor the assay for its consistency; they include the appropriate concentration of N-ethylmaleimide, a standard electrophile, oxygen removal procedures, the quantity of enzyme needed and a suitable preparation for use as a standard.

Cellular Assays for ROS Production and Oxidative Stress

The origins of PM-induced ROS in the target cells are from mixed subcellular sources (Nel, 2005; Xia et al., 2006b; Hiura et al., 1999). These include: (i) catalytic conversion of PAHs to quinones by cytochrome P450 1A1 in the endoplasmic reticulum; (ii) quinone and transition metal redox cycling that could involve NADPH dependent P450 reductase in microsomes; (iii) mitochondrial perturbation leading to electron leakage in the inner membrane (Hiura et al., 2000; Xia et al., 2004); (iv) NADPH oxidase activation on the cell membrane or the phagosome of macrophages.

The cellular response to oxidative stress includes protective as well as injurious events (Xia et al., 2006b; Li et al., 2003b; Xiao et al., 2003). Proteome and biological analysis of target cell responses to PM-induced oxidative stress led to the characterization of a hierarchical oxidative stress model, which posits that at a lower level of oxidative stress (Tier 1), cells generate protective antioxidant and detoxification enzymes by acting on a genetic response element that require the bZIP transcription factor, Nrf2 (Li et al., 2004; Li et al., 2002a; Xiao et al., 2003). Nrf2 drives the antioxidant response element (ARE) in the promoter of phase II genes, leading to the expression of antioxidant and cytoprotective enzymes (Li et al., 2000; Li et al., 2004). A number of these phase II enzymes in lung target cells have been shown to be responsive to DEP, ambient UFP and organic DEP extracts (Li et al., 2000; Li et al., 2004; Li et al., 2002b). These include HO-1, glutathione-S- transferase (GST), NADPH quinone oxidoreductase (NQO1), catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and UDP-glucoronosyltransferase (UGT). These phase II enzymes protect against oxidative stress injury (Tiers 2 and 3), such that a reduced or compromised Tier 1 response may promote oxidant PM injury. Clinically, a compromise in Tier 1 responses can occur due to phase II enzyme polymorphisms in phase II genes or null genotypes. For instance, the GST M1 null genotype predisposes atopic people to asthma, as well as to an enhanced allergic inflammatory response by DEP challenge in the nose (Gilliland et al., 2004). Conversely, the induction of a phase II response may help people to adapt to a polluted environment, and may explain why only a relatively small number of people in a population develop adverse health effects in response to a sudden rise in ambient PM levels. Moreover, adaptation can explain why repeated low-dose CAPs exposures fail to elicit persistent lung inflammation.

If Tier 1 protection fails, a further increase in oxidative stress could lead to the generation of pro-inflammatory (Tier 2) or cytotoxic (Tier 3) effects at the cellular level. Tier 2 responses are linked to the activation of intracellular signaling pathways that impact cytokine and chemokine gene promoters (Li et al., 2003b; Xia et al., 2006b). An example is activation of the MAP kinase cascades. These cascades are responsible for the expression and activation of AP-1 transcription factors (e.g., c-Jun and c-Fos), which play a role in the transcriptional activation of pro-inflammatory genes, such as the genes encoding for cytokines, chemokines and adhesion molecules (Wang et al, 2005). Tier 3 responses involve mitochondrial perturbation by pro-oxidative chemicals (Li et al., 2003a, Xia et al, 2004). Although the *in vivo* significance of the mitochondrial pathway is uncertain, it has been demonstrated in tissue culture cells that PM interference in mitochondrial electron transfer can contribute to ROS production (described above) as well as the induction of apoptosis. Intact ultrafine particles as well as organic chemicals that have been extracted from DEP can mimic these effects (Li et al., 2003a; Hiura et al., 2000).

A series of cellular assays have been developed that reflect each tier of oxidative stress, and have been shown to be useful for comparing oxidant injury and by a number of ambient and engineered nanoparticles (Nel et al., 2006; Xia et al., 2006a). While useful for the comparison of the oxidant potential of a range of particles, from a practical perspective the most useful analysis has been to screen for heme oxygenase 1 (HO-1) expression at protein as well as RNA level (Li et al., 2000). In addition, the antioxidant response element in the promoter of that gene has been demonstrated to be useful as a luciferase reporter gene that can be used to compare different particle types or aliphatic, aromatic and polar chemical compounds that are extracted from PM (Li et al., 2004). In a comparison of coarse, fine and ultrafine particles in the Los Angeles basin, we have shown that there is a good correlation between HO-1 expression and the oxidative potential of the particles as determined by DTT analysis (Li et al., 2003a). Moreover, the higher PAH content of ultrafine particles calculated on a per mass basis showed an excellent correlation with the higher DTT activity of these particles (abiotic test), as well as their ability to induce HO-1 expression (biological test) (Li et al., 2003a).

PARTICLE-ASSOCIATED ORGANICS AND OXIDATIVE STRESS

Francelyne Marano and Armelle Baeza-Squiban

The fine and ultrafine airborne particles generated by the burning of fossil fuels contain a large amount of organic compounds including polyaromatic hydrocarbons (PAH) and are the most abundant components of $PM_{2.5}$ in urban areas such as Paris. Diesel engine vehicles are a major source. In a kerbside station in Paris more than 50% of particles were close to the ultrafine range ($\leq 0.26\ \mu$m) likely due to the influence of the traffic (Baulig et al., 2004). Chemical analysis of $PM_{2.5}$ collected in a kerbside and a background station in Paris revealed that PAH are twice as abundant in the kerbside station. We have also observed variations of PAH according to the seasons probably due to chemical reactions with atmospheric oxidants. However, PAH are only a part of the organic component of PM and they do not greatly influence the soluble organic fraction (SOF) measured after dichloromethane extraction that appear to be between 10 and 12% of the mass of the particles whatever the station. The samples of $PM_{2.5}$ were also found to differ in their metal contents. Heavy metals (Cd and Pb) are more important at the background station and transition metals (Fe and Cu) at the kerbside station. The evaluation of hydroxyl radical formation, as indicated by DMPO-OH adducts detected by EPR, appears to be a good indicator of the presence of these metals and of their ability to induce oxidative stress (Baulig et al., 2004).

Bioavailability of Organic Compounds

The presence on particles of organic compounds able to participate in the generation of oxidative stress and inflammatory response raises the question of their bioavailability and their metabolisation in the lung. Diaz-Sanchez et al. have published numerous studies on the role of DEP and their associated

polyaromatic hydrocarbons in the induction of allergic airway diseases (Riedl and Diaz-Sanchez, 2005). However the identification of the chemical components involved in these biological effects and the understanding of the underlying mechanisms are still imperfect. Such studies are difficult because of great variability in the chemical composition of PM according to its emission sources, age and site of sampling. For these studies, we have used a human bronchial epithelial cell line (16 HBE) and primary cultures of nasal human epithelial cells. In these cells, foreign substances are detoxified by two sequential reaction processes: namely Phase I and Phase II metabolic enzymes. Among the members of the *CYP* gene family (Phase I), *CYP1A1* is known to be induced by PAH through a receptor-dependent mechanism. The cytosolic aryl hydrocarbon receptor (AhR) when bound by PAH, translocates to the nucleus, heterodimerizes with another partner and activates the transcription of CYP1 family genes through binding to the xenobiotic response element. Native DEP and PM and their respective extracts act as activators of the AhR, inducing CYP1A1 expression and activity (Bonvallot et al., 2001; Baulig et al., 2003a). As shown in Figure 7A DEP and their organic extract induce a transient CYP1A1 mRNA expression in human bronchial epithelial cells (HBE) similar to B(a)P whereas carbon black particles have no such effect (Baulig et al., 2003a).

The genes of the Phase II metabolic pathway (GST, NQO-1) are regulated in a concerted manner at the transcriptional level through the antioxidant-responsive element (ARE)/electrophile-responsive element. The transcription factor Nrf2 is central to ARE-mediated gene expression. DEP induce the translocation of Nrf2 to the nucleus of HBE cells, increase nuclear protein binding to the ARE (Baulig et al., 2003a) as well as NQO1 expression as shown in Figure 7B. These results provide evidence that organic compounds are bioavailable as they induce phase 1(CYP 1A1) and Phase 2 (NQO-1) gene expression.

Organic Compounds and Oxidative Stress

Evidence for the involvement of oxidative stress in the effects of organic compounds came from the initial observation that the mortality resulting from lung edema after intratracheal administration of whole DEP into mice was suppressed by pretreatment with polyethylene glycol-modified superoxide dismutase (Sagai et al., 1993) and that it was limited with methanol-washed DEP. Many recent data have shown that organic compounds are a source of ROS. Indeed, we have measured a pro-oxidant status using various specific fluorescent probes in airway epithelial cells treated either with DEP, PM or their corresponding organic extract whereas carbon black particles or solvent-extracted particles do not have such an effect (Baulig et al., 2003a, b; Baulig et al., 2004). For example, increased ROS production determined by the dichorofluorescein fluorescence was observed in HBE cells exposed for 4 hours to DEP, urban $PM_{2.5}$ sampled in Paris, their respective extracts giving a fluorescence signal similar to native particles (Figure 8). A pro-oxidant status

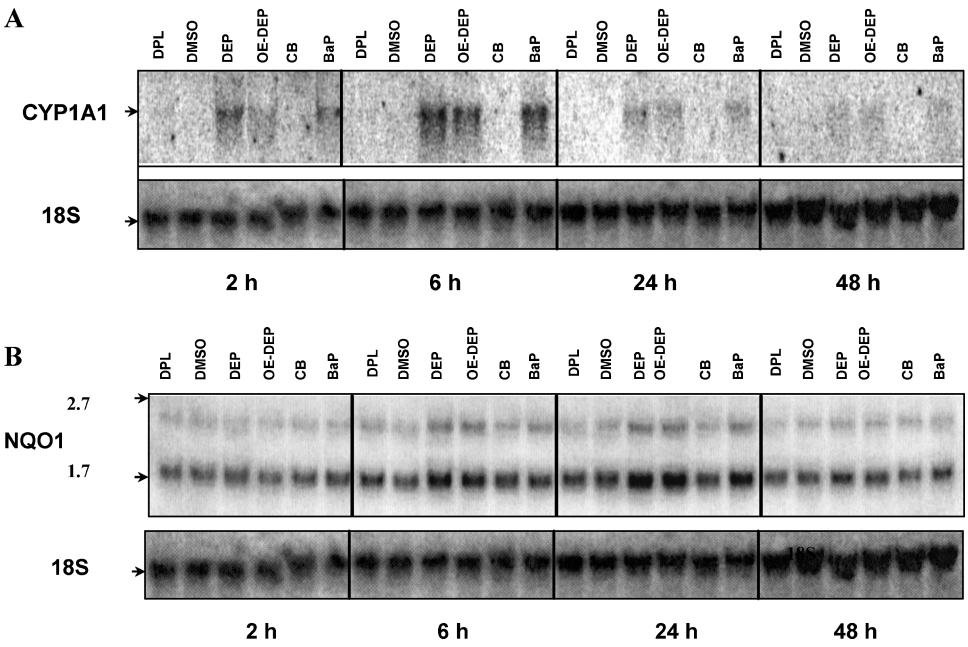

FIG. 7. Induction of cytochrome P-450 1A1 (CYP1A1) and NADPH quinone oxidoreductase 1 (NQO-1) gene expression in HBE cells (A and B respectively). Cells were treated or not with DEP (10 μg/cm^2), carbon black (10 μg/cm^2) organic extracts of DEP (OE-DEP), 10 μg/mL) or benzo(a)pyrene (B(a)p, 3 μM). RNA (30 μg) was extracted from cells after 2, 6, 24 or 48 h of treatment, electrophoresed, Northern-blotted and then incubated with a ^{32}P-labeled cDNA probe for CYP1A mRNA, NQO-1 mRNA or 18S RNA. (From Baulig et al., 2003b).

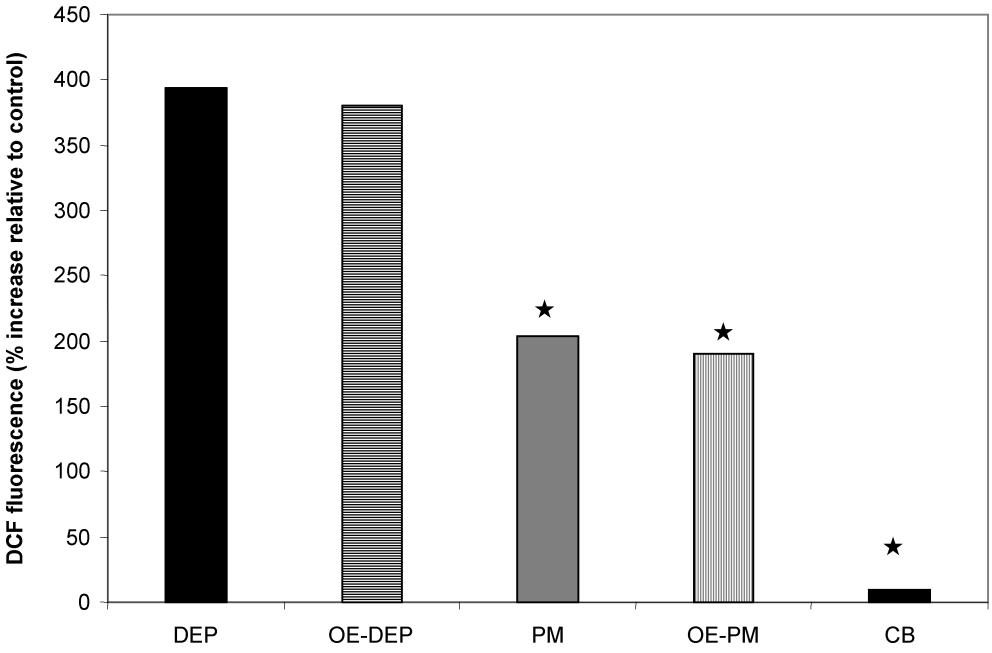

FIG. 8. Dichlorofluorescein (DCF) fluorescence intensity in human bronchial epithelial cells treated with diesel exhaust particles (DEP, 10 μg/cm^2) or their corresponding organic extract (OE-DEP), Paris urban $PM_{2.5}$ (PM, 10 μg/cm^2) or their corresponding organic extract (OE-PM), or carbon black particles (CB, 10 μg/cm^2). The cells were loaded with 2′,7′-dichlorofluorescein-diacetate (H2DCF-DA) at 20 μM for 20 mi and then treated or not with the toxics for 4 h. The DCF fluorescence was measured by cytometry. Results are expressed in% of increase of DCF fluorescence relative to control.

is known to induce cellular specific responses which allow cells to face an oxidant insult. By a genomic approach, the expression profiles of proinflammatory genes induced by DEP, PM or their organic extracts show a differential expression of cytokine genes such as IL 1α, GRO α and amphiregulin, a ligand of the EGF receptor(Baulig et al., 2003a, Blanchet et al., 2004). Their increased expression was confirmed by RT PCR and/or Northern blot and an increased release was also observed. The organic fraction of particles is mainly involved in these responses. The release of proinflammatory cytokines induced by PM or DEP occurs after triggering transduction pathways including nuclear factor (NF)κB activation and mitogen activated kinase (MAPK) phosphorylation (Bonvallot et al., 2000, 2001). Moreover, transduction pathways as well as cytokine secretions were inhibited by the antioxidants such as N-acetyl-cystein or DMTU suggesting the role of oxidative stress. (Boland, 1999, 2000).

In conclusion, these results show that ROS production is a central event to explain the biological effects of PM and DEP. For DEP, bioavailable organic compounds are the main source of intracellular ROS production which induces nuclear factors and gene activations and, consequently, biological responses such as proinflammatory cytokines secretion. The measure of intracellular ROS could be a good indicator of a potential biological response. For PM, the presence of heavy and transition metals, which are likely involved in the production of ROS in the biological fluids, could be measured by an abiotic test such as EPR.

PARTICLE TOXICOLOGY TESTING—ANIMAL STUDIES AND OXIDATIVE STRESS

Ingeborg M. Kooter and Flemming R. Cassee

In recent years, numerous toxicological studies have documented the capacity of inhaled particulate matter (PM) to cause oxidative stress both within the lung and systemically, and related this capacity to the health effects observed in exposed subjects. In recent years several animal studies have been performed at our institute to specifically investigate the role of oxidative stress as a mechanism for air pollution-induced health effects. The overall objective was to select a set of oxidative stress markers which can serve as indicators for health effects caused by air pollution mixtures.

The specific objective of the first study (Kooter et al., 2005) performed was to gain insight into the roles of a wide range of genes in the mechanisms of ambient particulate matter induced health effects. Particular attention has been paid to immediate oxidative stress in the lung. Therefore total lung RNA was isolated from spontaneously hypertensive male rats between 2 to 40 h after exposure to reference urban PM (EHC-93; 10 mg/kg body weight). Our results showed that exposure to PM generated a time-dependent pattern of gene expression. From the

8799 genes or expressed sequence tags tested on the Affymetrix chips, 132 genes were up or down regulated shortly after exposure (i.e., 2–6 h), whereas after 15–21 h and 24–40 h, 46 and 56 genes showed altered expression, respectively. Focusing on events immediately after exposure (<6 h), 99 of the 132 genes were solely expressed here. These include genes involved in an oxidative stress response HO-1, metallothioneins, and thioredoxin reductase), an inflammatory response (macrophage inflammatory protein-2, and tumor necrosis factor alpha), transcription factors belonging to the activator protein-1 family, and genes involved in cardiovascular functions (Kooter et al., 2005). This study, although not representing an ambient situation, was used to identify plausible biological pathways causing initial injury due to PM.

In a second study, gene expression due to ozone, a well-known oxidative stressor, was studied in a transcription-coupled repair-deficient mouse model. *Csb* knockout mice were exposed to 0.8 ppm ozone for 8 h, and examined for a wide range of biological parameters in the lung at the gene expression, protein, and cellular level (Kooter et al., 2007). Biological responses to ozone were observed in repair-deficient mice, such as biochemical increases of polymorphonuclear neutrophils, alkaline phosphatase, macrophage-inflammatory protein-2 and tumor necrosis factor-alpha in bronchoalveolar lavage fluid (BALF), and changed gene expression (up-regulation of oxidative-stress-related genes). From the 20000 oligonucleotides tested, 134 genes were up-regulated and 198 genes were down regulated, mainly immune response related genes. Up regulation of oxidative stress related genes involved genes with known function in glutathione metabolism, metallothionein-1, thioredoxin reductase and heme oxygenase-1 (Kooter et al., 2007).

Thus, in both studies, the HO-1 gene turned out to be up regulated, despite the difference in exposure and species. Since HO-1 is a well known antioxidant enzyme which catalyses the oxidation of heme into the anti-oxidants biliverdin, CO, and free iron we were interested to see whether protein and activity levels would be affected as well. Therefore the HO-1 protein levels were investigated in BALF and lung homogenate of rats that have been exposed to different concentrations ambient particles ($PM_{2.5}$). A maximum of the HO-1 protein level was found at around 600 $\mu g/m^3$ PM (Kooter et al., 2006). This suggests that cytoprotective pathways are induced at relatively low levels of oxidative stress, which may constitute the first tier of a hierarchical oxidative stress response (Xiao et al., 2003). We also found a remarkably good ($r^2 = 0.79$) correlation between the amount of HO-1 protein in BALF and the amount of HO-1 protein present in lung homogenate. Since HO-1 is a membrane bound enzyme, the amount of protein present in the BALF likely represents degradation products, whereas the fraction measured in the lung homogenate most likely represents active HO-1.

We have verified the non-monotonic dose response curve found for HO-1 in the BALF of CAP-exposed rats by another study wherein rats were exposed for 6 months to diesel engine exhaust (DE) at 4 different concentrations as described by Seagrave et al. (2005). It was very clear that the HO-1 response, having its maximum at 30 $\mu g/m^3$, follows the same pattern as that of the total glutathione response. As a next step, knowing that HO-1 is an antioxidant enzyme, we measured HO-1 activities using a gas chromatographic method for quantifying the CO produced *in vitro* by frozen lung tissue homogenates, heme and NADPH. HO-1 activities in lung tissue and BALF of exposed rats were measured (Vreman and Stevenson, 1988). For the BALF a doubling in the HO-1 activity is found after 4 h, whereas for lung homogenate a doubling in activity is found 24 hours following an intratracheal instillation of 7.5 mg/kg EHC-93. We therefore conclude that monitoring the HO-1 activity levels after exposure to air pollutants could be a valuable readout for the oxidative stress response preceding health effects observed in exposed subjects.

In another recent experiment, we explored in a time course analysis the hypothesis that oxidative stress occurs shortly after a 2 h exposure to PM in rat lung. Rats were exposed for 2 h to 1.9 mg/m^3 PM as part of diesel engine exhaust gases and parameters were analysed at 4, 18, 24, 48 and 72 hours after the exposure. Overall, a decrease in protein in BALF was observed at all time points. Successive in time the following oxidative stress parameters were affected (Table 2); the GSH/GSSG ratio was decreased at 18 h, whereas at the same time as well as after 24 h the protein level of HO-1 increased in BALF, while HO-1 activity increased at 24h in lung homogenate, and finally the amount of the antioxidant uric acid is increased at 24 h. An increase of the inflammatory markers was only seen after 48 h (IL-6 and TNF-α). Surprisingly, an increased neutrophil influx was not observed in this study, suggesting that the main source of oxidative stress derives from the diesel PM themselves, and not from products released by cells that respond after exposure to PM. Overall, the results of this study also support the hierarchical oxidative stress model (Figure 1) in response to the diesel engine exhaust exposure.

Most *in vivo* studies on PM toxicity have used short-term exposure (<3 days). Very little is known about the role of oxidative stress in prolonged exposures (weeks, months). This, however, is of great importance since the largest burden on human health comes from chronic exposure to PM and co-pollutants. In a yet

TABLE 2
Oxidative stress and inflammation response in BALF of rats exposed to diesel PM

Time point (h)	4	18	24	48	72
GSH/GSSG	0	−	0	0	0
HO-1 protein	0	+	++	+	+
HO-1 activity*	0	0	+	0	0
Uric Acid	0	0	+	0	0
IL-6	0	0	0	++	+
TNF-α	0	0	0	++	++

*Measured in lung homogenate.

unpublished study, we have exposed healthy F344 rats for 4 and 13 weeks (6 hr/day; 5 days/wk) by inhalation to concentrated $PM_{2.5}$. Although very little effects were noted (average concentrations 200–250 $\mu g/m^3$) a decrease in anti-oxidant levels (GSH) were noted after 4 wks. Although normal healthy animals can usually easily adapt to oxidative stress, this signal indicates that a semi-continuous oxidative stress may result in other adverse health effect such as increased inflammation as seen in this study.

The potential of inhaled PM to cause oxidative stress in the lung and other organs, such as the blood, can be related to the health effects observed in exposed subjects. Therefore, a measure of the oxidative potential of PM should be considered by selecting a more health relevant PM indicator than mass alone. In addition, by relating this 'integrative' measure of PM reactivity with detailed chemical analysis of PM samples it will be possible to identify those PM fractions and PM components that contribute most significantly to the observed health effects associated with PM. Our studies support the idea that HO-1 can serve as a powerful indicator of oxidative stress and biomarker. The correlation between *in vitro* and *in vivo* findings needs to be resolved. As indicated in Table 3, HO-1 expression is well correlated with the abiotic DTT assay. Our findings support the hypothesis that (a battery of) abiotic oxidant capacity assays are predictive for *in vivo* toxicity of (nano) particles.

THE DISRUPTION OF IRON HOMEOSTASIS AND ITS ROLE IN OXIDATIVE STRESS

Andy Ghio

Ambient air pollution particles include a temporally and spatially shifting combination of particles originating from both natural and anthropogenic sources. While the specific component of this particulate matter (PM) responsible for the biological effect of ambient air pollution particles has not been determined, oxidative stress has been accepted as the ultimate mechanism (Kelly, 2003; Risom et al., 2005). Several components of ambient air pollution particles (i.e. ultrafines, organic constituents, biological components, metals, and acid sulfates) have been demonstrated to have the capacity to effect a biological response in a cell, tissue, and living system. These same components have been associated with an oxidative stress presented by PM (Ghio and Cohen, 2005). The production of oxidants following exposure to particles results in a cascade of dependent cell signaling, transcription factor activation, mediator release, inflammation, and fibrosis. Interruption of the oxidative stress can either diminish or eliminate the biological effect of PM both *in vitro* and *in vivo*.

As a result of its interactions with O_2, its tendency towards donor-acceptor complex formation, and its abundance in nature, iron was selected in molecular evolution to carry out a wide range of biological functions in cells and tissues. Those same chemical properties which allow iron, with either a labile or reactive coordination site available, to function as a catalyst in the reactions of molecular oxygen make it a threat to life via the generation of oxidants:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$$
$$Fe^{2+} + O_2^- + 2H^+ \rightarrow Fe^{3+} + H_2O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + .OH + OH^-$$

While cells and tissues must obtain iron to catalyze homeostatic functions, oxidants generated by the metal have a capacity to damage biological molecules. There results a very delicate balance of iron in any cell or tissue with concentrations of available metal only great enough to meet the homeostatic requirements. Evidence suggests that a disruption of iron homeostasis in the lung and in the living system can be an initial event in both the generation of an oxidative stress and the biological effects which follow exposure to PM. The introduction of a solid-liquid interface, ultrafines, organic constituents, biological components, metals, and acid sulfates can all disrupt the normal homeostasis in an exposed host (Ghio and Cohen, 2005). Therefore, this proposed mechanism would be common to all particles whether or not they contain metal at the time of the exposure. Evidence of the disruption in normal iron homeostasis after exposure to a particle can be observed as the formation of a ferruginous body on examination of tissue by light microscopy. The production of a ferruginous body minimizes the oxidative stress following particle exposure by sequestering iron associated with the PM in host ferritin (i.e. a less reactive state for the metal) (Olakanmi et al., 1993). This host response to decrease oxidative stress after PM exposure is comparable to that following infection (Bullen et al., 1974).

An association between a disruption in iron homeostasis by PM and their biological effects in a cell, tissue, and living system could explain the observed differential toxicity of ultrafines, fine, and coarse particles (i.e. greater surface area predicts increased metal complexation and oxidative stress). In addition, it could assist in understanding disparities in host susceptibility. Many groups found to have greater susceptibility to the health effects of inhaled particles demonstrate elevated metal stores, including the elderly, newborn, diabetics, and those with pre-existing disease. These populations contain increased stores of metal in some defined equilibrium with other cell sources and therefore may reflect greater concentrations of iron available in a host for mobilization which subsequently can present an oxidative stress to the host (Ford and Cogswell, 1999; Say et al., 2002). A greater injury is therefore predicted to result in these individuals.

NANOTOXICOLOGY RESEARCH AT THE NATIONAL INSTITUTE FOR OCCUPATIONAL SAFETY AND HEALTH (NIOSH)

Vincent Castranova

Nanoparticles, having at least one dimension <100 nm, exhibit physicochemical properties dramatically different from fine-sized particles of the same composition. The possible health

TABLE 3
Comparison of acellular and cellular tests to assess the oxidative potential of ambient PM fractions

Test	Requirements							
Acellular	A	B	C	D	E	F	Test principle	Reference
EPR[1]	+	−	−	+	+	+	Scavenging of hydroxyl radicals with e.g. DMPO. Will be made as an online system	
DTT	++	++	−	++	++	++	Ability of PM to catalyze electron transfer from a reducing source (DTT or ascorbate) and the generation of superoxide and hydroxyl radicals, respectively. While the DTT assay is not directly affected by metals, the ascorbate acid assay is. The abiotic DTT assay correlates well with cellular heme oxygenase (HO-1) expression	Li et al. (2003b), Cho et al. (2005)
Antioxidant depletion from a synthetic respiratory tract lining containing ascorbate, urate and reduced glutathione		++		+	++	++	Oxidative consumption of anti-oxidants due to their reduction of metals and quinones, allied to their scavenging of superoxide/hydrogen peroxide. Sensitive to metals and organic (quinone) radicals. Predictive of macrophage arachidonic acid release in response to PM, with evidence of predictive value in a small-scale retrospective epidemiological study. Metal versus non-metal oxidative potential quantifiable through the use of specific metal chelators.	Mudway et al. (2004, 2005), Zielinski et al. (1999), Kunzli et al. (2006)
Salicylate → DHBA	+	++	−	+	++	+	Formation of DHBA formation from salicylate + hydroxyl radical in the presence of soluble transition metals	
Free fatty acid e.g. thiobarbituric acid assay (TBA reative products)	+	−	−	+	+	+	Measurement of oxidized fatty acids	
Biosensors/microchip	?	+	?	?	−	−	Inclusion of a range of test on a chip	
Cellular							Measurement of oxidize fatty acids	
Glutathione depletion	−	+/−	+	+	−	+	HPLC analysis of cell content (cytosol)	
HO-1 activity/CO production	−	++	+/−	++	+	+	HO-1 is an anti-oxidant and is considered as a very sensitive marker for oxidative stress. Detection of the release CO might be possible in vitro systems	Li et al. (2003b, 2004)
Cell with specific reporter genes (macrophages)	−	++	−	+	−	+	Activation of luciferase results in light production as function of oxidative stress	Li et al. (2004)
Cellular ROS production	−	++	−	+	−	+		Xia et al. (2006a)

[1]Can also be done in the presence of cells.

A. Rapid, preferably online system; B. Sensitive to detect small changes as well as low oxidative potential; C. Clear relationship with human health effects; D. Added value to current PM metrics (including quantification ability); E.Cheap (value for money); F. Currently available for routine analysis.

effects of exposure to nanoparticles are likely to be very different from those of fine particles of the same composition, since the nanoparticles exhibit a high surface area, have a high deposition in the lung, and have the potential to translocate from the lung to systemic tissue. As with ambient PM, oxidant generation is considered an important mechanism driving adverse effects resulting from exposure to engineered nanoparticles, and measurement of oxidant generating capacity has been suggested as an important component of a screening strategy for nanotoxicology (Oberdorster et al., 2005; Nel et al., 2006).

In 2004, NIOSH initiated a research program in nanotoxicology, which has expanded to eight projects in 2006. The major research issues addressed by these projects are:

(1) Measurement of the deposition of nanoparticles in the lung; determination of the sites of deposition; quantitation of the movement of nanoparticles from deposition sites to the interstitium within the alveolar septa; and evaluation of the ability of nanoparticles to translocate to systemic sites.
(2) Measurement of the pulmonary toxicity of nanoparticles and determination of the role of particle composition, redox potential, shape, and coatings on this toxicity.
(3) Measurement of possible cardiovascular effects of nanoparticles after pulmonary exposure.
(4) Measurement of the response of the skin to dermal exposure to nanoparticles.
(5) Determination of possible neural uptake and resultant responses in the brain from pulmonary exposure to nanoparticles.
(6) Evaluation of various dose metrics, such as mass, particle number, or particle surface area, to investigate biological response.

In a study of the pulmonary deposition and translocation of nanoparticles, Robert Mercer and colleagues developed a method to label single-walled carbon nanotubes (SWCNT) with ultrafine colloidal gold prior to pharyngeal aspiration of these nanoparticles into the lungs of mice (Mercer et al., 2005). The distribution of gold-labeled SWCNT was evaluated histologically by light microscopy of silver-stained lung sections. Large agglomerates of SWCNT material deposited at the terminal bronchioles and the proximal alveolar. However, more dispersed structures were seen in the interstitium of the distal alveolar septa. Dispersed SWCNT structures were not avidly phagocytized by alveolar macrophages, but rapidly (within 3 hours post – exposure) migrated into the alveolar interstitial spaces. The lack of *in vivo* phagocytosis of SWCNT correlates with the failure of SWCNT to be engulfed by macrophages *in vitro* and the failure to activate a respiratory burst upon exposure (Kagan et al., 2006).

Anna Shvedova is leading an investigation of pulmonary toxicity of carbon nanotube particles in which she and colleagues (Shvedova et al., 2005) investigated the pulmonary reactions to pharyngeal aspiration of SWCNT (purified to remove contaminating iron) in a mouse model. These purified SWCNT did not generate oxidant species in acellular or cellular *in vitro* systems. Pulmonary exposure to SWCNT resulted in a rapid but transient inflammatory and oxidant stress response, which increased within 1–3 days following aspiration and returned toward control 1 week post-exposure. In contrast, deposition of agglomerates of SWCNT resulted in the formation of granulomas, characterized by collagen deposition which was significant 1 week post-exposure and progressed through 2 months after exposure. Deposition of dispersed SWCNT structures resulted in diffuse intersitial fibrosis, which was of rapid onset (1 week post-exposure) and progressed through the 2 month course of evaluation. Therefore, *in vitro* redox potential failed to predict the *in vivo* fibrogenic response to purified SWCNT.

Dale Porter and Val Vallyathan are principal investigators of a project entitled "Pulmonary toxicity of metal oxide nanospheres and nanowires." Results indicate that exposure of alveolar macrophages to SnO_2 nanoparticles *in vitro* induced the generation of hydroxyl radicals, which can be detected by electron spin resonance spectroscopy. Experiments are ongoing to evaluate the role of shape in the pulmonary reaction of rats to exposure.

In a study of the role of carbon nanotubes in cardiopulmonary inflammation, Petia Simeonova and colleagues have demonstrated that oxidant stress was evident in cardiac and aortic tissue 1 week after pulmonary exposure of mice to purified SWCNT (Li et al., 2005). This oxidant stress was noted as induction of message for hemoxygenase-1 (HO-1) in cardiac and aortic tissue and oxidative damage to aortic mitochondrial DNA. Simeonova and colleagues (Li et al., 2006) also reported that multiple pulmonary exposure to SWCNT significantly increase plaque formation in systemic vessels in an atherosclerotic susceptible mouse model on a high fat diet compared to mice on a high fat diet with no nanoparticle exposure.

Vincent Castranova is principal investigator of a project entitled "Systemic microvascular dysfunction effects of ultrafine vs fine particles" in which he and colleagues (Nurkiewicz et al., 2006) have shown that pulmonary exposure to levels of fine TiO_2, which did not cause marked pulmonary inflammation or damage, decreased the ability of microvessels in the systemic circulation to respond to vasodilators. This dysfunction was associated with adherence of polymorphonuclear leukocytes to the microvessel walls and production of reactive oxygen species in the microvessels. On an equivalent deposited mass basis, ultrafine. TiO_2 was nearly an order of magnitude more potent in inducing systemic microvascular dysfunction than fine-sized TiO_2 (Nurkiewicz et al., 2007).

In a further study focussing upon the dermal effects of nanoparticles (P.I.s Anna Shvedova and Min Ding) results indicate the unpurified SWCNT (contaminated with iron) induced the generation of hydroxyl radicals by human keratinocytes *in vitro*. This oxidant generation was associated with cytotoxicity (Shvedova et al., 2003). Removal of the contaminating iron by acid treatment reduced the generation of oxidant stress. Exposure of mouse skin to unpurified SWCNT caused dermal

inflammation (Murray et al., 2006). In addition, exposure of epidermal cells to nanometal oxide particles generated hydroxyl radicals and caused oxidant – dependent activation of MAP kinase signaling pathways (Ding et al., 2006).

James Antonini, Diane Miller, and James O'Callaghan are leading a study of neurotoxicity after pulmonary exposure to welding fumes containing manganese. Evidence indicates that pulmonary exposure to stainless steel welding fume caused lung damage and increased the susceptibility of exposed rats to pulmonary infection (Antonini et al., 2006). Experiments are in progress to determine if brain responses are associated with welding fume exposure.

Results of a project entitled "Particle surface area as a dose metric" (P.I.s Vincent Castranova and Victor Robinson) thus far indicate that dispersal of nanoparticles is critical to the evaluation of *in vitro* or *in vivo* pulmonary reactivity. Alveolar lining fluid has been found to be effective in significantly decreasing the size of agglomerates for ultrafine TiO_2 or carbon black without masking the reactivity of the nanoparticles (Sager et al., 2007). Improved dispersion of ultrafine carbon black particles prior to intratracheal instillation of rats significantly increased the inflammatory and cytotoxic potential of the particles compared to suspension in saline, where μm-sized agglomerates were produced (Shvedova et al., 2007). In another experiment, *in vitro* cytotoxicity in response to fullerenes was compared to unpurified SWCNT produced by the high pressure carbon monoxide or laser ablation technique. All three nanoparticles generated hydroxyl radical in the presence of bronchial epithelial cells. However, the association between radical generation and cytotoxicity was not perfect (Shvedova et al., 2007).

In summary, NIOSH has an active and expanding research program in nanotoxicology. Effects on the lung, skin, brain, and cardiovascular system are being investigated following exposure to carbon nanotubes, nanometal oxides, and carbon black. NIOSH, under the direction of its Nanotechnology Research Center, is conducting projects in nanoparticle measurement and characterization, exposure assessment, promulgation of effective controls and good handling practices, and risk assessment, in addition to nanotoxicology, in an effort to assure the safe production and use of nanomaterials in the workplace. Although the oxidant generation potential of engineered nanoparticles appears to be an important factor in predicting biological response, its predictive value is not perfect, with failure to predict the pulmonary interstitial fibrotic response to purified SWCNT being a case in point.

CONSENSUS STATEMENT OF CONCLUSIONS AND RECOMMENDATIONS

The capacity of inhaled particles to elicit oxidative stress *in vivo* has been proposed as being central to the adverse health effects reported in exposed populations. Much effort has been put into understanding the mechanisms that link exposure to PM and the health effects as observed in many epidemiological studies. Oxidative stress seems to be a (but not necessarily the only) crucial process that drives adverse health effects. Oxidative stress can be defined as a change to living cells (and thereby the organs and tissues composed of those cells) caused by reactive oxygen (or nitrogen) species, which include (but are not limited to) superoxide, hydrogen peroxide hydroxyl radical, singlet oxygen atoms, or peroxynitrite. It is also defined as an imbalance between reactive oxidant species (ROS) and antioxidants, with the former prevailing. Oxidative stress could be damaging but is in essence a homeostatic mechanism that also includes protective responses that can be referred to as Tier1 (see contribution by Nel et al. in this paper). In general, people can deal with low levels of exogenous oxidants including PM through their ability to generate protective responses as an integral component of the biology of oxidative stress. However, in some cases, for instance when the body fails to restore the redox homeostasis, people can become more susceptible to the harmful effects of oxidant stress. The fact that only a small subset of the population is susceptible to the adverse health effects of PM demonstrates that the majority of people do adapt to the oxidative stress it causes. During this meeting a variety of assays were discussed that attempt to quantify the capacity of ambient particulate matter to cause damaging oxidation reactions in the lung and extra-pulmonary organs. In the discussion of these methods several issues were raised, which need to be considered when attempting to establish a mechanistic linkage between the particle oxidative potential and particle-induced health effects. The following section summarizes these issues and also considers how best to measure the oxidative potential of PM. There are two main issues to consider:

(a) Are new predictive tests for hazard assessment of particles useful for developing abatement policies based on emission reductions from specific sources?
(b) Is the development of an alternative metric for PM toxicity, other than mass or number concentration useful for regulatory purposes?

A test that reflects the inherent toxicity of a given sample of particles has the potential to be useful in both roles.

Sampling and Pre-Treatment of Particulate Matter

The ambient atmosphere is a dynamic system, in which the mixture of air pollutants changes over time (diurnally, between days and seasons). Unless direct and online methods are available, particulate matter must first be collected before it can be assayed for physical characteristics, chemical composition, and oxidative potential. The fluctuation of important atmospheric parameters that influence the ambient PM concentrations (and hence human exposure), such as the emission strengths of particle sources, temperature, season, time of day, relative humidity, wind direction and speed, and mixing height, presents a significant challenge to obtaining a valid time-integrated collection that reflects the real-world situation. Yet, relatively high quantities of particles are often needed for chemical and biological analyses. Often, high volume filter and cascade impactor samplers are used that allow size fractionated

sampling on small surfaces over a long period. Once PM has been collected, vigorous methods are needed to remove the PM from the collection substrate (filters, foams) into suspension. Depending on the nature of the PM chemical composition (i.e., its content of labile species content) as well as the subsequent analysis to be conducted, this type of collection may or may not introduce substantial sampling/extraction artifacts. Novel approaches have addressed this problem by collecting particles in a fluid using a combination of particle concentration, followed by impaction and centrifugation as physical principles (Kim et al., 2001a; Kim et al., 2001b). Whilst there is evidence that such methods retain the size distribution and chemical composition of the atmospheric sample, there is currently no way of assessing whether oxidative potential of the collected material is affected.

In the case of airborne PM (as opposed to bulk samples of engineered nanoparticles) there is a need to extract the particles from the filter or impaction substrate in order to carry out testing for oxidative potential. Avoidance of filter materials that are themselves active in the tests (e.g. glass fibre and quartz) is essential. It is, however, difficult to assess whether a suspension/extraction method is wholly efficient in extracting those components of the particles that exert oxidative potential. The "biosampler" method that traps concentrated particles in aqueous suspension (Kim et al., 2001a,b) circumvents many of these problems.

Assessment of Oxidative Potential

As described above, various tests exist for the determination of PM oxidative potential (hazard screening). These consist of abiotic chemical assays that can easily be adapted for use in the field and methods that require cultured cells and have been linked to oxidative stress *in vivo*. These permit the examination of PM-induced oxidative stress at a number of levels:

(a) at the molecular level: (bio)chemical reactions
(b) at subcellular level: mechanistic *in vitro* studies
(c) at tissue/organ level: permitting complex interactions between oxidative stress and inflammatory effects to be examined

Requirements for Test of Oxidative Potential of Particles

In order to serve as a valuable and predictive test some requirement can be defined for an assay. It should be:

A. Rapid, preferably an online system
B. Sufficiently sensitive to detect small changes in, as well as low levels of, oxidative potential
C. There should be a clear relationship with human health effects
D. It should provide added value to the currently available PM metrics (including quantification ability)
E. It should be low-cost (value for money)
F. Currently available for routine analysis

Biological Effective Dose

Each test can provide useful information on oxidative potential of particles. Ultimately, however one would like to link ambient PM concentrations to a Biological Effective Dose (see section by Donaldson). Whilst oxidative potential may integrate various PM characteristics (size, surface area and composition) into a single biologically relevant measure of toxicity, in terms of its risk to the population, the size of the particles also needs to be considered in the context of airway deposition: For example particles in the accumulation mode ($0.1\ \mu m < d < 2.5\ \mu m$) have substantially lower deposition efficiencies than smaller or larger particle fractions. Regional deposition (i.e. the location of deposition in the respiratory system) is also an issue for some health outcomes. In that case, a hazard index (HI) may be given as final outcome of a test or battery of tests once deposition efficiency has been taken into account. One further advantage of this classification is that HI could be adapted for estimating risk in susceptible groups e.g. patients with COPD and asthma who tend to have higher local deposition of PM depending on size.

Timescale

People are continuously exposed to PM. This can result in both acute effects due to short-term increases in concentrations as well as effects that develop more slowly due to chronic exposures (as well as everything that ranges in between). Account also needs to be taken of the ability of the intact organism to induce repair mechanisms that are not present in cellular or acellular systems that do not take these dynamic exposure conditions into account. In such systems, a single dose is delivered to the test system at one time and will interact with the system for a distinct duration. It is also possible to show that prior exposure with the low dose of PM can protect against the cytotoxic or pro-inflammatory effects of a large dose of PM. Consequently, extrapolation is needed to account for the difference in dose and dose rate in real life to reflect the value of existing PM metrics in air quality networks. The sensitivity and hence the averaging time should therefore at least approach 1 hr as used for PM mass concentrations in some networks. This need for short averaging times is underlined by observations that the PM mix changes in composition (as well as oxidative potential) during the day. Integration of short averaging times over a longer period can subsequently be predictive for adverse health effects due to prolonged exposure to PM. Oxidative stress will be predictive for health effects due to short term and long term exposures to PM.

Proposed Test Methods

Acellular Procedures

Various acellular systems are available (see previous sections), some of which correlate well with one another (EPR and anti-oxidant depletion). The major advantages of these methods are that they are high throughput and relatively cheap. On the

other hand, there is no biological interaction and therefore false negative findings may result: some samples may contain material that needs the interaction with the organism before they are activated, e.g. a combination of organics with iron from the body (see Ghio), or to drive the generation of reactive oxygen species such as with endotoxin, polyaromatic hydrocarbons etc. Other examples include the subcellular localization of the particles and the possibility that this could lead to targeting of organelles that can contribute to ROS production, e.g., mitochondria. False negative findings due to the presence of protective mechanisms are also possible

Cellular Systems

As outlined above the use of acellular models only permits the inherent oxidative activity of particles to be assessed. For the determination of the total oxidative potential, interaction with cells is necessary for the reasons outlined above; oxidative stress being defined as stress imposed on cells by oxidative materials. Several systems have been described in the previous sections that have demonstrated their potential to evaluate the oxidative potential of PM samples. A major drawback is that at present these systems cannot easily be applied in routine analysis or in field studies for logistical and practical reasons.

Points of Agreement

(a) It was generally agreed that measurement of particulate oxidative potential represented a valuable additional method for assessing the relative toxicity of particulate matter to humans.

(b) There is clearly a need for more studies examining the linkage between particle oxidative potential with adverse human health outcomes, to demonstrate that these measurements have biological plausibility. The practical link between particle toxicity and epidemiology has to take into consideration the susceptible human subsets. The majority of people may actually mount an effective Tier 1 response that blunts the pro-inflammatory and cytotoxic effects of PM, which could explain why the majority of small group exposure studies yield insignificant results because they may not include enough susceptible people. If biomarkers for susceptibility could be found, it should be possible to perform the epidemiological studies in which susceptible subgroups (minority) can be compared with non-susceptible (majority) people.

(c) Relevant methods for establishing PM toxicity encompass a variety of study approaches including: (1) human inhalation studies; (2) animal inhalation studies, in appropriate models; (3) animal *in vivo* instillation experiments; (4) in vitro cell challenges and (5) the use of *in vitro* acellular systems. While *in vivo* studies are preferable for health risk assessment, it was recognized that large-scale screening exercises were only possible – both logistically and in terms of cost, using simple cell models, with limited but established endpoints and acellular systems. This again emphasizes the necessity of establishing mechanistic linkage between cellular and acellular toxicological endpoints with biological endpoints in the whole animal/individual context. Despite the desirability of performing *in vivo* studies, valuable information can be gained from the low cost acellular tests and these offer a valuable screening mechanism that may need to be followed by cellular or *in vivo* tests.

(d) For cellular tests, there was a general preference for immortalized epithelial and macrophagic cell-lines rather than primary cells. This was driven by practical, rather than purely scientific considerations

(e) There was no overall consensus as to the best acellular test system. There was, however, an appreciation that simple models such as the DTT and ascorbate oxidation tests, which permit discrimination between different redox components, are preferred. The use of these assays was envisaged as a initial screening step for identifying nanoparticulate materials for more detailed examination in cell and animal models. The limitations of the DTT test are that this assay measures a variety of substances that are probably semivolatile and if the collection method were not geared towards preserving these chemicals, the predictive value of the test would be diminished or could introduce artefacts that make the interpretation difficult. In addition, it may not provide an accurate assessment of the role of transition metals. The DTT assay is a better reflection of oxidative stress potential in ultrafine and fine compared to coarse particles in the Los Angeles basin. The credit of potency of these particle types could vary according to their sources in other places in the world.

(f) It was agreed that there is considerable value in intercomparison of different test methods on identical particle samples. The use of appropriate controls materials for experiments, and protocols for the preparation of homogeneous particle suspensions was also emphasized, as were procedures to prevent contamination of studied materials. It was also agreed that further evaluation of the transferability of individual test methods between laboratories would be highly valuable.

POSTSCRIPT: WIDER RELEVANCE OF OXIDATIVE STRESS POTENTIAL TESTING

Roy M. Harrison

As indicated elsewhere within this document, air quality metrics such as $PM_{2.5}$ and PM_{10} are used as a crude surrogate for biologically effective dose rate in air quality management. This is justified by the fact that epidemiology has revealed robust relationships between the $PM_{2.5}$ and PM_{10} metrics and adverse human health effects. Such relationships show a remarkable degree of spatial and even temporal (as far as can currently be judged) consistency. Concentration-response relationships show close similarity when determined in locations where particle major component composition is known to vary

substantially (Harrison and Yin, 2000). More recent studies using harmonised methodology have revealed heterogeneity that may be related to factors such as the fractional contribution of road traffic to airborne concentrations (Katsouyanni et al., 2001) and the prevalence of air conditioning as an attenuating factor for personal exposures relative to concentrations measured out-of-doors (Janssen et al., 2002).

Despite the broad consistency of epidemiological findings irrespective of particle major component composition, toxicological tests, such as the oxidative stress potential tests described in this paper, suggest that the toxicity of airborne particulate matter may derive predominantly from components such as transition metals and polycyclic aromatic hydrocarbons and their derivatives that comprise only a minor proportion of the total mass. If this finding can be extrapolated from toxicological tests to clinically-significant impacts on exposed humans, this will have profound implications for air quality management.

Air quality policy in relation to particulate matter is focussed upon reducing airborne concentrations of $PM_{2.5}$ and PM_{10} in order to comply with concentration limit values, or in future in Europe, exposure reduction targets. If it is the case that particulate matter toxicity is driven largely by minor components (in terms of mass) then such a policy could prove relatively ineffective if it were to focus on those components or emission sources contributing substantially to total mass, but containing only a very small fraction of the most toxic components, relative to other sources. If, on the other hand, toxicology or epidemiology were able to identify with confidence those chemical components (and/or size fractions) of the overall particulate matter mass which contributed most to adverse health effects, then for most cost-effective abatement strategies focussing on the major sources of those components can be envisaged.

How then should science advance in order to inform policy? One of the conclusions of this workshop relates to the need to examine the linkages between particle oxidative potential and adverse human health outcomes. This will require carefully constructed epidemiological studies that study sufficient subjects to overcome the problem that the vast majority, having good antioxidant defences, may not be susceptible to adverse consequences. If, however, it can be demonstrated that one or more oxidative stress potential tests are better predictors of adverse health outcomes then more traditional mass metrics, there may be a case to base air quality policy upon such tests. This could operate at a number of levels:

- the tests could be used to identify those physicochemical properties of particulate matter (e.g. chemical components, particle size or surface area) responsible for adverse health outcomes;
- the tests could provide a ranking of primary emission sources in terms of toxicity per unit mass, and sources of secondary particle precursors could be ranked on the basis of minor component emissions and/or secondary particle toxicity;
- abatement policy could focus most heavily upon sources emitting particles containing the components contributing most to health hazard;
- air quality standards and objectives could be set in terms of oxidative stress potential, as measured by a standard test protocol. Measurement networks would be reconfigured to collect samples appropriate to such tests, or to operate the tests on a continuous real-time basis.

Oxidative stress potential tests may also have a role to play in regulation of the burgeoning nanotechnology industry. Some products such as carbon black and titanium dioxide have been produced in large tonnages in nanoparticle form for many years and are known to be of low hazard to the workforces that produce them. The toxicity of newly engineered nanoparticles is far less certain, and screening tests for new nanoparticle (and nanotube) products are urgently needed. Whilst recognising that oxidative stress potential may not be predictive of all possible adverse health outcomes (e.g. pulmonary fibrosis associated with purified SWCNT — see above), tests based upon oxidative potential may prove to be an invaluable tool for initial screening and classification of the relative hazard of such materials. However, an essential pre-requisite to their adoption for such purposes should be confirmation through carefully designed *in vivo* and epidemiological studies that the tests are reliable predictors of adverse health outcomes in humans.

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