Inherent Redox Properties of Diesel Exhaust Particles: Catalysis of the Generation of Reactive Oxygen Species by Biological Reductants

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The toxicity of diesel exhaust particles (DEP) can be due to the particle itself, extractable components, or both. Many studies focus on the biological properties of DEP-extractable components although it is possible that chemical properties inherent to the DEP itself can lead to toxicity. Thus, an examination of the chemistry inherent to DEP was carried out. Herein, we report that DEP are capable of catalyzing the consumption of O2 (monitored using a Clarke electrode) by ascorbate and thiols leading to the generation of reactive oxygen species. Consistent with the idea that DEP are capable of catalyzing the generation of reactive oxygen species, they were also found to catalyze DNA strand breakage via an O2- and reductant-dependent process. Significantly, extraction of DEP with either organic solvent (methylene chloride) or acid (aqueous HCl) did little to abrogate this chemistry. Finally, using electron paramagnetic spectrometry (EPR), DEP were found to have paramagnetic properties. The paramagnetic character of DEP may be important to their ability to catalyze the formation of reactive oxygen species and at least partially responsible for their toxicity. These findings indicate that studies that primarily consider or examine particle extracts as the toxic components of DEP may be insufficient in describing the toxicity associated with DEP exposure.

Key Words: diesel exhaust particles; DNA cleavage; free radical; oxidative stress.
These reports indicate that the size distribution of the airborne particulate matter—including DEP—may be an important aspect of their toxicity since ultrafine PM are capable of traversing cell membranes leading to mitochondrial damage. Thus, we have examined some of the chemical properties of DEP so we can begin to define them chemically and increase our understanding of their inherent toxicity.

In this study, we specifically address the possibility that DEP are capable of catalyzing the generation of ROS that can lead to a toxic insult to exposed cells. Since abnormal generation of ROS is known to be deleterious to cells, DEP-catalyzed generation of ROS may be an important aspect of the toxicity associated with DEP exposure. Herein, we find that DEP are capable of catalyzing the generation of ROS, a process that can lead to, among other things, DNA damage. Of particular note, DEP whose easily extractable components have been removed by organic solvent or acid washes, maintain the ability to catalyze the generation of ROS, indicating an inherent activity of the particle itself.

MATERIALS AND METHODS

Reagents. DEP were obtained from Prof. Yoshito Kumagai (Tskuba University, Japan) and were collected as previously described (Sagai et al., 1993). NADH and glutathione were purchased from Sigma (St. Louis, MO). Hydrogen peroxide was also purchased from Sigma. 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was purchased from Aldrich (Milwaukee, WI). 1,4-Dithio-DL-threitol (DTT), Agarose and Chelex100 were purchased from BioRad (Hercules, CA). Biological dyes were purchased from Promega (Madison, WI). All other chemicals and reagents were purchased from commercial suppliers and were of the highest purity available.

Preparation of native DEP, organic washed DEP, and acid washed DEP. Preparation of DEP suspensions used in the O2-consumption assays was accomplished by adding the appropriate amount of DEP to a volume of 1 M potassium phosphate buffer (pH 7.4). The heterogeneous suspension was then subjected to sonication for 2 min using a Branson Sonifier 250 (Danbury, CT) set at a 10% duty cycle and output control of 2. The sonicated suspensions could then be injected via syringe into the reactions vessels (vide infra). Methylene chloride washed DEP were prepared as follows. DEP were extracted with dichloromethane using a 1:5 (w/v) ratio of DEP to solvent. The suspension was then centrifuged for 10 min at 850 × g at 4°C. The DEP pellet was then separated from the organic extract and the extract dried using a nitrogen gas stream. The extract residue was resuspended in 0.1 ml of DMSO for further analysis. The DEP after extraction were air-dried and resuspended in 1 M of phosphate buffer. Extraction of DEP with aqueous acid for further analysis. The DEP after extraction were air-dried and resuspended in 1 M phosphate buffer (pH 7.4). In one neck of the flask, the Clarke electrode was inserted using a gas-tight adapter such that the electrode surface was in contact with the solution. The other two necks of the flask were capped with rubber septa through which solutions could be injected. No headspace gas was present. When solutions were injected into the flask through one of the septa, an open needle was inserted through the other septa to allow the displaced solution to leave the flask. The solution was stirred throughout the experiment using a magnetic stirrer. Reducing agents were injected into the flask using a syringe to final concentrations of 250 μM or 500 μM. Oxygen consumption was then monitored for 10 min. After 10 min, 5 mg of DEP in 1 ml of 1 M potassium phosphate buffer was injected into the flask and the rate of O2 consumption monitored. The rate of O2 consumption was determined by monitoring the decrease in the detector response over time and by assuming the initial concentration of O2 in air-saturated buffer to be 245 μM. O2 consumption rate is reported as μM/min/mg DEP.

Plasmid DNA nicking assay. DEP-dependent oxidative damage to DNA was assessed by using a plasmid DNA nicking assay. Briefly, each reaction mixture (final volume of 20 μl) contained 200 ng pUC 19 DNA in 100 mM potassium phosphate buffer, 500 μg/ml of DEP (native DEP, organic washed DEP and acid washed DEP), and 500 μM of ascorbic acid. The sample was incubated with agitation (150 RPM) for 2 h at room temperature. After incubation, 4 μl of a Blue/Orange 6X loading dye was added to the sample to stop the reaction. The samples were then loaded onto a 1.3 % agarose gel containing ethidium bromide and run at 100 V for 1 h at room temperature in 1 M Tris-acetate-EDTA buffer (TAE). Typhoon 9410 (blue laser module) with ImageQuant software (Amersham Biosciences, Piscataway, NJ) was used to perform densitometric analysis of the separated bands and to quantify the amount of supercoiled and open circular DNA. The results were expressed as the percent of open circular form over the sum of supercoiled and open circular forms (linear DNA was not observed). Since ethidium bromide binds “nicked” DNA better than supercoiled DNA, a correction factor for the supercoiled form of 1.4 fold was used to account for these differences (for example, Ohshima et al., 1999). Experiments were performed in triplicate.

RESULTS

O2 Consumption

DEP are reported to generate reduced oxygen species in vitro and in vivo. However, this phenomenon is not well characterized. For example, the nature of the biological reductant or the important properties of the particles have not been examined in detail. Thus, in an attempt to characterize this chemistry, DEP catalyzed reduction of O2 by a series of reducing agents was monitored using a Clarke electrode. Thus, the rate of O2 consumption elicited by NADH, ascorbate, Trolox, glutathione (500 μM), and dithiothreitol (DTT) (250 μM) in the presence of 5 mg of DEP was determined. In the absence of DEP, the reductants exhibited very little or no O2 consumption (as measured for...
the first 10 min). However, the addition of DEP caused significant oxygen consumption for all reductants, especially ascorbate and DTT (Fig. 1). GSH and Trolox displayed the least effect and DEP alone did not cause any O₂ consumption. These results indicate that DEP are capable of catalyzing the reduction of O₂ by various reductants.

**The Effect of DEP Extraction on O₂ Consumption**

In order to determine whether the O₂-consuming properties of DEP observed above were due to extractable species or the particles themselves, native DEP were washed with either methylene chloride (to remove easily extractable organics) or with 1 M HCl (to remove acid extractable metals) and examined for their ability to catalyze the reduction of O₂ by various reductants. As shown in Figure 2, washing the native DEP with methylene chloride or acid only slightly diminished their ability to catalyze O₂ reduction by DTT. Acid wash of the native DEP was unable to diminish native DEP reactivity by more than about 25%. The acid washed native DEP exhibited similar effects using ascorbate as the reductant (data not shown). Consistent with the results of previous studies, the organic extract also contains redox activity (Kumagai and Shimojo, 2001; Li et al., 2002). These results suggest that DEP contain inherent reactivity that is either difficult or impossible to extract with an organic solvent or aqueous acid.

**DEP and DNA Damage**

The DEP-catalyzed and reductant-dependent consumption of O₂ (demonstrated above) indicates the generation of ROS. In order to evaluate the possibility that this chemistry can lead to the oxidative damage of biological macromolecules, we examined the ability of DEP to catalyze oxidative DNA damage. Using the common DNA-nicking assay, we found that indeed DEP are capable of catalyzing ascorbate-dependent generation of oxidizing species, as measured by the conversion of supercoiled plasmid DNA to an open-circular form (Fig. 3A). Consistent with our observation that particles washed with organic solvent or acid were still capable of catalyzing O₂ reduction, washed particles were also able to catalyze DNA-nicking in a qualitatively identical fashion (data not shown). Densitometric analysis of the data indicates that a 3-fold increase in the open-circular form is generated in the presence of DEP compared to ascorbate alone (Fig. 3B). DEP in the presence of DTT showed similar results as those shown with ascorbate (data not shown).

**Analysis of DEP by Electron Paramagnetic Resonance Spectrometry (EPR)**

The reactivity exhibited by DEP indicates that they can perform redox chemistry in which the DEP are capable of accepting electrons from reducing agents and pass them on to O₂ to...
generate reduced oxygen species. The oxidations observed using the DNA-nick assay are suggestive of chemistry associated with these reduced O$_2$ species. This chemistry requires the DEP to participate in odd-electron or radical chemistry. Thus, an attempt was made to detect and/or monitor the presence of paramagnetic species within the DEP. An initial examination of the DEP by EPR revealed that there is a stable paramagnetic species (approx. 2.0 G) that was resistant to organic solvent and aqueous acid extraction (Fig. 4) and thus appeared to be associated with the particle itself, not a dissociable, extractable species.

**DISCUSSION**

DEP toxicity has been the subject of numerous studies since exposure to these particles can be significant (Sauvain et al., 2003; Steenland et al., 1998; Zhu et al., 2002). It is clear that exposure to DEP can manifest itself as a number of toxicological endpoints (Ma and Ma, 2002; McClellan, 1987; Nel et al., 1998; Rudell et al., 1996; Sydbom et al., 2001). The heterogeneous and chemically complex nature of these particles makes delineation of the detailed chemical mechanisms of their toxicity difficult. It is likely that the chemistry of DEP toxicity is the result of the reactivity of numerous chemical species participating in various reactions, ultimately leading to the disruption of cellular function. The recent findings that DEP and other PM can cross cellular membranes and reach intracellular targets (Boland et al., 1999; Li et al., 2003) warrants examination of the chemistry of the particles themselves and not just extractable organic compounds associated with them. In this study we find that DEP are capable of catalyzing the reduction of O$_2$ by the biologically relevant reductants NADH, ascorbate and the vicinal dithiol DTT (Reactions 1 and 2).

\[
X_{\text{[reduced]}} + \text{DEP}_{\text{[oxidized]}} \rightarrow X_{\text{[oxidized]}} + \text{DEP}_{\text{[reduced]}} \quad \text{(Reaction 1)}
\]

\[
X = \text{Biological reductant (i.e., ascorbate, NADH, etc.)}
\]

\[
\text{DEP}_{\text{[reduced]}} + \text{O}_2 \rightarrow \text{DEP}_{\text{[oxidized]}} + \text{O}_2^2/\text{H}_2\text{O}_2
\quad \text{(Reaction 2)}
\]

Interestingly, the water soluble Vit. E analog, Trolox, and GSH showed the least activity while ascorbate and DTT displayed the highest (Fig. 1). The differences in the reactivity of these species are at this time difficult to rationalize using, for example, reduction potentials since the intimate details of the reactions are not known (i.e., outer-sphere versus inner-sphere electron transfers or nucleophilic addition-elimination chemistry). Moreover, major differences exist in the accessibility of these agents to possible reactive centers on DEP. Regardless, it is worth noting that ascorbate and vicinal thiols are particularly adept at performing the above described redox chemistry.

Importantly, the redox properties of DEP appear to be intrinsic as the majority of the catalytic properties remain even after multiple extractions with methylene chloride or aqueous acid. The intrinsic redox properties of the DEP are not necessarily
covalently associated with the particle but rather, are considered to be inherent since they are not readily extracted under conditions which will be far more stringent than what can occur biologically. The idea that the toxicity of DEP can be due to the particles themselves as well as to the extractable components was demonstrated recently when Yanagisawa and coworkers (2003) reported that the DEP core, rather than organic extractables, was primarily responsible for the aggravation of LPS-mediated lung injury. From a toxicological perspective, this chemistry of DEP can be deleterious for several reasons. Depletion of intracellular reducing equivalents can change the redox status of a cell. It has been proposed that changes in cellular redox status towards greater oxidation can initiate cell signaling machinery leading to apoptosis and/or necrosis (Schafer and Buettner, 2001). The catalytic nature of the DEP chemistry and the ability to generate ROS would lead to a prediction that they can be extremely proficient in altering intracellular redox status. Myriad studies have established the toxicity

FIG. 3. (A) Conversion of supercoiled to open-circular DNA in the presence of ascorbate (500 μM). Lane 1, DNA only; lane 2, native DEP; lane 3, methylene chloride extracted DEP; lane 4, acid washed DEP; lane 5, ascorbate; lane 6, ascorbate + native DEP; lane 7, ascorbate + organic washed DEP, and line 8, ascorbate + acid washed DEP. (B) Densitometric analysis of the results shown in panel A. DNA damage was expressed as the percent of the open circular form of the total DNA (supercoiled and open circular). Due to the increased affinity of ethidium bromide for nicked forms of DNA compared to supercoiled forms, a factor of 1.4 was used to correct for this.
associated with excessive ROS generation (for one of many treatments of this topic, see Halliwell and Gutteridge, 1999). In this study, we find that DEP are capable of catalyzing DNA damage in the presence of a reductant, including DTT or ascorbate.

It was found that DEP contain a stable and prevalent paramagnetic species. This finding is not surprising since other researchers have reported EPR signals indicative of paramagnetic organic species in cigarette tar and extracts (Stone et al., 1995; Zang et al., 1995), airborne particles (Dellinger et al., 2001), and even in C_{60} fullerene preparations (Paul et al., 2002). The EPR signal in DEP is similar to those previously reported for semiquinone radical species in PM$_{2.5}$ (mean aerodynamic diameter < 2.5 microns) particulate matter (Dellinger et al., 2001) and cigarette tar extracts (Stone et al., 1995). It should not be surprising that similar signals are detected in PM$_{2.5}$ since they likely include DEP (whose average size, 0.1–0.3 nM, would indicate their presence in the PM$_{2.5}$ fraction). Since semiquinone radical species have been implicated in the generation of ROS in biological systems via redox cycling (Stone et al., 1995), it may well be that a non-dissociable semiquinone radical species associated with the DEP is at least partially responsible for the generation of ROS by the reductants tested in this study.

This work begins to provide definition and characterization of the inherent chemistry of DEP. Recent studies indicating intracellular localization of DEP, and ultrafine PM, underscore the importance of establishing the toxicologically relevant chemistry of these particles. The results of this study indicate that DEP are themselves reactive entities which can catalyze the reduction of O$_2$ by a variety of reducing agents, including biologically relevant reductants. This reactivity appears to be an intrinsic part of the particles since methylene chloride or aqueous acid extraction of DEP did not significantly alter their reactivity.

FIG. 4. ESR spectra of DEP after various treatments.
Evidence for oxidative damage to DNA in the presence of DEP has also been observed. Finally, EPR analysis of the particles indicates that they contain paramagnetic species which are likely to be semiquinones, and which may participate in the redox processes. To be sure, it will be difficult to extrapolate the conditions of these chemical studies to actual in vivo DEP exposure. However, the results of this study indicate the possibility of particle-dependent chemical processes that can contribute, along with biologically extractable components, to the overall toxicity of DEP.

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