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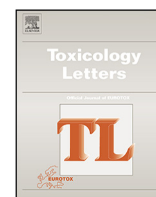

# Disruption of the integrity and function of brain microvascular endothelial cells in culture by exposure to diesel engine exhaust particles

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## HIGHLIGHTS

- Exposure to DEPs induced higher oxidative stress in HBMVEC cells.
- DEPs induce cell death and disrupt the function and integrity of HBMVEC cells.
- Data indicate a potential role of DEPs in neurotoxicities.

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## ABSTRACT

Diesel exhaust particles (DEPs), a by-product of diesel engine exhaust (DEE), are known to produce pro-oxidative and pro-inflammatory effects, thereby leading to oxidative stress-induced damage. Given the key role of DEPs in inducing oxidative stress, we investigated the role of DEPs in disrupting the integrity and function of immortalized human brain microvascular endothelial cells (HBMVEC). To study this, HBMVEC cells were exposed to media containing three different concentrations of DEPs or plain media for 24 h. Those exposed to DEPs showed significantly higher oxidative stress than the untreated group, as indicated by the glutathione (GSH) and malondialdehyde (MDA) levels, and the glutathione peroxidase and glutathione reductase activities. DEPs also induced oxidative stress-related disruption of the HBMVEC cells monolayer, as measured by trans-epithelial electrical resistance. Taken together, these data suggest that DEPs induce cell death and disrupt the function and integrity of HBMVEC cells, indicating a potential role of DEPs in neurotoxicities.

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## 1. Introduction

Diesel engine exhaust (DEE) is a complex mixture of organic and inorganic gases (NOx, SOx, CO), particulate matters (PMs), quinones, PAHs, and transition metals. Diesel exhaust particles (DEPs), a by-product of DEE, are one of the major components of airborne particulate matter in the urban environment. They are composed of carbon, heavy carbohydrates, hydrated sulfuric acid, polycyclic aromatic hydrocarbons (PAHs), and their derivatives: quinones, semi-quinones, and trace amounts of heavy metals such as iron, copper, chromium, and nickel (Bai et al., 2001; Vouk and Piver, 1983; Hartz et al., 2008). DEPs are easily respirable and capable of being deposited in the airways and the alveoli. They can pass through the respiratory tract to enter the circulation, reaching extra pulmonary tissues and having the potential to translocate to other tissues, including the brain by passing through the blood–brain barrier (BBB) (Oberdörster et al., 2002, 2004; Elder et al., 2006; Sugamata et al., 2006). It is believed that they can translocate to the brain *via* two routes, either through translocation along the olfactory nerve or by crossing the lung-blood barrier and the BBB (Oberdörster et al., 2005; Peters et al., 2006). Peters et al. (2006) have demonstrated that erythrocytes can uptake particles that range in size from 0.02 μm to 0.20 μm. It is believed that these particles can penetrate circulating cells, like erythrocytes, and translocate to other organs, including the brain.

Translocation to and accumulation of ultrafine particles in the brain (Oberdörster et al., 2004) are a concern owing to their potential neurotoxic consequences. Epidemiological studies have demonstrated a positive association between particulate matter

*Abbreviations:* CNS, central nervous system; DEE, diesel engine exhaust; DEPs, diesel exhaust particles; GSH, glutathione; GPx, glutathione peroxidase; $H_2$DCF-DA, 2′,7′-dichlorofluorescein diacetate; MDA, malondialdehyde; NPM, *N*-(1-pyrenyl)-maleimide; PBS, phosphate buffered saline; ROS, reactive oxygen species.

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and a number of diseases that affect the respiratory and cardiovascular systems, as well as the central nervous system (CNS) (Dockery et al., 1993; Pope et al., 1995; Sarnat et al., 2001; Nel et al., 1998; Diaz-Sanchez et al., 1997; Li et al., 1996). Even though the effects of DEPs on the lungs and cardiovascular system are relatively well-known, and can be linked to oxidative stress in some cases, limited information is available on the effect of DEPs on CNS.

In the last few years, a body of evidence has indicated that ultrafine particulate matter may cause neurodegenerative diseases like strokes, Parkinson's, and Alzheimer's. (Hirtz et al., 2007). Histological evidences indicate neurodegeneration in both canine and human brains that are exposed to high ambient PM levels. In addition, *in vivo* studies in mice have demonstrated the presence of oxidative stress, toxicity, and inflammation in brain tissue upon inhalation of particulate matter (Peters et al., 2006; Campbell et al., 2005; Elder et al., 2006; Kleinman et al., 2008; Veronesi et al., 2005; Oberdörster et al., 2004, 2005; Block et al., 2004; Hartz et al., 2008). This is further supported by *in vitro* studies that reported neurotoxic effects on specific brain cells and BBB disruption upon exposure to DEE particles (Block et al., 2004; Hartz et al., 2008; Long et al., 2007). In addition, free radical activity on the PM particle's surface has the potential to disrupt the tight junctions and facilitate particle translocation by damaging the BBB (Peters et al., 2006). Some of the chemical compounds in DEPs, such as quinones, PAHs, and transition metals, may induce reactive oxygen species (ROS) due to their ability to disrupt electron transfer in the inner mitochondrial membrane.

Translocation and accumulation of DEPs in the brain raises concerns about serious health consequences since free radical production and oxidative stress are implicated in the pathogenesis of different neurodegenerative disorders. The need for investigation of the role of DEPs in CNS damage is pressing because of rapidly increasing air pollution worldwide. In lieu of studies supporting the role of DEPs in oxidative stress-induced damage, we evaluated the role of DEPs in inducing oxidative stress in HBMVEC cells and disrupting their integrity and function.

## 2. Materials and methods

### 2.1. Materials

DEPs were purchased from NIST (SRM 1650b) (Gaithersburg, MD, USA). *N*-(1-pyrenyl)-maleimide (NPM) was obtained from Sigma–Aldrich (St. Louis, MO). High performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were bought from Sigma–Aldrich (St. Louis, MO).

### 2.2. Culture of human brain microvascular endothelial cells (HBMVEC) and toxicity studies

As an *in vitro* BBB model, immortalized human brain endothelial cells, HBMVEC (a gift from Dr. Pierre Courard), were seeded in 25 $cm^2$ tissue culture flasks coated with type 1 rat tail collagen (Sigma–Aldrich, St. Louis, MO) and maintained in EBM-2 medium in humidified 5% $CO_2$/95% air at 37 °C. Culture medium was changed twice a week and endothelial cells at passages 28–34 were used in this study. All assays were performed in triplicate and each experiment was repeated three times. EBM-2 medium (Lonza, Walkersville, MD) was supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin, and 2.5% fetal bovine serum (FBS), as recommended by the manufacturer. This fully supplemented medium was designated as Microvascular Endothelial Cell Medium-2 (EBM-2MV, herein referred to as EBM-2 medium). For dosing cells with DEPs, we used serum-free and growth-factor-free medium for all experiments instead of the fully supplemented media described above. Cells were treated with DEPs for 24 h for all the studies except for intracellular ROS measurements (3 h). DEPs were suspended in phosphate buffered saline (PBS), vortexed, and sonicated for 30 min to give a DEP stock solution concentration of 2 mg/ml. In order to test dose-dependency, a DEP working solution was prepared by diluting the stock DEP solution in a serum-free EBM-2 medium. These concentrations of DEPs were selected based on the reconciliation of the *in vivo* PM exposures, measured in micrograms per cubic meter ($\mu g/m^3$), with the tissue culture concentrations of DEP chemicals, and measured in micrograms per milliliter ($\mu$g/ml). The biologically relevant tissue culture concentration of DEP ranges from 0.2 to 20 $\mu g/cm^2$ which corresponds to 1.4–143 $\mu$g/ml (Li et al., 2003). The DEP particle suspension in the tissue culture medium was reported to contain particles between 40 nm and 2.5 $\mu$m, with a mean particulate diameter of approximately 400 nm (Carero et al., 2001). NIST reports the mean particle size diameter to be 180 nm after 24 h of sonication.

### 2.3. Determination of cell viability

The effect of DEPs on the viability of HBMVECs was assessed using the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI). MTS tetrazolium was reduced by mitochondrial dehydrogenase into a colored formazan product in proportion to the number of living cells. HBMVECs ($3 \times 10^4$ cells/well) were seeded in a 96-well tissue culture plate for a day. The medium was then discarded, and the cells were treated with DEPs (10, 25, 50 $\mu$g/ml) in serum-free medium for 24 h. CellTiter 96® AQueous One Solution Reagent (20 $\mu$l/well) was added to the wells and the plate was incubated at 37 °C for 1 h in a humidified atmosphere of 5% $CO_2$/95% air, and then centrifuged to get rid of the DEPs. The MTS formazan product was measured by determining the absorbance of the supernatant (100 $\mu$l) at 490 nm using a 96-well plate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

### 2.4. Intracellular ROS measurement

Intracellular ROS generation was measured using a well-characterized probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Wang and Joseph, 1999). DCFH-DA was hydrolyzed by esterases to dichlorofluorescin (DCFH), which was trapped within the cell. This nonfluorescent molecule was then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. A DCFH-DA stock solution (in methanol) of 10 mM was diluted 500-fold in HBSS without serum or any other additive to yield a 20 $\mu$M working solution. Cells were washed twice with HBSS and then incubated with a DCFH-DA working solution for 1 h in a dark environment (37 °C incubator). The cells were washed twice with serum-free EBM-2 medium, and varying concentrations of DEPs (10, 25, 50 $\mu$g/ml) were added to cells for 3 h. After this the cells were washed twice with serum-free medium and 100 $\mu$L of serum-free media was added to each well. Then, the fluorescence was determined at 485 nm excitation and 520 nm emission, using a microplate reader (FLUOstar, BMG Labtechnologies, Durham, NC, USA).

### 2.5. Determination of GSH

Intracellular endothelial cell GSH content was determined by reverse phase HPLC, according to the method developed in our laboratory (Ridnour et al., 1999). After treatment, HBMVEC cell samples were homogenized in SBB. Twenty microliters of this homogenate were added to 230 $\mu$l of HPLC grade water and 750 $\mu$l of NPM (1 mM in acetonitrile). The resulting solutions were incubated at room temperature for 5 min. The reaction was stopped by adding 5 $\mu$l of 2 N HCl. The samples were then filtered through a 0.45 $\mu$m filter (Advantec MFS, Inc. Dulin, CA, USA) and injected onto the HPLC system. An aliquot of 2.5 $\mu$l of the sample was injected for analysis using a Thermo Finnigan TM Spectra SYSTEM SCM1000 Vacuum Membrane Degasser, Finnigan TM SpectraSYSTEM P2000 Gradient Pump, Finnigan TM SpectraSYSTEM AS3000 Autosampler, and Finnigan™ SpectraSYSTEM FL3000 Fluorescence Detector ($\lambda_{ex}$ = 330 nm and $\lambda_{em}$ = 376 nm). The HPLC column was a Reliasil ODS-1 $C_{18}$ column (Column Engineering, Ontario, CA, USA). The mobile phase (70% acetonitrile and 30% water) was adjusted to a pH of 2.5 through the addition of 1 ml/l of both acetic and *o*-phosphoric acids. The NPM derivatives were eluted from the column isocratically at a flow rate of 1 ml/min.

### 2.6. Determination of malondialdehyde (MDA)

MDA content was determined as described by Draper et al. (1993). Briefly, the cell pellets were homogenized in SBB. To 0.350 ml of cell homogenate, 0.550 ml of 5% trichloroacetic acid (TCA) and 0.100 ml of 500 ppm butylated hydroxytoluene (BHT) in methanol were added. The samples were then heated in a boiling water bath for 30 min. After cooling on ice, the samples were centrifuged. The supernatant fractions were mixed 1:1 with saturated thiobarbituric acid (TBA). The samples were again heated in a boiling water bath for 30 min. After cooling on ice, 0.50 ml of each sample was extracted with 1 ml of *n*-butanol and centrifuged to facilitate the separation phases. The resulting organic layers were first filtered through a 0.45 $\mu$m filter and then analyzed using the Shimadzu HPLC system with a fluorescence detector. Excitation wavelength and emission wavelength were set at 515 nm and 550 nm, respectively. The column was 100 × 4.6 mm i.d. $C_{18}$ column (3 $\mu$m packing material, Astec, Bellefonte, PA). Twenty microliter samples were injected for analysis. The mobile phase consisted of 69.4% 50 mM sodium phosphate buffer (pH 7.0), 30% acetonitrile, and 0.6% THF. The flow rate of the mobile phase was 1.0 ml/min. The concentrations of the TBA-MDA complex in the mixture were determined by using the calibration curve obtained from a malondialdehyde bis(dimethyl acetal) standard solution.

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