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Diesel exhaust particle exposure causes redistribution of endothelial tube VE-cadherin

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ABSTRACT

Whether diesel exhaust particles (DEPs) potentially have a direct effect on capillary endothelia was examined by following the adherens junction component, vascular endothelial cell cadherin (VE-cadherin). This molecule is incorporated into endothelial adherens junctions at the cell surface, where it forms homodimeric associations with adjacent cells and contributes to the barrier function of the vasculature (Dejana et al., 2008; Venkiteswaran et al., 2002; Villasante et al., 2007). Human umbilical vein endothelial cells (HUVECs) that were pre-formed into capillary-like tube networks *in vitro* were exposed to DEPs for 24 h. After exposure, the integrity of VE-cadherin in adherens junctions was assessed by immunofluorescence analysis, and demonstrated that increasing concentrations of DEPs caused increasing redistribution of VE-cadherin away from the cell–cell junctions toward intracellular locations. Since HUVEC tube networks are three-dimensional structures, whether particles entered the endothelial cells or tubular lumens was also examined. The data indicate that translocation of the particles does occur. The results, obtained in a setting that removes the confounding effects of inflammatory cells or blood components, suggest that if DEPs encounter alveolar capillaries *in vivo*, they may be able to directly affect the endothelial cell–cell junctions.

1. Introduction

Epidemiological studies demonstrate an association between short-term increases in ambient air particulates and adverse cardiovascular events within a 48 h timespan, especially in the elderly or those with pre-existing coronary artery disease (Peters et al., 2001a,b, 2004; Pope and Dockery, 2006; Zanobetti and Schwartz, 2005). Vascular dysfunction is one of the potential mechanisms that may mediate the cardiovascular effects of exposure to particulate matter. Impaired endothelial-dependent vasodilation occurs in healthy young adults exercising near roadways (Rundell et al., 2007) and in older adults exposed to traffic at bus stops (Dales et al., 2007). Groups with pre-existing vascular dysfunction, such as diabetics (O'Neill et al., 2005), have an increased susceptibility to exposure to ambient particulate matter, and an exacerbated inflammatory response is observed in those with non-functional phase II enzyme variants (Gilliland et al., 2004). In addition to the lung's inflammatory response, pulmonary edema has been noted from exposure to particulates (Inoue et al., 2006; Nemmar et al., 2007; Sagai et al., 1993; Singh et al., 2004), indicating increased pulmonary vascular permeability. The presence of microalbumin in bronchoalveolar lavage fluid also supports the observation of vascular leakage into the lung. This can occur as early as 4 h after exposure, as shown in mice who involuntarily aspirated automobile DEPs (Singh et al., 2004). The exact molecular mechanism for how DEPs increase vascular permeability is not known, but one potential cause may be events initiated by direct contact of DEPs with alveolar endothelial cells. A small percentage of inhaled particles have been shown to reach the lung and gain access to the circulation, ending up in several compartments of the body (Brown et al., 2002; Geiser et al., 2005; Kreyling et al.,

Abbreviations: DEPs, diesel exhaust particles; HUVECs, human umbilical vein endothelial cells; $PM_{2.5}$, particulate matter with diameters equal to, or less than 2.5 μm; VE-cadherin, vascular endothelial cell cadherin.

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2002, 2009; Nemmar et al., 2001, 2002a; Oberdorster et al., 2004; Semmler et al., 2004; Shimada et al., 2006; Takenaka et al., 2006). Importantly, translocated particles may directly contribute to the increases in thrombotic activity that are observed in hamsters and humans after DEP exposure (Nemmar et al., 2002b, 2003), explaining some of the observed short-term adverse cardiovascular events.

Inflammatory mediators produced by many cell types likely contribute to the alveolar capillary leakage that results from DEP inhalation, and this complicates the identification of DEP-induced factors from individual cell types. We hypothesize that one of the effects of DEPs is a direct contribution to vascular permeability via disruption of endothelial cell–cell junctions. Barrier function is controlled by both the tight junctions (for review see Spring, 1998) and the adherens junctions (Corada et al., 2001), with adherens junctions controlling tight junctions via vascular endothelial (VE)-cadherin (Taddei et al., 2008). VE-cadherin is an endothelial specific cadherin of adherens junctions that regulates not only vascular permeability, but also leukocyte transmigration (Corada et al., 1999, 2001; Gotsch et al., 1997). To eliminate the contribution of the inflammatory cell response on vascular cell–cell junctions, and thereby examine the direct effect DEPs have on capillary structures, an *in vitro* culture system of pre-formed endothelial tubes was employed. The primary objective of this study was to use VE-cadherin as a marker of cell junction integrity in a system structurally related to capillaries, examining whether disruption of these molecules is one potential mechanism for how short-term (24 h) DEP exposures might induce an increase in vascular permeability in the lung.

2. Materials and methods

2.1. Diesel exhaust particles (DEPs)

Diesel exhaust particles (DEPs) were collected from a Japanese automobile engine by Dr. Masaru Sagai, who subsequently provided them to researchers at UCLA. Our group obtained them as a gift from Dr. David Diaz-Sanchez, formerly of UCLA. The particles have been characterized and used extensively (Bai et al., 2001; Inoue et al., 2006; Ito et al., 2000; Kumagai et al., 1997; Sagai et al., 1993; Singh et al., 2004). DEP powder (0.1 g) was suspended in 10 ml in PBS, 0.05% Tween-80 to make a 10 mg/ml DEP stock solution. Particles were then dispersed to achieve a particle size of $PM_{2.5}$ (2.5 μm diameter and smaller) by vortexing for 3 min, then sonicating at 60 Hz for 5 min. To determine the range of sizes, an aliquot was fixed with 4% paraformaldehyde for examination at 630× magnification (Leica TCS SP2 Spectral Confocal Microscope). A more accurate assessment was made by dynamic light scattering using a Zetasizer Nano ZS90 with Malvern DTS software version 5.10 (Malvern Instruments, Malvern, MA). With this technique, particles are placed in a laser beam. The intensity of the scattered light fluctuates at a rate that is dependent upon the size of the particles, with smaller particles moving more rapidly. Analysis of the intensity fluctuations yields the velocity of the particles' Brownian motion. The particle size is then determined using the Stokes–Einstein equation for diffusion of spherical particles though liquid. Specifications were: temperature, 25 °C; material refractive index, 1.59; material absorption, 0.01; dispersant refractive index, 1.33; viscosity, 0.8881 centipoise; measurement position, 4.65 (mm). Six runs (120 s/run) were performed to determine mean particle diameter. For cell exposures, dilutions of the stock suspension to 1, 10 or 100 μg/ml in medium were made immediately after vortexing and sonicating. Additional concentrations of 5 and 50 μg/ml DEPs were prepared prior to modified LDH assays.

2.2. Endothelial cell culture

Medium used was EBM-2 Bulletkit medium (Lonza), an endothelial cell growth medium which contains 2% FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin, and GA-1000 as purchased. In addition, since the DEPs were dissolved in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4.), 0.05% Tween-80, the medium was also supplemented to the same concentration with phosphate buffered saline and Tween-80, thereby minimizing differences between non-DEP-exposed controls and DEP-treated samples. In all cases below, the term "medium" refers to medium plus PBS–Tween-80.

Normal human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Lonza Walkersville, Inc.) and used at passages 5–15. Cells were always plated at a density of 156 cells per mm^2. This translates to 6×10^4 cells per well on the 12 well plates and 1.5×10^5 cells per well on the 6 well plates. Cultures were incubated in a 5% CO_2 atmosphere at 37 °C in a volume of medium proportional for the cell number, to insure that culturing parameters were always comparable between different well sizes. Medium was changed every day.

For monolayer cultures, HUVECs were plated on plastic tissue culture dishes. When used for assembling tube structures, cells were plated on the basement membrane substratum, Matrigel, a liquid at 4 °C which becomes solid at room temperature or above. LDEV-free Matrigel (BD Biosciences) at 10 mg/ml, 4 °C, was added to plates to completely coat the bottoms of 12-well (3.8 cm^2/well) or 6-well (9.6 cm^2/well) culture dishes residing on ice. Matrigel-coated dishes were transferred to the incubator to allow the substratum to solidify at 37 °C for 30 min before adding cells.

2.3. Preliminary assessments of endothelial tubes

Tube formation time was determined by seeding HUVECs onto Matrigel-coated dishes, and incubating them at 37 °C for 1, 2, 4, 6, 12 and 24 h. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. DAPI (1 ml/well of 300 nM final concentration in PBS) was used to stain nuclei in the samples after fixing. Phase contrast microscopy (Zeiss-Axiovert 40 Inverted Microscope) evaluated tube formation, and showed that by 12 h tube formation was totally complete, i.e., every DAPI stained nucleus resided in a cell participating in a tubular structure. For DEP exposure experiments, endothelial cells were plated on plastic (to form monolayers) or on Matrigel (to form tubes) at a density of 156 cells/mm^2 for 12 h, allowing the Matrigel samples to complete tube network formation. This 12 h post-plating time was defined as the "zero" time point in experiments. Endothelial tubes were incubated with either 0 μg/ml DEP (i.e., no DEP), or 1, 10 or 100 μg/ml dispersed DEPs in medium, unless otherwise indicated. Cultures were incubated at 37 °C for 24 h after the zero time point unless otherwise indicated.

Endothelial cell behavior as monolayers and as tube network cultures was compared. Proliferation was measured using the MTS assay, measuring mitochondrial enzyme activity via conversion of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and phenazine methosulfate to formazan (MTS kit, Promega). Three plates of cells were assayed at the zero time point (12 h after plating for both monolayers and endothelial tubes), another set of 3 at the 24 h post-zero time point, and a third set at 48 h post-zero time point. Cells were rinsed 3 times with cold PBS, then a mixture of 60 μl water-soluble kit reagent plus 300 μl fresh medium was added to each well for a 1 h incubation at 37 °C in the dark. Supernatants (100 μl/well) were collected and the absorbance of the generated formazan was measured at 490 nm. This absorbance reflects the total number of cells in each sample, and allows calculation of doubling time.

2.4. Modified LDH cytotoxicity assay to detect cell survival after DEP exposure

DEP cytotoxicity to endothelial tubes was evaluated using the CytoTox-Homogeneous Integrity Assay Kit (Promega), a method that measures cytosolic lactate dehydrogenase (LDH) released into medium when cells are lysed. The method was adapted to remove particles and dead cell-derived-LDH from the living cells after DEP exposure, to ensure the DEPs would minimally interfere with the assay. Plates of formed endothelial tubes were exposed to medium alone (the no DEPs control) or medium containing DEPs (1, 5, 10, 50, and 100 μg/ml medium) for a 24 h 37 °C incubation. Next, medium (containing LDH from dead cells and floating cell debris) was aspirated from the cultures. Endothelial tube cells were then washed 3 times in cold PBS. Cells were collected by centrifugation and lysed for 1 h in 200 μl lysis solution (Promega) following the manufacturer's instructions. The relative fluorescence (described in RFUs, relative fluorescence units) of LDH was measured at 490 nm. Unexposed cultures were used as the positive control, defining the maximum amount of LDH potentially released. This value was defined as 100%. With the adaptation described, the absorbance levels of DEP-exposed samples represent cells surviving the exposure, and were expressed as a percentage of the control unexposed sample.

2.5. VE-cadherin immunofluorescence

HUVECs (6×10^4 cells/well, plated at 156 cell/mm^2 density) were seeded onto Matrigel-coated 2-well chamber slides for tube formation prior to DEP exposures for 24 h. After exposure, endothelial tubes were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Nonspecific reactivity of HUVECs was blocked by addition of 2% normal goat serum with 0.02% sodium azide (NaN_3) in PBS for 1 h at room temperature. The endothelial tube cells were then incubated with primary anti-human VE-cadherin (BD Biosciences) monoclonal antibody at a 1:50 dilution (20 μl in 1 ml blocking buffer, i.e., 2% normal goat serum) for 1 h at room temperature. Goat anti-mouse secondary antibody labeled with Alexa 488 (green color, Jackson Immuno Research) was used at a 1:100 dilution (10 μl in 1 ml PBS) for 1 h at room temperature. When wide field (epifluorescence) microscopy was used, nuclei were stained by incubating endothelial tubes in 300 nM DAPI for 5 min at room temp, followed by washing with PBS/Tween for 5 min. When confocal microscopy was used, nuclei were stained by adding 1 ml 20 μM DRAQ5 (Alexis) for 10 min at room temperature to each well. Slides were covered with Prolong Gold (Invitrogen) anti-fade mounting media and incubated at 4 °C overnight. All images were observed at 100× and 400× magnifications on an epifluorescence microscope