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DIESEL particulate exposed macrophages alter endothelial cell expression of eNOS, iNOS, MCP1, and glutathione synthesis genes

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ABSTRACT

There is considerable debate regarding inhaled diesel exhaust particulate (DEP) causing impairments in vascular reactivity. Although there is evidence that inhaled particles can translocate from the lung into the systemic circulation, it has been suggested that inflammatory factors produced in the lung following macrophage particle engulfment also pass into the circulation. To investigate these differing hypotheses, we used *in vitro* systems to model each exposure. By using a direct exposure system and a macrophage-endothelial cell co-culture model, we compared the effects of direct DEP exposure and exposure to inflammatory factors produced by DEP-treated macrophages, on endothelial cell mRNA levels for *eNOS*, *iNOS*, *endothelin-1*, and *endothelin-converting-enzyme-1*. As markers of oxidative stress, we measured the effects of DEP treatment on glutathione (GSH) synthesis genes and on total GSH. In addition, we analyzed the effect of DEP treatment on *monocyte chemo-attractant protein-1*. Direct DEP exposure increased endothelial GCLC and GCLM as well as total GSH in addition to increased *eNOS*, *iNOS*, and *Mcp1* mRNA. Alternatively, inflammatory factors released from DEP-exposed macrophages markedly up-regulated endothelial *iNOS* and *Mcp1* while modestly down-regulating *eNOS*. These data support both direct exposure to DEP and the release of inflammatory cytokines as explanations for DEP-induced impairments in vascular reactivity.

1. Introduction

Exposure to traffic-related air pollution has been associated with cardiopulmonary mortality, ischemic heart disease, dysrhythmias, heart failure, and cardiac arrest (Dockery et al., 1993; Pope et al., 2002, 2004a,b; Hoek et al., 2001, 2002). A particularly hazardous component of traffic-related air pollution is fine ambient particulate matter ($PM_{2.5}$), which in many urban regions is in large part derived from diesel engines (Lewtas, 2007). Furthermore, numerous studies have demonstrated that short-term increases in $PM_{2.5}$ are associated with increased emergency room visits for many acute cardiovascular related events such as myocardial infarction and ischemic heart failure (Dominici et al., 2006; Peters et al., 2001a,b).

Diesel exhaust particulate (DEP) makes up a significant portion of ambient $PM_{2.5}$, which is defined as particles with an aerodynamic diameter of 2.5 μm or less ($PM_{2.5}$). This size range of PM is of most concern to human health as it has the ability to penetrate deep into the lung when inhaled, and thus is not efficiently removed by mucociliary clearance mechanisms (EPA, 2003). Instead, these particles are cleared by macrophage engulfment. Macrophage engulfment of DEP can lead to the production of proinflammatory cytokines (Maier et al., 2008; Sawyer et al., 2010) and it has been demonstrated that diesel exhaust (DE) inhalation causes neutrophilic airway inflammation (Nordenhäll et al., 2000; Salvi et al., 1999; Stenfors et al., 2004), and activation of antioxidant response pathways within the airway and lungs (Mudway et al., 2004; Pourazar et al., 2005). In addition, this induction of local airway and lung inflammation has been suggested to be a critical event

Abbreviations: $PM_{2.5}$, particulate matter; DEP, diesel exhaust particulate; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; Mcp1, monocyte chemoattractant protein 1; ROS, reactive oxygen species; NO, nitric oxide; $ONOO^-$, peroxynitrite; BH4, tetrahydrobiopterin; Edn-1, endothelin-1; ECE1, endothelin converting enzyme 1; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; AREs, antioxidant response elements; DMSO, dimethylsulfoxide; PBS, phosphate buffered saline; FBS, fetal bovine serum; RT-PCR, reverse transcriptase real time polymerase chain reaction; ANOVA, analysis of variance; SEM, standard error of the mean; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GSH, glutathione; IL1β, interleukin 1β; GMCSF, granulocyte macrophage colony stimulating factor; IL6, interleukin 6; TNFα, tumor necrosis factor α; Noxs, NAD(P)H oxidases.

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underlying systemic vascular effects associated with prolonged DE inhalation. These effects on the lung have been associated with systemic vascular inflammation and increases in serum proinflammatory cytokine concentrations (Stenfors et al., 2004; Sun et al., 2005, 2008; Törnqvist et al., 2007). Although there is considerable discussion regarding the systemic vascular effects of proinflammatory cytokines produced in the lungs, there is also strong evidence that a small fraction of inhaled particles are capable of translocating from the alveolar space into capillaries and pulmonary venules, allowing them to gain access to the systemic and coronary circulations (Nemmar et al., 2001, 2002, 2004). In this exposure scenario, DEP could influence vascular reactivity by directly interacting with the vascular endothelium.

DEP is a known pro-oxidant and its induction of pulmonary inflammation is thought to be largely due to its ability to incite oxidative stress. A small fraction of translocated DEP would likely have limited ability to directly interact with endothelial cells unless there was substantial translocation into the vasculature. Thus, because there is only limited particle interaction with vascular endothelium, it is likely that inflammatory factors released from the inflamed lung have a greater potential to produce this impairment in vascular reactivity. Accordingly, a significant amount of research has been focused on how inflammatory factors generated in the lung can disrupt endothelial nitric oxide synthase (eNOS) function, in that eNOS becomes 'uncoupled' following oxidation of its cofactor tetrahydrobiopterin (BH4). Although eNOS uncoupling may explain some of the effects on vascular function mediated by DE, there have been suggestions that $PM_{2.5}$ inhalation can alter mRNA expression for several vasoactive genes, including: *endothelin 1*, *endothelin receptor B*, *endothelin converting enzyme 1* (*ECE1*), *eNOS*, and *inducible NOS* (*iNOS*) (Thomson et al., 2005, 2007).

To investigate the potential role of direct DEP exposure to vascular endothelium versus exposure to DEP-induced proinflammatory factors, we compared gene expression in endothelial cells following direct DEP exposure and following exposure to macrophage-derived inflammatory factors when these cells were co-cultured with DEP-treated macrophage cells. By using this *in vitro* system, we modeled what would occur in endothelial cells if there were translocation of DEP from the lung into the systemic circulation, or if inflammatory factors generated in the lung entered the systemic circulation. In addition, in this report we investigated how DEP influences monocyte chemoattractant protein 1 (*Mcp1*) mRNA levels and, as a marker of oxidative stress, the mRNA levels of the catalytic (*Gclc*) and modifier (*Gclm*) subunits of the rate limiting enzyme in GSH synthesis, glutamate cysteine ligase (GCL).

Due to the large body of evidence that proinflammatory factors can influence gene expression for many vasoactive genes, and because it is unlikely that sufficient amounts of DEP are capable of translocating from the lung into the vasculature to cause widespread systemic changes in gene expression, we hypothesized that in our co-culture model gene expression changes would be more consistent with a vasoconstrictive/inflamed phenotype, characterized by a decrease in the expression of *eNOS*, while simultaneously upregulating expression of *iNOS*, *Edn1*, and *ECE1*. In addition, we also hypothesized that, as DEP is a pro-oxidant, direct DEP exposure would produce an upregulation of the antioxidant genes *Gclc* and *Gclm* and this will lead to a compensatory increase in total GSH.

Here, we demonstrate that direct DEP exposure can up-regulate *eNOS* (*Nos3*), *iNOS* (*Nos2*), *Gclc*, and *Mcp1* mRNAs in mouse endothelial cell line, but that co-culture of endothelial cells with DEP-exposed murine macrophages down-regulates *eNOS* while subsequently upregulating *iNOS* and *MCP1* to a much greater extent than seen with direct DEP exposure. Although DEP can directly produce oxidative stress, these findings support the view that the generation of inflammatory factors is likely the more significant pathway for DEP-induced changes in vasoactive gene expression.

2. Materials and methods

2.1. Cell culture

The simian virus 40 transfected mouse lymph node endothelial cell line SVEC4-10 and the mouse macrophage cell line RAW264.7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured following ATCC guidelines. For co-culture studies, RAW264.7 and SVEC4-10 cells were cultured in a 1:1 mixture of DMEM and RPMI media with 10% FBS plus antibiotics (100 units/ml penicillin; 100 μg/ml streptomycin). RAW264.7 cells were plated on Transwell inserts (Costar, City, ST) with a membrane pore size of 0.2 μm, and placed into six well-plates containing adherent SVEC4-10 cells. When using transwell inserts, soluble secreted factors from both macrophage cells and endothelial cells can pass freely between both sides of the insert membrane, but due to size exclusion, aggregates of DEP are not able to pass through the membrane. Having DEP-exposed macrophage cells within the insert and endothelial cells below, we were able to model the effects that DEP-induced, macrophage dependent inflammation would have on endothelial gene expression.

2.2. Exposure and DEP collection

$PM_{2.5}$ was collected from a Cummins diesel engine operating under load. Particles were collected from the outflow duct from the University of Washington diesel exhaust exposure facility. The fine particulate matter size distributions are very similar to aged diesel exhaust a few hundred meters away from a major roadway; these particles and exposure facility characteristics have been previously described (Gould et al., 2008). DEP were suspended in DMSO (2.5%) then further diluted in PBS (97.5%) to a 10 mg/ml stock solution. Cells were plated into six well culture plates and were dosed with appropriate volumes of DEP suspension to achieve the desired concentration. DEP stock solutions were sonicated for 1 min prior to all dosing. All control wells were dosed with an equivalent volume of the 2.5% DMSO, 97.5% PBS solution as a solvent control.

2.3. Fluorogenic 5′ nuclease-based assay and quantitative RT-PCR

The Center for Ecogenetics Functional Genomics Laboratory at the University of Washington developed fluorogenic 5′ nuclease-based assays to quantitate the mRNA levels of specific genes. RNA was isolated using Qiagen RNeasy kit according to the manufacturer's protocol. Reverse transcription was performed using total RNA and the SuperScript® III First-Strand Synthesis System, also according to the manufacturer's established protocol (Invitrogen, Carlsbad, CA). For gene expression measurements, 2 mL of cDNA were included in a PCR reaction (12 mL final volume) that also consisted of the appropriate forward (FP) and reverse (RP) primers, probes and TaqMan Gene Expression Master Mix (Applied Biosystems Inc., Foster City, CA). The PCR primers and the dual-labeled probes for the genes were designed using Primer Express v.1.5 software (ABI) Several genes were assessed using the inventoried TaqMan® Gene Expression Assays mix according to the manufacturer's protocol (ABI). Amplification and detection of PCR amplicons were performed with the PRISM 7900 system (ABI) with the following PCR reaction profile: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, and 62 °C for 1 min. Beta-actin amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and Beta-actin mRNA expression levels were utilized as an internal control to normalize the data.