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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.

Keywords

Diesel exhaust particulate; nitric oxide synthase; endothelin; glutathione; inflammation

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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, Pope et al., 2004a, Pope et al., 2004b, Hoek et al., 2001, Hoek et al., 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, Peters et al., 2001b).

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 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, Sun et al., 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., 2002, Nemmar et al., 2001, Nemmar et al., 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., 2007, Thomson et al., 2005).

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 wide-spread 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*, *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 *MCPI* 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.

Materials and Methods

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 ug/ml streptomycin). RAW264.7 cells were plated on Transwell inserts (Costar, City, ST) with a membrane pore size of 0.2 μ m, and placed into 6 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.

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 6 well culture plates and were dosed with appropriate volumes of DEP suspension to achieve the desired concentration. DEP stock solutions were sonicated for 1

minute 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.

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 30sec, 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.

Glutathione and GCL subunit protein levels

GCLM and GCLC protein levels were detected with the use of rabbit polyclonal antisera raised against ovalbumin conjugates of peptides specific to each subunit, using previously described procedures (Thompson et al., 1999). Total GCLM and GCLC levels were determined by Image J software and normalized to β -actin. For GSH analysis, clarified cell homogenates prepared in TES/SB buffer (20 mM Tris, 1mM EDTA, 0.25M Sucrose/ 20mM Sodium Borate) were diluted 1:1 with 10% 5-sulfosalicylic acid, incubated on ice for 10 min, and then centrifuged at 13,000 rpm in a microcentrifuge for 2 min to obtain deproteinated supernatants. Twenty-five-microliter aliquots of the supernatants were added to a 96-well black microtiter plate in triplicate followed by addition of 100 μ l of 0.2M N-ethylmorpholine/0.02M NaOH. Following the addition 50 μ l of 0.5N NaOH, total GSH was derivatized by adding 10 μ l of 10 mM naphthalene-1,2-dicarboxaldehyde (NDA). The reaction was incubated at room temperature for 30 min, and the fluorescence intensity of NDA-GSH measured at λ_{ex} 472 and λ_{em} 528, and quantified by interpolation on a standard curve constructed with NDA-conjugated GSH in TES/SB:10% sulfosalicylic acid (1:1).

Statistical Analysis

Quantitative real-time PCR data were analyzed using Prism (Graphpad Software, La Jolla, CA). Differences were determined by ANOVA followed by a Dunnett's post-hoc test. All error bars in figures represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Results

DEP concentrations of 0, 20, and 100 μ g/ml were chosen based on previous *in vitro* cytotoxicity assays we conducted demonstrating that these DEP have very little effect on SVEC4-10 or RAW264.7 cell viability at these concentrations (Supplemental Figure 1). Treatment of SVEC4-10 with 100 μ g/ml DEP for 24 hours caused less than a 20% reduction in cell metabolic activity and no increase in cell death, as determined by reduction of 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan and uptake of the cell membrane impermeable DNA stain propidium iodide, respectively (Supplemental Figure 1).

Effect of direct DEP treatment on endothelial eNOS, iNOS, Edn1, and ECE-1 expression

Although DEP did not induce overt cytotoxicity, direct DEP treatment to a confluent culture of SVEC4-10 cells led to a robust increase in *eNOS* and *iNOS* mRNA levels at both 3 and 6 hours (Figure 1, panels A and B). Interestingly, direct DEP treatment produced a decrease in *Edn1* mRNA levels at both 3 and 6 hours at the high dose of 100 mg/ml while the levels of *ECE-1* mRNA were not altered (Figure 1, panels C and D).

Effect of DEP treatment on co-cultured macrophages and endothelial cells

In order to test the ability of factors secreted by DEP exposed macrophages to induce changes in gene expression in vascular endothelial cells, we co-cultured RAW264.7 mouse macrophage cells on transwell inserts and placed them into multiwell plates that had been seeded with SVEC4-10 mouse endothelial cells. In this way, DEP exposure could primarily be limited to the macrophages within the insert, while soluble factors smaller than 0.2 μm could freely pass back and forth between the two cell types. First, we analyzed gene expression of known proinflammatory cytokine mRNAs within the RAW264.7 macrophage cell line following 6 and 24 hr treatment with DEP while in co-culture with SVEC4-10 endothelial cells. DEP treatment produced a dramatic increase in *TNF α* , *IL6*, *IL1 β* , and *Gmcsf* mRNA levels (Figure 2). For all genes except *TNF α* , mRNA expression was dramatically higher at 6 hr compared to 24 hr. Although *IL6*, *IL1 β* , and *Gmcsf* mRNA levels reached upwards of a 100-700 fold increase compared to vehicle treated controls, the levels of *TNF α* mRNA reached no more than an 8 fold increase over controls (Figure 2). This discrepancy is likely due to the differences in background gene expression of *TNF α* and *IL6*, *IL1 β* , and *Gmcsf*. The mRNA levels of *TNF α* in vehicle treated controls were dramatically higher than any other gene since *IL6*, *IL1 β* , and *Gmcsf* mRNA levels were barely detectable in vehicle controls.

Following confirmation that proinflammatory cytokine mRNAs were being upregulated in the macrophages after treatment with DEP, we next evaluated the effects of DEP treatment to macrophages placed in co-culture with endothelial cells on mRNA levels in the latter cell type. In contrast to the effects observed when endothelial cells were directly exposed, DEP treatment in co-culture produced a downregulation of *eNOS* mRNA levels in the SVEC4-10 cells. Nonetheless, as with direct DEP exposures, there was still an increase in *iNOS* mRNA when these endothelial cells were co-cultured with DEP exposed macrophages (Figure 3, panels A and B). The depression of *eNOS* mRNA levels in endothelial cells following DEP treatment of macrophages in co-culture was dose dependent and seemed to no longer be present at 24 hr. In addition, upregulation of *iNOS* mRNA seemed to be more robust at 6 hr compared to 24 hr. Consistent with what had been observed for the direct DEP exposure model, *Edn1* mRNA was down-regulated in the endothelial cells at the high DEP dose when they were co-cultured with DEP exposed macrophages at both 6 and 24 hr (Figure 3, panel C).

Effects of DEP on glutathione (GSH) and GSH synthesis genes *Gclc* and *Gclm*

GSH is an important intracellular antioxidant, and has been postulated to regulate DEP-induced inflammation and vascular function. We thus determined the effects of DEP on endothelial *Gclc* and *Gclm* mRNA levels, total GSH, and GCLC and GCLM protein levels following direct DEP exposure or with DEP-treated macrophages in a co-culture. We demonstrated that direct exposure of endothelial cells to DEP caused a significant upregulation of *Gclc* mRNA (Figure 4, panel A). This trend was also present for *Gclm*

mRNA but the slight increase at the high dose at 6 hours did not reach statistical significance (Figure 4, panel B). Interestingly, in contrast to direct exposures, there were no changes in endothelial *Gclc* and *Gclm* mRNA levels at either 6 or 24 hr following DEP treatment of macrophages in the co-culture model (Figure 4, panels C and D).

We next determined total GSH in endothelial cells following 24 hr DEP treatment in the direct and co-culture models. Direct DEP treatment produced a significant increase in GSH at both 20 and 100 $\mu\text{g/ml}$ (Figure 5). These data are in agreement with the *Gclc* and *Gclm* mRNA expression data, and support the role of GCL expression as a potentially limiting factor in GSH synthesis. Interestingly, in the co-culture model GSH was significantly increased in the high-dose treatment (Figure 5), even though mRNA levels for *Gclc* or *Gclm* were not increased (Figure 4, panels C and D).

In order to determine if changes in *Gclc* and *Gclm* mRNA levels were accompanied by changes in their respective proteins, we measured GCLC and GCLM protein levels by Western blot analysis in endothelial cells following 24 hr DEP treatment in direct and macrophage co-culture models. Western blot analysis showed that both GCLC and GCLM protein levels were significantly increased at 100 $\mu\text{g/ml}$ in the direct exposure model (Figure 6, panels A and B). Although not significant, this trend was also present at the 20 $\mu\text{g/ml}$ dose. Matching the mRNA expression data, DEP treatment in the co-culture model did not increase GCLC protein levels (Figure 6, panel C). In contrast, DEP treatment in the co-culture model actually produced a significant decrease in GCLM protein levels at the high dose (Figure 6, panel D), which was consistent with the slight but non-significant trend for lower *Gclm* mRNA levels seen in the endothelial cells exposed under similar conditions (Figure 4, panel D).

Effects from media soluble fraction of DEP

In order to determine if these changes in endothelial mRNA levels were due to either macrophage derived soluble factors, or soluble factors present on DEP, or possibly ultrafine DEPs able to pass through the 0.2 μm pore opening in the transwell inserts, we analyzed the mRNA levels of *eNOS*, *iNOS*, *Edn1*, and *ECE-1* with and without RAW264.7 cells present in the transwell insert. With the addition of DEP to the insert in the absence of RAW264.7 cells, endothelial cells would be exposed to a possible soluble fraction of the DEP but not to any macrophage derived mediators. In the absence of RAW264.7 macrophage cells, addition of DEP to the transwell insert caused a slight, but significant decrease in *eNOS* mRNA at 6 hours in SVEC4-10 cells adherent to the bottom of the well (Figure 7, panel A). Although no other changes were significant, there were trends of decreasing mRNA levels for *Edn1* and *ECE1* at 6 hr. However, the decrease in *eNOS* mRNA and the decreasing trends in *Edn1* and *Ece1* mRNAs seen at 6 hr were not present at 24 hr (data not shown). Although DEP addition to the insert alone did produce a small but significant decrease in *eNOS* mRNA, *iNOS* mRNA levels were not altered unless the macrophage cells were present (Figure 7, panel B), indicating that macrophage derived factors are critical in producing this response.

Effects of endothelial derived factors on macrophage inflammation

After we identified that macrophage derived factors are critical for producing the upregulation of *iNOS* in endothelial cells, we investigated whether factors released from endothelial cells were able to alter macrophage mRNA responses to DEP exposure. Thus, we analyzed mRNA levels in RAW264.7 cells cultured in transwell inserts, in the absence or presence of SVEC4-10 cells cultured below. We observed that at the highest DEP dose, the mRNA levels of *IL1 β* , *Gmcsf* and *IL6* were significantly elevated in RAW264.7 cells when co-cultured with SVEC4-10 cells, compared to RAW264.7 cells cultured alone (Figure 8, panels A, B and C). These results suggested that soluble factors released from

endothelial cells are able to exacerbate the macrophage cytokine response induced by DEP. Interestingly this increased response was not observed for *TNF α* mRNA levels (Figure 8, panel D).

Effects of DEP on monocyte chemoattractant protein-1 (Mcp-1) expression in endothelial cells

Mcp-1 has been shown to increase the inflammatory activation of macrophage cells (Deshmane et al., 2009). We thus measured the *Mcp-1* mRNA levels in endothelial cells following direct DEP treatment, DEP treatment in co-culture, and following DEP-treatment in co-culture when macrophage cells were not present. We observed that direct DEP treatment to endothelial cells produces a small but significant increase in *Mcp-1* mRNA expression at the high dose at 6 hours (Figure 9, panel A). Although this response was significant, it was only a 1.7-fold increase over controls. Strikingly, endothelial *Mcp-1* mRNA levels were dramatically higher when these cells were co-cultured with DEP-exposed macrophage cells, reaching nearly an 8-fold increase over vehicle treated controls (Figure 9, panel B). To determine if this increase in *Mcp-1* expression was due to macrophage derived soluble factors, we measured *Mcp-1* expression following DEP addition to the transwell insert when macrophages are not present. No increase was seen in *Mcp-1* mRNA levels under these conditions, indicating that the upregulation seen in DEP-treated co-cultures was dependent upon soluble factors released from DEP-treated macrophage cells.

Discussion

In this study, we attempted to use *in vitro* models to investigate the potential effects of diesel exhaust exposure on endothelial gene expression. Understanding how inhaled fine particulate matter (PM_{2.5}) can induce systemic vasoconstrictive effects is an important research question with broad implications for public health and regulatory policy. The exact mechanisms responsible for this effect of PM_{2.5} are debated. One aspect of this debate is the comparative role of particles translocating from the lung into the systemic circulation directly targeting the endothelium versus inflammatory factors produced in the lung entering the systemic circulation and targeting the endothelium. Here, we demonstrate that both direct DEP treatment to endothelial cells and DEP treatment to macrophages co-cultured with endothelial cells have effects on the mRNA levels of *eNOS*, *iNOS*, *endothelin-1* (*Edn1*), and *monocyte chemoattractant protein-1* (*Mcp1*).

In our direct exposure model, we first observed that DEP caused a marked increase in the expression of both *eNOS* and *iNOS* mRNAs. Although the transcriptional regulation of *iNOS* is largely mediated by the transcription factor NF κ B, regulation of *eNOS* is thought to be mediated by a continual feedback from available nitric oxide (NO), whereby available NO is reciprocally related to *eNOS* transcription (Zhen et al., 2008). Our finding that direct DEP exposure increases not only *iNOS* but also *eNOS* mRNA expression suggests that DEP may cause a depletion in available NO, possibly through the production of reactive oxygen species (ROS) such as superoxide anion radical, which can react with NO to produce the highly reactive molecule peroxynitrite (ONOO⁻). Our results indicate that the increase in *eNOS* mRNA seems to be decreasing at 6 hours compared to 3 hours, also suggesting that the simultaneous upregulation of *iNOS* mRNA and subsequently protein, is likely leading to an increase in NO and producing a negative feedback on *eNOS* transcription.

In our *in vitro* models we also investigated the ability of DEP to influence the expression of endothelin-1. Endothelin-1 (ET-1) is derived from the vascular endothelium, is a potent vasoconstrictor, and numerous reports have demonstrated that human and animal exposures to PM_{2.5} causes an increase in plasma ET-1 as well as expression of *Edn1* mRNA in the

lung (Peretz et al., 2008, Thomson et al., 2007, Thomson et al., 2005). Although we hypothesized that DEP treatment would likely increase *Edn1* expression, direct DEP treatment produced a significant decrease in *Edn1* expression at both 3 and 6 hr at the high dose and in addition we observed no effect on *endothelin-converting-enzyme-1 (ECE-1)* mRNA levels.

In our co-culture model, we exposed macrophage cells to DEP in a transwell insert, cultured simultaneously with endothelial cells attached below in a tissue culture plate. Using this *in vitro* model, we could investigate the potential of DEP-induced macrophage derived soluble factors to influence endothelial gene expression. We demonstrated that DEP treatment did produce a robust upregulation of inflammatory cytokine mRNAs in macrophages. Upon analysis of endothelial gene expression, we observed that DEP-treated macrophages released factor(s) that caused a decrease in *eNOS* mRNA levels while simultaneously upregulating *iNOS* mRNA expression. As discussed above, *iNOS* mRNA induction is largely mediated by the transcription factor NFκB. In contrast, the downregulation of *eNOS* mRNA is possibly due to negative feedback caused by an increase in available NO, likely iNOS derived. These results suggest that DEP-induced inflammatory factors can increase *iNOS* expression in the endothelium, and this could have a negative feedback effect on the level of *eNOS* expression.

Interestingly, we further demonstrate that DEP-induced production of inflammatory factors also have a down regulating effect on *Edn1* as well as *ECE-1*. This is an interesting finding as it is in direct opposition to what has been observed following *in vivo* exposures. Although the mechanism of decreased *Edn1* expression following exposure to DEP and DEP-induced inflammatory factors is not clear, these data suggest that the observed increase in ET-1 within plasma and increased expression of *Edn1* in the lung following *in vivo* PM_{2.5} exposure is likely not due to direct PM_{2.5} exposure, or exposure to inflammatory factors, but likely due to secondary effects on factors that can directly increase ET-1, e.g. angiotensin, thrombin, and NO.

A primary factor that has been attributed to the induction of inflammation and impairments in vascular reactivity observed following PM_{2.5} inhalation is the onset of oxidative stress. A critical antioxidant in regulating oxidative stress is the intracellular antioxidant glutathione (GSH). GSH is a tripeptide thiol that is synthesized in a two-step process, the rate limiting of which is catalyzed by glutamate cysteine ligase (GCL). To investigate how direct DEP and DEP-exposed macrophages influence endothelial oxidative stress, we determined the effect of DEP treatment within both models of exposure on the transcriptional regulation of the antioxidant synthesis genes, the subunits of GCL, *Gclc* and *Gclm*. In addition, we also determined the effect of DEP exposure on total GSH as well as total Gclc and Gclm protein level within endothelial cells. The analysis of *Gclc* and *Gclm* mRNA expression can be a direct marker of oxidative stress as both *Gclc* and *Gclm* genes contain antioxidant response elements (AREs; also know as electrophile response elements, EpREs) within their 5' promoter region and are transcriptionally regulated by the activation of the transcription factor NF-E2-Related-Factor 2 (Nrf2) (Ungvari et al., 2011, Bea et al., 2003, Bea et al., 2009). As such, increased expression of *Gclc* and *Gclm* mRNAs can be markers of increased oxidative stress (Thompson et al., 1999). As we had hypothesized, direct DEP exposure caused a significant upregulation of *Gclc* mRNA at 3 and 6 hours at the high dose, and although it was not significant, *Gclm* mRNA levels showed a similar trend. In the co-culture of DEP-treated macrophages with endothelial cells there was no change in either *Gclc* or *Gclm* mRNAs, and if any trend were present, the data would suggest a decrease in expression. In agreement with the mRNA expression data, upon analysis of total GSH, we found that GSH levels significantly increased at both the medium and high DEP doses following 24 hr of direct treatment. Interestingly, we found that total GSH was also

increased in the high dose following 24 hr of co-culture treatment. This is an interesting result as it indicates that GSH levels can increase without an upregulation in *Gclc* and *Gclm* mRNAs. To determine if the changes in mRNA expression translated to a change in protein levels, we analyzed GCLC and GCLM proteins by Western blot. The results from this analysis support the mRNA expression data since a 24 hr direct DEP treatment increased both GCLC and GCLM, while a 24 hr co-culture does not change total GCLC, but does produce a significant decrease in total GCLM.

The finding that total GSH increases within endothelial cells following exposure to DEP in the co-culture system in the absence of simultaneous increases in GCLC or GCLM proteins may suggest that although there may be insufficient oxidative stress occurring to induce an Nrf2 mediated transcriptional event, other mechanisms of increased GCL activity may be occurring. We have previously demonstrated that GCL activity can be rapidly increased following exposure to oxidative stress due to an increase in GCL holoenzyme formation (Krejsa et al., 2010). It is possible that although the inflammatory factors released from macrophages treated with DEP do not directly cause a rapid increase in oxidative stress, they are capable of increasing the activity and expression of other enzymes that are capable of producing ROS, such as NADPH oxidases following TNF α exposure (Gao et al., 2007). This could then lead to activation of GCL and increased GSH synthesis. Indeed this mechanism of inflammatory factor induced activation of NADPH oxidases and superoxide production has been suggested to be an important factor mediating vascular oxidative stress and impairment in vascular reactivity following PM_{2.5} inhalation (Cherng et al., 2010, Kampfrath et al., 2011, Sun et al., 2005, Sun et al., 2008). Alternatively, uptake of cysteine can be rate limiting for GSH synthesis in some cell types, and cysteine transporter expression can increase under conditions of oxidative stress (Susanto et al., 1998). Regardless of the mechanism, the increase in GSH found in the co-culture model, may be responsible for the down-regulation of *Gclm* protein via negative feedback caused by elevated GSH levels.

Although our model focuses on the investigation of soluble factors released from macrophages targeting the endothelium, we also investigated the potential for factors secreted by endothelial cell to influence changes in the macrophages. To do this, we investigated the inflammatory response following DEP exposure within macrophages, with and without endothelial cells present in the well below the transwell insert. Strikingly, we observed that macrophage cells have a dramatically increased response to DEP for the expression of *IL6*, *IL1 β* , and *Gmcsf* when the endothelium is present. This result suggests that there is a soluble factor released from the endothelial layer that is capable of exacerbating the DEP-induced inflammatory response. This is potentially an important finding as it suggests an endothelium-derived factor that can increase macrophage inflammation. To investigate one potential factor, we looked at the expression of *Mcp1* mRNA in the endothelial cells. MCP-1 is known to exacerbate macrophage inflammation and it is an important factor influencing the progression of atherosclerosis (Deshmane et al., 2009). We demonstrated that there was only a slight increase in *Mcp1* mRNA expression at the high DEP dose when directly applied to the endothelial cells. Alternatively, in the co-culture system DEP-treated macrophages produce a dramatic upregulation of *Mcp1* mRNA in the endothelial cells, a clear marker of endothelial inflammation. To demonstrate that this response was due to macrophage-derived factors, we can clearly show that when macrophage cells are not present, *Mcp1* mRNA was not upregulated.

A study was recently published investigating the inflammatory effects of preconditioned medium from DEP-exposed human monocyte-derived macrophages (MDMs) on human umbilical vein endothelial cells (HUVECs) (Shaw et al., 2010). Their findings are very consistent with ours in that DEP-exposed macrophage medium produced a dose dependent

increase in MCP-1 excreted by the HUVEC cultures. They also demonstrated that direct DEP treatment to HUVEC cultures has no effect on MCP-1 concentrations found in the media. Although our findings indicate that direct DEP at 100 µg/ml induced a slight increase in *Mcp1* gene expression at 6 hours, it is dramatically less than the greater than 8 fold increase in *Mcp1* gene expression found in the co-culture system. Shaw et al. (2010) also investigated the role of TNFα in the induction of endothelial MCP-1. With the use of the soluble TNF receptor Etanercept, which binds to TNFα and limits its biological activity, they demonstrated that TNFα is responsible for nearly 90% of the preconditioned media's induction of endothelium derived MCP-1. The findings of Shaw and co-workers are very important and they are very consistent with our findings and hypotheses. One advantage of their research is that all exposures and testing were conducted in human derived macrophage and endothelial cells. Although research with human derived cells is critical for a better understanding of the effects of DEP-induced human cardiovascular effects, many of our biological models use mice as a surrogate and it is important that we demonstrated these same effects in mouse derived cells and tissues.

In conclusion, our results provide evidence that direct treatment of DEP to endothelial cells produce a different profile of gene expression changes compared to the effects observed when endothelial cells are co-cultured with DEP-treated macrophage cells. In addition, the profile of gene expression using our co-culture model is more consistent with gene expression changes observed during whole animal DE exposure, indicated by a decrease in *eNOS* expression and an increase in *MCP1* and *iNOS* expression. Collectively, these results provide an important 'proof of concept' demonstrating that DEP-exposed macrophages secrete soluble factors that are capable of producing these changes in mRNA levels and further suggest a critical role for the inflammatory responses in the lung as mediators of the impaired systemic vascular reactivity seen in humans when exposed to ambient PM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of all 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
Edn1	Endothelin1
ECE-1	Endothelin Converting Enzyme 1
GCL	Glutamate Cysteine Ligase subunits
GCLC	Glutamate Cysteine Ligase catalytic subunits
GCLM	Glutamate Cysteine Ligase modifier subunits
AREs	Anti-oxidant 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 1 β
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
IL6	Interleukin 6
TNFα	Tumor Necrosis Factor α
Noxs	NAD(P)H oxidases

Highlights

- Diesel exhaust particulate (DEP) has been suggested to traverse alveolar barriers.
- But, DEP vascular effects may be related to the release of inflammatory cytokines.
- We found that direct DEP exposure to endothelium perturbed cell function.
- Endothelial responses were greater when co-cultured with DEP exposed macrophages.
- DEP vascular effects are likely due to release of soluble factors by macrophages.

Figure 1.

Real-time PCR assessment of mRNA levels for *eNOS*, *iNOS*, *Edn1*, and *Ece1* normalized to β -actin in SVEC4-10 cells after 3 and 6 hr of direct DEP exposure. Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 2.

Real-time PCR assessment of mRNA levels for proinflammatory cytokines *Tnfa*, *Il6*, *Il1β*, and *Gmcsf* normalized to β -actin in RAW264.7 macrophage cells following 6 and 24 hr DEP exposure in the co-culture model. Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 3. Real-time PCR assessment of mRNA levels for *eNOS*, *iNOS*, *Edn1*, and *Ece1* normalized to β -actin in SVEC4-10 cells following 6 and 24 hr DEP treatment in the co-culture model. Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 4.

Real-time PCR assessment of mRNA levels for *Gclc* and *Gclm* normalized to β -actin in SVEC4-10 cells after 3 and 6 hr direct DEP exposure (panels A and B) as well as 6 and 24 hr DEP treatment in the co-culture model (panels C and D). Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 5.

Total GSH normalized to total protein in SVEC4-10 cells following 24 hr direct DEP treatment (panel A) and 24 hr DEP treatment in the co-culture model (panel B). Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 6.

Total GCLC and GCLM protein levels normalized to β actin measured by Western blot after 24 hr direct DEP exposure (panels A, B, and C) and 24 hr DEP exposure in the co-culture model (panels D, E, and F). Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 7.

Real-time PCR assessment of mRNA levels for *eNOS*, *iNOS*, *Edn1*, and *Ece1* normalized to β -actin in SVEC4-10 cells after 6 hr DEP treatment in the co-culture model with and without RAW264.7 cells present in the transwell insert. Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 8.

Real-time PCR assessment of mRNA levels for proinflammatory cytokines *Tnfa*, *Il6*, *Il1β*, and *Gmcsf* normalized to β -actin in RAW264.7 macrophage cells following 6 hr DEP treatment in the co-culture model, with and without SVEC4-10 cells present below in the tissue culture plate. Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

Figure 9.

Real-time PCR assessment of mRNA levels for *Mcp1* normalized to β -actin in SVEC4-10 cells after 3 and 6 hr of direct DEP exposure (panel A), 6 and 24 hr DEP exposure in the co-culture model (panel B), and 6 hr DEP exposure in the co-culture model with and without RAW264.7 cells present in the transwell insert. Error bars represent SEM. *, **, *** = Significant difference from the matched control at P-values of < 0.05, 0.01 and 0.001, respectively.

A. Direct DEP to Endothelium

B. DEP-treated macrophage co-culture

Figure 10.

Schematic diagram indicating the proposed mechanism of DEP-induced effects in endothelial cells following direct DEP-exposure (panel A) and when co-cultured with DEP-treated macrophages (panel B).