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EPIDERMAL GROWTH FACTOR RECEPTOR ACTIVATION BY DIESEL PARTICLES IS MEDIATED BY TYROSINE PHOSPHATASE INHIBITION

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Abstract

Exposure to particulate matter (PM) is associated with increased cardiopulmonary morbidity and mortality. Diesel exhaust particles (DEP) are a major component of ambient PM and may contribute to PM-induced pulmonary inflammation. Proinflammatory signaling is mediated by phosphorylation-dependent signaling pathways whose activation is opposed by the activity of protein tyrosine phosphatases (PTPases) which thereby function to maintain signaling quiescence. PTPases contain an invariant catalytic cysteine that is susceptible to electrophilic attack. DEP contain electrophilic oxy-organic compounds that may contribute to the oxidant effects of PM. Therefore, we hypothesized that exposure to DEP impairs PTPase activity allowing for unopposed basal kinase activity. Here we report that exposure to 30 $\mu\text{g}/\text{cm}^2$ DEP for 4 hrs induces differential activation of signaling in primary cultures of human airway epithelial cells (HAEC), a primary target cell in PM inhalation. In-gel kinase activity assay of HAEC exposed to DEPs of low (L-DEP), intermediate (I-DEP) or high (H-DEP) organic content showed differential activation of intracellular kinases. Exposure to these DEP also induced varying levels of phosphorylation of the receptor tyrosine kinase EGFR in a manner that requires EGFR kinase activity but does not involve receptor dimerization. We demonstrate that treatment with DEP results in an impairment of total and EGFR-directed PTPase activity in HAEC with a potency that correlates with the organic content of these particles. These data show that DEP-induced EGFR-phosphorylation in HAEC is the result of a loss of PTPase activities which normally function to dephosphorylate EGFR in opposition to baseline EGFR kinase activity.

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Keywords

diesel exhaust particle; inflammation; protein tyrosine phosphatase; epidermal growth factor receptor

Introduction

Diesel exhaust particles (DEP) are ubiquitous air contaminants in ambient and occupational settings (Lloyd and Cackette, 2001). The composition of DEP is complex and variable consisting of an elemental carbon core with adsorbed organic compounds, as well as small amounts of sulfate, nitrate, metals and other trace elements (Wichmann, 2007). The organic fraction of DEP varies, ranging from 2–50% of the total particle mass, and has been associated with differential pulmonary toxicity and mutagenicity in cell and animal models (Li *et al.*, 2002; DeMarini *et al.*, 2004; Singh *et al.*, 2004).

In human studies, acute exposure (1 hr) to freshly generated DEP has been shown to induce acute pulmonary inflammation characterized by increased levels of neutrophils, B-lymphocytes, and the inflammatory mediators, histamine and fibronectin in bronchoalveolar lavage fluid (Salvi *et al.*, 1999). Another study reported an increased expression of the proinflammatory cytokine IL-8 in bronchial mucosal biopsies obtained from healthy human volunteers exposed to DEP for 1 hr (Pourazar *et al.*, 2005). It is also well established that DEP induces the expression of proinflammatory cytokines in cultured cell systems (Bonvallot *et al.*, 2001; Baulig *et al.*, 2003; Matsuzaki *et al.*, 2006). Taken together, these studies support the notion that exposure to DEP can induce pulmonary inflammation.

Expression of proinflammatory signaling molecules is controlled by phosphorylation-dependent signaling cascades wherein activated kinases function to phosphorylate downstream signaling molecules. In the case of tyrosine kinases, the activities of these enzymes are opposed by that of protein tyrosine phosphatases (PTPases) which thereby function to maintain signaling quiescence (Stoker, 2005). The PTPases constitute a superfamily of enzymes which contain conserved Cys, Arg, and Asp residues critical for catalysis (Barford *et al.*, 1994). The microenvironment of the PTPase active site cleft lowers the pKa of the catalytic cysteine residue to < 6, allowing it to exist in its thiolate anion (R-S⁻) form at physiological pH (Peters *et al.*, 1998). This property renders PTPases highly susceptible to electrophilic attack (Denu and Tanner, 1998; Takakura *et al.*, 1999; Kikuno *et al.*, 2006; Iwamoto *et al.*, 2007).

We have previously shown that divalent zinc (Zn²⁺), another component of ambient PM, induces EGFR activation and upregulation of NF B-dependent IL-8 expression in human airway epithelial cells (Kim *et al.*, 2006; Tal *et al.*, 2006). Moreover, we reported that Zn²⁺ exposure did not increase EGFR kinase activity but rather, impaired EGFR-directed PTPase activity, allowing for ligand-independent activation of the EGFR (Tal *et al.*, 2006). A recent study showed that a specific organic constituent of PM, 1,2-napthoquinone, impairs the tyrosine phosphatase PTP1B leading to sustained EGFR signaling (Iwamoto *et al.*, 2007). However, the link between PTPase inhibition and EGFR activation has not been made for particulate exposures. Here we show that DEP exposure induces EGFR-dependent

phosphorylation through a mechanism involving the inactivation of EGFR-directed PTPase activity in primary human airway epithelial cells, a principal target cell of inhaled PM.

Methods

Preparation of DEP.

Three DEP samples were examined. The first, DEP with low organic content (L-DEP), was obtained from the National Institute of Sciences and Technology (NIST 2975; Donaldson, Minneapolis, MN). The material was collected using a diesel forklift and hot bag filter system. L-DEP contains 2.0 % (wt/wt) extractable organic matter by dichloromethane extraction (Singh *et al.*, 2004). The second sample, DEP with intermediate organic content (I-DEP), was generated in June 2005 at the U.S. Environmental Protection Agency (Research Triangle Park, NC) with the use of a 30-kW (40 hp) four-cylinder Deutz BF4M1008 diesel engine connected to a 22.3-kW Saylor Bell air compressor. I-DEP contains 18.9% (wt/wt) extractable organic matter by dichloromethane extraction (Dr. Seung-Hyun Cho, personal communication). The third particle, DEP with high organic content (H-DEP), was generated using a light-duty (2,740 cc), 4-cylinder, 4JB1-type Isuzu diesel engine with torque load of 6 kg/m generated by an ECDY dynamometer (Meiden-Sya, Tokyo, Japan) and collected previously described by Sagai *et al.* (Sagai *et al.*, 1993). H-DEP contains 26.3 % (wt/wt) extractable organic matter by dichloromethane extraction (Singh *et al.*, 2004). Carbon Black (CB) with an approximate surface area of 50 m²/g (CC-1150 Columbian Chemical Company) was used as a particle control.

Cell culture and treatment.

Primary normal human airway epithelial (HAEC) cells were obtained from normal adult human volunteers by brush biopsy of the mainstem bronchus using a cytology brush during fiberoptic bronchoscopy, conducted under a protocol approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina at Chapel Hill. HAEC cells were initially plated in supplemented Bronchial Epithelial Cell Basal Medium (BEGM; 0.5 ng/ml human epidermal growth factor, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 0.5 µg/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 µg/ml gentamycin, 50 ng/ml amphotericin-B, 52 µg/ml bovine pituitary extract, and 0.1 ng/ml retinoic acid) (Clonetics; San Diego, CA) on tissue culture plates (Falcon; Fisher Scientific, Raleigh, NC) coated with human collagen, grown to confluence, and then passaged 2 or 3 times in BEGM on ordinary tissue culture plates. Cells were then growth factor starved in un-supplemented BEBM for 9–15 hours prior to particle treatment. 300 µg/ml L-DEP, I-DEP, H-DEP and carbon black suspensions (Columbian Chemicals Company; Marietta, GA) were freshly prepared in BEBM by water bath sonication for 10 minutes. HAEC were exposed to a final concentration of 30 µg/cm² for 4 hr. 100 mM H₂O₂ and 100 mM vanadate were mixed at room temperature to produce 50 mM pervanadate (PV) stock (Sigma Chemical Co; St. Louis, MO). HAEC were treated with 50 µM PV for 30 min.

In-gel kinase activity assay.

Protein kinase activities in cell lysates fractionated by SDS-PAGE were measured as described by Wang and Erikson (Wang and Erikson, 1992). Briefly, cells were lysed in a

low-salt buffer containing 1% Triton X-100, 25 mM Tris, pH 7.5, 2 mM EGTA, 10% glycerol, 1 mM PMSF, 1 mM sodium metavanadate, 10 mM sodium fluoride, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Lysates were loaded onto standard 11% SDS-polyacrylamide gels containing 250 µg/ml myelin basic protein (MBP). 100 µg of sample protein was loaded per well. After running, the gels were washed sequentially with 20% 2-propanol-50 mM Tris (pH 8.0), 50 mM Tris (pH 8.0)-0.05% 2-mercaptoethanol (*buffer A*), and 6 M guanidine hydrochloride in *buffer A*, followed by repeated washings in 0.04% Tween in *buffer A* overnight at 4°C. Phosphorylation of MBP was carried out by adding 10 ml of 40 mM HEPES (pH 8), 2 mM dithiothreitol (DTT), 100 µM EGTA, 5 mM MgCl₂, 25 µM ATP, and 250 µCi [γ -³²P]ATP for 60 min at room temperature. The gel was then washed extensively with 5% TCA-1% sodium pyrophosphate, dried, and exposed to film. Data shown are representative of at least 2 experiments.

Western Blotting.

Cells were extracted with RIPA lysis buffer consisting of phosphate-buffered saline (pH 7.4) containing 1% NP-40, 0.5% deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail sets I and II, and protease inhibitor cocktail set III (Calbiochem; San Diego, CA). 50 µg of sample protein was mixed with one volume of SDS-PAGE loading buffer containing, 0.125 M Tris [pH 6.8], 4% SDS, 20% glycerol, 10% β -mercaptoethanol, and 0.05% bromophenol blue. The samples were heated for 1 min at 95°C and run on adjacent lanes of 4–20% Tris-Glycine Gradient pre-cast gels (Lonza; Basel, Switzerland) with pre-stained molecular weight markers in Tris-glycine-SDS buffer (Bio-Rad; Richmond, CA). Electrophoresed proteins were electroblotted onto nitrocellulose paper. Blots were blocked with 5% non-fat milk, washed briefly, and incubated overnight with antiphosphotyrosine primary rabbit antibodies (Cell Signaling; Beverly, MA) in 5% BSA. HRP-goat anti-rabbit was used as a secondary antibody and a non-specific EGFR primary antibody was used to normalize for loading variability (Santa Cruz Biotech.; Santa Cruz, CA). Protein bands on the membrane were detected using chemiluminescence reagents and film as per manufacturer's instructions (Amersham; Piscataway, NJ). In some cases, blots were stripped and reblotted using a commercially available stripping reagent (Chemicon International). Blots were digitized using a Fujifilm LAS-3000 with Multigauge software (Fujifilm U.S.A., Valhalla, NY). Western blotting results shown are representative of three or more experiments. Graphical representation of blot densities obtained from three separate experiments is also shown.

EGFR dimerization.

A431 cells were cultured in Dulbecco's minimum essential medium (DMEM) with high glucose supplemented with 10% fetal bovine serum and gentamicin (5 µg/ml) and deprived of serum for 12–18 hr prior to treatment in DMEM. Following particle exposure (as previously described) or treatment with 200 ng/ml EGF for 15 min, cells were washed with ice-cold PBS and treated with 1 ml of 2.5 mM Bis(Sulfosuccinimidyl)suberate (BS₃; Pierce, Rockford, IL) in PBS for 30 min at room temperature. The cross-linking reaction was stopped by incubating with PBS containing 20 mM Tris, pH 7.5, for 15 min, and the cells were scraped into 100 µl of PBS and centrifuged at 1000 × *g* for 5 min at 4°C. The pellet was resuspended in 50 µl RIPA buffer containing anti-protease and anti-PTPase inhibitor cocktail, sheared with a syringe, and subjected to Western blotting using a mouse anti-

human-EGFR antibody cocktail that recognizes the extracellular domain of the EGFR (Santa Cruz).

Protein tyrosine phosphatase activity assay.

DEP-treated HAEC were harvested in a specialized glove box flushed with argon with a final concentration < 2% oxygen. HAEC were lysed using a Phosphatase Lysis Buffer containing 100 mM HEPES, 0.2% NP-40, 20 µg/ml PMSF and centrifuged at 850 G for 5 min to remove cellular debris. Lysates were subsequently centrifuged at 20,000 G for 20 min to remove visible particles. Supernatants were normalized for protein content. 10 µg of cell lysates were used to determine total PTPase activity using a 96 EnzChek Tyrosine Phosphatase Assay Kit as per the manufacturer's directions (Molecular Probes; Carlsbad, CA). Fluorescence was measured over time using excitation at 355 ± 20 nm and emission at 460 ± 12.5 nm using a PolarStar Optima microplate reader (BMG Labtech; Durham, NC). Data are shown as % control from 3 independent experiments. Significance was determined by one-way ANOVA with a Dunnett's Multiple Comparison post test (n=3).

Exogenous EGFR dephosphorylation assay.

Active EGFR (86kDa) was induced to autophosphorylate by incubation at room temperature for 5 min in Mg^{2+} /ATP cocktail. DEP-treated HAEC were harvested in a glove box flushed with argon with a final concentration < 2.0% oxygen. HAEC were lysed using a Phosphatase Lysis Buffer (previously described) containing 10 µM compound 56 (c56; Calbiochem, San Diego, CA) and centrifuged at 850 G for 5 min to remove cellular debris. Lysates were subsequently microcentrifuged at 20,000 G for 20 min to remove visible particles. In an anaerobic environment, cell lysates were normalized for protein content and 100 µg of protein was brought up in 35 µl of phosphatase buffer composed of 25 mM HEPES pH 7.2, 50 mM NaCl and 2.5 mM EDTA and 10 µM c56. 100 µg of harvested protein in 35 µl was added to the reaction mixture containing 115 µl of PTPase buffer, 10 µM c56 and 20 µl of phosphorylated EGFR substrate (0.2 µg; Upstate) which was incubated at 30°C with mixing and sampled at 0 (prior to lysate addition), 3, 10, and 30 minutes. Each sample was placed in 15 µl 4X loading buffer on ice and heated for 1 min at 100°C then subjected to SDS-PAGE and Western Blotting as previously described to assess the change in phosphorylation over time. Band intensities were analyzed using Kodak Software System and graphical representations of the optical densities corresponding to the blot shown are shown (n=3). Significance was determined by one-way ANOVA. Dephosphorylation curves were fit by nonlinear regression analysis of the band intensities (%CT), using a model of one-phase exponential decay. Nonlinear regression analyses were performed using Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

Exposure to DEP of varying organic-content induces differential kinase activation in HAEC.

The organic content of DEP has been suggested to be a determinant of its toxicity (Singh *et al.*, 2004). Preliminary time-course and dose-response experiments showed that 30 µg/cm² H-DEP treatment for 4 hr produced maximal EGFR phosphorylation in HAEC (Data not

shown). In order to obtain a general assessment of the role of organic content on DEP-induced activation of intracellular signaling pathways, protein extracts prepared from HAEC exposed for 4 hours to 30 $\mu\text{g}/\text{cm}^2$ of DEP of low- (L), intermediate- (I), or high- (H) organic-content were subjected to an in-gel kinase activity assay. As shown in Figure 1, exposure to L-DEP, I-DEP or H-DEP induced differential kinase activation profiles in HAEC. For example, extracts prepared from HAEC treated with H-DEP showed the presence of a distinct kinase of approximately 45 kDa, which is altogether absent in L-DEP and I-DEP treated cells. In contrast, treatment with L-DEP resulted in the loss of a kinase with a molecular weight of approximately 70 kDa. Carbon black (CB) was used as a negative control for the effect of organic-free particulate exposure, and the data show that CB exposure did not result in noticeable changes in kinase activity relative to controls treated with media alone. These data showed that DEP with varying organic content can differentially activate intracellular kinase activity in HAEC.

The EGFR is a critical receptor tyrosine kinase that regulates cell growth, survival, differentiation, apoptosis, and inflammation (Bazley and Gullick, 2005). Therefore, to determine whether DEP exposure triggers EGFR activation, we first measured levels of phospho EGFR in HAEC exposed to 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP or H-DEP for 4 hr using Western Blotting with a phosphosite-specific antibody. As shown in Figure 2, exposure to H-DEP induced a marked increase in EGFR phosphorylation levels relative to control. In contrast, L-DEP or I-DEP exposure resulted in relatively weak EGFR phosphorylation, while CB failed to increase phospho-EGFR levels above control levels. As expected, treatment with pervanadate (PV), a potent inhibitor of PTPase activity, induced marked EGFR phosphorylation.

Exposure to DEP induces EGFR kinase-dependent EGFR phosphorylation in HAEC.

To characterize the mechanism of its activation, we next examined the functional requirements for DEP induced activation of the EGFR in HAEC. HAEC were pretreated with the potent EGFR kinase inhibitor, c56 (10 μM for 60 min), vehicle control (DMSO), or 1 $\mu\text{g}/\text{ml}$ of the EGFR blocking antibody, LA-1 for 1 hr prior to exposure with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr, 50 μM PV for 30 min or 100 ng/ml EGF for 15 min. EGFR kinase inhibition significantly blocked H-DEP-induced EGFR phosphorylation in HAEC (Figure 2). P-EGFR levels induced by L-DEP and I-DEP were also suppressed. As expected, c56 treatment also prevented EGF-mediated EGFR phosphorylation. Interestingly, c56 pretreatment in control HAEC completely ablated basal (control) levels of EGFR phosphorylation suggesting that EGFR autophosphorylation is the dominant mechanism of EGFR activation in untreated HAEC. We also observed that c56 pretreatment failed to block CB and PV-mediated EGFR phosphorylation. While this does not rule out transphosphorylation of the receptor, these data suggest that, in the absence of EGFR-directed PTPase activity, basal EGFR kinase activity is required for EGFR phosphorylation. In comparison, while LA-1 pretreatment effectively blocked EGF-mediated EGFR phosphorylation (Figure 2), it diminished DEP induced EGFR phosphorylation only partly suggesting that blocking the EGFR ligand binding site is ineffective in preventing H-DEP and L-DEP induced EGFR phosphorylation. Taken together, these data suggest that DEP

induces EGFR phosphorylation by a ligand-independent mechanism that requires EGFR kinase activity.

DEP-induced EGFR phosphorylation does not induce receptor dimerization.

Ligand-dependent EGFR activation results in homo- or hetero-dimerization of the receptor (Gunther *et al.*, 1990). As an independent assessment of the possibility that DEP induced-EGFR phosphorylation is initiated through an extracellular ligand-like mechanism, we next examined the possibility that DEP treatment induces EGFR dimerization. To increase the likelihood of detecting EGFR dimerization should it occur with DEP exposure, we performed these experiments using A431 cells, a skin carcinoma cell line which overexpresses EGFR and displays a high density of surface EGFR molecules (Samet *et al.*, 2003). EGFR dimerization was measured in intact A431 cells treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr, or with 200 ng/ml EGF for 15 min. The presence of EGFR dimers was measured following the addition of a cross-linking agent (2.5 mM BS₃) by Western blotting. As expected, activation by EGF resulted in EGFR dimerization in A431 cells (Figure 3). However, exposure to L-DEP, I-DEP, or H-DEP did not result in detectable EGFR dimerization. Similarly, CB and PV treatments failed to induce EGFR dimerization. Taken together with the receptor blocking experiments, these data suggest that DEP-mediated EGFR phosphorylation occurs through a mechanism that is independent of ligand-stimulation and receptor dimerization.

Exposure to DEP inhibits PTPases in HAEC.

Our previous work with Zn²⁺-induced activation of EGFR demonstrated ligand-independent activation of the EGFR secondary to PTPase inactivation. To assess whether DEP can induce a similar effect in HAEC, we measured PTPase activity in lysates obtained from HAEC exposed to 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr, 50 μM PV for 30 min or media alone. To prevent non-specific oxidation of cellular PTPases lysates were handled in an anaerobic chamber as described previously (Meng *et al.*, 2005). Total PTPase activity was then measured over time using a synthetic substrate and the data are shown as the ratio of PTPase activity in treated versus untreated controls at 6 min (Figure 4) and the rates of dephosphorylation over the entire assay period (Table 1). Treatment with L-DEP- or H-DEP resulted in a marked and statistically significant (* $p < 0.05$ and ** $p < 0.01$) impairment of PTPase activity in comparison to lysates obtained from control cells (Figure 4 and Table 1). Moreover, consistent with observed EGFR phosphorylation trends noted earlier (Figure 2), H-DEP was a more potent inhibitor of PTPase activity relative to L-DEP, whereas I-DEP exposure did not show an effect on PTPases. Consistent with their respective effects on EGFR phosphorylation, treatment with CB did not result in significant impairment of PTPase activity ($p > 0.05$) while treatment with PV induced pronounced impairment in cellular PTPase activity relative to control ($p < 0.01$).

Exposure to DEP impairs EGFR-directed PTPase activity in HAEC.

We next determined whether DEP-induced EGFR phosphorylation is linked to the inhibition of PTPases that regulate the phosphorylation status of the receptor. EGFR dephosphorylation rates were measured in lysates prepared from HAEC exposed to 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr, or treated with 50 μM pervanadate for 20 min or media alone.

Recombinant P-EGFR was added to cell lysates and the reaction mixture was sampled at 0, 3, 10, and 30 min and analyzed by Western blotting. Time-dependent dephosphorylation of the exogenous P-EGFR substrate could be observed clearly in control HAEC lysates over the 30 min assay period, with a marked decrease in levels of P-EGFR observed by 10 min (Figures 5A and 5B). In marked contrast, exposure to H-DEP resulted in a significant impairment in the rate of exogenous EGFR dephosphorylation at each time point. Similarly, treatment with L-DEP also induced a measureable impairment in EGFR dephosphorylation. Notably, the magnitude of the effect of H-DEP and L-DEP on impairment of EGFR-directed PTPase activity exceeded the effects of PV, the general PTPase inhibitor (Fig. 5B). In the case of H-DEP, this observation supports the findings observed Figure 4 and Table 1. In further agreement with the data shown in Figure 4 and Table 1, there were no differences noted in EGFR-directed PTPase activity in lysates obtained from HAEC treated with I-DEP or CB (Fig. 5A and 5B).

Discussion

The mechanisms by which PM induces adverse health effects are not well understood. Lung epithelial cells are directly exposed to inhaled particles and are a significant source of inflammatory mediators. We have previously reported that exposure to Zn^{2+} or DEP induces proinflammatory signaling in lung epithelial cells (Kim *et al.*, 2006; Cao *et al.*, 2007a; Cao *et al.*, 2007b; Kim *et al.*, 2007). In the case of Zn^{2+} , we have shown that impairment in tyrosine kinase-directed PTPase activity was the initiating event in Zn^{2+} -induced inflammatory mediator expression (Kim *et al.*, 2006; Tal *et al.*, 2006). Here we report that exposure to DEP induces ligand-independent EGFR phosphorylation through a mechanism that involves impairment of EGFR-directed PTPase activity in HAEC.

A recent study examining the role of basal PTPase activity in signaling showed that pharmacological inhibition of kinases activated by insulin leads to rapid dephosphorylation of downstream phosphosubstrates (Zhander *et al.*, 2006). This mechanism, termed “dephosphorylation by default” by the authors, demonstrates that impairment of PTPases is sufficient to allow phosphorylation by basal tyrosine kinase activity to accumulate. Our data showing that exogenous P-EGFR is rapidly dephosphorylated in lysates prepared from HAEC treated only with an EGFR kinase inhibitor is in agreement with this concept. Furthermore, our finding that exposure to DEP impairs EGFR-directed PTPase activity supports the notion that frank kinase activation is not required for the initiation of phosphorylation-dependent signaling in HAEC treated with DEP. In addition, our results imply that an impairment of EGFR-directed PTPase activity(ies) is sufficient to enable an accumulation of basal EGFR phosphorylation, leading to downstream phosphorylation-dependent signaling pathways. Interestingly, we have recently observed that treatment with H-DEP and L-DEP but not I-DEP for 4 hr results in increased expression of the proinflammatory mediator IL-8 in HAEC, arguing that PTPase impairment is a pivotal event in signaling that leads to pro-inflammatory gene expression. Moreover, recent findings in our laboratory have also shown that L-DEP-induced increases in IL-8 expression can be blocked with EGFR kinase inhibitors, evincing the toxicological relevance of EGFR activation in HAEC exposed to DEP (Tal, unpublished observations).

The thiolate cysteine residue required for PTPase catalytic activity is highly susceptible to electrophilic attack. Recently, a series of studies has provided the first mechanistic evidence supporting the notion that environmentally relevant reactive quinones and aldehydes can directly inactivate protein tyrosine phosphatase 1B (PTP1B) activity by a covalent modification of reactive cysteines (Iwamoto *et al.*, 2007; Seiner *et al.*, 2007). Notably, treatment of A431 cells with 1,2-naphthoquinone was shown to arylate two reactive cysteinyl residues in PTP1B, thereby impairing its activity and leading to the prolonged and irreversible activation of EGFR (Iwamoto *et al.*, 2007). These studies are in keeping with the concept that DEP-associated oxy-organics can directly contribute to proinflammatory signaling through a mechanism involving EGFR activation and suggest a possible mechanism by which H-DEP induces PTPase inhibition in our system.

In addition to arylation or acylation of reactive cysteines, DEP-associated oxy-organics may indirectly impair PTPase activity via redox cycling. Two main families of compounds, polycyclic aromatic hydrocarbons (PAHs) and quinones, are absorbed on diesel particles (Baulig *et al.*, 2003) and are thereby delivered to the airway epithelium with inhaled PM. In addition to irreversible oxy-organic adduct formation, DEP-associated quinones and reactive PAH metabolites can generate reactive oxygen (ROS) and nitrogen species (RNS) that reversibly inactivate PTPases by the formation of $-S-OH$ or $-S-NO$ derivatives (Li *et al.*, 2002; Barrett *et al.*, 2005; Chiarugi and Buricchi, 2007). ROS are also formed during the NADPH-cytochrome P450 reductase-mediated metabolism of DEP-associated quinones to semiquinone radicals. PM-associated PAHs are first metabolized by cytochrome P450s and peroxidases to oxidized derivatives such as epoxides, diols, and redox-cycling quinones. This source of oxidants has been implicated in the toxicity associated with H-DEP (Xia *et al.*, 2004) which contains 26.3 % (wt/wt) extractable organic matter (EOM) (Singh *et al.*, 2004). However, this mechanism does not explain L-DEP-induced PTPase inhibition since L-DEP has a low (approximately 2.0 % (wt/wt)) EOM (Singh *et al.*, 2004), arguing that the potency with which DEP activate EGFR is not correlated with its organic content and likely involves other classes of compounds associated with the particles. This alternative view is further supported by the inability of I-DEP (18.9% (wt/wt) EOM; (Singh *et al.*, 2004)) treatment to impair PTPase activity in our system. Thus, the exact mechanism of inactivation of PTPase activity by DEP is likely complex and will require further investigation. Similarly, while we failed to detect EGFR dimerization in HAEC exposed to DEP, it should be noted that EGF stimulates cell proliferation in airway epithelial cells, yet this growth factor has been shown to impair proliferation in A431 cells (Gill and Lazar, 1981; Barnes, 1982). Thus, it is possible that differences in downstream signaling between these cell types influenced these results.

PM-associated metal ions may represent an additional mechanism responsible for DEP-induced PTPase inhibition. We have previously shown that exposure to residual oil fly ash, a metallic ash that contributes to the PM mass in some airsheds, can disregulate phosphoprotein metabolism by inactivating PTPase activity in airway epithelial cells (Gavett *et al.*, 1997; Samet *et al.*, 1997; Kodavanti *et al.*, 1998; Gavett *et al.*, 2003). Common soluble metallic components associated with PM include Fe^{3+} , Cu^{2+} , Zn^{2+} , $V^{3+/5+}$ and $Cr^{3+/6+}$ (Gavett *et al.*, 1997). Redox-cycling metals, such as Fe^{3+} , Cu^{2+} , $V^{3+/5+}$ and $Cr^{3+/6+}$, can generate ROS capable of inactivating PTPases through the Haber-Weiss reaction (Koppenol,

2001). $V^{3+/5+}$, Fe^{3+} and Cu^{2+} are reportedly present in L-DEP at concentrations of 20, 100 and 300 ppm, respectively (Park *et al.*, 2006). Thus, depending on their speciation, it is possible that these metals contribute to L-DEP induced PTPase inhibition and EGFR signaling effects in HAEC. Moreover, Zn^{2+} , which is found at a concentration of 400 ppm in L-DEP (Park *et al.*, 2006), and $V^{3+/5+}$ are potent direct inhibitors of PTPase activity (Samet *et al.*, 1999; Tal *et al.*, 2006), suggesting an additional mechanism by which L-DEP exposure impairs EGFR-dephosphorylation in HAEC.

Inflammatory responses are thought to be a critical feature of many of the adverse effects of PM exposure, including morbidity and mortality (Brook *et al.*, 2003). Through studies aimed at elucidating the mechanism of PM-induced EGFR activation, we show here that exposure to DEP impairs EGFR-directed PTPase activity in HAEC. These findings provide evidence for an initiating mechanism through which DEP exposure induces the expression of proinflammatory proteins such as IL-8 and COX-2.

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Figure 1.

(A) Exposure to DEP induces differential kinase activation in HAEC. Cells were treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or carbon black (CB) or media control (CT) for 4 hr. 50–100 μg cell lysate was loaded onto a gel containing 250 $\mu\text{g}/\text{ml}$ MBP and analyzed for kinase activity by the addition of 5 mM MgCl_2 , 25 μM ATP, and 250 μCi [λ ^{32}P]ATP. Image contrast was optimized to show bands of intermediate molecular weights. The results shown are representative of two experiments. (B) Cells were treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB or media (CT) for 4 hr or 50 μM pervanadate (PV) for 30 min. Cells were lysed and the state of EGFR phosphorylation was detected by Western Blotting using a phosphorylation-specific antibody directed against tyrosine 845 and a general EGFR antibody, that recognizes the extracellular domain of the EGFR, was used as a loading control.

Figure 2.

DEP exposure induces ligand-independent EGFR kinase dependent EGFR phosphorylation. Following pretreatment with 10 µM of the EGFR kinase inhibitor compound 56 (c56), vehicle control (DMSO) or 1 µg/ml of the EGFR blocking antibody, LA-1 for 1 hr, HAEC were exposed to 30 µg/cm² L-DEP, I-DEP, H-DEP or CB or media for 4 hr, 50 µM PV for 30 min or 100 ng/ml EGF for 15 min. Lysates were analyzed for EGFR phosphorylation via Western Blotting with phospho-specific antibodies. Total EGFR protein was assessed by use of a general EGFR antibody. The results shown are representative of three experiments.

Figure 3.

DEP-induced EGFR phosphorylation does not require receptor dimerization. Analysis of EGFR dimerization was measured in intact A431 cells treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB or media for 4 hr or 200 ng/ml EGF for 15 min. Cells were then rinsed and treated with 2.5 mM BS_3 for 30 min lysed and subjected to Western blotting using an EGFR antibody that recognizes the extracellular domain of the EGFR. The results shown are representative of three or more experiments.

Figure 4.

DEP exposure impairs total PTPase activity in lysates obtained from HAEC exposed to DEP. HAEC were treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr or 50 μM PV for 30 min and harvested in an anaerobic environment to prevent non-specific oxidation. 10 μg of protein extracts were loaded onto a PTPase activity kit and fluorescence was measured at 6 min using excitation at 355 ± 20 nm and emission at 460 ± 12.5 nm. Data are expressed as the % of control of picomoles of substrate hydrolyzed per mg of protein. Total PTPase activity in cells treated with L-DEP, H-DEP or PV were statistically significant from control samples (* $p < 0.05$, ** $p < 0.01$). HAEC exposed to I-DEP and CB did not result in significant impairments in total PTPase activity. Significance was determined by one-way ANOVA with a Dunnett's Multiple Comparison post test ($n=3$).

Figure 5.

Exogenous EGFR dephosphorylation was inhibited in lysates obtained from HAEC exposed to high and low but not intermediate organic-containing DEP *in vitro*. (A) HAEC were treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr or 50 μM PV for 30 min. In an anaerobic environment, 0.2 μg of active, phosphorylated EGFR substrate was mixed with 100 μg of cellular lysate and the reaction was sampled at 0, 3, 10, and 30 min. Lysates were analyzed for EGFR dephosphorylation over time via Western Blotting with phospho-specific anti-EGFR antibodies. The results shown are representative of three experiments. (B) A graphical representation of the optical densities obtained from three independent experiments. Band intensities were analyzed using Multigauge Software and shown here as % control of the intensity of the band area minus the background, curves were fit by non-linear regression analysis and significance was determined by one-way ANOVA with a Dunnett's Multiple Comparison post test (n=3).

Table 1.

DEP exposure impairs total PTPase activity in lysates obtained from HAEC exposed to DEP. HAEC were treated with 30 $\mu\text{g}/\text{cm}^2$ L-DEP, I-DEP, H-DEP or CB for 4 hr or 50 μM PV for 30 min and harvested in an anaerobic environment to prevent non-specific oxidation. 10 μg of protein extracts were loaded onto a PTPase activity kit and fluorescence was over time using an excitation at 355 ± 20 nm and emission at 460 ± 12.5 nm. Linear regression was used to obtain the rates of dephosphorylation (picograms substrate hydrolyzed min^{-1} mg protein $^{-1}$). Total PTPase activity in cells treated with L-DEP, H-DEP or PV were statistically significant from control samples (* $p < 0.05$, ** $p < 0.01$). Significance was determined by one-way ANOVA with a Dunnett's Multiple Comparison post test ($n=3$).

Treatment	Dephosphorylation rate (picomoles substrate hydrolyzed min^{-1} mg protein $^{-1}$)
CT	12.7 + 0.98
L-DEP	10.4 + 0.50*
I-DEP	12.1 + 1.07
H-DEP	5.9 + 2.33**
CB	12.3 + 0.88
PV	7.8 + 1.49**