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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 μg/cm^2 DEP for 4 h 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 is independent of 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.

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 to 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 h) 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 h (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 p*K*a 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).

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We have previously shown that divalent zinc (Zn^{2+}), another component of ambient PM, induces EGFR activation and upregulation of NFκB-dependent IL-8 expression in human airway epithelial cells (Kim et al., 2006; Tal et al., 2006). Moreover, we reported that Zn^{2+} 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 (2740 cm^3), 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 as previously described by 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 m^2/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 h 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 min . HAEC were exposed to a final concentration of 30 $\mu g/cm^2$ for 4 h . 100 mM H_2O_2 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 $MgCl_2$, 25 μM ATP, and 250 μCi [γ-^{32}P]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. A 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 h 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 (BS_3; 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