INVESTIGATING TYROSINE PHOSPHATASES AS TARGETS OF AIR POLLUTANTS

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ABSTRACT

Tamara Tal: Investigating Tyrosine Phosphatases as Targets of Air Pollutants

(Under the direction of James M. Samet)

Exposure to ambient particulate matter (PM) is associated with elevated rates of morbidity and mortality. Inflammation is thought to be a central mechanism by which PM exposure induces adverse health effects. In lung epithelial cells, a principle target of inhaled PM, 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. Therefore, we hypothesized that exposure to oxidative, electrophilic or metal cation components of ambient PM would impair PTPase activity allowing for unopposed basal tyrosine kinase activity. Here we report that exposure to the ubiquitous PM components Zn or diesel exhaust particles (DEP) induce activation of the receptor tyrosine kinase Epidermal Growth Factor Receptor (EGFR) in primary human airway epithelial cells. This phosphorylation event occurs by a ligand-independent mechanism that requires EGFR kinase activity. We also show that exposure to Zn or DEP impair the activity of PTPases, which function to dephosphorylate the EGFR. These data provide a mechanism by which disparate components of ambient PM can similarly activate proinflammatory signaling in human lung cells. In summary, these data show that PM-induced EGFR-phosphorylation in human airway epithelial cells is the result of a loss of PTPase activities that normally function to dephosphorylate EGFR in opposition to baseline EGFR kinase activity.

DEDICATION

For Harriet Block Wasser, Robert Arden Wasser,

Anne Luree, Rony, Jonathan Arden and Ariel Ben Tal,

and Kathleen and David Arden Wasser

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I would like to first express my deepest gratitude to my mentor, Dr. James M. Samet, who has provided me with outstanding training and mentorship over the last five years. My training experience would not have been possible without his guidance and I am deeply grateful for the multitude of ways in which he has prepared for the next steps in my scientific career. Next I express my sincere gratitude to the members of my committee, Dr. Cordeiro-Stone, Dr. Graves, Dr. Jaspers, Dr. Singleton and Dr. Samet for their continuing scientific guidance. I would especially like to acknowledge Dr. Graves for his mentorship throughout the Zn^{2+} project, Dr. Cordeiro-Stone for her scientific, professional, and personal support as well as Dr. Jaspers who has allowed me to shamelessly take advantage of her open office door for fresh ideas, professional guidance and long standing support.

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This experience would have not been possible without my family, whose appreciation for education coupled with their deep love and support guided me here.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

LIST OF ABBREVIATIONS

A549 cells: Cultured airway epithelial cells

Ap: Apical

AP-1: Activator Protein 1

BEAS2B cells: cultured human airway epithelial cell line

BEBM: HAEC growth factor depleted medium

BEGM: HAEC epithelial growth medium

BL: Basolateral

C/EPBβ: CCAAT/enhancer-binding protein β

CB: Carbon black

EGF: Epidermal growth factor

EGFP: Enhanced green fluorescence protein

EGFR: Epidermal growth factor receptor

DEP: Diesel exhaust particle

DMEM: Dulbecco's modified eagle's medium

DSP: Dual specificity phosphatase

DTT: Dithiothreitol

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPCR: G-Protein Coupled Receptor

GSH: Glutathione

GSSG: Diglutathione

HAEC: Human airway epithelial cells

H-DEP: High organic-containing DEP (Japanese DEP)

I-DEP: Intermediate organic-containing DEP (DEP3)

IL-8: Interluekin-8

KBM: Keratinocyte basal medium

KGM: Keratinocyte growth medium

L-DEP: Low organic-containing DEP (NIST SRM 2975)

LPA: Lysophosphatidic acid

MAPK: Mitogen-activated protein kinase

MMP12: matrix metalloelatase

MOI: Multiplicity of infection

Nanoparticles: PM with a diameter < 0.1 microns

ND: Nanodiamond

NFκB**:** Nuclear factor κB

PAH: Polycyclic aromatic hydrocarbon

PDGFR: Platelet Derived Growth Factor Receptor

PM: Particulate matter

$PM_{2.5}$: ultrafine PM, with a diameter < 2.5 microns

PM_{10}: coarse PM, with a diameter < 10 microns

PT: Pyrithione

PTP1B: Protein tyrosine phosphatase 1B

PTPase: Protein tyrosine phosphatase

PTB domain: Phosphotyrosine binding domain

PV: Peroxyvanadate

ROFA: Residual oil fly ash

ROS: Reactive oxygen species

SH: Src-homology domains

SHP1: SH2 domain-containing phosphatase 1

siRNA: Small interference RNA

STAT 3: Signal transducers and activators of transcription 3

TACE: TNF-α-converting enzyme

TNF: Tumor necrosis factor

TLR: Toll-like receptor

V: Vanadium

Zn^{2+}: Zinc

Chapter I: Background and significance

1-A Particulate Matter (PM) as a public health concern

Numerous epidemiological studies have associated PM inhalation with adverse health effects including diminished lung function, morbidity and mortality (Dockery *et al.*, 1993; Koenig *et al.*, 1993; Schwartz, 1994). In particular, exposure to PM may account for as many as 500,000 deaths worldwide each year (U.N., 1994; Nel, 2005). A recent study has shown that the onset of myocardial infarction was three times as likely for those individuals exposed to traffic within one hour of the heart attack (Peters *et al.*, 2004). A similar association between ambient particulate matter and the rate of hospitalization for congestive heart failure has also been reported (Wellenius *et al.*, 2005).

While the correlation between PM inhalation and cardiopulmonary events is well established, the means by which inhaled particles exert deleterious effects on the cardiovascular system remains unclear. It has been proposed that particle inhalation may directly induce alterations in cardiac autonomic function thereby causing changes in heart rate variability and increasing the likelihood of myocardial infarctions (Rhoden *et al.*, 2005). It has also been hypothesized that particles deposited in the terminal region of the lung promote pulmonary inflammation triggering a subsequent release of blood-borne mediators into the circulatory system which are thought to either induce or contribute to pre-existing atherosclerosis, arrhythmia, and vascular dysfunction [reviewed in (Brook *et al.*, 2003), Figure 1-1]. As a primary target of inhaled pollutants, the airway epithelium is capable of initiating or augmenting pulmonary inflammatory defenses by synthesizing a number of mediators that can cause chemotaxis and activation of inflammatory cells (Fujii *et al.*, 2001). These mediators promote local

and systemic inflammation that is thought to culminate in cardiovascular dysfunction [reviewed in (Bai *et al.*, 2006)].

Figure 1-1

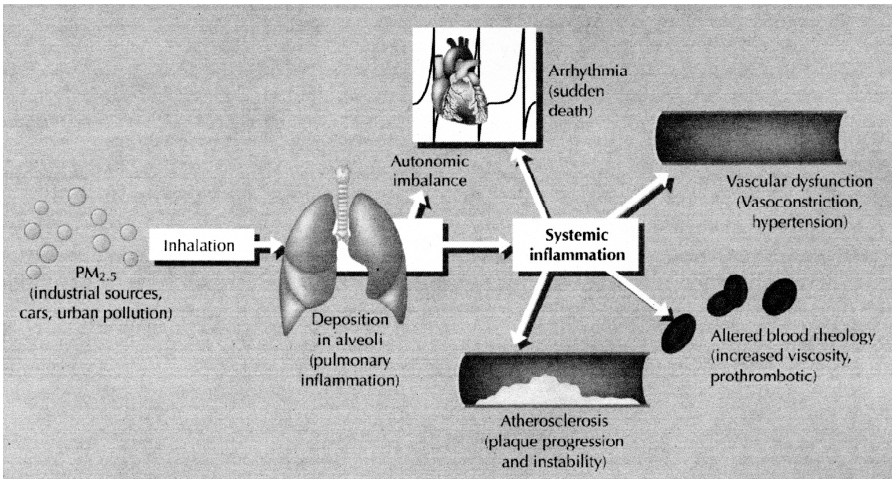


Figure 1-1 *PM and cardiovascular disease*. Inhalation of $PM_{2.5}$ produces pulmonary inflammation leading to alterations in autonomic balance in addition to systemic inflammation capable of triggering acute and chronic cardiovascular disease (Brook *et al.*, 2003).

1-B Composition of ambient PM

PM is a ubiquitous air pollutant particle with adsorbed metals, nitrates, sulfates and organic compounds. PM is produced from both natural and anthropogenic sources including waste incineration, vehicular exhaust, power generation, tire wear, wildfires and agricultural practices. Not surprisingly, ambient PM varies greatly in its size, composition, and toxicity. This variation makes it difficult to identify particle types or components that cause biological toxicity [Figure 1-2 from (Seagrave *et al.*, 2006)].

1-C PM and pulmonary inflammation

In human studies, exposure to diesel exhaust particles (DEP) has been shown to induce acute pulmonary inflammation characterized by increased levels of neutrophils, B-lymphocytes, and the inflammatory mediators, histamine and fibronectin (Salvi *et al.*, 1999). Biopsies revealed an upregulation of the endothelial adhesion molecules ICAM-1 and VCAM-1 suggesting a possible mechanism by which initiation of DEP-induced recruitment of inflammatory cells occurs (Salvi *et al.*, 1999). Inflammation-associated cytokine expression is another validated means of detecting pulmonary inflammation. Exposure to the coarse fraction (PM10) of Concentrated Air Particles (CAPs), obtained in Chapel Hill, induced upregulation of TNF-α, IL-6, and COX-2 in alveolar macrophages, and IL-8, TNF-α, and COX-2 in primary human airway epithelial cells (HAEC) (Becker *et al.*, 2005). Exposure to the fine fraction (PM2.5) of CAPs collected in Dunkerque, France was similarly shown to induce expression of the inflammatory cytokines TNF-α, IL-1β, IL-8, IL-6, GM-CSF, and Transforming Growth Factor-beta (TGF- β) in cultured epithelial cells (Dagher *et al.*, 2005). Taken together, these studies show that exposure to a wide variety of PM can induce pulmonary inflammation which is hypothesized to contribute to the presentation of cardiovascular pulmonary inflammation which is hypothesized to contribute to the presentation of cardiovascular disease associated with PM inhalation (Brook *et al.*, 2003). Although these studies describe similar

Figure 1-2

Figure 1-2A-C

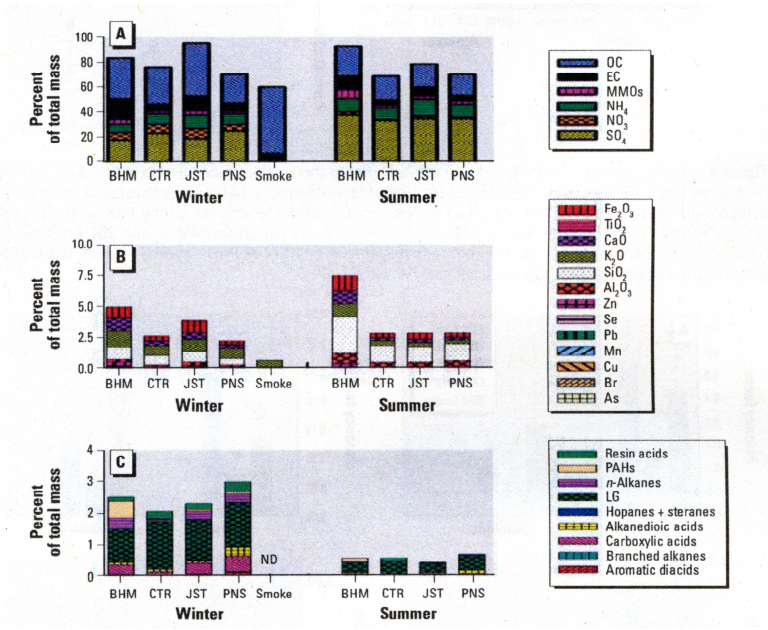


Figure 1-2D

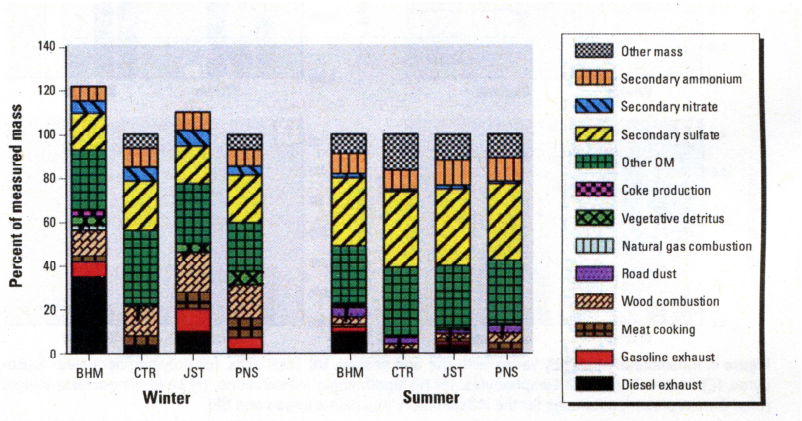


Figure 1-2 *Composition of ambient PM.* Season and site related differences were noted in particle composition (Figure 1-2A), PM-associated metals (Figure 1-2B) and classes of PM-associated organic compounds between 2 rural (CTR and PNS) and 2 urban (BHM and JST) sites in the Southeastern U.S. sampled in the Summer and Winter of 2004 (Seagrave *et al.*, 2006). Source apportionment analyses were performed demonstrating that diesel and gasoline derived PM substantially contributed to PM generated in urban versus rural areas (Figure 1-2D).

inflammatory responses to particle challenge, the mechanism by which particles induce pulmonary inflammation remains unresolved.

1-D Proinflammatory signaling pathways

The MAPKs consist of parallel kinase cascades leading to the activation of the four Serine/Threonine MAPKs (ERK, JNK, p38 and ERK5/BMK) (Figure 1-3). Activation of these signaling cascades functions to drive diverse cell fates including proliferation, differentiation, apoptosis, growth and inflammation (Zhang and Dong, 2007). MAPK activity is regulated by the phosphorylation status of a conserved Threonine-X-Tyrosine sequence present in the MAPK activation loop (Chen *et al.*, 2001). When appropriately phosphorylated, MAPK can activate a diverse set of transcription factors via phosphorylation on regulatory Serine and Threonine residues.

Transcription factor activation stimulates its translocation to gene promoters as well as recruitment of cofactors and the transcriptional complex to modulate the expression of genes involved in proliferation, migration, inflammation, apoptosis, or differentiation (Chen *et al.*, 2001). In the case of inflammatory signaling in lung epithelial cells, MAPK-mediated activation of the transcription factors NFκB, AP-1 and STAT-3 leads to the generation of proinflammatory signaling molecules such as IL-8, IL-6, TNF-α, COX-2 and GM-CSF in response to physiological and toxicological stimuli (Hayden and Ghosh, 2008).

A number of PM components have been associated with increased expression of proinflammatory signaling cytokines in vivo, in cells and in vitro(Salvi et al., 1999; Bonvallot et al., 2001; Fujii et al., 2001; Li et al., 2002; Pourazar et al., 2005; Kim et al., 2006; Matsuzaki et al., 2006; Cao et al., 2007). Perhaps most strikingly, a series of studies from the Sandstrom laboratory reported enhanced

Figure 1-3

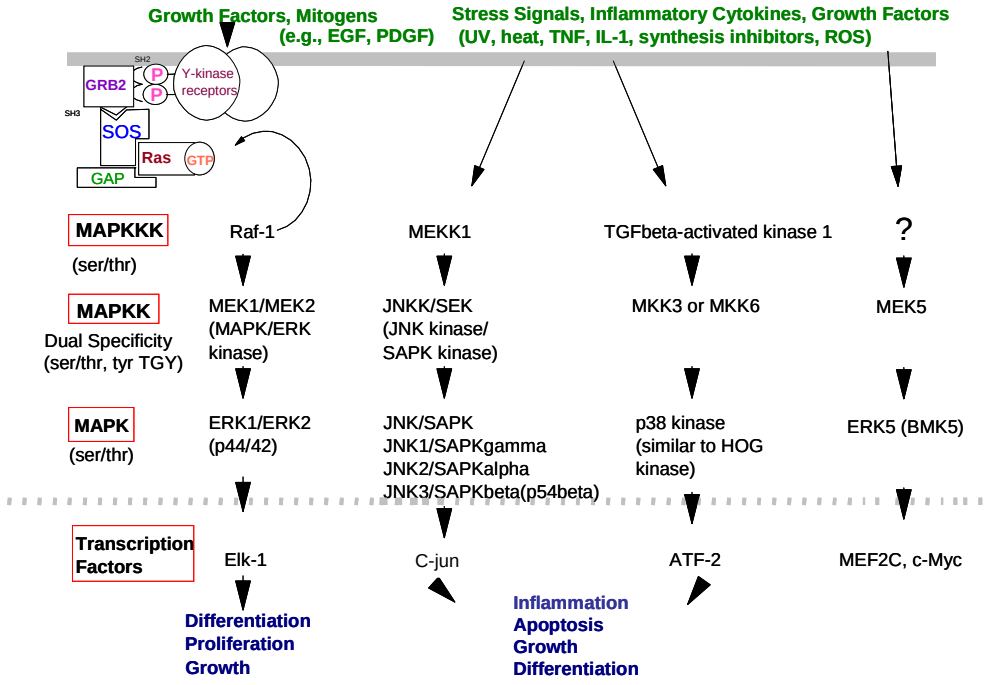


Figure 1-3 *Mitogen activated protein kinase (MAPK) signaling pathways.* MAPK pathways constitute a large kinase network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to sequential activation of a MAPK module. After MAPKs are activated either in the cytoplasm or in the nucleus, they regulate transcription by modulating the function of a target transcription factor via phosphorylation of Serine and Threonine residues.

expression of the proinflammatory cytokines IL-13 (Pourazar *et al.*, 2004), IL-8 and Gro-α (Salvi *et al.*, 2000) in bronchial biopsies obtained from humans exposed to DEP. Pourazar et al. later used archived biopsies and reported three important findings (Pourazar *et al.*, 2005). First, DEP exposure resulted in an increase in the nuclear translocation of NFκB and AP-1, demonstrating a means by which DEP exposure can induce expression of proinflammatory cytokines. Second, activation (determined by enhanced phosphorylation) of the upstream MAPKs JNK and p38 in response to DEP exposure was demonstrated by immunohistochemical staining. Third, total tyrosine phosphorylation was significantly increased in biopsies obtained from DEP exposed subjects. Taken together, these data demonstrate that DEP exposure stimulates MAPK signaling, characterized by increased tyrosine phosphorylation, leading to activation of NFκB and AP-1 and synthesis of proinflammatory cytokines. While the pathways involved in PM-induced proinflammatory signaling have been described, much less is known about the mechanisms by which PM initiates phosphorylation-dependent proinflammatory signaling.

1-E Epidermal growth factor receptor

As mentioned previously, the MAPK cascades can be activated by the receptor tyrosine kinase EGFR (Citri and Yarden, 2006) and some reports suggest that PM-induced proinflammatory signaling begins at the level of receptor tyrosine kinases (Wu *et al.*, 1999; Wu *et al.*, 2002). EGFR (ERBB1) is one of four ERBB family members capable of undergoing homo- and hetero-dimerization. With some exceptions (ERBB2 is unable to bind ligand and ERBB3 lacks a functional kinase domain), ERBB family members are structurally defined by the presence of an extracellular ligand-binding domain, a single transmembrane spanning domain and cytoplasmic juxtamembrane, kinase and C-regulatory domains all of which are reported to contribute to the complex regulation of receptor activity (Landau and Ben-Tal, 2008).

Upon ligand stimulation, the EGFR undergoes receptor homo- or hetero-dimerization, activation of receptor kinase activity, and autophosphorylation of key tyrosine residues in the Src-homology (SH)-2 and protein tyrosine binding (PTB) domains (Gale *et al.*, 1993). In particular, EGFR's carboxy-terminus contains three major ($Tyr^{1068,\ 1173,\ and\ 1148}$) and two minor ($Tyr^{992\ and\ 1086}$) tyrosine autophosphorylation sites that are differentially phosphorylated following receptor activation by ligand or transphosphorylation by other stimuli [Figure 1-4 and (Keilhack *et al.*, 1998)]. Phosphorylation of these sites results in the recruitment of SH-2-, or PTB-containing proteins, such as Grb2, activation of RAS and the MAPK pathways (Gale *et al.*, 1993). EGFR phosphorylation is regulated by a number of Protein Tyrosine Phosphatases which function to dephosphorylation the EGFR returning the receptor to its inactive form (Table 1-1).

One critical mechanism through which ERBB activity is regulated is by receptor dimerization (Figure 1-5). It was long believed that in the absence of ligand, EGFR dimerization was prevented (with the exception of ERBB2). However, random, ligand-independent dimerization of ERBB family members has recently been reported [reviewed in (Warren and Landgraf, 2006)]. In particular, a recent study showed that in the absence of ligand, the majority of EGFR and ERBB2 receptors form dimers (Liu *et al.*, 2007). Another study proposed that the formation of the ligand-less dimers actually facilitate the formation of active dimers. This hypothesis is supported by their finding that ligand-independent dimers bind EGF with markedly greater affinity than the monomers (Teramura *et al.*, 2006). The authors speculate that the formation of the ligand-independent dimers primes the complex for activation by decreasing the area through which the necessary components must diffuse (Teramura *et al.*, 2006). Regulation of EGFR activity by PTP1B is also reported to be both spatially and temporally regulated in cells (Haj *et al.*, 2002). In particular, EGFR and PTP1B expression was shown to be restricted to the plasma membrane or endoplasmic reticulum, respectively, in resting

Figure 1-4

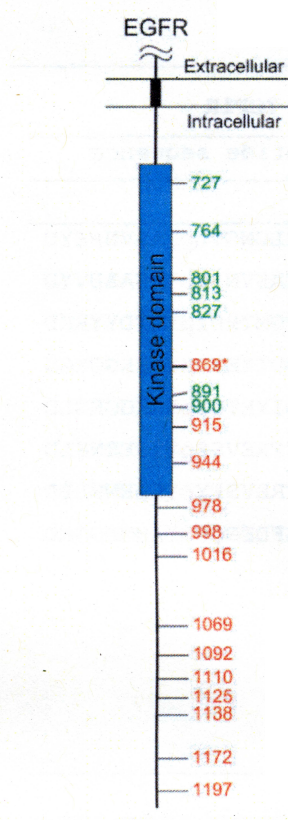


Figure 1-4 *The Epidermal growth factor receptor phosphorylation sites.* The EGFR consist of an extracellular ligand-binding domain, a single transmembrane spanning domain, and cytoplasmic kinase and C-regulatory domains containing a number of physiologically relevant tyrosine phosphorylation sites (shown in red) (Kaushansky *et al.*, 2008).

Table 1-1

PTPase	Classification	Cell type	Reference
LAR	Receptor PTPase	McA-RH777 rat hepatoma cells	(Kulas *et al.*, 1996)
RPTPσ	Receptor PTPase	A431 cells	(Suarez Pestana *et al.*, 1999)
SHP-1	Intracellular PTPase	[1]293T cells or COS cells and [2]A431 cells	(Tenev *et al.*, 1997; Keilhack *et al.*, 1998)
PTP1B	Intracellular PTPase	COS cells	(Liu and Chernoff, 1997)
TCPTP	Intracellular PTPase	COS cells	(Tiganis *et al.*, 1999)
SHP-2	Intracellular PTPase	[1]SHP-2 (-/-) wt chimeras and[2]COS cells	(Qu *et al.*, 1999; Fina *et al.*, 2006)
DEP-1/Scc1	Receptor PTPase	C. elegans	(Berset et al., 2005)

Table 1-1 *PTPases that regulate EGFR.*

Figure 1-5

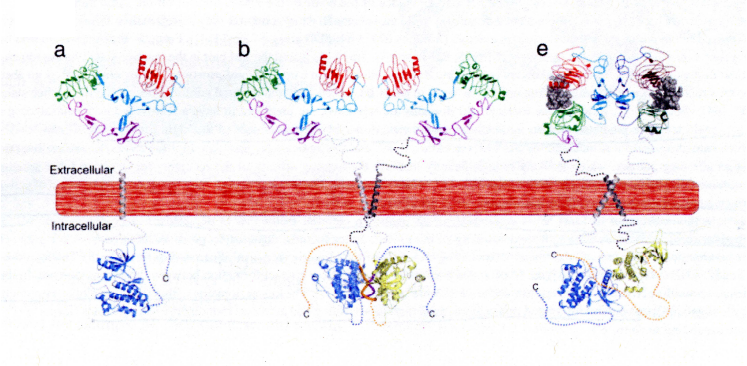


Figure 1-5 *Ligand-dependent EGFR activation.* The EGFR forms ligand-less dimmers with other members of the ERBB recepter family. Upon ligand binding to the extracellular ligand binding domain, the dimmer undergoes a conformational change assuming an asymmetric conformation of the intracellular kinase and C-regulatory domains. This allows one member of the dimer to phosphorylate the other on Tyrosine residues (Zhang and Dong, 2007).

cells. Upon receptor activation, EGFR internalization and subsequent dephosphorylation was shown to co-localize with PTP1B.

Researchers have recently solved the conformation of the EGFR active site providing deeper understanding of EGFR-dependent signaling. Appropriate interactions between the external, transmembrane, juxtamembrane, kinase and C-terminal domains result in the formation of an asymmetric dimer in which one kinase domain activates its partner [Figure 1-5 and (Zhang and Dong, 2007)]. This mechanism of receptor autophosphorylation is relevant to the exploration of PM induced signaling effects in primary human lung cells where autophosphorylation is a major mechanism by which EGFR activation occurs [(Tal *et al.*, 2006), Chapter II and Tal, unpublished observations, Chapter III].

The EGFR is activated by multiple ligand-independent mechanisms (Figure 1-6). Src tyrosine kinase mediates transphopshorylation of the EGFR at $Tyr^{845 \text{ and } 1101}$ leading to activation of the receptor's tyrosine kinase activity (Tice *et al.*, 1999). Moreover, Src-mediated EGFR transactivation has been reported has been reported in a number of cell types including A431 cells (Samet *et al.*, 2003), B82 mouse lung fibroblasts (Wu *et al.*, 2002), and rat cardiac fibroblasts (Chen *et al.*, 2006). Inactivation of EGFR-directed PTPase activity is another ligand-independent mechanism by which EGFR activation occurs where impairment in PTPase activity is sufficient to activate EGFR-dependent signaling in the presence of low levels of basal EGFR activity (Zhande *et al.*, 2006).

1-E.1 Evidence for PM induced EGFR activation

Recently, there have been several studies linking PM exposure to EGFR activation. First, our lab has shown that Zn^{2+} exposure induces EGFR phosphorylation by cell-type specific mechanisms involving Src kinase-mediated transactivation (Samet *et al.*, 2003) or by inhibition of EGFR-directed PTPase activity [(Tal *et al.*, 2006), Chapter 2] in cultured BEAS2B cells or primary human lung cells, respectively. In support of a PTPase driven mechanism of EGFR activation, we have also reported

Figure 1-6

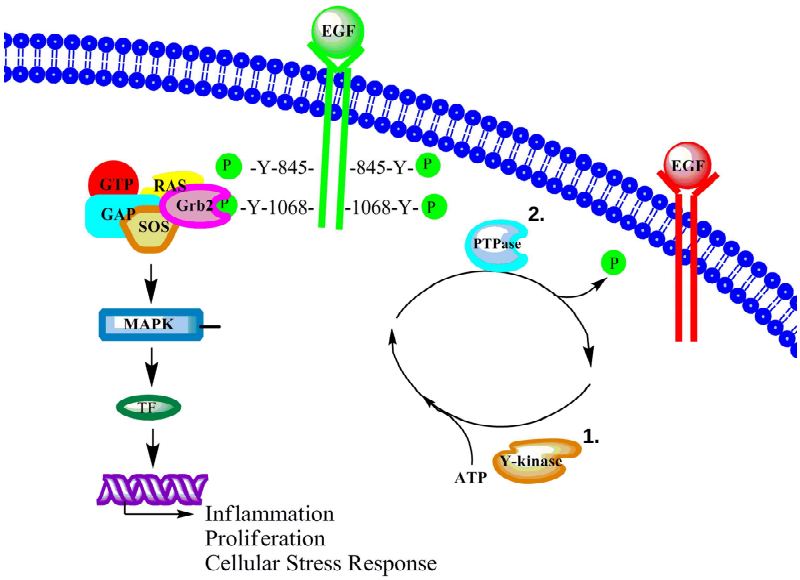


Figure 1-6 *Ligand-independent EGFR activation.* Tyrosine kinase-mediated transactivation (1) or inhibition of EGFR-directed PTPase activity (2) results in ligand-independent EGFR phosphorylation.

that exposure to a low-organic containing DEP (NIST SRM 2975) (Cao *et al.*, 2007b) or high-organic containing DEP (Tal, unpublished observations, Chapter III) similarly impairs EGFR-directed PTPase activity, resulting in the activation of EGFR dependent signaling. Carbon nanoparticles are also reportedly capable of stimulating EGFR dependent activation of ERK1/2 and JNK in human bronchial or rat epithelial cells, respectively (Sydlik *et al.*, 2006; Unfried *et al.*, 2008). Additionally, a series of papers has recently described a mechanism by which PM exposure results in sustained proinflammatory signaling involving EGFR activation. In human bronchial epithelial cells (16HBE), $PM_{2.5}$ or DEP treatment induced EGFR- and Erk-dependent expression of amphiregulin, a ligand of the EGFR (Blanchet *et al.*, 2004). In support of these data, it was subsequently reported that PM-induced amphiregulin is secreted toward the basolateral side where the EGFR is expressed in differentiated airway epithelial cells (Rumelhard *et al.*, 2007a). The authors propose that PM induces EGFR-activation directly, leading to expression of amphiregulin, and indirectly, through amphiregulin induced EGFR-dependent signaling, thereby prolonging proinflammatory signaling through the induction of GM-CSF expression (Blanchet *et al.*, 2004; Rumelhard *et al.*, 2007a; Rumelhard *et al.*, 2007b). A more recent paper, from the Sanstrom laboratory using archived bronchial biopsies from human subjects exposed to diesel exhaust, provides the first *in vivo* data showing a significant increase in both total and phosphorylated EGFR (Tyr^{1173}) (Pourazar *et al.*, 2008). Taken together, these studies support the concept that PM exposure impacts EGFR-dependent signaling.

In addition to proinflammatory signaling, activation of EGFR-dependent signaling leads to a diverse set of cellular outcomes including proliferation, differentiation, growth, and apoptosis (Figure 1-3). Recently, a series of studies have elucidated a mechanism of EGFR-dependent wound healing following damage to the lung epithelium (Vermeer *et al.*, 2003; Vermeer *et al.*, 2006a; Vermeer *et al.*, 2006b). These studies demonstrate that the EGFR is located on the basolateral domain while the soluble receptor ligand heregulin-α is restricted to the apical membrane. Tight junctions between

adjacent epithelial cells form a barrier between the apical and basolateral domains thereby restricting diffusion of soluble ligands. However, following mechanical injury or disruption of the tight junctions by Ca^{2+} chelation, heregulin-α gained access to the basolateral domain and induced EGFR kinase activation and cellular proliferation to repair the damaged monolayer. In addition to physiologically relevant signaling, inappropriate activation of EGFR-dependent signaling is involved in numerous pathological outcomes including cancer progression [reviewed in (Milanezi *et al.*, 2008)] and inappropriate mucin production that has been implicated in a number of hypersecretory disesases including asthma, chronic obstructive pulmonary disease, and cystic fibrosis [reviewed in (Nadel and Burgel, 2001)].

1-F Protein Tyrosine Phosphatases

Tyrosine kinase activity is opposed by protein tyrosine phosphatases (PTPases) which function to maintain signaling quiescence (Stoker, 2005). PTPases are characterized by a highly conserved 11-residue signature motif, I/VHCXAGXXR(S/T)G, which contains required Cys and Arg residues necessary for catalysis (Barford *et al.*, 1994). The PTPase catalytic pocket has a low pKa (<6). Therefore, the catalytic thiol group (R-SH) exisits as a thiolate anion (R-S-) at physiological pH (Peters *et al.*, 1998). The thiolate anion can thereby initiate catalysis by nucleophilic attack of phosphate-bearing substrates.

It is estimated that the human genome codes for over 100 proteins in the PTPase superfamily with additional diversity introduced by alternative promoters and splice sites and post-translational modifications (Tonks, 2006). The PTPase superfamily is subdivided into multiple classes based upon their structure and substrate specificities (Figure 1-7). The classical PTPases function to dephosphorylate phospho-tyrosine bearing substrates. This class of PTPases includes receptor-like and cytosolic PTPases. Receptor-like PTPases, such as CD45 and LAR, contain an extracellular

Figure 1-7

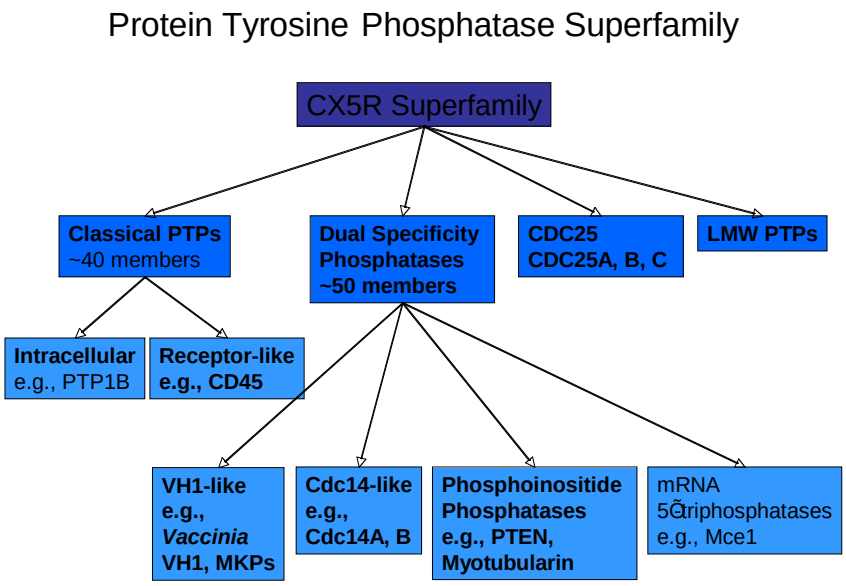


Figure 1-7 *Protein tyrosine phosphatase superfamily.*

ligand-binding domain, a transmembrane domain and two tandem intracellular PTPase domains. The catalytic activity resides in the proximal D1 domain while the distal D2 PTPase domain is reported to be involved in enzymatic specificity and stability (Streuli *et al.*, 1990). Cytosolic classical PTPases, such as PTP1B, SHP1, and PTP-PEST, contain regulatory regions that flank the catalytic site and control enzymatic activity directly- by interactions with the active site or by influencing substrate specificity- and spatially- by directing subcellular localization (Wang *et al.*, 2003; Tonks, 2006). The second main class of PTPases is the cytosolic Dual Specificity Phosphatases (DSPs). DSPs are defined by their ability to dephosphorylate phospho-serine and phospho-threonine residues in addition to phospho-Tyrosine bearing substrates. The best characterized sub-division of DSPs, the MAPK phosphatases, inactivate MAPKs by dephosphorylating the signature Tyrosine-X-Threonine sequence in the MAPK activation loop (Owens and Keyse, 2007). The Cdc25 PTPases constitute the third class of PTPases that function to dephosphorylate conserved Threonine and Tyrosine residues on cyclin-dependent kinases (Wang *et al.*, 2003). In the case of Cdc25 PTPases, dephosphorylation of substrate kinases results in their activation and drives progression through the cell cycle (Millar and Russell, 1992). The fourth class of PTPases, the low molecular weight PTPases, is less well understood but has been reported to regulate a number of tyrosine kinase receptors including the platelet derived-, vascular endothelial-, and fibroblast-growth factor receptors (Wang *et al.*, 2003). Finally, there are some members of the PTPase superfamily that have non-protein targets, including mRNA, complex carbohydrates and inositol phospholipids (Ross *et al.*, 2007).

1-F.1 Regulation of PTPase activity

PTPase superfamily members 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 ($R\text{-}S^-$) form at physiological

Figure 1-8

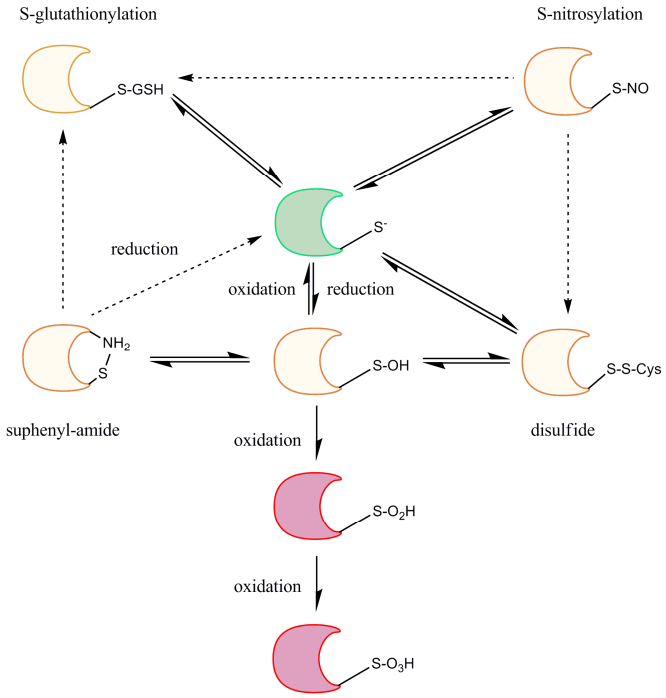


Figure 1-7 *Regulation of PTPase activity*. PTPases are characterized by a signature motif [I/V]HCXXGXXR[S/T], which contains an invariant Cys residue that is essential for catalysis. The environment of the active site confers an unusually low pKa on this Cysteine residue, which therefore is present as a thiolate anion at neutral pH (green). However, the low pKa also renders this residue highly susceptible to a series of oxidations, with concomitant reversible (yellow) and irreversible (red) inhibition of PTPase activity. A number of reversible, post-translational modifications of the catalytic cysteine have also been reported.

pH (Peters *et al.*, 1998). This renders PTPases highly susceptible to inactivation by oxidation (Denu and Tanner, 1998; Takakura *et al.*, 1999) (Figure 1-8). A primary oxidation to a sulphenyl derivative (R-SOH) reversibly inactivates PTPase activity. PTPases are protected from further oxidation by mechanisms involving intra- and inter-molecular disulfide formation, sulphenyl-amide formation or S-glutathionylation (Heneberg and Draber, 2005; Salmeen and Barford, 2005). The addition of reducing agents, such as dithiothreitol (DTT), reverts inactivated sulphenic acids to active thiolate anion groups. In particular, we have observed a reversal of H_2O_2-mediated inhibition of human recombinant PTP-1B activity following treatment with DTT (Tal, unpublished observations). In the presence of strong oxidizing agents, such as peroxyvanadate or peroxynitrite, subsequent stepwise oxidations from the sulphenic (SOH) to the sulphinic (SO_2H) and sulphonic or cysteic acid (SO_3H) groups occur. Those PTPases that become doubly or triply oxidized are terminally inhibited (Takakura *et al.*, 1999). Interestingly, transient EGF-mediated EGFR activation, concurrent with ROS generation and reversible PTPase inactivation, was shown to induce lateral EGFR activation, supporting the notion that reversible inactivation of PTPases by a single oxidation event is not a toxicological effect, but rather a required means for activation of phospho-dependent signaling cascades (Reynolds *et al.*, 2003).

1-F.2 Evidence for PM-PTPase interactions

In order to address whether PM exposure inhibits PTPase activity and in light of the varied mechanisms by which PTPase activity is regulated, it is useful to discuss the components of PM and how they might contribute to PTPase inactivation. There are two broad mechanisms by which components of PM could potentially interact with PTPases. These include both indirect inhibition, through PM-induced formation of reactive oxygen species, and direct inhibition by PM associated metals and nucleophilic organic compounds.

1-F.2A Indirect PTPase inhibition via PM-induced oxidative stress

Numerous studies have sought to identify the mechanism by which particle inhalation induces local and systemic inflammation. Particle mass, size and surface area, metallic and organic contents, acids, sulfates, nitrates, elemental carbon, and co-pollutants have been investigated and oxidative stress has emerged as a leading mechanism by which PM elicits pulmonary toxicity (Gurgueira *et al.*, 2002; Brook *et al.*, 2003; Li *et al.*, 2003; Risom *et al.*, 2005). Oxidative stress, induced by the imbalance of oxidant formation and elimination, is tightly regulated by both enzymatic (e.g. superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic (e.g. α-tocopherol and glutathione) defenses. Oxidative stress is characterized by glutathione (GSH) depletion and diglutathione (GSSG) accumulation triggering the activation of redox-sensitive signaling pathways and culminating in the expression of cytoprotective- and inflammation-associated genes (Xiao *et al.*, 2003). Generation of reactive species is not limited to pathological outcomes but instead is a critical contributor to immunological host defense (Geiszt *et al.*, 2003) and a proposed mediator of phospho-dependent signaling progression (Reynolds *et al.*, 2003). Interestingly, reactive species is commonly described in response to growth factor-mediated activation of transmembrane receptors (Nakashima *et al.*, 2005) and has been implicated in PM-related signaling aberrations (Kim *et al.*, 2006; Tal *et al.*, 2006).

PM is thought to exert oxidative stress on the lung by presenting or stimulating cells to produce reactive species via its metals, organics (semi-quinones and hydrocarbons), lipopolysaccarides, and ultrafine constituents (Tao *et al.*, 2003). Studies using residual oil fly ash (ROFA) have demonstrated that pulmonary inflammation is attributable to water-soluble metal constituents (Gavett *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). Mechanistically, redox-cycling metals, such as Fe^{3+}, Cu^{2+}, $V^{3+/5+}$ and $Cr^{3+/6+}$, can generate ROS by Fenton-type chemistry and act as catalysts by Haber-Weiss reactions (Koppenol, 2001). Fe^{3+} in particular, is reported to be a primary contributor to DEP-induced H_2O_2 generation (Park *et al.*, 2006).

In addition to metals, bioavailable organic compounds have been reported to contribute to oxidant effects induced by PM exposure (Xia *et al.*, 2004). Two main families of compounds, polycyclic aromatic hydrocarbons (PAHs) and quinones, are adsorbed on diesel particles (Baulig *et al.*, 2003) and are thereby delivered to the airway epithelium following inhalation. Both reactive PAH metabolites and redox-cycling quinones generate reactive oxygen species. In particular, NADPH-cytochrome P450 reductase reduces quinones to semiquinone radicals which in turn, reduce oxygen to $O_2^{\cdot-}$ and become reoxidized to the original quinone. PM-associated PAHs are metabolized by cytochrome P450s and peroxidases to oxidized derivatives such as epoxides, diols, and redox-cycling quinones [reviewed in (Li *et al.*, 2002)]. A body of work has been produced supporting the notion that the organic fraction of DEP, via ROS generation, is the primary mediator of PM-associated inflammation/toxicity (Li *et al.*, 2002; Li *et al.*, 2003; Li and Whorton, 2003). However, it must be noted that most studies cited in these particular reviews used a Japanese diesel particle (H-DEP), which reportedly contains approximately 50% organics and as such, is not representative of most ambient DEP. The chemical composition of DEP is influenced in part by the age of the engine, type of fuel, load characteristics, lube oil composition, presence and efficiency of control devices and the sampling procedures (Wichmann, 2007). Not surprisingly, pulmonary toxicity, adjuvancy, and mutagenicity in response to DEP exposure vary greatly among different DEPs (Table 1-2).

1-F.2B Direct PTPase inhibition

In addition to indirect inhibition by PM-induced oxidative stress, a growing body of literature supports the notion that both metallic and organic PM components can directly impair PTPase activity by forming inhibitory electrostatic interactions with the catalytic cysteine or by covalent modification of catalytic and regulatory residues, respectively.

Table 1-2

Particle	Source	Organic content	DCM Extractable organic mass	Pulmonary toxicity	Mutagenic properties	Metal content (%)				
						Fe	Zn	Ni	Cu	V
NIST-SRM 2975	Diesel powered forklift	5%*	2%*	+*PMNs	+†	.033	.032	0	0	0
DEP-3	Diesel engine with a compressor	41%¥	18.9%¥	Adjuvants¥	n/a	.078	.115	0	.001	0
Sagai DEP	Automobile	50%*	26.3%*	+* MACs Adjuvant¥	+†	.004	.030	0	0	0

Table 1-2 *Diesel exhaust particles.* Differences in organic content, pulmonary toxicity, adjuvancy, and mutagenecity have been reported in DEPs obtained from different sources [*(Singh *et al.*, 2004), †(DeMarini *et al.*, 2004), ¥(Stevens *et al.*, 2008), and ‡Dr. Seung-Hyun Cho US EPA, personal communication]. For metal content analysis, 1.0 ml 3 N HCL was used to determine ionizable concentrations of iron (Fe), zinc (Zn), vanadium (V), nickel (Ni), copper (Cu), and vanadium (V) by inductively coupled plasma optical emission spectroscopy as previously described (Ghio *et al.*, 2003).

We and others have reported that Zn^{2+}, a common PM constituent, may be implicated in the toxicity associated with PM inhalation (Kodavanti *et al.*, 2002; Samet *et al.*, 2003; Okeson *et al.*, 2004; Tal *et al.*, 2006; Kim *et al.*, 2007). Zn^{2+} is unable to undergo redox cycling yet is a known inhibitor of PTPases (Haase and Maret, 2003; Haase and Maret, 2005), including a broad spectrum of PTPases present in airway epithelial cells (Samet *et al.*, 1999). A mechanism of direct inhibition has been proposed wherein Zn^{2+} blocks PTPase activity by binding to the catalytic cysteine and to neighboring histidine or aspartate residues present in the highly conserved active site (Haase and Maret, 2003). More recently, the authors suggest that Zn^{2+}-coordinated thiolates can additionally participate in redox cycling generating disulfide and terminally oxidized sulfur moieties (Krezel *et al.*, 2007). However, treatment of Zn^{2+}-exposed HAEC with the strong reductant and weak Zn^{2+} chelator, dithiothreitol (DTT), but not other structurally unrelated anti-oxidants, reverses Zn^{2+}-mediated inhibition of EGFR-directed PTPase activity (Tal, unpublished observations). This suggests that Zn^{2+}-mediated PTPase inhibition may occur through a mechanism dependent upon direct metallic attack of the PTPase catalytic site, rather than indirectly inhibiting PTPases by oxidation. Interestingly, exposure to nucleophilic aldehydes (commonly associated with ambient PM) was reported to mobilize cellular Zn^{2+} from metallothionein and thionein at levels sufficient to reduce PTPase activity in HepG2 cells (Hao and Maret, 2006).

The second mechanism by which PM components are known to directly inhibit PTPase activity has recently surfaced in a series of studies that provided the first mechanistic evidence that environmentally relevant reactive quinones and aldehydes can directly inactivate PTP1B activity by a covalent modification of reactive cysteines (Iwamoto *et al.*, 2007; Seiner *et al.*, 2007). Seiner *et al.* reported that the reactive aldehyde acrolein inhibited PTP1B activity through conjugate addition to the catalytic cysteine *in vitro* (Seiner *et al.*, 2007). Notably, treatment of A431 cells with 1,2-napthoquinone was shown to arylate two reactive cysteinyl residues in PTP1B, thereby inhibiting its activity and leading to the prolonged and irreversible activation of EGFR (Iwamoto *et al.*, 2007).

Taken together, these studies are in keeping with the concept that DEP-associated oxy-organics can inhibit PTPase activity and thereby directly contribute to proinflammatory signaling. In support of this, activation of vanilloid receptor 1 leading to contraction of tracheal smooth muscle in guinea pigs exposed to 1,2-napthoquinone was blocked when tracheal tissue was pretreated with the EGFR kinase inhibitor PD153035 (Kikuno *et al.*, 2006).

1-G Conclusions

Exposure to ambient PM is associated with elevated rates of morbidity and mortality. Toxicological and epidemiological studies have implicated multiple particle constituents as active agents of the toxicology of PM. Inflammatory responses have been reported to be a feature of many of the adverse effects of PM exposure (Brook *et al.*, 2003). We have previously shown that *in vitro* exposure to PM constituents, such as the divalent metal cation Zn^{2+}, or carbonaceous ultrafine particles, activates multiple phosphorylation-dependent signaling pathways, including the EGFR signaling cascade [(Wu *et al.*, 1999; Kim *et al.*, 2005; Tal *et al.*, 2006), Chapter II]. Moreover, PM-induced EGFR activation leads to increased expression of inflammatory mediators such as COX-2 and the chemokine IL-8 in human airway epithelial cells [(Cao *et al.*, 2007a), Tal, unpublished observations, Chapter III]. Through studies aimed at elucidating the mechanism of PM-induced signaling, we have shown that exposure to a variety of metallic components of ambient PM blunts the activity of cellular PTPases. Based on the mechanisms by which PTPase activity is regulated, these data suggest that metallic, oxidant or electrophilic constituents of ambient PM can similarly induce kinase-dependent proinflammatory signaling in HAEC through inhibition of kinase-directed PTPase activity.

1-G.1 Hypothesis and specific aims of the doctoral research

We hypothesized that ambient PM constituents, including DEP and Zn^{2+}, activate phosphorylation-dependent signaling pathways through inhibition of PTPases that function to maintain signaling

quiescence and moreover, that an inhibitory interaction between PM components and PTPases results in the activation of proinflammatory signaling in lung epithelial cells.

This hypothesis was addressed in this dissertation research through three specific aims. **First, we characterized the effects of Zn^{2+} on EGFR signaling in HAEC by examining the role of Src and EGFR kinase activities and PTPase inhibition in Zn^{2+}-induced EGFR activation.** Because PTPases can be inhibited by electrophilic organic compounds, we hypothesized that other components of ambient PM such as DEP would similarly inhibit EGFR-directed PTPase activity leading to sustained EGFR-dependent signaling. **Therefore, with the goal of improving the understanding of the relationships between biologically active constituents of DEP and mechanisms of toxicity, we investigated the effects of DEP with varying organic content on EGFR activation and EGFR dephosphorylation in HAEC.** Preliminary studies revealed that the same exposures to PM constituents that led to signaling disregulation characterized by EGFR activation and impairment in EGFR-directed PTPase activity, resulted in elevated expression of proinflammatory mediators in HAEC. Therefore, we also hypothesized that exposure to PM leads to proinflammatory mediator expression as a consequence of disruption of cell signaling by PTPase inhibition. **In order to examine the link between PM-induced signaling and ensuing proinflammatory responses, we determined the effect of PM constituents on signaling pathways that regulate expression of proinflammatory mediators (such as IL-8) and examined the role of signal transduction disregulation involving PTPase inhibition in PM-induced IL-8 expression in HAEC.**

Chapter II: Inhibition of protein tyrosine phosphatase activity mediates epidermal growth factor receptor signaling in human airway epithelial cells exposed to Zn^{2+}

The main findings of Specific Aim 1 (Chapter II) were published in "Tal, T. L., Graves, L. M., Silbajoris, R., Bromberg, P. A., Wu, W., and Samet, J. M. (2006). Inhibition of protein tyrosine phosphatase activity mediates epidermal growth factor receptor signaling in human airway epithelial cells exposed to Zn^{2+2+}. *Toxicology and Applied Pharmacology* **214**, 16-23."

TT contributed to the manuscript as follows. TT, JS, PB, and LG conceived of the study design; TT performed all experiments; and TT, JS, and PB wrote the manuscript.

2-A Abstract

Epidemiological studies have implicated zinc in the toxicity of ambient particulate matter (PM) inhalation. We previously showed that exposure to metal-laden PM inhibits protein tyrosine phosphatase (PTP) activity in human primary bronchial epithelial cells (HAEC) and leads to Src-dependent activation of EGFR signaling in B82 and A431 cells. In order to elucidate the mechanism of Zn^{2+}-induced EGFR activation in HAEC, we treated HAEC with 500 µM $Zn^{2+}SO_4$ for 5-20min and measured the state of activation of EGFR, c-Src and PTPs. Western blots revealed that exposure to Zn^{2+} results in increased phosphorylation at both trans- and auto-phosphorylation sites in the EGFR. Zn^{2+} mediated EGFR phosphorylation did not require ligand binding and was ablated by the EGFR kinase inhibitor PD153035, but not by the Src kinase inhibitor PP2. Src activity was inhibited by Zn^{2+} treatment of HAEC, consistent with Src-independent EGFR transactivation in HAEC exposed to Zn^{2+}. The rate of exogenous EGFR dephosphorylation in lysates of HAEC exposed to Zn^{2+} or V^{4+} was significantly diminished. Moreover, exposure of HAEC to Zn^{2+} also resulted in a significant impairment of dephosphorylation of endogenous EGFR. These data show that Zn^{2+} -induced activation of EGFR in HAEC involves a loss of PTP activities whose function is to dephosphorylate EGFR in opposition to baseline EGFR kinase activity. These findings also suggest that there are marked cell-type specific differences in the mechanism of EGFR activation induced by Zn^{2+} exposure.

2-B Introduction

Zinc (Zn^{2+}) is a ubiquitous metallic constituent of ambient particulate matter (PM) (Chang *et al.*, 2000; Claiborn *et al.*, 2002). Epidemiological studies have associated PM inhalation with adverse health effects including diminished lung function, morbidity and mortality (Dockery *et al.*, 1993; Koenig *et al.*, 1993; Schwartz, 1994; Samet *et al.*, 2000). In occupational settings, inhalation of zinc oxide is known to cause Metal Fume Fever, an acute flu-like syndrome accompanied by airway inflammation and production of TNF-α and IL-6 (Nemery, 1990; Blanc *et al.*, 1993). Animal and *in vitro* studies have shown that Zn^{2+} exposure results in increased synthesis of inflammatory mediators, such as chemokines and cytokines (Kodavanti *et al.*, 2002; Richter *et al.*, 2003; Riley *et al.*, 2003) whose expression is regulated by phosphorylation-dependent signaling cascades.

Zn^{2+} is known to affect a variety of cellular proteins and phosphorylation-dependent signaling pathways including the Epidermal Growth Factor Receptor (EGFR) (Wu *et al.*, 1999), Tropomyosin-related kinase (Hwang *et al.*, 2005), the Mitogen Activated Protein Kinase (Samet *et al.*, 1998) and insulin/insulin-like growth factor-1 (Haase and Maret, 2003) pathways. These signaling pathways originate at the level of receptor tyrosine kinases whose phosphorylation status is regulated by opposing protein tyrosine phosphatase (PTP) activity (Ostman and Bohmer, 2001). We have previously shown that exposure to Zn^{2+} can inhibit PTP activity in Human Airway Epithelial Cells (HAEC) (Samet *et al.*, 1999).

The EGFR is a 170-kDa transmembrane glycoprotein that mediates the mitogenic response of cells to a variety of polypeptides including EGF and transforming growth factor alpha (Korc *et al.*, 1987). Structurally, the receptor contains an extracellular ligand binding domain, an intracellular tyrosine kinase domain, and a C-terminal region harboring several tyrosine residues which undergo trans- and autophosphorylation upon receptor activation (Ullrich *et al.*, 1984; Hsu *et al.*, 1990; Margolis *et al.*, 1990). Following ligand stimulation, EGFR undergoes homo- or heterodimerization consequently

activating the receptor's intrinsic kinase activity (Ullrich and Schlessinger, 1990). Transphosphorylation of activating tyrosine residues by Src kinase has also been shown to activate the receptor (Samet *et al.*, 2003). Both traditional, ligand stimulated receptor activation and transactivation events prompt increased receptor kinase enzymatic efficiency (Cooper and Howell, 1993).

We have previously reported that Zn^{2+} activates EGFR in human epidermoid carcinoma cells (A431), B82 mouse lung fibroblasts (B82L), and primary HAEC (Wu *et al.*, 1999; Wu *et al.*, 2002; Samet *et al.*, 2003). The signaling mechanism by which Zn^{2+} induces EGFR phosphorylation was shown to be secondary to Src kinase activation in both A431 and B82L cells (Wu *et al.*, 2002; Samet *et al.*, 2003). It must be noted however, that striking cell-type dependent variability exists within signaling pathways (Lakshminarayanan *et al.*, 1998). In order to study Zn^{2+} exposure in a highly relevant model of PM inhalation in the present study, we have examined the effect Zn^{2+} exposure on EGFR in primary HAEC cultures. We report here that Zn^{2+} exposure induces EGFR activation in HAEC through a mechanism which does not involve Src activation but rather, inhibition of EGFR-directed PTP activity.

2-C Methods

Reagents

Tissue culture media, supplements, and supplies were obtained from Clonetics (San Diego, CA). Phosphate-buffered saline, tissue culture media, and reagents were purchased from GibcoBRL (Gaithersburg, MD); bis[sulfosuccinimidyl]suberate (BS^3) was obtained from Pierce (Rockford, IL). Protease inhibitors, phosphatase inhibitors, PD153035, 4-amino-5-(4-chlorophenyl)-7-(tbutyl)pyrazolo[3,4-d]pyrimidine (PP2), Compound 56 (c56), and EGF were purchased from Calbiochem (San Diego, CA). Detergents, metal salts, human collagen, Nonidet P-40, PolyGlu:Tyr(4:1), 2-β-mercaptoethanol, dithiothreitol (DTT), 1-hydroxypyridine-2-thione (pyrithione) and common laboratory reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture flasks were purchased from Falcon (Fisher Scientific, Raleigh, NC). The Src assay kit, active EGFR, magnesium/ATP cocktail and Fer Kinase were purchased from Upstate (Lake Placid, NY). Polyacrylamide was obtained from Roche (Indianapolis, IN). Electrophoresis supplies such as molecular mass standards, polyacrylamide and buffers were purchased from Bio-Rad (Richmond, CA). PAGEr Duramide precast gels were obtained from Cambrex (Rockland, ME). Luminescence reagents and [^{32}P]-γ-adenosine triphosphate (ATP) (10mci/ml) were purchased from Amersham Biosciences (Piscataway, NJ). Phospho-EGFR (Tyr^{845}/Tyr^{1068}) and phospho-Src (Tyr^{527}/Tyr^{416} antibodies were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-mouse, donkey anti-goat IgG, C-terminal EGFR antibody, protein A-agarose, agarose conjugated EGFR, and broad specificity Src antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

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 (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) (BEGM) on tissue culture plates coated with human collagen (Sigma), grown to confluence, and then passaged 2 or 3 times in BEGM on ordinary tissue culture plates. Solutions of 100 mM Zn^{2+} and 100 mM V^{4+} were prepared in distilled water and used as stocks for dilution into serum free BEGM. The final concentration of both metals was 500 µM, a concentration previously shown to be acutely non-cytotoxic (Samet *et al.*, 1998).

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 purchased from Calbiochem. Each sample was normalized for a protein content of 30-100 µg then mixed with one volume of SDS-PAGE loading buffer containing, 0.125 M Tris [pH 6.8], 4% SDS, 20% glycerol, 10% ß-mercaptoethanol, and 0.05% bromophenol blue. The samples were heated for 1 min at 95°C and run on adjacent lanes of 11% SDS-PAGE gels or 4-20% Tris-Glycine Gradient pre-cast gels (Cambrex) with pre-stained molecular weight markers in Tris-glycine-SDS buffer. Electrophoresed proteins were electroblotted onto nitrocellulose paper. Blots were blocked with 5% non-fat milk, washed briefly, and incubated overnight with HRP-

conjugated antiphosphotyrosine primary antibodies. HRP-goat anti-rabbit and HRP-donkey anti-goat were used as secondary antibodies. A non-specific EGFR primary antibody, which has lower binding efficiency when EGFR is highly phosphorylated, was used to normalize for loading variability. Protein bands on the membrane were detected using chemiluminescence reagents and film as per manufacturer's instructions (Amersham Pharmacia Biotech) and exposed on high-performance chemiluminecence film (Amersham Pharmacia Biotech). In some cases, blots were stripped and reblotted using a commercially available stripping reagent (Chemicon International, Temecula, CA). Blots were digitized using a Kodak EDAS 120 System (Rochester, NY). Western blotting results shown are representative of three or more experiments.

Src Kinase Activity Assay

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 Phenyl methane sulfonyl fluoride (PMSF) (Calbiochem, San Diego, CA), 1 mM sodium metavanadate, 10 mM sodium fluoride, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Following preclearing with protein-A agarose, Src kinase was immunoprecipitated from 500 µg cell lysate using anti-Src kinase monoclonal antibody (Santa Cruz) for 1 h at 4°C. The immunoprecipitates were then washed with lysis buffer followed by a Src reaction buffer provided in the Src activity kit (Upstate, Lake Placid, NY). Src kinase activity was assayed in kinase buffer containing 6µCi [^{32}P]-γ-ATP and Src substrate peptide as per the manufacturer's instructions. The reaction was incubated for 10 min at 30°C with vigorous agitation in an Eppendorf ThermoMixer (Brinkman Instruments, Westbury, NY) and was subsequently terminated by the addition of 20 µl of 40% (final) TCA, and a fraction was absorbed onto P81 cellulose phosphate paper (Src Activity Kit, Upstate) then washed extensively with 1% phosphoric acid. Radioactivity retained on the P81 paper was quantified by liquid scintillation counting (LKB Wallace, Gaithersberg, MD).

Radiolabeling of [^{32}P]PolyGlu:Tyr(4:1)

A total of 200 µg PolyGlu:Tyr was radiolabeled using 1 µg of recombinant FER kinase (Upstate, Lake Placid, NY) in the presence of 200 µCi [^{32}P]-γ-ATP for 1 hour at 30° C in 300 µl of a buffer consisting of 10 mM $MgCl_2$, 50 mM NaCl and 0.1 mM ATP. The substrate was precipitated by adding TCA solution to 20% wt/vol and centrifuging at 12,000 × g for 5 min. The pellet was washed three times in 10% TCA and the substrate was resuspended at 10 µg/ml in 2 M Tris, pH 8.0.

In-Gel Tyrosine Phosphatase Activity Assay

The in-gel phosphatase activity assays were carried out using a modification of a method described elsewhere (Burridge and Nelson, 1995). Protein extracts were prepared as for Western blots (described previously), except that samples were not boiled. The samples were then subjected to SDS-PAGE on 11% polyacrylamide gels containing [^{32}P]PolyGlu:Tyr (approximately 1.5 million cpm/40 ml gel). The proteins were then renatured by removing SDS with 20% isopropanol, followed by extensive washing of the gels with 0.04% Tween-40 in Tris, pH 8.0. Clear bands indicative of tyrosine phosphatase activity were visualized by autoradiography using a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Exogenous EGFR Dephosphorylation Assay

Active EGFR (86kDa) was induced to autophosphorylate by incubation at room temperature for 5 min in Mg^{2+}/ATP cocktail. HAEC at ~80% confluency were starved for 8-14 hours prior to treatment with 500 µM Zn^{2+} or V^{4+} in 4µM pyrithione for 20min and harvested in a specialized Phosphatase Lysis Buffer containing 100 mM HEPES, 0.2% NP-40, 20 µg/ml PMSF and 10 µM c56. 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. 100 µg of harvested protein in 35 µl was added to the reaction mixture containing 115 µl of PTPs buffer, 10 µM c56 and 20 µl of phosphorylated EGFR substrate (10 ng/µl) which was incubated at 30°C with mixing and sampled at 0 (prior to lysate addition), 5, 10, and 20 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.

Endogenous EGFR Dephosphorylation Assay

HAEC at ~80% confluency were starved for 8-14 hours prior to treatment with 20 ng/ml EGF to induce full receptor phosphorylation. After washing with room temperature PBS, cells were treated with 500 µM Zn^{2+} or V^{4+} in 4 µM pyrithione for 20 min and then exposed to 10 µM c56 to inhibit further EGFR kinase activity. Cells were harvested, in lysis buffer (as previously described) without phosphatase inhibitors, at 10, 30, and 90 seconds and cellular lysates were subjected to SDS-PAGE and Western Blotting using phospho-specific antibodies enabling detection of EGFR activation over time. Separate blots were analyzed for optical densities using Kodak Software System. Optical densities are shown as percentage of individual control.

2-D Results

2-D.1 *Zn^{2+}-induced EGFR phosphorylation in human airway epithelial cells (HAEC) requires EGFR kinase domain activity but not EGFR ligand binding*

To study the effects of Zn^{2+} exposure on EGFR signaling in HAEC, we first examined EGFR phosphorylation in HAEC treated with 500 µM Zn^{2+} for 5 and 20 min using phospho site-specific antibodies. As shown in Fig. 2-1A, Zn^{2+} treatment induced a significant increase in EGFR phosphorylation at both Tyr^{1068} and Tyr^{845} which was evident as early as 5 min. The total EGFR antibody used for normalization has reduced binding efficiency for phosphorylated EGFR. As reported elsewhere (Haase and Maret, 2003), the Zn^{2+}-specific ionophore pyrithione was found to reduce inter-experiment variability in HAEC responsiveness to Zn^{2+} exposure and, therefore, all subsequent experiments in this study were conducted in the presence of 4 µM pyrithione unless otherwise noted.

We have previously shown in B82L (Wu *et al.*, 2002) and A431 cells (Samet *et al.*, 2003) that Zn^{2+}-induced EGFR activation is independent of EGFR kinase activity, being mediated via transphosphorylation by Src. To characterize the mechanism by which Zn^{2+} activates EGFR in HAEC, these cells were pretreated with the potent EGFR kinase inhibitor, c56 prior to exposure with 500 µM Zn^{2+} or 20 ng/ml EGF for 20 min. In contrast to observations in B82L and A431 cells, EGFR kinase inhibition significantly blocked Zn^{2+}-mediated EGFR activation in HAEC. As expected, c56 pretreatment completely abolished EGF-induced EGFR phosphorylation (Figure 2-1B).

To test the role of the extra-cellular ligand-binding domain of the EGFR, HAEC were pretreated with a blocking antibody prior to stimulation with Zn^{2+}. Blocking the EGFR ligand binding domain ablated EGF-induced EGFR phosphorylation but had no discernible effect on EGFR phosphorylation of either site in response to Zn^{2+} (Figure 2-1C).

Figure 2-1

Figure 2-1A

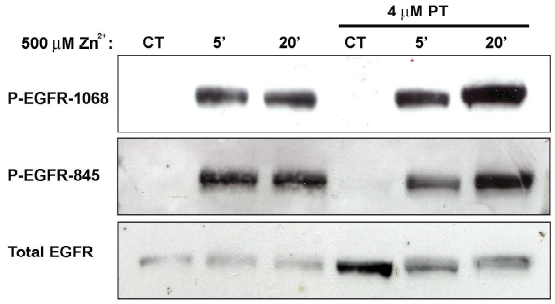


Figure 2-1B

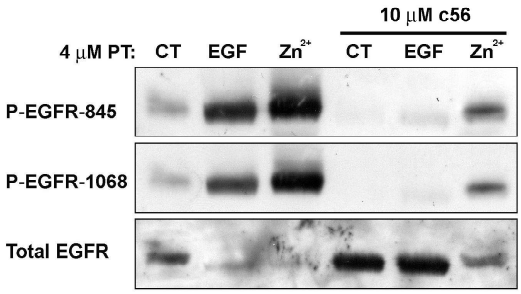


Figure 2-1C

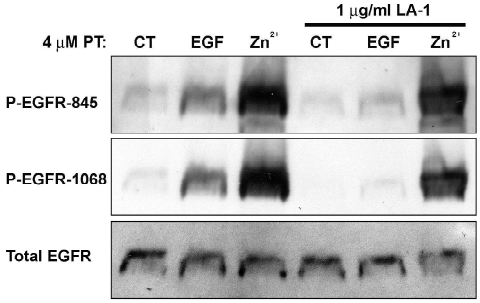


Figure 2-1. *Zn^{2+}-induced EGFR activation in Human Airway Epithelial Cells (HAEC) requires EGFR kinase domain activity but not external ligand binding.* 2-1A: HAEC were treated with 500 μM Zn^{2+} for 5 and 20 min in the presence and absence of the Zn^{2+}-membrane ionophore, pyrithione (PT). Cells were harvested and analyzed for EGFR activation by Western Blotting using phospho-specific antibodies. The antibody used for normalization has reduced binding efficiency for phosphorylated EGFR. 2-1B: Following pretreatment with 10 μM Compound 56 (c56) or vehicle control (DMSO) for 1 h, HAEC were treated with 500 μM Zn^{2+} or 20 ng/ml EGF in media containing 4 μM PT for 20 min. Lysates were analyzed for EGFR activation via Western Blotting with phospho-specific antibodies. 2-1C: HAEC were pretreated with 1 μg/ml of the EGFR blocking antibody, LA-1 for 1 hr. Cells were then exposed to 500 μM Zn^{2+} or 20 ng/ml EGF in media containing 4 μM PT for 20 min. EGFR activation was assessed via Western Blotting with phospho-specific antibodies. The results shown are representative of three or more experiments.

2-D.2 *Zn^{2+}-mediated EGFR activation does not require Src kinase activity in HAEC*

The data above showed that Zn^{2+}-induced EGFR phosphorylation required receptor kinase activity but not external ligand binding, and suggested that Src activity is not involved in EGFR activation of HAEC exposed to Zn^{2+}. To further clarify its role in Zn^{2+}-induced EGFR activation in HAEC, we directly examined the effect of Zn^{2+} exposure on Src activation in these cells. Immunoblotting with phosphospecific anti-Src antibodies showed no changes in levels of P-Tyr^{527} Src or P-Tyr^{416} Src in HAEC exposed to 500 µM Zn^{2+} for 5 or 20 min relative to untreated controls (Figure 2-2A). In addition, pretreatment of HAEC with the Src kinase activity inhibitor PP2 was partially effective in blocking Zn^{2+}-induced EGFR phosphorylation at Tyr^{1068} and Tyr^{845} by 20 min (Figure 2-2B). We subsequently examined the effect of Zn^{2+} treatment on Src kinase activity in HAEC. As shown in Figure 2-2C, exposure to Zn^{2+} resulted in a marked reduction in Src kinase activity as early as 5 min, with nearly complete inhibition by 20 min. The inhibitory effect of Zn^{2+} treatment on Src activity was also pronounced in the absence of pyrithione (Figure 2-2C) These results confirmed that Zn^{2+}-induced EGFR phosphorylation is independent of Src kinase activity in HAEC.

2-D.3 *Exposure to Zn^{2+} inhibits EGFR-specific Protein Tyrosine Phosphatases (PTP) in HAEC.* Phosphorylation-dependent signal transduction pathways are regulated by the opposing activities of kinases and phosphatases, and we previously reported that exposure to Zn^{2+} and the non-specific PTP inhibitor vanadium (V^{4+}) inhibits protein tyrosine phosphatases in a human airway epithelial cell line (Samet *et al.*, 2003). We therefore examined the possibility that Zn^{2+}-induced EGFR activation is driven by an inhibition of the tyrosine phosphatase activity(ies) which normally opposes baseline EGFR autophosphorylation activity. Lysates from HAEC treated with 500 µM Zn^{2+} or 20 ng/ml EGF for 20 min were subjected to in-gel phosphatase activity analyses on ^{32}P-polyGlu:Tyr-impregnated acrylamide gels. As seen in Figure 2-3, Zn^{2+} treatment inhibited PTPs of molecular weights ranging from 15 to 250 kDa in HAEC, while stimulation with EGF had no discernible effect.

These data confirmed that exposure to Zn^{2+} can decrease PTP activity in HAEC. In order to examine EGFR-specific dephosphorylation, we first measured the dephosphorylation activity in lysates prepared from HAEC exposed to Zn^{2+}, V^{4+} or media alone. Dephosphorylation activity in control HAEC against exogenous P-EGFR could be observed clearly over the assay period, with a marked decrease in phosphorylation of Tyr^{1068} and Tyr^{845} observed by 20 min (Figure 2-4A). By comparison, exposure to 500 μM Zn^{2+} for 20 min resulted in a significant impairment in the rate of exogenous EGFR dephosphorylation in HAEC (Figure 2-4A and 2-4B). As expected, V^{4+} treatment of HAEC also induced marked inhibition of EGFR dephosphorylation activity (Figure. 2-4A).

Figure 2-2

Figure 2-2A

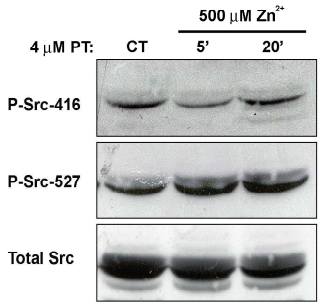


Figure 2-2B

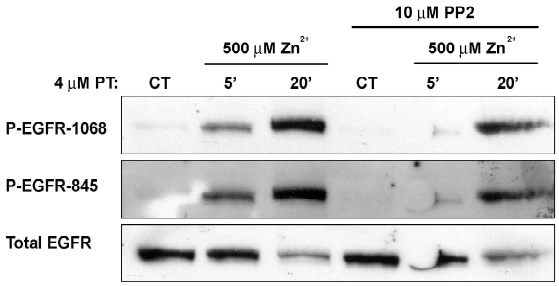


Figure 2-2C

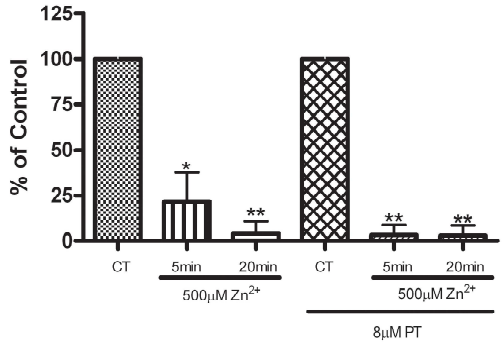


Figure 2-2. *Zn^{2+}-mediated EGFR activation does not require Src kinase activity in HAEC.* 2-2A: HAEC were treated with 500 μM Zn^{2+} and 4 μM PT for 5 and 20 min. Cellular lysates were subjected to SDS-PAGE and Western Blotting using phospho-specific antibodies directed against Src activation residues. The results shown are representative of three or more experiments. 2-2B: Following pretreatment with 10 μM PP2 or vehicle control (DMSO) for 1 h, HAEC were treated with 500 μM Zn^{2+} in media containing 4 μM PT for 5 and 20 min. Lysates were analyzed for EGFR activation via Western Blotting with phospho-specific antibodies. The results shown are representative of three or more experiments. 2-2C: HAEC were treated with 500 μM Zn^{2+} for 5 and 20 min in the presence and absence of 8 μM PT. 500 μg of cellular lysates were precleared with 20 μl protein-A agarose for 30 min and immunoprecipitated (IP) using a broad specificity Src monoclonal antibody for 1 h at 4°C. The precipitated protein was assessed for Src kinase activity. Shown are means ± SE of three independent experiments. *P<0.05 and **P<0.01 compared with control.

Figure 2-3

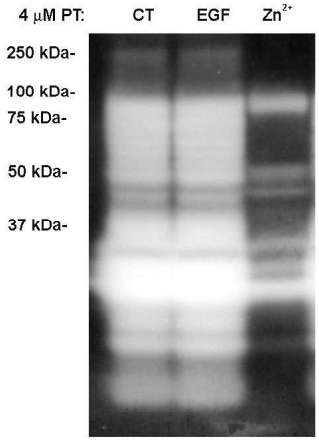


Figure 2-3. *Zn^{2+} inhibits Protein Tyrosine Phosphatases (PTPases) in HAEC.* HAEC were treated with 500 μM Zn^{2+} or 20 ng/ml EGF in media containing 4 μM PT for 20 min. 40-80 μg of protein extracts were prepared as for Western Blots, except that samples were not boiled. The samples were then subjected to SDS-PAGE on 11% polyacrylamide gels containing [^{32}P]PolyGlu:Tyr (approximately 1.5 million cpm/40 ml gel). Following protein renaturation, clear bands indicative of tyrosine phosphatase activity were visualized by autoradiography using a Molecular Dynamics PhosphorImager. The results shown are representative of three or more experiments.

Figure 2-4

Figure 2-4A

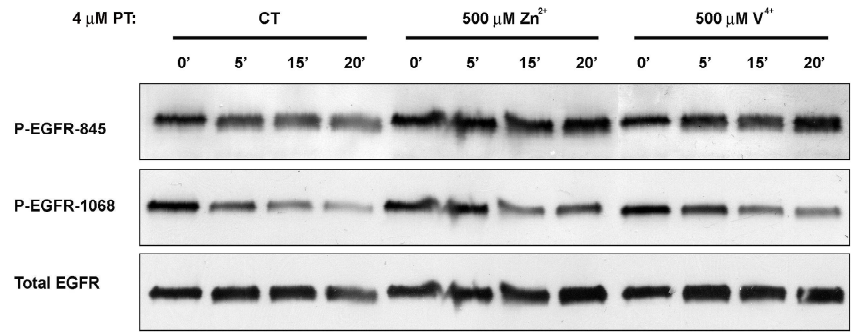


Figure 2-4B

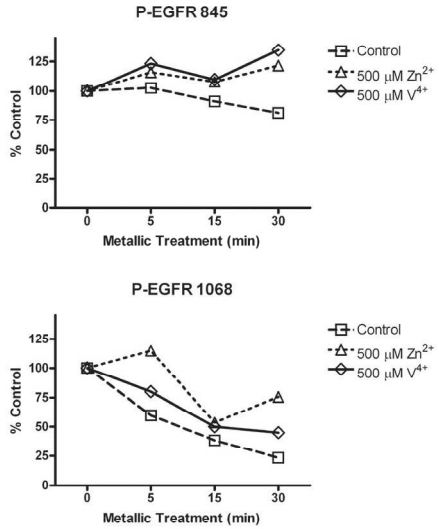


Figure 2-4. *Exogenous EGFR Dephosphorylation was inhibited in lysates obtained from HAEC exposed to Zn^{2+} in vitro.* 2-4A: HAEC treated with 500 μM Zn^{2+} or V^{4+} in 4 μM PT for 20 min. 1 ng/μl active, phosphorylated EGFR substrate was mixed with 60-100 μg of cellular lysate and the reaction was sampled at 5, 15, and 20 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 or more experiments. 2-4B: A graphical representation of the optical densities corresponding to the blot shown.

To corroborate the findings obtained using exogenous EGFR and HAEC cell lysates, we next performed separate experiments in which the effect of Zn^{2+} on phosphatase activity toward endogenous EGFR was assayed in intact HAEC. HAEC were first stimulated with EGF to induce maximal autophosphorylation of endogenous EGFR at multiple sites. Following treatment with 500 uM Zn^{2+}, 500 uM V^{4+} or 20 ng/ml EGF for 20 min, a fast-acting EGFR kinase inhibitor (c56) was added to the cells and the rate of EGFR dephosphorylation was then measured using immunoblotting (Figure 2-5A). As shown in Figure 2-5B and 2-5C, endogenous EGFR dephosphorylation at both sites in untreated HAEC was strikingly fast, with over 80% of the P-Tyr^{1068} signal being lost within 10 s. In comparison, the rate of P-Tyr^{1068}EGFR and P-Tyr^{845}EGFR dephosphorylation was markedly diminished in Zn^{2+}-exposed cells (Figure 2-5B). HAEC treatment with V^{4+}, used as a reference inhibitor of PTP activity, also resulted in a pronounced impairment of EGFR dephosphorylation at both sites examined (Figure 2-5B).

Figure 2-5

Figure 2-5A

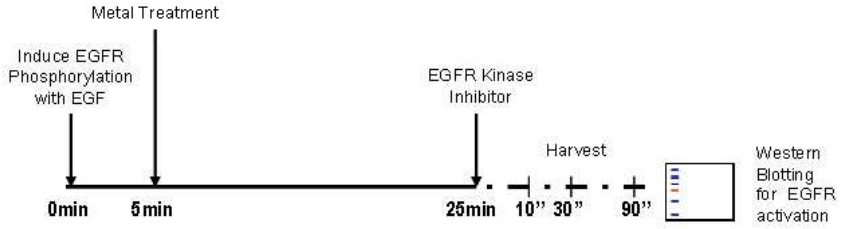


Figure 2-5B

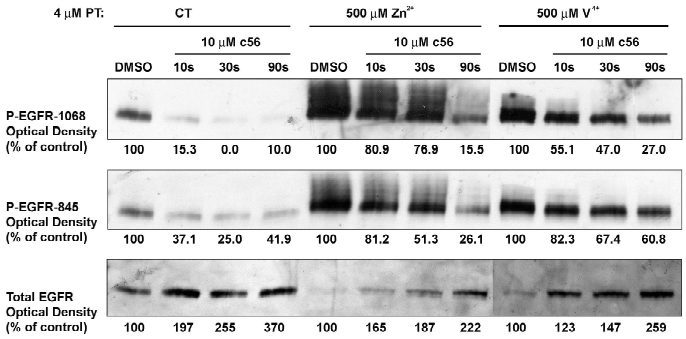


Figure 2-5C

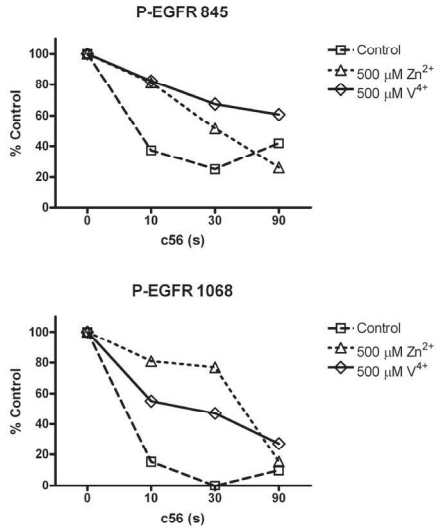


Figure 2-5. *EGFR dephosphorylation is inhibited by Zn^{2+} in intact HAEC.* 2-5A: A schematic displaying the experimental protocol. 2-5B: HAEC were treated with 20 ng/ml EGF for 5 min to induce maximal receptor phosphorylation. Cells were subsequently exposed to 500 μM Zn^{2+} or V^{4+} in 4 μM PT for 20 min followed by 10 μM c56 treatment to inhibit further EGFR kinase activity. Cells were then harvested at 10, 30, and 90 seconds. Lysates were subjected to SDS-PAGE and Western Blotting using phospho-specific anti-EGFR antibodies. Band intensities were analyzed using Kodak Software System. Optical densities shown were normalized to the DMSO value within each treatment group. The results shown are representative of 5 or more experiments. 2-5C: A graphical representation of the optical densities corresponding to the blot shown.

2-E Discussion

We have previously reported that exposure to Zn^{2+} induces EGFR phosphorylation in A431 (Samet *et al.*, 2003) and B82L cells (Wu *et al.*, 2002) through a mechanism that involves transactivation of the receptor by Src. In this study we examined the pathway that leads to EGFR activation in primary cultures of the human airway epithelium and report a Src-independent mechanism wherein the initiating event appears to be not kinase activation, but rather inhibition of tyrosine phosphatase activity by Zn^{2+} (See Appendix B).

The lack of involvement of Src in EGFR phosphorylation induced by Zn^{2+} in HAEC is supported in this study by several lines of evidence. First, EGFR phosphorylation in HAEC exposed to Zn^{2+} is not accompanied by an alteration in the levels or pattern of phosphorylation at activating (Tyr^{416}) and inhibiting (Tyr^{527}) sites on Src. Second, by 20 min**,** Zn^{2+}-induced EGFR phosphorylation minimally blocked by pretreatment of HAEC with the Src kinase inhibitor PP2. Third, EGFR kinase activity is necessary for Zn^{2+} induced EGFR phosphorylation. Perhaps the most compelling data in the case against a role for Src in EGFR phosphorylation in Zn^{2+}-treated HAEC, is the fact that Src kinase activity is actually inhibited under the same conditions in which EGFR phosphorylation is increased during exposure to Zn^{2+}. Thus the data show that Src kinase activity is not required for Zn^{2+}-mediated EGFR activation. However, these data do not rule out the possibility that other PP2-sensitive kinases contribute to Zn^{2+}-mediated EGFR transphosphorylation. It is also possible that the required EGFR kinase activity is itself partially inhibited by PP2. Zn^{2+} is also known as a competitive inhibitor of Csk (Sun and Budde, 1999), a tyrosine kinase that functions to suppress Src activity and is structurally very similar to Src. Moreover, Zn^{2+} inhibits Ck2 activity *in vitro* (W. Wu, personal communication), and therefore, Zn^{2+} likely acts as a competitive inhibitor of all tyrosine kinases by displacing required Mg^{2+} ions from specific metal binding sites (Sun and Budde, 1999).

Here we report that the mechanism of Zn^{2+}-induced EGFR activation in HAEC is clearly distinct from that previously reported in the A431 skin carcinoma and B82L mouse lung fibroblast cells. In those cell lines, Zn^{2+} exposure was found to induce a Src-dependent and EGFR-kinase independent transphosphorylation of EGFR (Wu *et al.*, 2002; Samet *et al.*, 2003). The mechanistic explanation for these differences is not known, however is it possible to speculate that cell type differences in permeability to Zn^{2+} result in temporal variations in the inactivation of PTPs and the activation of Src.

The highly conserved PTP active site requires that the catalytic cysteine be in the thiolate form in order to allow efficient transfer of substrate phosphates. Due to its unique microenvironment, the catalytic cysteine has a low pK_a (Zhang and Dixon, 1993) enabling activity at physiological pH but also rendering the active thiolate anion highly susceptible to oxidation by ROS (Salmeen and Barford, 2005), and possibly also to attack by metal ions like Zn^{2+} (Appendix A). Transient and reversible inhibition of tyrosine phosphatases is now recognized as an essential event in growth factor receptor signaling (Meng *et al.*, 2004). ROS-mediated PTP inhibition has been shown to occur in a variety of cell types in response to EGF (Deyulia and Carcamo, 2005), PDGF (Meng *et al.*, 2002) and insulin receptor (Meng *et al.*, 2004) binding. The target of the inhibition is the thiolate group in the catalytic cysteine of the PTP, which is oxidized to the inactive sulfenyl derivative.

Zn^{2+} is a known inhibitor of PTPs (Haase and Maret, 2003), including a broad spectrum of PTPases present in HAEC (Samet *et al.*, 1999). A mechanism of direct inhibition has recently been proposed wherein Zn^{2+} blocks PTP activity by binding to the catalytic cysteine and to neighboring histidine or aspartate residues present in the highly conserved active site (Haase and Maret, 2003). Treatment of Zn^{2+} exposed HAEC with the reductant dithiothreitol reversed Zn^{2+}-mediated EGFR activation demonstrating the transient nature of the proposed mechanism of PTP inactivation (Tal, Appendix i). Interestingly, Maret and colleagues have proposed a physiological role for intracellular Zn^{2+} stores in the inhibition of PTPs during receptor-mediated signaling. According to this model, tightly regulated fluctuations in intracellular Zn^{2+} are induced by oxidation of metallothionein cysteine residues

associated with Zn^{2+} causing the release of free Zn^{2+} at concentrations sufficient to inhibit PTPs and thereby regulate phosphorylation dependent signaling pathways in a reversible manner (Haase and Maret, 2003).

In untreated HEAC, growth factor induced receptor phosphorylation was extensively reversed within 10 s of the cessation of kinase activity, demonstrating the tight control that PTPs exert over receptor tyrosine kinase signaling. The ability of Zn^{2+} exposure to induce a prolongation of EGFR phosphorylation in the absence of kinase activity illustrates the susceptibility of phosphoprotein catabolism to inhibition by Zn^{2+}. Interestingly, our data may show differential rates of dephosphorylation at tyrosine residues 1068 and 845, suggesting the involvement of multiple PTPs in this regulatory event. The number of PTPs which exert activity on EGFR is not currently known. Implicated candidates include PTP1B (Flint *et al.*, 1997; Haj *et al.*, 2003), LAR (Kulas *et al.*, 1996), PTP-σ (Suarez Pestana *et al.*, 1999), SHP-1 (Keilhack *et al.*, 1998), and T-cell PTP (Tiganis *et al.*, 1998). Additional studies will be required to identify the specific EGFR-directed PTP(s) whose activity is inhibited in HAEC exposed to Zn^{2+}.

The data presented in this study suggest that signal transduction events induced by exposure to Zn^{2+} are initiated not by the direct induction of kinase activation, but through the inhibition of critical phosphatases whose activities oppose baseline kinase activity and normally function to maintain signaling quiescence in resting cells. It was not a goal of this study to model the conditions of Zn^{2+} exposure experienced by HAEC in vivo. Nonetheless, it is interesting to note that serum concentrations of Zn^{2+} are approximately 25 μM, and certain biological compartments (e.g., synaptic space) can exceed 300 μM Zn^{2+} (Chen *et al.*, 1997; Huang, 1997). Thus, the exposure conditions used in this study, which did not induce overt cytoxicity, may be relevant from a toxicological as well as a pathophysiological standpoint. Given the pervasive presence of Zn^{2+} in ambient air, and the broad role that EGFR plays in signaling processes within cells, these findings may be relevant as a mechanism of PM inhalation toxicity.

Chapter III: Epidermal growth factor receptor activation by diesel particles is mediated by tyrosine phosphatase inhibition

The main findings of Specific Aim 2 (Chapter III) are currently under revision for resubmission to Toxicology and Applied Pharmacology “Tamara Tal, Philip Bromberg, Yu-Mee Kim, and James Samet. Epidermal growth factor receptor activation by diesel particles is mediated by tyrosine phosphatase inhibition.”

TT contributed to the manuscript as follows. TT, JS, and PB conceived of the study design; TT performed all experiments; and TT and JS wrote the manuscript.

3-A 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 $\mu g/cm^2$ 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 assays 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.

3-B 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 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 pKa of the catalytic cysteine residue to < 6, allowing it to exist in its thiolate anion ($R\text{-}S^-$) 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 (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 though a mechanism involving the inactivation of EGFR-directed PTPase activity in primary human airway epithelial cells, a principal target cell of inhaled PM.

3-C Materials and Methods

Preparation of DEP. Thee 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) organic matter extractable by dichloromethane (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) 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 as previously described by Sagai et al. (Sagai *et al.*, 1993). H-DEP contains 26.3 % (wt/wt) organic matter extractable by dichloromethane (Singh *et al.*, 2004).

Cell culture and treatment. Primary normal human airway epithelial (HAEC) cells were cultured as described earlier (Chapter II). Prior to particle treatment, cells were growth factor starved in un-supplemented BEBM for 9-15 h. L-DEP, I-DEP, H-DEP and carbon black suspensions (Columbian Chemicals Company; Marietta, GA) were freshly prepared at 300 µg/ml in BEBM by water bath sonication for 10 min. HAEC were exposed to a final concentration of 30 µg/cm^2 for 4 h. H_2O_2 (100 mM) and vanadate (100 mM) 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). In each well, 100 µg of sample protein was loaded and the gel was submitted to electrophoriesis at 20 V for overnight at 4°C. After running, the gels were washed sequentially with (i) 20% 2-propanol-50 mM Tris (pH 8.0), (ii) 50 mM Tris (pH 8.0)-0.05% 2-mercaptoethanol (*buffer A*), (iii) 6 M guanidine hydrochloride in *buffer A*, followed by repeated washings in (iv) 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 dithiotheitol (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.

Western Blotting. Western Blotting was performed as earlier described (Section 2-C). Graphical representation of blot densities obtained from thee 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 with DEP in DMEM. Following particle exposure, 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 incubation in 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 a cocktail of anti-protease and anti-PTPase inhibitors, 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. Samples containing 10 µg of cell lysates were used to detemine total PTPase activity using a 96 EnzChek Tyrosine Phosphatase Assay Kit, as per manufacturer's directions (Molecular Probes; Carlsbad, CA). Fluorescence was measured over time using excitation at 355 $\pm$ 20 nm and emission at 460 $\pm$ 12.5 nm. Data are shown as percent control of untreated from 3 independent experiments. Two-way ANOVA with a Bonferroni post-test was used to determine significance.

Exogenous EGFR dephosphorylation assay. The exogenous EGFR dephosphorylation assay was performed as described Section 2-C. Band intensities were analyzed using Kodak Software System.

3-D Results

3-D.1 *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). 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 $\mu g/cm^2$ 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 3-1, 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 activation 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.

Figure 3-1

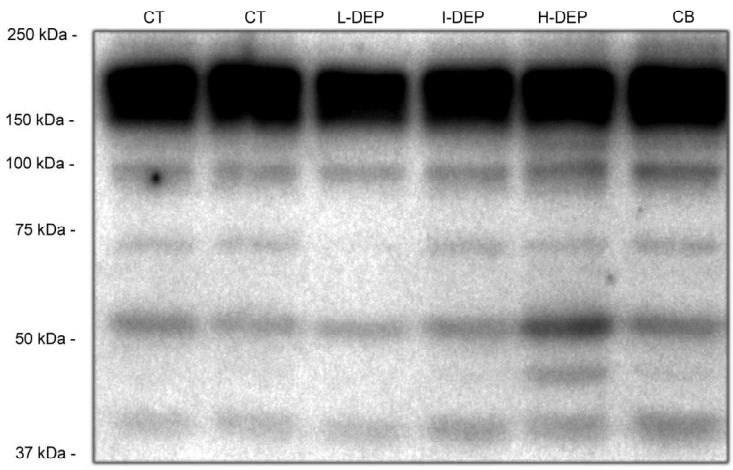


Figure 3-1. *Exposure to DEP induces differential kinase activation in HAEC.* Cells were treated with 30 µg/cm^2 L-DEP, I-DEP, H-DEP or carbon black (CB) or media control (CT) for 4 h. Samples containing 50-100 µg cell lysate were loaded onto a gel containing 250 µg/ml MBP and analyzed for kinase activity by the addition of 5 mM $MgCl_2$, 25 µM ATP, and 250 µCi [gamma^{32}P]ATP. Image contrast was optimized to show bands of intermediate molecular weights. The results shown are representative of two experiments.

3-D.2 *Exposure to DEP induces EGFR kinase-dependent EGFR phosphorylation 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 µg/cm^2 L-DEP, I-DEP or H-DEP for 4 h using Western Blotting with a phosphosite-specific antibody. As shown in Figure 3-2A, 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. Time-course and dose-response experiments showed that 30 ug/cm^2 H-DEP treatment for 4 h produced maximal EGFR phosphorylation in HAEC (data not shown).

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) or vehicle control (DMSO) prior to exposure with 30 µg/cm^2 L-DEP, I-DEP, H-DEP or CB for 4 h, 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 3-2B). 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. As expected, we observed that c56 pretreatment failed to block PV-mediated EGFR phosphorylation. While these results do not rule out transphosphorylation of the receptor, they suggest that, in the absence of EGFR-directed PTPase activity basal EGFR kinase activity is required for EGFR phosphorylation.

Figure 3-2

Figure 3-2A

Figure 3-2B

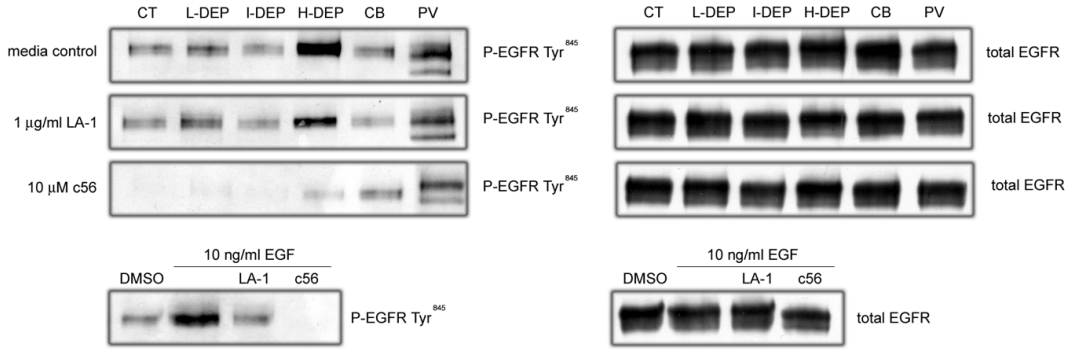


Figure 3-2. *DEP exposure induces EGFR kinase dependent EGFR phosphorylation*. 3-2A: Cells were treated with 30 µg/cm^2 L-DEP, I-DEP, H-DEP or CB or media (CT) for 4 h or 50 µM pervanadate (PV) for 30 min. Cells were lysed and the state of EGFR phosphorylation was detected by Western Blotting using a phosphorylation-specific antibody directed against tyrosine 845. 3-2B: 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 h, HAEC were exposed to 30 µg/cm^2 L-DEP, I-DEP, H-DEP or CB or media for 4 h, 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. The results shown are representative of thee experiments.

3-D.3 *DEP-induced EGFR phosphorylation does not require access to the EGFR ligand binding domain or receptor dimerization.* To further characterize the functional requirements for DEP-induced EGFR phosphorylation, we investigated the role of the EGFR extracellular ligand-binding domain. HAEC were pretreated with a blocking antibody (clone LA-1, 1 µg/ml for 60 min) prior to exposure with 30 µg/cm^2 L-DEP, I-DEP or H-DEP for 4 h, 50 µM PV for 30 min or 100 ng/ml EGF for 15 min. LA-1 pretreatment effectively blocked EGF-mediated EGFR phosphorylation (Figure 3-2B). In comparison, LA-1 pretreatment diminished DEP induced EGFR phosphorylation only partially. These data suggest that blocking the EGFR ligand binding site is ineffective in preventing H-DEP and L-DEP induced EGFR phosphorylation.

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 though 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 µg/cm^2 L-DEP, I-DEP, H-DEP or CB for 4 h, or with 200 ng/ml EGF for 15 min. The presence of EGFR dimers was then measured in protein extracts by Western blotting following the addition of a cross-linking agent (2.5 mM BS_3). 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 though a mechanism that is independent of ligand-stimulation and receptor dimerization.

Figure 3-3

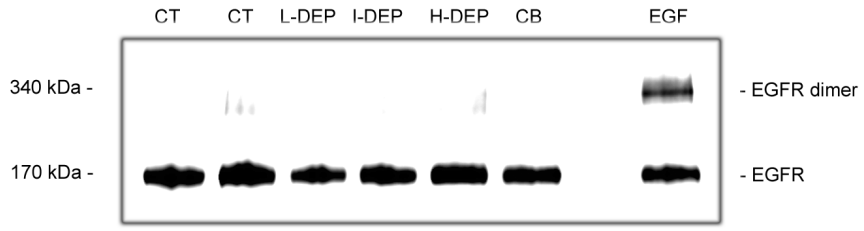


Figure 3-3. *DEP-induced EGFR phosphorylation does not require receptor dimerization.* Analysis of EGFR dimerization was measured in intact A431 cells treated with 30 µg/cm^2 L-DEP, I-DEP, H-DEP or CB or media for 4 h or 200 ng/ml EGF for 15 min. Cells were then rinsed and treated with 2.5 mM BS_3 for 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 thee or more experiments.

3-D.4 *Exposure to DEP inhibits PTPases in HAEC.* Our previous work with Zn^{2+}-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 µg/cm^2 L-DEP, I-DEP, H-DEP or CB for 4 h, 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 using a synthetic substrate and the data are shown as the ratio of PTPase activity in treated versus untreated controls (Figure 3-4). Treatment with L-DEP- or H-DEP resulted in a marked and statistically significant ($p < 0.01$) impairment of PTPase activity in comparison to lysates obtained from control cells (Figure 3-4). Moreover, consistent with observed EGFR phosphorylation trends noted earlier (Figure 3-2A), 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 ($p < 0.01$).

Figure 3-4

Figure 3-4A

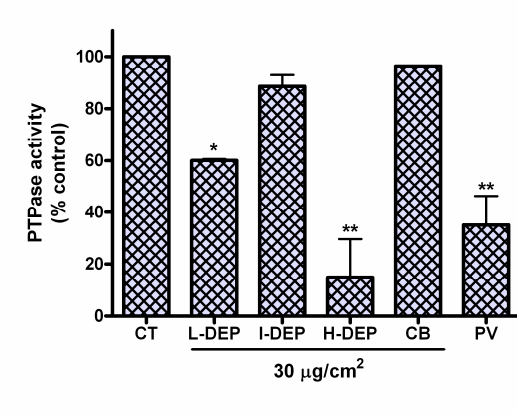


Figure 3-4B

Treatment	Dephosphorylation rate (picomoles substrate hydrolyzed min^{-1} mg protein^{-1})
CT	85.57
L-DEP	71.07*
I-DEP	83.88
H-DEP	44.27**
CB	83.80
PV	58.50**

Figure 3-4. *DEP exposure impairs total PTPase activity HAEC lysates.* HAEC were treated with 30 μg/cm^2 L-DEP, I-DEP, H-DEP or CB for 4 h or 50 μM PV for 30 min and harvested in an anaerobic environment to prevent non-specific oxidation. Protein extracts (10 μg) were loaded onto a PTPase activity kit and fluorescence was measured at using excitation at 355 $\pm$ 20 nm and emission at 460 $\pm$ 12.5 nm at 0-18 min**.** Data are expressed as the ratio of picomoles of phosphate released in treated versus control samples at 6 min (3-4A) and rates of dephosphorylation over the entire assay period are shown (3-4B). Total PTPase activity in cells treated with L-DEP, H-DEP or PV were statistically significant from control samples ($P < 0.001$). 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 Bonferroni post-test (n=3).

3-D.5 *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 µg/cm^2 L-DEP, I-DEP, H-DEP or CB for 4 h, 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 significant decrease in levels of P-EGFR observed by 10 min (Figures 3-5A and 3-5B). In marked contrast, exposure to H-DEP resulted in a marked 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. 3-5B). In agreement with the data shown in Figure 3-4, there were no differences in EGFR-directed PTPase activity in lysates obtained from HAEC treated with I-DEP or CB (Fig. 3-5A and 3-5B).

Figure 3-5

Figure 3-5A

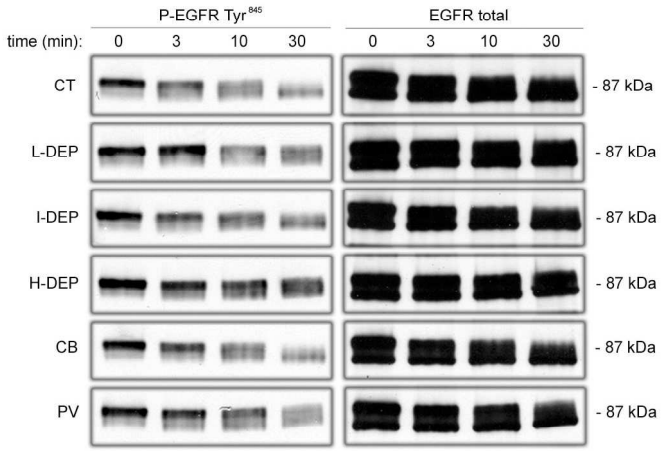


Figure 3-5B

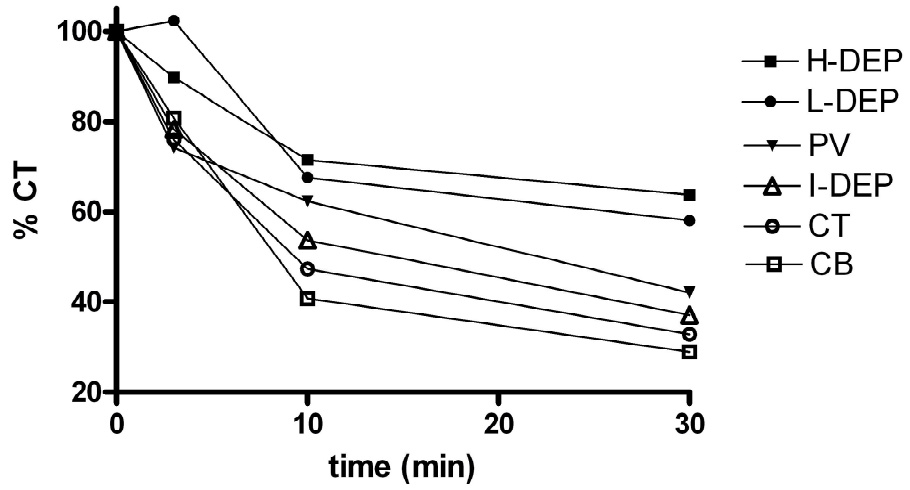


Figure 3-5. *Exogenous EGFR dephosphorylation was inhibited in lysates obtained from HAEC exposed to DEP containing high and low but not intermediate organic content in vitro*. 3-5A: HAEC were treated with 30 µg/cm^2 L-DEP, I-DEP, H-DEP or CB for 4 h or 50 µM PV for 30 min. In an anaerobic environment, 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 thee experiments. 3-5B: A graphical representation of the optical densities obtained from thee independent experiments (error bars omitted for clarity). Band intensities were analyzed using Multigauge Software and shown here as % control of the intensity of the band area minus the background (n=3).

3-E 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 though 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 (Zhande *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 h 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).

In order to characterize the mechanism by which DEP induces EGFR phosphorylation, we employed a variety of tactical approaches. First, we showed that treatment with an EGFR kinase inhibitor completely abolished DEP-mediated EGFR phosphorylation (Figure 3-2B). This suggests that EGFR autophosphorylation is a central mechanism by which DEP activates the receptor. We next sought to determine whether DEP stimulated ligand-dependent or -independent activation of the EGFR. Although DEP fails to induce EGFR dimerization, these data were obtained in the A431 cell line (Figure 3-3B). A431 cells were used because they grossly overexpress the EGFR (Samet *et al.*, 2003) however, these data may not be indicative of what occurs in lung epithelial cells. In support of this, the data shown in Figure 3-3A suggests that L-DEP and H-DEP induce EGFR phosphorylation by different mechanisms. Treatment with an EGFR-ligand binding domain blocking antibody (LA-1) fails to block L-DEP induced EGFR phosphorylation. Furthermore, EGFR activation induced by pervanadate treatment, a known inhibitor of PTPase activity, is similarly unaffected by LA-1 pretreatment. These data support the notion that L-DEP induces EGFR phosphorylation by a ligand independent mechanism possibly involving inhibition of EGFR-directed PTPase activity. In comparison, LA-1 partially ablates H-DEP induced EGFR phosphorylation suggesting that H-DEP activates the receptor by multiple ligand- dependent and –independent mechanisms.

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-napthoquinone 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 though 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-cytochome P450 reductase-mediated metabolism of DEP-associated quinones to semiquinone radicals. PM-associated PAHs are first metabolized by cytochome 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 EOM content. 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

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 though 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). Though 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 though which DEP exposure induces the expression of proinflammatory proteins such as IL-8 and COX-2.

Chapter IV: Investigating the transcriptional regulation of IL-8 expression in human airway epithelial cells exposed to diesel exhaust

The main findings of Specific Aim 3 (Chapter IV) will be submitted for publication in Cell Signaling upon completion. “Tamara Tal, Steve Simmons, Robert Silbajoris, Ram Ramabhadran, Philip Bromberg, and James Samet. Distinct regulation of IL-8 expression in BEAS2B cells exposed to diesel exhaust particles.”

TT contributed to the manuscript as follows. TT, SS, RR, JS, and PB conceived of the study design; TT performed all experiments; and TT wrote the manuscript.

4-A Abstract

Particulate matter (PM) exposure induces adverse health effects, leading to cardiopulmonary morbidity and mortality. Inflammation is thought to be a central mechanism by which PM exposure exerts deleterious health effects. Diesel exhaust particles (DEP) are a ubiquitous component of urban ambient PM. Exposure to DEP induces inflammatory signaling characterized by MAP kinase-mediated activation of NFκB and AP-1 *in vitro* and in bronchial biopsies obtained from human subjects exposed to DEP. NFκB and AP-1 activation results in the upregulation of genes involved in promoting inflammation in lung epithelial cells, a principle target of inhaled DEP. IL-8 is a proinflammatory chemokine synthesized by lung epithelium in response to environmental pollutants. Although DEP exposure is known to cause increases in IL-8 mRNA levels, the mechanism by which this occurs is not well understood. Here we show that exposure to DEP with varying physicochemical compositions differentially induces IL-8 expression in primary human lung epithelial cells and BEAS2B cells. Here we show that treatment with a low organic-containing DEP (L-DEP) stimulates IL-8 expression by an NFκB-dependent mechanism. In contrast, we report that exposure to a high organic-containing DEP (H-DEP) induces IL-8 expression independently of NFκB. These data suggest that DEP induces proinflammatory signaling by multiple mechanisms in BEAS2B cells.

4-B Introduction

Epidemiological studies have consistently demonstrated an association between exposure to ambient particulate matter (PM) and adverse respiratory and cardiovascular health effects particularly with regards to fine particles with an aerodynamic diameter < 2.5 μM (Peters *et al.*, 2001; Peters *et al.*, 2004). Diesel exhaust particles (DEP), generated from diesel powered engines, are ubiquitously present in $PM_{2.5}$ derived from urban areas (Lloyd and Cackette, 2001). DEP contain a carbon core with adsorbed organic compounds, such as polycyclic aromatic hydrocarbons and quinones (Wichmann, 2007). These compounds or their reactive metabolites can redox cycle thereby generating reactive oxygen species (ROS) (Li *et al.*, 2002; Baulig *et al.*, 2003; Li *et al.*, 2003). In support of these findings, activation of redox-sensitive transcription factors, such as NFκB and AP-1 and their upstream map kinases (MAPKs) p38 and JNK, has been reported in bronchial biopsies obtained from human subjects exposed to diesel exhaust (Pourazar *et al.*, 2005).

Airway epithelial cells, a principle target cell of inhaled DEP, synthesize and secrete a number of chemical mediators capable of activating and recruiting inflammatory cells. It has been previously shown that DEP induces the expression of proinflammatory cytokines in cultured cell systems including IL-8, IL-1α, GM-CSF, and Gro-α (Bonvallot *et al.*, 2001; Matsuzaki *et al.*, 2006; Baulig *et al.*, 2007). In agreement with these findings, enhanced epithelial expression of IL-8, IL-13 and Gro-α was reported in bronchial biopsies obtained from human subjects exposed to DEP (Salvi *et al.*, 2000; Pourazar *et al.*, 2005). Taken together, these studies support the notion that DEP exposure induces pulmonary inflammation mediated by MAPK signaling, activation of NFκB and AP-1, and synthesis of proinflammatory cytokines.

IL-8 is a potent neutrophilic activator and chemotaxin agent that is secreted by airway epithelial cells and often used as a biological marker of environmentally-induced pulmonary inflammation (Strieter, 2002). In particular, IL-8 expression is increased in epithelial cells exposed to ambient PM, the

metallic PM components Zn and V (Samet *et al.*, 1998; Kim *et al.*, 2006), as well as DEP (Salvi *et al.*, 1999; Salvi *et al.*, 2000; Mudway *et al.*, 2004) and residual oil fly ash (ROFA) particles (Carter *et al.*, 1997) and the atmospheric contaminant ozone (Jaspers *et al.*, 1997b; Jaspers *et al.*, 1997a). Although some information concerning DEP-induced activation of upstream redox-sensitive signaling pathways has been reported (Takizawa *et al.*, 1999; Pourazar *et al.*, 2005), the exact mechanism by which DEP exposure induces IL-8 expression remains unclear.

The IL-8 gene contains multiple 5' regulatory elements in its promoter, including binding sites for NFκB, AP-1, AP-2, AP-3, CCAAT/enhancer binding protein β (C/EBP β), interferon regulatory factor 1, and a glucocorticoid response element [(Mukaida *et al.*, 1989; Strieter, 2002) and Figure 4-1]. Exposure to a high organic-containing DEP (H-DEP) has been previously shown to induce NFκB-dependent IL-8 expression in the human epithelial cell line BEAS2B (Takizawa *et al.*, 1999). However, we have preliminary data suggesting that H-DEP induces increased IL-8 mRNA levels by an NFκB-independent mechanism in BEAS2B cells (Tal, unpublished observations). Cell culture conditions may play a role in this apparent discrepancy, as Takizawa et al. cultured their cells in serum, which contains 25 ng/ml EGF among other components. Furthermore, in contrast to the data presented here, cells were not serum starved prior to treatment with DEP.

In addition to H-DEP, a low-organic containing model DEP (L-DEP; NIST SRM 2975) can reportedly stimulate proinflammatory signaling (Singh *et al.*, 2004; Cao *et al.*, 2007a; Cao *et al.*, 2007b), suggesting that DEP may also activate redox-independent mechanisms by which aberrant signaling occurs in lung epithelial cells. Therefore, in the present study, multiple DEPs with different physicochemical characteristics were used in an attempt to unravel the transcriptional regulation of IL-8 expression in lung epithelial cells exposed to DEP.

Figure 4-1

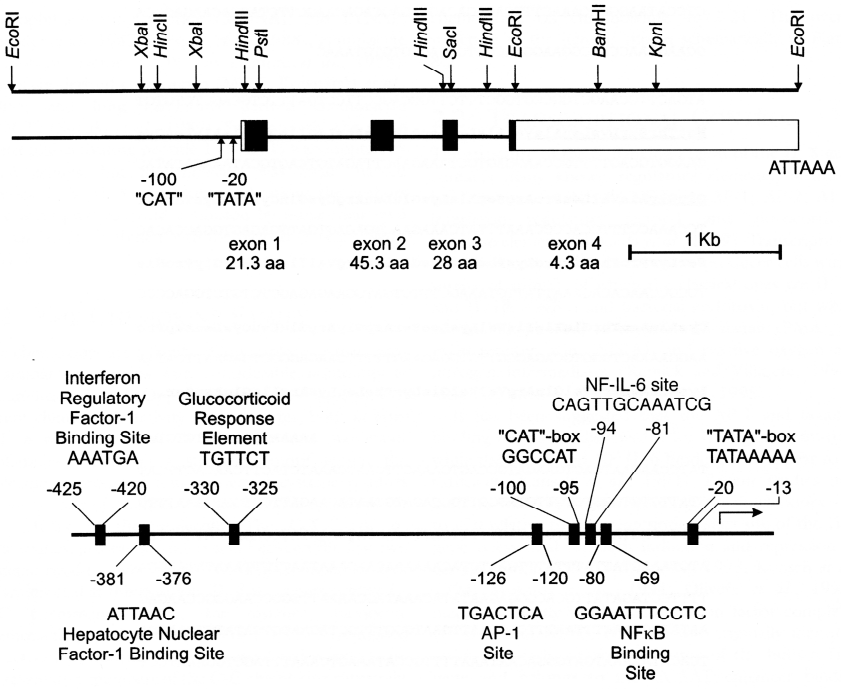


Figure 4-1 *The IL-8 gene.* Mire-Sluis and Thorpe. Cytokines 1998

4-C Materials and Methods

Preparation of DEP. L-DEP, I-DEP, H-DEP, and CB were used in the current study. The preparation of these particles is described in detail in the methods section of Chapter III (Section 3-C).

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 and cells were cultured as previously reported [(Tal *et al.*, 2006), Section 2-C]. HAEC were growth factor starved in un-supplemented BEBM for 9-15 h prior to particle treatment. BEAS2B cells were derived by transformation of human airway epithelial cells with an ad12-SV40 adenovirus construct (Reddel *et al.*, 1988). BEAS2B cells (subclone S6) were obtained from the Human Studies Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and maintained in keratinocyte growth medium (KGM, Cambrex Bioproducts, Clonetics Division, San Diego, CA). Cells were then growth factor starved in un-supplemented KGM for 9-15 h prior to particle treatment. L-DEP, I-DEP, H-DEP and carbon black suspensions (Columbian Chemicals Company; Marietta, GA) were freshly prepared at 100 µg/ml in BEBM or KBM by water bath sonication for 10 minutes. HAEC or BEAS2B cells were exposed to a final concentration of 10 $\mu g/cm^2$ for 4 h.

Real-Time Quantitative PCR. Relative gene expression in HAEC and BEAS2B cells were quantified using Real-Time Quantitative PCR. Total RNA was isolated using a Qiagen kit (Qiagen, Valencia, CA) and reverse transcribed to generate cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Oligonucleotide primer pairs and fluorescent probes for IL-8, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), firefly luciferase (FLuc), and enhanced green fluorescent protein (EGFP) were designed using a primer design program (Primer Express,

Applied Biosystems) and obtained from Integrated DNA Technologies (Coralville, IA). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems), primer/probe sets of interest and TaqMan Universal PCR Master Mix (Applied Biosysytems). The relative abundance of IL-8 and GAPDH mRNA levels were determined from standard curves generated from a serially diluted standard pool of cDNA prepared from cultured human airway epithelial cells. The relative abundance of GAPDH mRNA was used to normalize levels of IL-8 mRNA. The relative abundance of FLuc and EGFP mRNA levels were determined from standard curves generated from serially diluted pGL3-basic and pHygroEGFP plasmids, respectively. To control for transduction efficiency, the relative abundance of EGFP mRNA was used to normalize levels of FLuc mRNA. Data shown are representative of 3 or more experiments.

Synthesis of lentiviral promoter reporters. Briefly, the IL-8 promoters were digested from the pGL2 parent vectors by BamHI and XhoI and cloned into the lenti transfer vector (Open Biosystems) between the BamHI and XhoI sites. The sequences for the IL-8 wildtype (IL-8 wt-FLuc) promoter and a promoter bearing a mutated NFκB response element (IL-8- NFκB-FLuc) have been previously published [(Jaspers *et al.*, 1999) and Figure 4-2]. The NFκB and AP-1 promoters were generated by annealing complementary oligos which were then cloned into the lenti transfer vector between the NheI and XhoI sites. The sequences for the NFκB and AP-1 promoters are as follows. For the tandem repeat NFkB (NFkB sites in bold)

G**GGGACTTTCC**GCTTG**GGGACTTTCC**GCTG**GGGACTTTCC**GCTG**GGGACTTTCC**GCT **GGGGACTTTCC**GCGGAGACTCAAGAGGGTATATAATG and for the tandem repeat AP-1 (AP-1 sites in bold)

GC**ATGACTCAGAG**AAACGCTGGAAGAAACGCAGGT**ATGACTCAG**TAGTGCGGACAGA GTGATAAACG**ATGACTCAG**GAGAAATAGGGGAGACAGCCCATAACTAGCCAATCACGT

Figure 4-2

Figure 4-2A IL-8 wt-FLuc

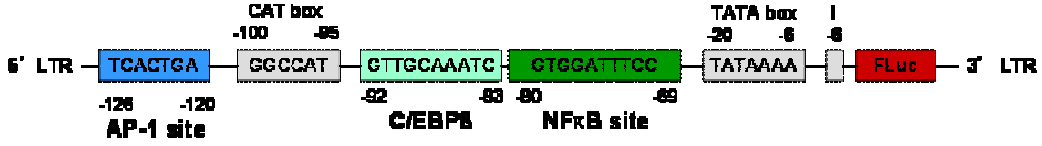


Figure 4-2B IL-8˙NFκB-FLuc

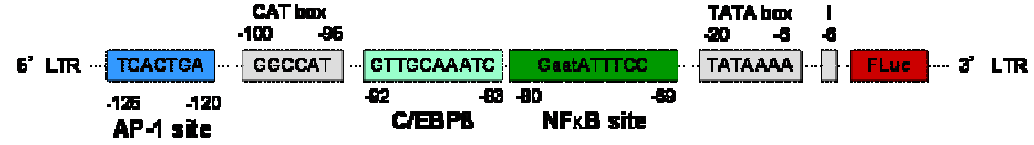


Figure 4-2C NFκBsynthetic-Fluc

Figure 4-2 *Lentiviral promoter reporters.* Schematics describing the IL-8 wildtype (4-2A), IL-8 promoter with a mutated NFκB binding site (4-2B) and a synthetic tandem repeat of the NFκB consensus sequence (4-2C).

AATCTGCTGCTTGCAATCAAAAAAACCACCGCTACCAGTATAAAAGGGGGGGAAGTCGT GTCTTACCGGGTTATCAGTCTT. All transfer vector constructs were confirmed by DNA capillary sequencing. HEK293T cells were co-transfected with purified transfer vector plasmids and lentiviral packing mix (Open Biosystems) by a calcium phosphate method. Sixteen h post-transfection, media was replaced with 6 ml fresh Dulbecco's modified eagle's medium (DMEM) + 5% fetal bovine serum and cells were incubated for 48 h. Media was harvested and centrifuged for 10 minutes at 5,000 g, and the supernatants were transferred to sterile microfuge tubes. Viral stocks were stored at -80 °C. To titer, 70,000 HEK293T cells stably expressing the rTTA3 (tet-off) transactivator were transduced with 25 µl of undiluted, 1:5, 1:25, 1:125, 1:625, and 1:3125 dilutions. Viral titer was determined 72 h post-transduction by counting red fluorescent colonies by fluorescent microscopy (red colonies are present due to rTTA3-mediated activation of secondary TRE-turboRED reporter within transgene) and back-calculating transducing units per ml stock.

Lentiviral promoter reporter assays. To determine an appropriate multiplicity of infection (MOI), BEAS2B cells were transduced for 72 h with MOI of 1, 2, 5, or 10 of IL-8 wildtype-firefly luciferase (IL-8 wt-FLuc) or a synthetic NFκB tandem repeat-FLuc (NFκB-FLuc). Following transduction, cells were challenged with 20 ng/ml Tumor necrosis factor α (TNF) for 4, 6, or 24 h and assessed for the presence of luciferase mRNA by RT-PCR. To obtain stably transduced cell lines, BEAS2B were transduced with IL-8wt-FLuc (MOI 5), IL-8-NFκB-FLuc (MOI 5), or NFκB synthetic-FLuc (MOI 5) for 72 h and carried for the duration of the study. All cells were co-transduced with EF1α-EGFP (MOI 10) in order to control for transduction efficiency. Transduced cells were treated with 10 µg/cm^2 L-DEP, I-DEP, H-DEP or CB or 20 ng/ml TNF for 4 h and assessed for promoter reporter activity by RT-PCR. Shown are data representative of 4 or more experiments.

Statistical Analysis. Data are presented as mean $\pm$ SE. Two-tailed paired Student's t test or one-way ANOVA was used to evaluate differences between control and treated groups; values of $P < 0.05$ were considered statistically significant.

4-D Results

4-D.1 *DEP exposure induces IL-8 expression in primary and immortalized human lung epithelial cells.* To study the effects of DEP exposure on proinflammatory signaling in lung epithelial cells, we first examined IL-8 expression in HAEC treated with 10 $\mu g/cm^2$ L-DEP, I-DEP, H-DEP, or CB for 4-24 h using RT-PCR. The data are expressed as the fold change over control normalized to GAPDH mRNA levels. As shown in Figure 4-3A, each DEP induced expression of IL-8 with varying potency and kinetics. Moreover, the rank potency by which these DEPs induce IL-8 expression is similar to that with which they induce phosphorylation of the Epidermal Growth Factor Receptor (EGFR) and impair EGFR-directed PTPase activity (Chapter III). Both L-DEP and I-DEP induced a time-dependent increase in IL-8 mRNA levels, whereas H-DEP-induced IL-8 expression peaked at 4 h and stabilized at an approximately 10-fold increase throughout the remainder of the time course. Carbon black (CB), used as a particle control, induced a time-dependent increase in IL-8 expression.

In order to assess the transcriptional regulation of DEP-induced increases in IL-8 expression, we next employed the immortalized lung epithelial cell line BEAS2B, which is amenable to stable transduction. First, non-transduced BEAS2B cells were treated with 10 $\mu g/cm^2$ L-DEP, I-DEP, H-DEP, CB or 20 ng/ml TNF for 4-24 h and IL-8 expression was assessed by RT-PCR. In agreement with Figure 4-3A, DEP induced IL-8 expression with a similar rank potency at 4 h (H-DEP > L-DEP > I-DEP) (Figure 4-3B). In contrast, while H-DEP exposure induced a robust induction in IL-8 mRNA 6 h post exposure, neither L-DEP, I-DEP or CB stimulated IL-8 expression at 6 h and all particles failed to induce IL-8 expression at 24 h (Figure 4-1B). As expected, TNF, a potent inducer of IL-8 expression, induced a robust increase in IL-8 mRNA at 4 and 6 h. Because the focus of this study is on early signaling events in response to DEP treatment and the similarities noted between DEP treated HAEC and BEAS2B cells at 4 h, the remainder of experiments were carried out in BEAS2B cells treated with particles for 4 h.

Figure 4-3

Figure 4-3A

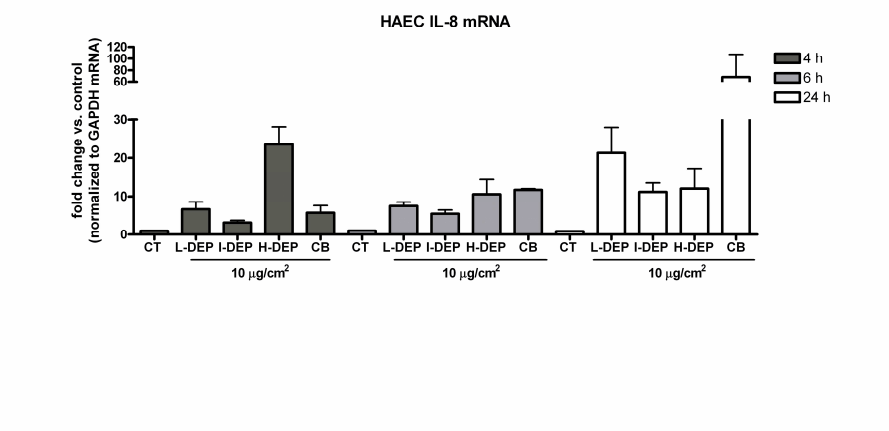


Figure 4-3B

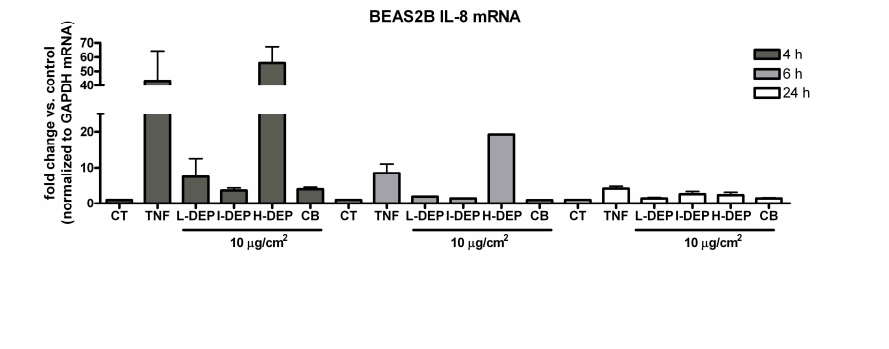


Figure 4-3. *Exposure to DEP induces differential IL-8 expression in lung epithelial cells.* 4-3A: Primary human airway epithelial cells (HAEC) were treated with 10 μg/cm^2 L-DEP, I-DEP, H-DEP or carbon black (CB) or media control (CT) for 4 h. Following RNA extraction and cDNA generation, samples were assessed for IL-8 expression by RT-PCR. 4-3B: BEAS2B cells were treated with 10 μg/cm^2 L-DEP, I-DEP, H-DEP or carbon black (CB), media control (CT), or 20 ng/ml TNF for 4 h and IL-8 mRNA levels were determined by RT-PCR. Data are normalized to GAPDH mRNA levels and expressed as the fold change over controls. The results shown are representative of thee or more experiments.

4-D.2 *L-DEP and H-DEP induce IL-8 promoter reporter activity in BEAS2B cells.* The data above show that L-DEP and H-DEP induced IL-8 expression in HAEC and BEAS2B cells at 4 h post exposure (Figure 4-3A and 4-3B). To further clarify their respective roles in DEP-induced IL-8 expression in BEAS2B cells, we examined the effect of DEP exposure on IL-8 promoter reporter activity directly in BEAS2B cells co-transduced with lentiviral promoter reporter constructs expressing the human wild type IL-8 and EF1α promoters linked to firefly luciferase and EGFP, respectively (IL-8 wt-FLuc and EF1α-EGFP). Because particles interfere with luminescence and fluorescence measurements, and luciferase protein expression requires 24 h of DEP exposure (Tal, unpublished observations) promoter reporter activity was assessed by RT-PCR 4 h following particle treatment. FLuc mRNA levels are normalized to EGFP mRNA levels and expressed as the fold change over controls. As shown in Figure 4-4A, a statistically significant elevation in promoter reporter activity was measured in cells exposed to 10 $\mu g/cm^2$ L-DEP or H-DEP. In contrast, both I-DEP and CB exposure failed to induce IL-8 promoter reporter activity. As expected, TNF exposure resulted in a pronounced increase in IL-8 promoter reporter activity.

To confirm that transduced cells appropriately respond to particle exposures by synthesizing IL-8, endogenous IL-8 mRNA levels were assessed in the same RNA pools initially probed for FLuc expression. In support increased promoter reporter activity (Figure 4-4A), both L-DEP and H-DEP exposure resulted in a 10-20 fold increase in endogenous IL-8 mRNA, respectively (Figure 4-4B). In comparison, both I-DEP and CB exposure failed to induce IL-8 expression whereas TNF induced a highly significant increase in IL-8 mRNA. Interestingly, although the magnitude of response to DEP exposure varies greatly when comparing promoter reporter activity to endogenous IL-8 expression, the relative potency with which individual DEPs induce these effects is conserved (Figure 4-4A and 4-4B).

Figure 4-4

Figure 4-4A

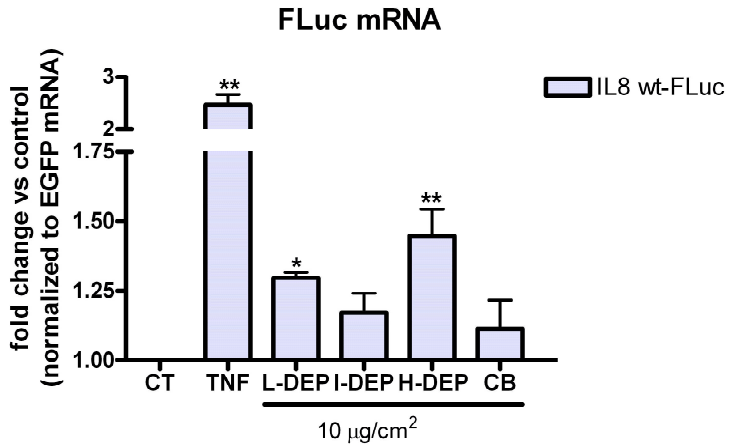


Figure 4-4B

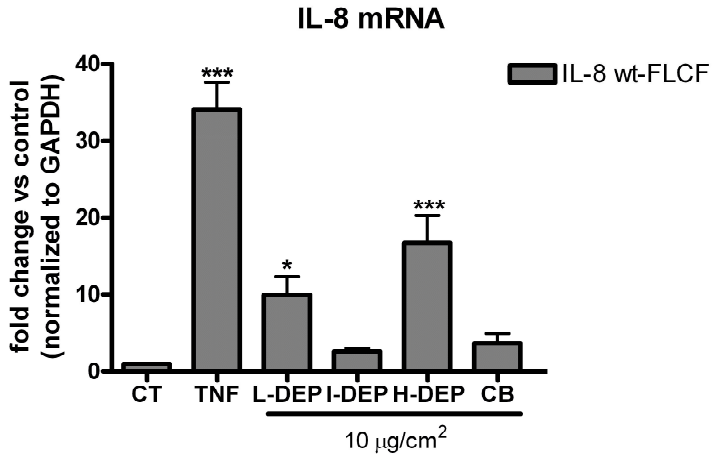


Figure 4-4. *DEP exposure induces IL-8 promoter reporter activity***.** BEAS2B cells were stably co-transduced with lentiviral promoter reporters carrying the human wildtype IL-8 promoter linked to firefly luciferase (IL-8 wt-FLuc) and EF1α-EGFP. Transduced cells were treated with 10 μg/cm^2 L-DEP, I-DEP, H-DEP or CB, media (CT) or 20 ng/ml TNF for 4 h and cDNA generated from cell lysates was analyzed for (4-4A) FLuc and EGFP or (4-4B) endogenous IL-8 and GAPDH by RT-PCR. FLuc and IL-8 mRNA levels were normalized to EGFP or GAPDH mRNA levels, respectively and the data is expressed as fold change over controls. Statistical significance was determined by one-way ANOVA with a Bonferronni's or Dunnett's Multiple Comparison Test (* $P < 0.05$, ***$P < 0.001$). The results shown are representative of thee or more experiments.

4-D.3 *L-DEP- but not H-DEP-induced IL-8 promoter reporter activity is NFκB dependent.* The IL-8 promoter contains putative binding sites for several transcription factors including NFκB, AP-1, and C/EPB1-β, among others [Figure 4-1 and (Jaspers *et al.*, 1999)]. H-DEP exposure has been previously reported to drive IL-8 expression by an NFκB-dependent mechanism (Takizawa *et al.*, 1999). We therefore examined the possibility that NFκB-mediated transcriptional activation was responsible for L-DEP and H-DEP induced IL-8 expression. To do so, BEAS2B cells were stably co-transduced with IL-8 lentiviral promoter reporter containing a mutated NFκB response element (IL-8$^-$NFκB-FLuc), and EF1α-EGFP. Transduced cells were exposed to 10 μg/cm^2 L-DEP, I-DEP, H-DEP, CB or 20 ng/ml TNF for 4 h and promoter activity was measured by RT-PCR as previously described. Mutant promoter reporter data was compared to IL-8 wt-FLuc data reported in Figure 4-4A. As shown in Figure 4-5A, L-DEP-induced wildtype promoter reporter activity was blunted in cells transduced with the mutant reporter suggesting that L-DEP-induced IL-8 expression is NFκB-dependent in BEAS2B cells. In comparison, H-DEP-induced promoter reporter activity was not statistically different between the wildtype and mutant promoters. TNF is known to induce NFκB-dependent IL-8 expression (Smith *et al.*, 1994). In agreement, transduction with the mutant promoter completely abolished promoter reporter activity (Figure 4-5A). In contrast, there were no statistically significant differences detected between the wildtype and mutant promoter reporter activity in cells exposed to I-DEP or CB (Figure 4-5A).

To confirm that transduced cells respond appropriately to treatment with DEP, endogenous IL-8 expression was measured in cells transduced with IL-8 wt-FLuc and IL-8$^-$ NFκB-FLuc. As shown in Figure 4-5B, L-DEP, I-DEP and H-DEP induce IL-8 expression with a similar rank potency as reported earlier (Figures 4-3B and 4-4B).

Although our observation that H-DEP induces IL-8 promoter reporter activity in cells transduced with the IL-8$^-$NFκB-FLuc lentiviral promoter reporter (Figure 4-5A) is at odds with a previously published

report (Takizawa *et al.*, 1999), the data presented here suggested that H-DEP induces IL-8 expression by an NFκB-independent mechanism. In order to confirm these findings, BEAS2B cells stably transduced with a lentiviral promoter reporter expressing tandem repeats of the NFκB consensus sequence was generated (NFκB-FLuc) and tested in response to DEP exposure. As shown in Figure 4-5C, exposure to H-DEP failed to induce NFκB promoter reporter activity. This is in agreement with earlier data showing that H-DEP induces IL-8 expression by an NFκB-independent mechanism (Figure 4-5A and 4-5C). By comparison, L-DEP treatment induced NFκB promoter reporter activity (Figure 4-5C). These findings align well with earlier observations that are suggestive of an NFκB-dependent mechanism by which L-DEP exposure results in increased IL-8 expression (Figure. 4-5A and 4-5C). As expected, TNF exposure induced robust NFκB promoter reporter activity. Increased NFκB (consensus sequence) promoter reporter activity was also observed in cells treated with I-DEP and CB. To confirm that transduced cells respond appropriately to treatment with DEP, endogenous IL-8 expression was next measured in cells transduced with NFκB-FLuc (Figure 4-5D). Although the magnitude of the response was greater in cells expressing NFκB-FLuc, the rank potency with which DEP exposure induced IL-8 expression was similar to that reported earlier (Figures 4-3B, 4-4B and 4-5B).

Figure 4-5

Figure 4-5A

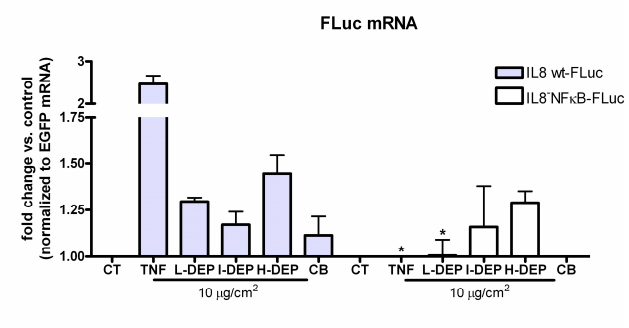


Figure 4-5B

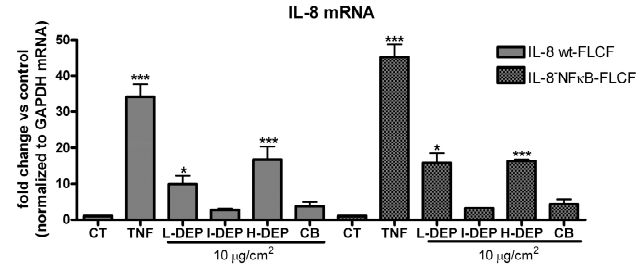


Figure 4-5C

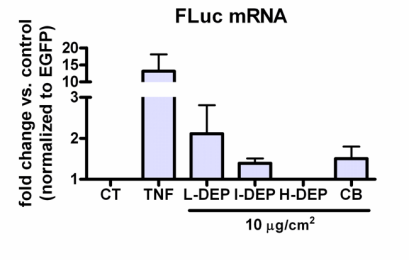


Figure 4-5D

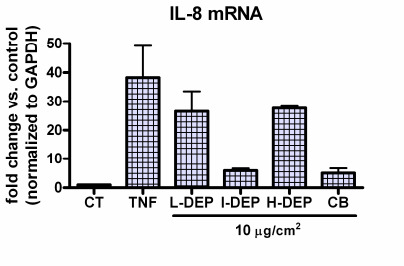


Figure 4-5. *L-DEP but not H-DEP exposure induces NFκB-dependent IL-8 expression in BEAS2B cells.* BEASE2B cells co-transduced with EF1α-EGFP and IL-8 wt-FLuc (data from 4-4A) and compared to data obtained from BEAS2B cells co-transduced with EF1α-EGFP and IL-8-NFκB (Figure 4-5A). Cells were exposed to 10 μg/cm^2 L-DEP, I-DEP, H-DEP or CB, media (CT) or 20 ng/ml TNF for 4 h and (4-5A) EGFP and FLuc or (4-5B) IL-8 and GAPDH mRNA levels were assessed by RT-PCR. FLuc and IL-8 mRNA levels were normalized to EGFP or GAPDH mRNA levels, respectively, and expressed as fold change over controls. Statistical significance was determined by a two-tailed Student's T Test or by one-way ANOVA with a Bonferronni's Multiple Comparison Test (*P < 0.05, ***P < 0.001). The results shown are representative of thee or more experiments. BEASE2B cells co-transduced with NFκB-FLuc and EF1α-EGFP were exposed to 10 μg/cm^2 L-DEP, I-DEP, H-DEP or CB, media (CT) or 20 ng/ml TNF for 4 h and (4-5C) EGFP and FLuc or (4-5D) IL-8 and GAPDH mRNA levels were assessed by RT-PCR. FLuc and IL-8 mRNA levels were normalized to EGFP or GAPDH mRNA levels, respectively, and expressed as fold change over controls. The results shown are representative of two experiments.

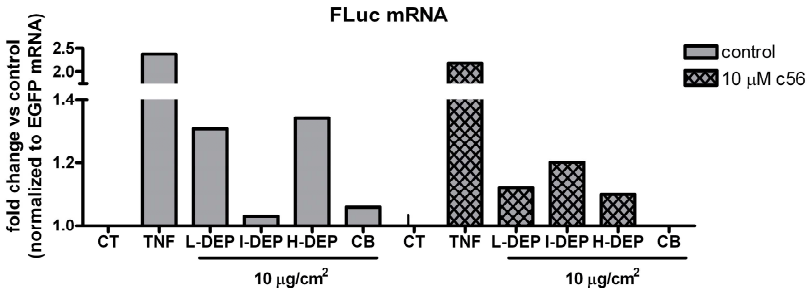
4-D.4 *Involvement of EGFR in DEP-induced IL-8 expression.* We and others have implicated EGFR in DEP-induced proinflammatory signaling effects (Blanchet *et al.*, 2004; Tal *et al.*, 2006; Cao *et al.*, 2007b; Rumelhard *et al.*, 2007a; Rumelhard *et al.*, 2007b; Pourazar *et al.*, 2008). It has been proposed that DEP associated electrophilic compounds (Iwamoto *et al.*, 2007), DEP-generated ROS, or ROS produced by cells in response to DEP exposure impair EGFR-directed PTPase activity leading to sustained EGFR activation (Tal *et al.*, 2006; Cao *et al.*, 2007b). However, the toxicological relevance of these findings in relation to DEP-induced proinflamatory signaling is unclear. To determine whether EGFR is involved in DEP-mediated increases in IL-8 expression, BEAS2B cells expressing IL-8 wt-FLuc were pretreated with an inhibitor of EGFR kinase activity (c56) for 1 h followed by exposure to 10 $\mu g/cm^2$ L-DEP, I-DEP, H-DEP, CB or 20 ng/ml TNF for 4 h and FLuc expression was measured by RT-PCR. As shown in Figure 4-6A, c56 pretreatment blunts L-DEP and H-DEP mediated increases in IL-8 promoter reporter activity. As expected, c56 is unable to block TNF-induced IL-8 promoter reporter activity. Interestingly, c56 pretreatment enhanced I-DEP's ability to induce IL-8 promoter reporter activity.

To further examine the role of the EGFR in DEP induced IL-8 expression we next determined whether c56 pretreatment was sufficient to prevent DEP induced increases in endogenous IL-8 mRNA in BEAS2B cells expressing IL-8 wt-FLuc. As shown in Figure 4-6B, c56 pretreatment markedly blocked L-DEP- and H-DEP-mediated IL-8 expression and weakly blocked I-DEP-induced IL-8 expression. An increase in IL-8 mRNA levels in response to CB was also diminished by c56 pretreatment. Interestingly, TNF-induced IL-8 expression was partially blocked by c56. TNF is known to induce IL-8 expression though TNF receptor mediated activation of NFκB. Although substantial crosstalk is reportedly involved in MAPK signaling (Pimienta and Pascual, 2007), our data showing that c56 blocks TNF induced IL-8 expression suggests that c56 may have downstream, off-target effects. Taken together, these data suggest that EGFR may be involved in DEP-induced IL-

8 expression although, in light of off-target concerns and the fact that these data are derived from a single experiment, these findings should be interpreted with caution.

Figure 4-6

Figure 4-6A



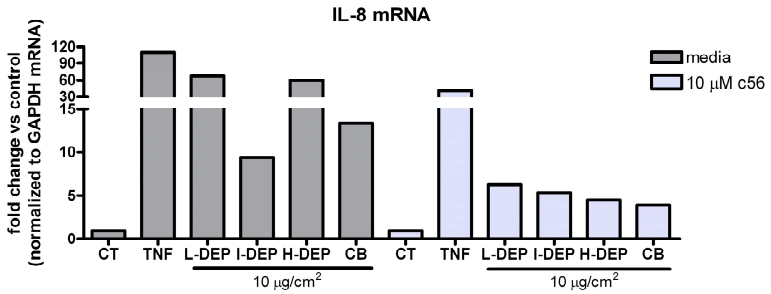


Figure 4-6B

Figure 4-6. *Characterizing the role of the EGFR in DEP-induced proinflammatory signaling.* BEASE2B cells co-transduced with IL-8 wt-FLuc and EF1α-EGFP were pretreated with 10 μM c56 or vehicle control for 1 h. Cells were then washed and treated with 10 μg/cm^2 L-DEP, I-DEP, H-DEP or CB, media (CT) or 20 ng/ml TNF for 4 h and (4-6A) EGFP and FLuc or (4-6B) IL-8 and GAPDH mRNA levels were assessed by RT-PCR. FLuc and IL-8 mRNA levels were normalized to EGFP or GAPDH mRNA levels, respectively, and expressed as fold change over controls (n = 1).

4-E Discussion

The mechanisms by which DEP induces adverse health effects are not well understood. Lung epithelial cells are directly exposed to inhaled DEP and are a significant source of inflammatory mediators. We have previously reported that exposure to L-DEP induces proinflammatory signaling in lung epithelial cells characterized by increased expression of IL-8 and Cox-2 (Cao *et al.*, 2007a; Cao *et al.*, 2007b). However, it is unclear whether the physicochemical properties of DEP determine proinflammatory signaling in lung epithelial cells. Moreover, the transcriptional regulation by which these signaling events occur are not well understood. Here we report that exposure to DEP with low or high organic content induces IL-8 expression by distinctly different mechanisms in BEAS2B cells.

While the mechanism by which DEP induces proinflammatory signaling in airway epithelial cells is not fully understood, information concerning the pathways involved has been reported. DEP-exposure has been shown to activate the redox-sensitive transcription factors AP-1 and NFκB (Takizawa *et al.*, 1999; Bonvallot *et al.*, 2001) in addition to their upstream, stress-related MAPKs, p38 and JNK, in human lung biopsies (Pourazar *et al.*, 2005). Activation of these molecules promotes the transcription of pro-inflammatory cytokines, triggering a pulmonary inflammatory response characteristic of PM exposure (Kim *et al.*, 2006). The data presented in Chapter IV suggest that L-DEP and H-DEP induce IL-8 expression by distinctly different mechanisms. In particular, we report that L-DEP triggers NFκB-dependent IL-8 transcriptional activation (Figures 4-3A and 4-3C). These findings are in agreement with studies demonstrating DEP-mediated NFκB activation (Takizawa *et al.*, 1999; Bonvallot *et al.*, 2001). In contrast, we report that H-DEP exposure induces IL-8 expression by an NFκB-independent mechanism.

Induction of IL-8 expression is controlled in part by an enhancer region upstream of the transcriptional start site (base pairs -126 to -72) [Figure 4-1 and (Jaspers *et al.*, 1999)]. As mentioned previously, the enhancer region contains cis-acting AP-1, NFκB, and C/EPB response elements

(Mukaida *et al.*, 1989; Strieter, 2002). All three elements are required for maximal transcriptional activation (Jaspers *et al.*, 1999).

H-DEP exposure has been previously reported to drive IL-8 expression by an NFκB-dependent mechanism in BEAS2B cells (Takizawa *et al.*, 1999). However, here we provide several lines of evidence showing that H-DEP induces NFκB-independent IL-8 transcription in BEAS2B cells. First, H-DEP-induced promoter reporter activity was not prevented by use of an IL-8 promoter reporter carrying a mutated NFκB response element (IL-8-NFκB-FLuc, Figure 4-5A). Second, DEP failed to induce NFκB promoter reporter activity in BEAS2B cells stably transduced with a lentiviral promoter reporter expressing tandem repeats of the NFκB consensus sequence (NFκB-FLuc, Figure 4-5C) Taken together, these data suggest that, in opposition to earlier published findings (Takizawa *et al.*, 1999), H-DEP induces NFκB-independent IL-8 expression. One possible explanation for the alternative mechanism of proposed here is the cells themselves. BEAS2B cells cultured in serum-supplemented KGM results in a morphologic shift to squamous epithelial cells (Jaspers, unpublished observations). The results reported in *Takizawa et al.* were obtained in BEAS2B cells cultured in serum (Takizawa *et al.*, 1999) and therefore, a different cell type than that which generated the data presented here (obtained in BEAS2B cells appropriately cultured in serum-free KGM).

In support of NFκB-independent mechanism by which exposure to H-DEP increases IL-8 mRNA levels, Zhao *et al.* showed that treatment with Lysophosphatidic Acid (LPA) resulted in the activation of its cognate GPCR, which in turn, stimulated transactivation of the EGFR by protein kinase Cδ (Zhao *et al.*, 2006). Activation of p38, JNK, NFκB, and AP-1 and increased expression of IL-8 in primary human airway epithelial cells was also observed in cells exposed to LPA. Interestingly, LPA-induced IL-8 expression, but not MAPK, AP-1, or NFκB activation, was almost completely blocked by downregulation of EGFR by siRNA or by pretreatment with an EGFR kinase inhibitor. These data demonstrate a role for EGFR in LPA-induced IL-8 expression that is

independent of AP-1 and NFκB (Zhao *et al.*, 2006). Moreover, this study highlights a cross-link between GPCR and EGFR receptors and provides a physiological role for GPCR/EGFR signaling in the synthesis of IL-8 (Zhao *et al.*, 2006). Future studies involve the use of lentiviral promoter reporters carrying a tandem repeat of the AP-1 or C/EPBβ consensus sequences to test the hypothesis that H-DEP induces IL-8 expression by an NFκB-independent mechanism.

Transcriptional activation is just one means by which cells regulate gene expression. Transcripts are additionally controlled via splicing, polyadenylation, mRNA transport (nuclear export, cytoplasmic compartmentalization, and polysomal localization), and by mRNA turnover (stabilization and destabilization) (Wilusz and Wilusz, 2004; Hu *et al.*, 2005; Garneau *et al.*, 2007; Ibrahim *et al.*, 2008). Gene regulation is controlled by the complex interaction of these varied regulatory events and it therefore seems likely that the relative contribution of transcriptional and posttranscriptional events differs greatly in response to different stressors. Strikingly, recent studies using cDNA microarrays to compare steady-state mRNA versus newly transcribed mRNA reveal shown that over half of all known stress-response genes are regulated by changes in mRNA stability (Fan *et al.*, 2002; Kawai *et al.*, 2004). One common feature found in transcripts that undergo rapid turnover, such as cytokines and cell-cycle associated proteins, is the presence of adenosine-uridine-rich elements (AUREs) in the 3' untranslated regions (3'UTRs) (Caput *et al.*, 1986). AUREs interact with a number of proteins thought to either impede or induce mRNA degradation (Garneau *et al.*, 2007).

A recent study reported that LPS-induced nitric oxide (NO.) generation stabilized a large set of mRNA transcripts by activation of Erk1/2 or p38 MAPKs (Wang *et al.*, 2006). Sequence analysis revealed an over-representation of AUREs in the 3'UTRs of transcripts stabilized by NO.-induced P38 MAPK signaling. In contrast, CU-rich elements (CUREs) were over-represented in a group of transcripts stabilized by Erk1/2-dependent signaling. Taken together, these data indicate that MAPK-specific signaling is involved in distinct mechanisms by which mRNA is stabilized. p38-dependent MAPK signaling in particular, has been implicated in regulating the mRNA half life of over 40

AURE-containing genes including IL-8 (Wang *et al.*, 2008). In support of this, we have observed that mRNA stabilization is the main mechanism by which nanodiamond particles induce IL-8 expression in the absence of transcriptional activation in HAEC (Silbajoris, in preparation). Therefore it is possible that DEP exposure induces IL-8 message stabilization in BEAS2B cells. However, our data showing that L-DEP and H-DEP induce IL-8 promoter reporter activity suggests that mRNA stability would be only one of multiple mechanisms by which DEP exposure increased IL-8 expression in our model.

We have previously shown that exposure to L-DEP, I-DEP, or H-DEP induces differential EGFR activation in HAEC [Tal, Submitted, Chapter III]. In support of these findings, here we report that DEP induces IL-8 expression and IL-8 promoter reporter activity with a similar rank potency (H-DEP > L-DEP > I-DEP). These data are in agreement with earlier reports showing that these DEPs have differential pulmonary toxicity (Singh *et al.*, 2004), mutagenic potential (DeMarini *et al.*, 2004), and adjuvant properties (Stevens *et al.*, 2008). Therefore, these data suggest that organic content is an inaccurate means of predicting particle toxicity and that the exact mechanism of signal disregulation by DEP is likely complex and will require further investigation.

We report that exposure to DEP induces EGFR activation and proinflammatory signaling characterized by increased IL-8 expression in HAEC and BEAS2B [Tal, submitted, Chapter III and Tal, unpublished observations, Chapter IV]. However, it is unclear whether EGFR-dependent signaling is causally related to IL-8 expression or if it instead, occurs in parallel. While there are some reports suggesting that EGFR activation induces IL-8 expression, the data is generally obtained through the use of EGFR kinase inhibitors which, as noted earlier, likely have off-target effects. The role of the EGFR in IL-8 expression will be discussed in detail in the following chapter (Chapter V).

In summary, DEP is a pervasive environmental contaminant known to activate proinflammatory signaling in lung epithelium. Here we provide evidence that DEP exposure can enhance expression of

IL-8 mRNA by distinct NFκB-dependent and –independent mechanisms in bronchial epithelial cells. Future work includes the use of AP-1 and C/EPBβ wildtype and mutant promoter reporters to further elucidate the mechanisms by which DEP exposure induces IL-8 expression in lung epithelial cells.

Chapter 5: Concluding Remarks

The research presented here was designed to determine whether exposure to ambient PM constituents activate tyrosine phosphorylation-dependent signaling pathways through inhibition of PTPases that function to maintain signaling quiescence in airway epithelial cells. In support of this, we have demonstrated that exposure to disparate PM components, including Zn^{2+} and DEP, induce phosphorylation of the EGFR by a similar mechanism. First, we show that Zn^{2+}- or DEP-mediated EGFR phosphorylation requires EGFR kinase activity. Second, through the use of a ligand-blocking antibody and dimerization assays, we report that Zn^{2+} and DEP induce EGFR phosphorylation independently of receptor dimerization. Third, we show that both Zn^{2+} and DEP exposure result in impairment of total and EGFR-directed PTPase activity. Taken together, these data support the notion that exposure to different components of ambient PM similarly blunt the activity of PTPases that exert their activity against the EGFR. Therefore, in the presence of low levels of basal kinase activity, an impairment in EGFR-directed PTPase activity would enable EGFR-dependent signaling to occur unopposed in lung epithelial cells (Figure 5-1).

Based on these findings, we hypothesized that inhibition of PTPase activity by PM exposure would result in the activation of phosphorylation-dependent signaling and culminate in the synthesis of proinflammatory mediators such as IL-8. In order to examine the link between PM-induced signaling and ensuing proinflammatory responses, we determined the effect of PM constituents on signaling pathways that regulate expression of IL-8. Here we report that DEP treatment results in the expression of IL-8 by mechanisms dependent and independent of NFκB in cultured lung epithelial cells.

Figure 5-1

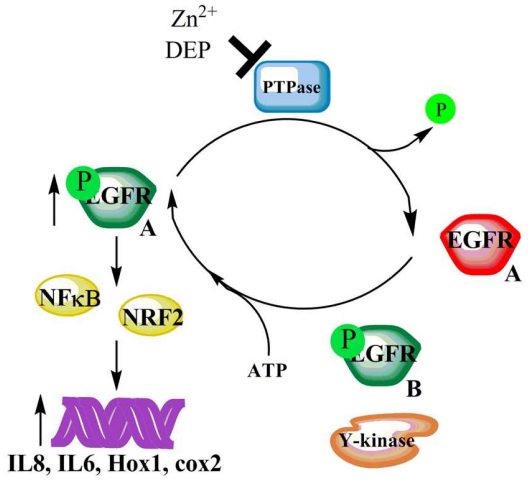


Figure 5-1 *Zn^{2+} and DEP induce EGFR-dependent signaling through inhibition of EGFR-directed PTPases.* Impairment in PTPases that function to regulate the EGFR results in an accumulation of basally phosphorylated EGFR leading to activation of MAPK pathways and upregulation in genes involved in inflammation, cell growth and differentiation, apoptosis, and mucin production.

5-A Toxicity associated with ambient PM inhalation

The cardiovascular risk associated with exposure to ambient particulates is well documented (Dockery *et al.*, 1993; Schwartz, 1994; Peters *et al.*, 2001; Peters *et al.*, 2004; Peters *et al.*, 2006). However, the mechanisms by which PM induces adverse health effects are not well understood. Although, oxidative stress, triggering proinflammatory signaling in the lung, has emerged as a leading mechanism by which ambient PM might trigger cardiopulmonary attack (Gurgueira *et al.*, 2002; Li *et al.*, 2002; Xiao *et al.*, 2003; Xia *et al.*, 2004), there are a number of other, less well studied mechanisms that may also contribute to the adverse health effects associated with PM exposure (Figure 1-1). In particular, inhaled ultrafine PM can reportedly bypass the lung completely by transposition through the lung epithelium, entering the cardiovascular system and thereby directly exerting deleterious effects on the heart (Geiser *et al.*, 2005). PM exposure has also been reported to induce cardiac oxidant stress by autonomic signaling resulting in significant functional alterations in the heart (Rhoden *et al.*, 2005). More recently, air pollution exposure has been associated with a disruption of the blood-brain barrier and neuroinflammation in the sudden death of children and young adults living in highly-polluted cities (Calderon-Garciduenas *et al.*, 2008). A disruption of the blood-brain barrier by DEP exposure involving inflammatory signaling and oxidant stress has recently been described in rat and mice brain capillaries (Hartz *et al.*, 2008) providing a novel mechanism by which ambient PM might induce deleterious neurological health effects.

5-B PM-induced mechanisms of toxicity

Numerous studies have sought to identify the mechanism by which particle inhalation induces local and systemic inflammation. Particle mass, size and surface area, metallic and organic contents, acids, sulfates, nitrates, elemental carbon, and co-pollutants have been investigated and oxidative stress has emerged as a pivotal mechanism by which PM elicits pulmonary toxicity (Gavett *et al.*, 1997; Gurgueira *et al.*, 2002; Kodavanti *et al.*, 2002; Li *et al.*, 2002; Brook *et al.*, 2003; Gavett *et al.*, 2003; Risom *et al.*, 2005).

PM exposure exerts oxidative stress on the lung by presenting or stimulating cells to produce reactive species via its metals, organics, lipopolysaccarides, and ultrafine constituents (Tao *et al.*, 2003). Among them, bioavailable organic compounds have been reported to contribute to oxidant effects induced by PM exposure (Xia *et al.*, 2004). Two main families of compounds, PAHs and quinones, are adsorbed on diesel particles (Baulig *et al.*, 2003) and both reactive PAH metabolites and redox-cycling quinones generate ROS via reduction by NADPH-cytochrome P450 reductase resulting in the formation of semiquinone radicals capable of reducing oxygen to $O_2^{\cdot-}$. A body of work has been produced supporting the notion that the organic fraction of DEP, via ROS generation, is the primary mediator of PM-associated toxicity (Li *et al.*, 2002; Li *et al.*, 2003; Li and Whorton, 2003).

In addition to bioavailable organics, studies using residual oil fly ash have demonstrated that pulmonary toxicity is attributable to water-soluble metal constituents (Gavett *et al.*, 1997; Kodavanti *et al.*, 1998; Gavett *et al.*, 2003). In particular, ubiquitous soluble metallic components such as Fe^{3+}, Cu^{2+}, $V^{3+/5+}$ and $Cr^{3+/6+}$, can generate ROS by Fenton-type chemistry and act as catalysts by Haber-Weiss reactions (Koppenol, 2001).

We and others have also reported that Zn^{2+}, a common PM constituent, may be implicated in the toxicity associated with PM inhalation (Kodavanti *et al.*, 1998; Soukup *et al.*, 2000; Kodavanti *et al.*, 2002; Okeson *et al.*, 2004). Although Zn^{2+} is unable to undergo redox cycling, the metal cation is a known inhibitor of PTPases (Kim *et al.*, 2006; Tal *et al.*, 2006), including a broad spectrum of PTPases present in HAEC (Samet *et al.*, 1999). Here we report that Zn^{2+}-mediated PTPase inhibition may occur through a mechanism dependent upon direct metallic attack of the PTPase catalytic site, rather that indirectly inhibiting PTPases by oxidation (Chapter II and Appendix A). As shown in Figure 1-2B, Zn is present in ambient PM, particularly in urbanized and industrialized areas although these data do not identify the speciation of Zn present (Seagrave *et al.*, 2006). Analysis of DEP (NIST SRM 1650, representative of DEP produced by heavy-duty diesel engines) by X-ray absorption fine structure and proton-induced X-ray emission and analysis revealed the presence of high concentrations of Zn^{2+} relative to other metals (Huggins *et al.*, 2000). Although DEP SRM 1650 was not used in the present studies, these data demonstrate that a characteristic DEP contains measurable concentrations of Zn^{2+} that may be involved in DEP's mode of action. In our model, pretreatment of cells with a Zn^{2+} chelating agent prior to DEP exposure would demonstrate whether Zn^{2+} contributes to DEP-induced toxicity. Additionally, future experiments include the determination of the Zn^{2+} content in L-DEP, I-DEP, and H-DEP by inductively coupled plasma mass spectrometry.

5-C Mechanisms of PM-induced PTPase inhibition

The data presented here supports the concept that structurally dissimilar components of ambient PM induce signal disregulation characterized by increases in phospho-EGFR levels, reduced rates of EGFR dephosphorylation and therefore, inhibition in EGFR-directed PTPase activity, and activation of proinflammatory signaling in HAEC and BEAS2B cells [Tal, Chapters I-III, (Kim *et al.*, 2006; Tal

et al., 2006; Cao *et al.*, 2007)]. A more thorough discussion detailing how Zn^{2+} and DEP potentially interact with PTPases can be found in Chapters I-III. Briefly, based on the literature and the data presented here, we suspect that Zn^{2+} and DEP inhibit PTPase activity by direct and indirect mechanisms. First, the nucleophilic catalytic cysteine present in all PTPases is susceptible to direct inhibitory interactions with a variety of PM components including electrostatic associations with metal cations, such as Zn^{2+} and $V^{3+/5+}$ (Stankiewicz *et al.*, 1995; Haase and Maret, 2005) and covalent modification by DEP-associated electrophilic organic compounds (Iwamoto *et al.*, 2007; Seiner *et al.*, 2007). Second, PTPases are indirectly inhibited via oxidation of the catalytic cysteine by DEP-associated quinones and PAHs (Li *et al.*, 2002; Xia *et al.*, 2004). Taken together, these studies provide a plausible mechanism by which Zn^{2+} and DEP exposure induce similar effects in our system.

Although the catalytic cysteine is often hypothesized to be susceptible to post-translational modification (Chiarugi and Buricchi, 2007), it is likely that other regulatory residues are targets of direct and indirect inhibition by Zn^{2+} and DEP. A recent report examining the effects of 1,2 napthoquinone on PTP1B activity demonstrated that nucleophilic histidine (His^{25}) and cysteine (Cys^{121}) residues, in addition to the catalytic cysteine (Cys^{215}), are susceptible to covalent modification by 1,2 napthoquinone (Iwamoto *et al.*, 2007). The authors speculate that although Cys^{121} is non-catalytic, it may act as a site of allosteric inhibition (Iwamoto *et al.*, 2007). In the case of Zn^{2+}, the metal cation is hypothesized to form inhibitory electrostatic interactions with multiple cysteine and histidine residues located near the catalytic groove (Haase and Maret, 2003).

There is accumulating evidence that PM-associated electrophilic aldehydes and quinones such as acrolein and 1,2 napthaquinone, respectively, can covalently modify critical cysteine and histidine residues thereby irreversibly inhibiting PTPase activity (Iwamoto *et al.*, 2007; Seiner *et al.*, 2007). While these data show that reactive organic compounds commonly found in ambient PM directly impair PTPase activity, it is not known whether these compounds inactivate PTPase activity when associated with particles. The data presented here demonstrate for the first time that DEP exposure

reduces the rate of EGFR-directed PTPase activity leading to sustained EGFR phosphorylation in HAEC (Tal, Submitted, Chapter III). However, these data can not distinguish the exact mechanism by which DEP inhibits PTPase activity. In particular, it is unclear whether DEP associated electrophilic compounds are covalently inhibiting PTPase activity or participating in redox cycling resulting in the oxidation of PTPases. Furthermore, the role of Zn^{2+} and other metal cations in DEP-mediated toxicity is not well understood.

To better understand how DEP impairs PTPase activity, airway epithelial cells can be treated with organic extracts obtained from different DEPs and levels of EGFR phosphorylation and as well as total and EGFR-directed PTPase activity can be measured to determine whether the biological activity resides in the organic extract. In order to determine whether the particles themselves or their soluble components mediate DEP-induced signaling effects, similar experiments can be conducted using particles or particle washes obtained from DEP "washed" in media for 4 h, then spun down and resolubilized. Furthermore, the role of soluble metals such as Zn^{2+} can be similarly assessed by treating HAEC with the Zn^{2+} chelating agent TPEN prior to DEP exposure. While the data generated from the aforementioned experiments will reveal some information about biologically active compartments present in DEP, more studies are needed to characterize the mechanism by which DEP interacts with cellular PTPases. To do so, cells could be pretreated with metal chelators or antioxidants or transfected with antioxidant expressing constructs to determine whether metals or oxidant stress is involved in DEP-induced signaling disregulation. The most rigorous means of identifying the inhibitory mechanism is by the identification of DEP-induced covalent modifications to PTPases by mass spectrometry (Iwamoto *et al.*, 2007).

5-D PM-induced activation of EGFR-dependent signaling

The data presented here and elsewhere demonstrate that exposure to Zn^{2+} and DEP induce activation of the EGFR and proinflammatory signaling characterized by increases in IL-8 expression in lung epithelial cells [Chapter III-IV and (Kim *et al.*, 2006; Tal *et al.*, 2006)]. However, it is unclear whether PM-induced EGFR-dependent signaling leads to upregulation of IL-8 in our system. A number of recent studies support the notion that EGFR activation causes an upregulation of IL-8. First, a recent study showed that IL-8 expression induced by exposure to the inflammatory mediator MMP12 was blocked in A431 cells treated with EGFR kinase inhibitors or EGFR siRNA (Quement *et al.*, 2008). Second, rhinovirus-induced IL-8 expression was blocked by pretreatment with EGFR kinase inhibitors and by overexpression of a kinase inactive EGFR (Liu *et al.*, 2008). Third, in agreement with these studies, exposure to phorbal 12-myristate 13-acetate, a model inflammatory agent, or gram-negative bacterial lipopolysaccharide was shown to induce EGFR-dependent IL-8 expression in airway epithelial cells (NCI-H2N2) (Nakanaga *et al.*, 2007). Taken together, these studies provide evidence supporting the direct role of the EGFR in increases in IL-8 mRNA levels in response to proinflammatory stimuli.

In addition to proinflammatory signaling, activation of EGFR-dependent signaling leads to a diverse set of cellular outcomes including proliferation, differentiation, growth, and apoptosis (Figure 1-3). Recently, a series of studies have elucidated a mechanism of EGFR-dependent wound healing following damage to the lung epithelium (Vermeer *et al.*, 2003; Vermeer *et al.*, 2006a; Vermeer *et al.*, 2006b). These studies demonstrate that following mechanical injury or disruption of the tight junctions by Ca^{2+} chelation, apically restricted EGFR ligand gained access to the basolateral domain and thereby inducing EGFR kinase activation and cellular proliferation to repair the damaged monolayer. In addition to physiologically relevant signaling, inappropriate activation of EGFR-dependent signaling is involved in numerous pathophysiological outcomes including cancer

progression [reviewed in (Milanezi *et al.*, 2008)] and inappropriate mucin production that has been implicated in a number of hypersecretory disesases including asthma, chronic obstructive pulmonary disease, and cystic fibrosis [reviewed in (Nadel and Burgel, 2001)].

5-E Identity of PTPases that regulate EGFR activity in lung epithelial cells

Previously published reports and data presented here (Chapters II-III) reveal that Zn^{2+} and DEP induce EGFR phosphorylation by a ligand-independent mechanism (Samet *et al.*, 2003; Tal *et al.*, 2006). In the case of Zn^{2+}, we report that Zn^{2+} exposure induces Src-dependent (Samet *et al.*, 2003) and Src-independent (Tal *et al.*, 2006) EGFR phosphorylation in A431 cells and HAEC, respectively. Moreover, we observed an impairment in cellular and EGFR-directed PTPase activity in HAEC exposed to Zn^{2+} and DEP [(Tal *et al.*, 2006), Chapter 2 and Tal unpublished observations, Chapter 3]. While these findings do not rule out the possibility the PM exposure induces transactivation of the EGFR by intracellular tyrosine kinases, they identify a common mechanism of PTPase inhibition by which disparate components of ambient PM similarly activate the EGFR. For PM-induced inhibition of EGFR-directed PTPase activity to result in downstream signaling in the absence of EGFR transactivation by cytosolic tyrosine kinases, low-levels of basal EGFR kinase activity required. In support of this, preventing EGFR autophosphorylation by c56 abolished EGFR phosphorylation in HAEC exposed to media (Figure 2-5A) suggesting that EGFR autophosphorylation is the dominant mechanism by which EGFR phosphorylation occurs under basal conditions.

Here we report that structurally dissimilar components of ambient PM (DEP and Zn^{2+}) inhibit EGFR-directed PTPase activity (Figures 2-4, 2-5, 3-4, and 3-5). Although many PTPases have been shown to regulate the phosphorylation status of the EGFR (Table 1-1), we have not identified specific PTPases that control phospho-EGFR levels and are impaired by PM exposure in our system. Recently, a new technique has been described that would allow for the identification of PTPases

inhibited by PM exposure that function to regulate EGFR activity (Li *et al.*, 2007). Briefly, the methodology reverses the role of the substrate and kinases in traditional in-gel tyrosine kinase activity assays (Figure 3-1). Rather than impregnating the gel with a kinase substrate and running lysates obtained from different treatment groups to determine their effect on cellular kinase activity, active kinases are impregnated in the gel to identify PTPases capable of dephosphorylating them (Li *et al.*, 2007). Future studies are currently being designed to identify PTPases that regulate the phosphorylation status of the EGFR and are inhibited by PM exposure by impregnating gels with phosphorylated/active EGFR.

5-F Summary

Exposure to ambient PM is associated with elevated rates of morbidity and mortality. Inflammation is thought to be a central mechanism by which PM exposure induces adverse health effects. In lung epithelial cells, a direct target of inhaled PM, exposure to ambient PM has been shown to activate tyrosine phosphoylation-dependent signaling culminating in the synthesis of proinflammatory mediators capable of recruiting and activating circulating immune cells. Here we report that exposure to the ubiquitous PM components Zn^{2+} or DEP induce activation of the receptor tyrosine kinase EGFR in human airway epithelial cells. This phosphorylation event occurs by a ligand-independent mechanism that requires EGFR kinase activity. We also show that exposure to Zn^{2+} or DEP impair the activity of PTPases that function to dephosphorylate the EGFR. Taken together, these data show that PM-induced EGFR-phosphorylation in human airway epithelial cells is the result of a loss of PTPase activities which normally function to dephosphorylate EGFR in opposition to baseline EGFR kinase activity. We additionally show that DEP exposure induces proinflammatory signaling characterized by increases in IL-8 expression. Moreover, we report that DEP exposure induces NFκB-dependent and NFκB-independent IL-8 expression in airway epithelial cells.

In summary, exposure to ambient PM, an extremely complex and heterogenous environmental pollutant is associated with an increased risk of cardiopulmonary morbidity and mortality. The data described in this dissertation support the concept that the components of PM, particularly metals and specific organic compounds, are primary contributors to particle-induced toxicity. Furthermore, our findings showing that two structurally dissimilar PM components (Zn^{2+} and DEP) induce signal disregulation characterized by EGFR kinase-dependent EGFR phosphorylation, inhibition of cellular PTPases including those PTPases that directly regulate the phosphorylation status of the EGFR, and induce proinflammatory signaling suggests that inhibition of PTPase activity is a unifying mechanism by which ambient air pollutants exert toxicological effects in airway epithelial cells.

Appendix A: Zn^{2+} directly impairs EGFR-dependent PTPases in HAEC

A-1 Background

We and others have reported that Zn^{2+}, a common PM constituent, may be implicated in the toxicity associated with PM inhalation (Gavett *et al.*, 1997; Kodavanti *et al.*, 2002; Tal *et al.*, 2006). Zn^{2+} is unable to undergo redox cycling yet is a known inhibitor of PTPases (Kim *et al.*, 2006; Tal *et al.*, 2006; Cao *et al.*, 2007), including a broad spectrum of PTPases present in HAEC (Samet *et al.*, 1999). A mechanism of direct inhibition has recently been proposed wherein Zn^{2+} blocks PTPase activity by binding to the catalytic cysteine and to neighboring histidine or aspartate residues present in the highly conserved active site (Haase and Maret, 2005). Treatment of Zn^{2+} exposed HAEC with the strong reductant and weak Zn^{2+} chelator, dithiothreitol (DTT) but not other structurally unrelated anti-oxidants, can reverse Zn^{2+}-mediated inhibition of EGFR-directed PTPase activity (Tal, unpublished observations, Appendix A). This suggests that Zn^{2+}-mediated PTPase inhibition may occur through a mechanism dependent upon direct metallic attack of the PTPase catalytic site, rather that indirectly inhibiting PTPases by oxidation.

A-2 Methods (*Cell culture, exogenous EGFR dephosphorylatrion assays, and Western blotting were performed as previously described in Sections 2-C, 3-C, and 4-C)*

Radiolabeling of [^{32}P]PolyGlu:Tyr(4:1)

A total of 200 µg PolyGlu:Tyr was radiolabeled using 1 µg of recombinant FER kinase (Upstate, Lake Placid, NY) in the presence of 200 µCi [^{32}P]-γ-ATP for 1 hour at 30° C in 300 µl of a buffer consisting of 10 mM $MgCl_2$, 50 mM NaCl and 0.1 mM ATP. The substrate was precipitated by adding TCA solution to 20% wt/vol and centrifuging at 12,000 × g for 5 min. The pellet was washed three times in 10% TCA and the substrate was resuspended at 10 µg/ml in 2 M Tris, pH 8.0.

Recombinant PTP1B activity assay

In a polypropalene 96 well plate, 0.1 µg of recombinant agarose conjugated PTP1B (Upstate) was added to 3 µg [^{32}P]PolyGlu:Tyr(4:1) diluted in a reaction buffer containing 20 mM Hepes pH7.4, 350 mM sucrose, and 150 mM KCl per well. To stop the reaction, 50 µl of the reaction mixture was sampled at 1, 5, 10, and 20 min and placed into wells containing 150 µl 10% TCA. Following the assay period, the plate was microcentrifuged at maximum speed for 10 min. 150 µl of the unpelleted mixture was removed and assessed for radioactivity by liquid scintillation counting.

A-3 Results

A-3A *Treatment with Chelators but not Reducing Agents Reverses Zn^{2+}-Mediated EGFR Phosphorylation.* Zn^{2+} has been postulated to influence phosphorylation dependent signaling pathways by forming both direct electrostatic interactions with highly conserved PTP residues thereby directly inhibiting enzymatic activity (Haase and Maret, 2005). Although Zn^{2+} is not a redox active metal, it is possible that treatment with Zn^{2+} induces the formation of reactive species, thereby indirectly causing PTP inhibition and activation of EGFR-dependent signaling. In order to determine whether Zn^{2+} exposure induces EGFR phosphorylation directly or indirectly, through the generation of oxidants, following exposure to 50μM Zn^{2+} for 30 min, HAEC were treated with the Zn^{2+} chelators TPEN or DTT or structurally dissimilar antioxidants, α-lipoic acid (LA) or N-acetyl cysteine (NAC). As shown in Figure A-1, TPEN and DTT but not NAC or LA were capable of reversing Zn^{2+}-mediated EGFR phosphorylation at $Tyr^{1068/845}$. These data suggest that Zn^{2+} induces Tyr-kinase phosphorylation by a mechanism independent of oxidant formation.

A-3B. *Zn^{2+}-mediated impairment of EGFR-directed PTPase activity is metal but not oxidant-dependent.* Phosphorylation-dependent signal transduction pathways are regulated by the opposing activities of kinases and phosphatases. We previously reported that exposure to Zn^{2+} inhibits a broad range of cellular PTPases in HAEC (Samet *et al.*, 1999) including those which regulate EGFR kinase activity (Tal *et al.*, 2006; Cao *et al.*, 2007). We have also recently shown that Zn^{2+}-mediated impairment of tyrosine kinase activity is not limited to the EGF-receptor but also the cellular kinases, ERK and JNK by a similar mechanism of impairment of PTPase activities (Kim *et al.*, 2006). Therefore, it is likely that the ability of Zn^{2+} to inhibit PTPase activity is conserved across the entire PTPase family. However, the mechanism which Zn^{2+}-impairs PTPase activity is unclear. As shown in Figure A-2 inhibition of human recombinant PTP1B activity by Zn^{2+} is reversed by DTT. We have reported previously that Zn^{2+} induce EGFR phosphorylation by a mechanism involving the

Figure A-1

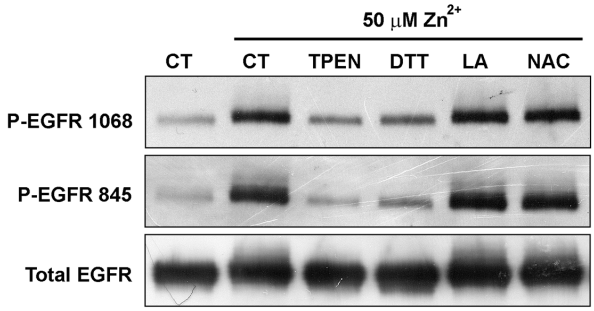


Figure A-1 *Treatment with Chelators by not Reducing Agents Reverses Zn^{2+}-Mediated EGFR Phosphorylation***.** HAEC were treat with 50 μM Zn^{2+} for 20 min the rinsed with room temperature PBS to remove excess Zn^{2+}. Cells were then treated with 100 μM TPEN, 1 mM DTT, 100 μM LA, or 10 mM LAC for 30 min. EGFR phsophorlyation was measured by Western blotting.

Figure A-2

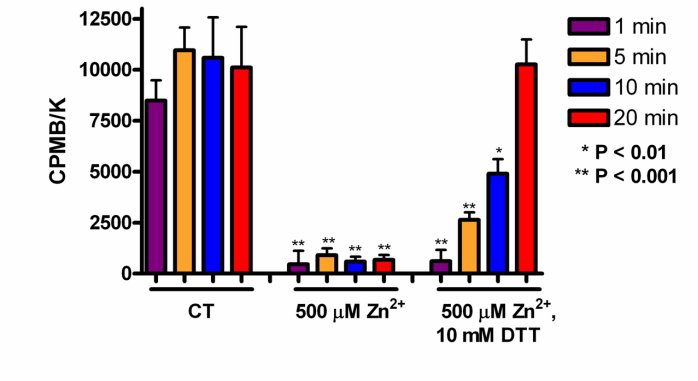


Figure A-2 *DTT reverses Zn^{2+}-mediated impairment in PTP1B activity.* Agarose conjugated human recombinant PTP1B was treated with 500 μM Zn^{2+} for 20 min then added to a reaction mixture containing 10 mM DTT and [^{32}P]PolyGlu:Tyr(4:1). The reaction mixture was sampled at 1, 5, 10, and 20 min and PTP1B activity was assessed by liquid scintillation. Statistical significance was determined by one-way ANOVA with a Dunnett's Multiple Comparison Test (* $P < 0.01$, **$P < 0.001$). The results shown are representative of four independent experiments.

We have reported previously that Zn^{2+} induce EGFR phosphorylation by a mechanism involving the inactivation of EGFR-directed PTPase activity. Therefore, we next examined the possibility that DTT treatment would reverse Zn^{2+}-induced impairment in EGFR-directed PTPase activity. As shown in Figure A-3, treatment with DTT but not NAC reversed Zn^{2+}-mediated inhibition of EGFR-directed PTPase activity further supporting the concept that Zn^{2+} inhibits PTPase activity by a mechanism that is independent of oxidant generation.

Figure A-3

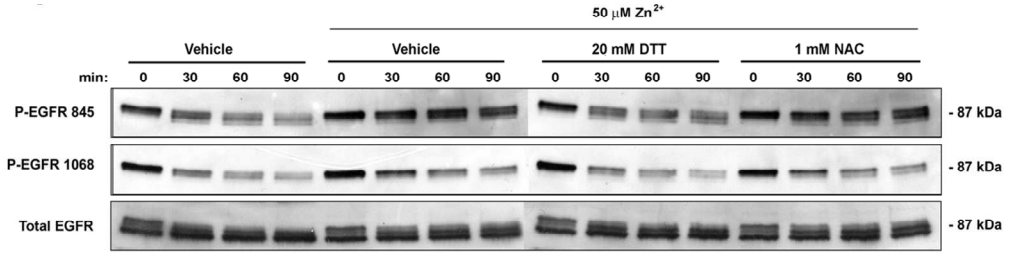


Figure A-3 *Inhibition of exogenous EGFR dephosphorylation by Zn^{2+} was reversed by DTT but not NAC in lysates obtained from HAEC exposed to Zn^{2+} in vitro.* HAEC treated with 50 μM Zn^{2+} in 4 μM PT for 20 min. Cells were rinsed then treated with 10 mM DTT or NAC. 1 ng/μl active, phosphorylated EGFR substrate was mixed with 60-100 μg of cellular lysate and the reaction was sampled at 5, 15, and 20 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 or more experiments.

Appendix B: Zn^{2+} induces EGFR phosphorylation in differentiated HAEC cultured under air-liquid interface

B-1 Background

The conducting region of the airways is lined with pseudo-stratified ciliated columnar epithelia forming the first line of defense against inhaled pathogens, particles and xenobiotics. Polarization, mediated by the appearance of tight and adheren junctional complexes, precedes- and is required for-differentiation (Ross *et al.*, 2007). Recently, a series of elegant studies have elucidated a mechanism of cellular regeneration following damage to the lung epithelium (Vermeer *et al.*, 2003; Vermeer *et al.*, 2006). These studies employed primary human airway epithelial cells differentiated under air-liquid interface culturing conditions. Briefly, EGFR is located on the basolateral surface in differentiated HAEC grown under air-liquid interface (HAEC-ALI) but not undifferentiated cells, while the soluble receptor ligand heregulin-α is restricted to the apical membrane. Tight junctions between adjacent epithelial cells form a barrier between the apical and basolateral domains thereby restricting diffusion of soluble ligands. However, following mechanical injury or disruption of the tight junctions by Ca^{2+} chelation, heregulin-α was liberated from the apical domain and induced basolaterally localized EGFR kinase activation and cellular proliferation to repair the damaged monolayer. It is unclear whether the mechanism of PM-mediated receptor tyrosine kinase activation will be affected by the state of cellular differentiation, junctional complex formation and subsequent receptor localization.

B-2 Methods Primary human bronchial epithelial cells were obtained from three different healthy, nonsmoking adult donors. The protocol and consent form were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. Cells were obtained by cytological brushing at bronchoscopy and expanded to passage three in bronchial epithelial growth medium (BEGM). Cells were plated on vitrogen-coated filter supports inserted into 12-well culture plates and maintained as described previously (Turi *et al.*, 2002). 500 nM retinoic acid was added to culture medium after cells reached 100% confluence to promote differentiation. Air-liquid interface culture was initiated 48 hours later by removing the apical medium, and basal medium containing 100 nM retinoic acid was used for the remainder of the culture period. The cells were maintained in ALI culture for 28 days prior to treatment with EGF or Zn^{2+} as previously described (Section 2-C). All other methods used in the following experiments were performed as previously described (Sections 2-C and 3-C).

B-3 Results

B-3A *EGF-mediated EGFR phosphorylation requires ligand access to the basolateral domain in HAEC differentiated under air-liquid interface.* As mentioned previously, *Zabner et al.* has elucidated a mechanism by which the respiratory epithelium regenerates following injury (Vermeer *et al.*, 2003). To verify our experimental model, we determined that HAEC-ALI expressed EGFR on the basolateral side by immunohistochemistry. As shown in Figure B-1A, EGFR is uniformly expressed on the surface of HAEC cultured on plastic and submerged by media. In contrast, following differentiation under air-liquid interface, EGFR expression is restricted to the basolateral domain (Figure B-1A). To further verify the experimental model, HAEC-ALI were treated with 50 ng/ml EGF for 20 min and assessed for the state of EGFR and Erk phosphorylation using phospho-specific antibodies directed against Erk Thr^{202}/Tyr^{204}. As expected, EGF-induced Erk phosphorylation was only observed in cultures that were exposed basolaterally (Figure B-1B). This suggests that EGFR localizes to the basolateral domain and also, is indicative of the presence of functional tight junctions within our experimental model. Additionally, pretreatment with the Ca^{2+} chelator EGTA disrupted Ca^{2+}-dependent tight junctions, thereby inducing phospho-Erk Thr^{202}/Tyr^{204} in apically stimulated cells (Figure B-1B). These experiments confirm previous reports in which receptor localization and cellular polarization (as evidenced by the restriction of soluble ligands implicating the presence of functional junctional complexes) indicate the presence of differentiated airway epithelium (Vermeer *et al.*, 2003).

B-3B Zn^{2+} *Induces kinase-dependent EGFR phosphorlyation in HAEC-ALI.* We have previously reported that Zn^{2+}-induced EGFR phosphorylation requires EGFR kinase activity in HAEC [Chapter II, (Tal *et al.*, 2006)]. Additionally, membrane permeability to Zn^{2+} but not EGFR ligand binding was required to initiate EGFR phosphorylation and downstream signaling [Chapter II, (Tal *et al.*, 2006)]. This suggests that Zn^{2+}-mediated EGFR phosphorylation requires metal cation access to the

Figure B-1

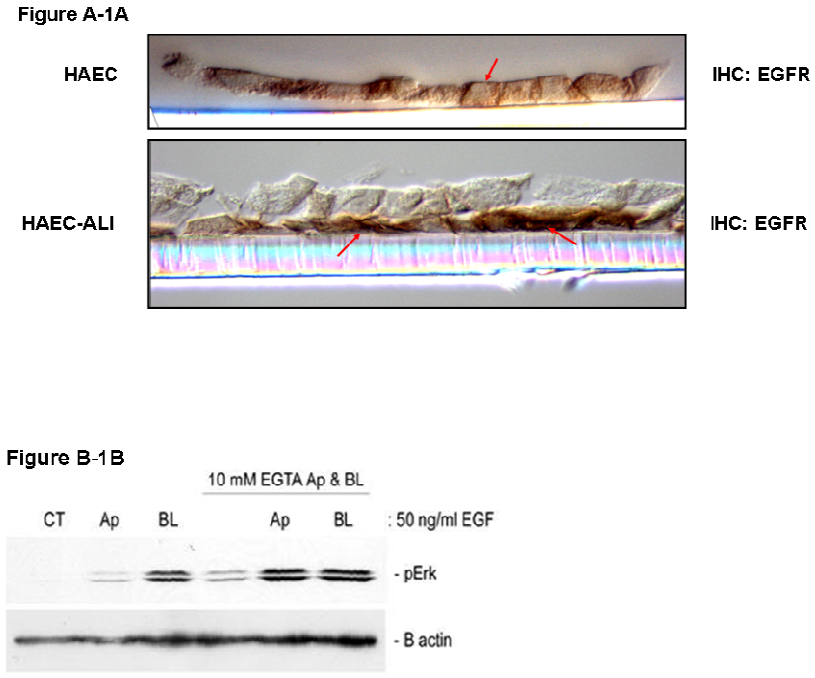


Figure B-1 *EGF-mediated EGFR phosphorylation requires ligand access to the basolateral domain in HAEC differentiated under air-liquid interface* B-1A. HAEC were cultured under submerged (HAEC) or air-liquid interface (HAEC-ALI) conditions for then EGFR expression was assessed by immunohistochemistry (red arrows). B-1B. HAEC-ALI were apically and basolaterally pretreated with 10 mM EGTA for 30 min followed by treatment with 50 ng/ml EGF for 20 min and Erk phosphorylation was assessed by Western blotting.

intracellular domain. To ascertain whether Zn2+-mediated, EGFR phosphorylation depends upon the state of cellular differentiation, HAEC-ALI were treated Apically (Ap) or Basolaterally (BL) with 50 μM Zn^{2+} in the presence or absence of pyrithione (PT) for 30 min and assessed for the state of EGFR and ERK phosphorylation by Western Blotting. Ap and BL treatment with Zn^{2+} induced robust phosphorylation of EGFR $Tyr^{845/1068}$ only when concomitantly exposed to PT (Figure B-2A). These data are in agreement with earlier findings demonstrating that Zn^{2+}-mediated EGFR phosphorylation requires intracellular labile Zn^{2+} in HAEC [Chapter 2 (Tal et al., 2006)] and HAEC-ALI (Figure B-2A).

We have previously reported that Zn^{2+} exposure induces EGFR kinase dependent EGFR phosphorylation in HAEC (Tal *et al.*, 2006). To determine whether a similar mechanism of EGFR activation in conserved in differentiated cells, HAEC-ALI were pretreated with the EGFR kinase inhibitor c56. As expected, pretreatment with the EGFR kinase inhibitor c56 blocked ligand-mediated receptor phosphorylation (Figure B-2B). As shown in Figure B-2A, c56 prevented Zn^{2+}-induced EGFR phsophorylation. Taken together these data suggest that Zn^{2+}-mediated EGFR kinase-dependent EGFR phosphorylation occurs independently of the state of cellular differentiation, junctional complex formation and subsequent receptor localization.

Figure B-2

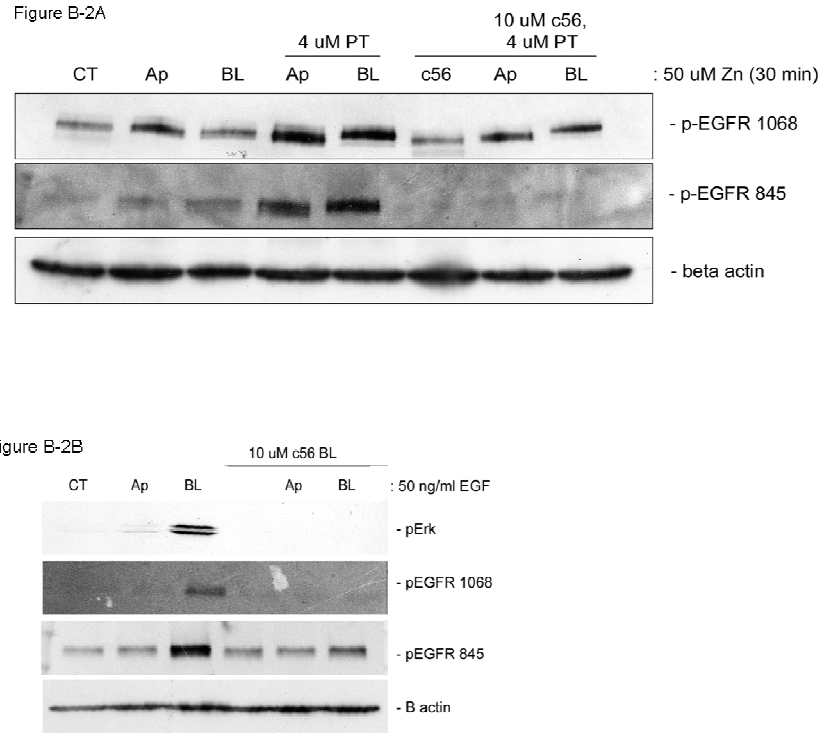


Figure B-2 Zn^{2+} **i***nduces kinase-dependent EGFR phosphorlyation in HAEC-ALI* B-2A. HAEC-ALI were pretreated with 10 µM c56 for 1 h then treated Ap or BL with 50 µM Zn^{2+} in the presence or absence of 4 µM PT for 30 min. EGFR phosphorylation was measured by Western blotting with phospo-specific antibodies. B-1B. HAEC-ALI were pretreated with 10 µM c56 for 1 h followed by apically or basolaterally treatment with 50 ng/ml EGF for 30 min and EGFR and Erk phosphorylation were assessed by Western blotting.

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