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Role of Neprilysin in Airway Inflammation Induced by Diesel Exhaust Emissions

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Abstract

In this study, we examined the role of neprilysin (NEP*), a key membrane-bound endopeptidase, in the inflammatory response induced by diesel exhaust emissions (DEE) in the airways through a number of approaches: in vitro, animal, and controlled human exposure. Our specific aims were (1) to examine the role of NEP in inflammatory injury induced by diesel exhaust particles (DEP) using *Nep*-intact (wild-type) and *Nep*-null mice; (2) to examine which components of DEP are associated with NEP downregulation in vitro; (3) to determine the molecular impact of DEP exposure and decreased NEP expression on airway epithelial cells' gene expression in vitro, using a combination of RNA interference (RNAi) and microarray approaches; and (4) to evaluate the effects on NEP activity of human exposure to DEE. We report four main results: First, we found that exposure of normal mice to DEP consisting of standard reference material (SRM) 2975 via intratracheal installation can downregulate NEP expression in a concentration-dependent manner. The changes were accompanied by increases in the number of macrophages and epithelial cells, as well as proinflammatory cytokines, examined in bronchoalveolar lavage (BAL) fluid and cells. *Nep*-null mice displayed increased and/or additional inflammatory responses when compared with wild-type mice, especially in response to exposure to the higher dose of DEP that we used. These in vivo findings suggest that loss of NEP in mice could cause increased susceptibility to injury or exacerbate inflammatory responses after DEP exposure via release of specific cytokines from the

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

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OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Wong SS, Sun NN, Hugh BM, Witten ML, Burgess JL. 2010. Acute changes in sputum collected from exposed human subjects in mining conditions. Inhal Toxicol 22: 479–485.

Dr. Simon S. Wong's 2-year study, "The Molecular Effects of Diesel Exhaust Particulates on Respiratory Neutral Endopeptidase," began in September 2007. Total expenditures were $190,289. The draft Investigators' Report from Wong and colleagues was received for review in January 2010. A revised report, received in May 2010, was accepted for publication in June 2010. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and the Review Committee's Critique.

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lungs. Second, we found evidence, using in vitro studies, that downregulation of NEP by DEP in cultured human epithelial BEAS-2B cells was mostly attributable to DEP-adsorbed organic compounds, whereas the carbonaceous core and transition metal components of DEP had little or no effect on NEP messenger RNA (mRNA) expression. This NEP downregulation was not a specific response to DEP or its contents because the change also occurred after exposure to urban dust (SRM 1649a), which differs in physical and chemical composition from DEP. Third, we also collected the transcriptome profiles of the cells through a 2 × 3 factorial design. DEP exposure upregulated 151 genes and downregulated 59 genes. Cells with decreased NEP expression (accomplished by transfecting an NEP-specific small interfering RNA [siRNA]) substantially altered the expression of genes (upregulating 17 and downregulating 14) associated with DNA/protein binding, calcium channel activities, and the cascade of intracellular signaling by cytokines. Data generated from the combined RNAi and microarray approaches revealed that there is a complex molecular cascade mediated by NEP in different subcellular compartments, possibly influencing the inflammatory response. Fourth, in a controlled human exposure study, we observed significant increases in soluble NEP in sputum after acute exposure to DEE, with an average net increase of 31%. We speculate that the change in NEP activity in sputum, if confirmed in larger epidemiologic investigations at ambient exposure levels to DEE, may provide a useful endpoint and promote insight into the mechanism of DEE-induced airway alterations.

INTRODUCTION

UNCERTAINTY ABOUT UNDERLYING MECHANISMS OF HEALTH EFFECTS INDUCED BY AMBIENT PARTICULATE MATTER

Particulate matter (PM), especially with an aerodynamic diameter ::: 2.5 μm ($PM_{2.5}$), is a major risk to human health. Its acute and chronic adverse effects at the ambient level, particularly the association between elevated levels of PM and morbidity or mortality, have been demonstrated epidemiologically (Dockery et al. 1992, 1994; Peters et al. 1997; Salvi et al. 1999; Samet et al. 2000a, b; Schwartz et al. 2002). Studies over a broad range of geographic areas indicate that with each increase of 10 μg/m^3 in ambient particles, daily mortality increases approximately 1% to 5% (Pope et al. 2002; Schwartz et al. 2002). The adverse effects are especially apparent in the young or old and in those with preexisting cardiopulmonary disorders (Venn et al. 2001; Bateson and Schwartz 2004; Stenfors et al. 2004; McCreanor et al. 2007). Small short-term increases in $PM_{2.5}$ levels have been associated with increases in symptoms of certain conditions such as asthma, bronchitis, and airway hyperresponsiveness. Although PM effects in the airways are commonly manifested by irritation, inflammation, and functional impairment (HEI Diesel Working Group 1995; Veronesi and Oortgiesen 2001; Pandya et al. 2002; Nel 2005), the precise mechanisms that control these responses to inhaled particulates are still poorly understood.

In vivo and in vitro experimental observations have suggested that the integrity of the targeted epithelium, especially its capacity to prevent or recover from oxidative injury, is central to the inflammatory reaction. Acute exposure to $PM_{2.5}$, or its components, has been shown to induce release of mediators such as interleukin 6 (IL-6), the chemokine IL-8, epidermal growth factor, and granulocyte-macrophage colony-stimulating factor (Boland et al. 2000). Long-term exposure results in damage identified histopathologically, such as

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bronchioli wall thickening, concomitant with the presence of numerous particles in the lungs of exposed individuals (Churg et al. 2003). At the molecular level, the proinflammatory responses are in part mediated through the activation of many signaling transduction pathways. These may involve transcription factors (nuclear factor-kappa B [NFKB2], activator protein-1 [AP-1], and the signal transducer and activator of transcription 3 [Stat3]) (Takizawa et al. 1999, 2003; Zhang et al. 2004; Cao et al. 2007) and mitogen-activated protein kinases (MAPKs) (Fahy et al. 2000; Hashimoto et al. 2000) via reactive oxygen species–dependent mechanisms (Baulig et al. 2003). While substantial progress has been made in understanding inflammatory pathogenesis after PM-induced injury, other regulatory mechanisms remain to be studied.

Macrophages, another cell type found in the lung, are also part of the first line of host defense and play a critical role in the cellular response of the airways to inhaled PM. They are located largely on the epithelial surfaces and act as a barrier to inhaled matter by phagocytosing particles and intracellularly processing them (Bowden 1984; Kreyling 1992). It has been shown that there is a direct, positive relation between the extent of PM exposure and the number of macrophages present in the lungs (Brain 1992). However, the macrophages' phagocytic and chemotactic functions are inhibited when the volume of the phagocytosed particles is greater than 60% of their total internal volume (Morrow 1988; MacNee and Donaldson 2003). In his 1988 study, Morrow observed that even if phagocytosed PM constituted only 6% of the total volume of macrophages, the ability of the airway macrophages to migrate to the mucociliary escalator was compromised (Morrow 1988). Phagocytosis of certain particles, such as silica and those from fire smoke, can damage or kill macrophages, releasing their contents, which then fuels an inflammatory response (Bowden 1984; Wong et al. 2004). Furthermore, activation of macrophages after PM phagocytosis releases copious amounts of oxygen radicals, neurokinins, proteases, proinflammatory cytokines, and growth-regulating proteins that may be involved in the pathogenesis of both acute and chronic lung inflammation (Oberdörster et al. 1992; Zhou et al. 2007). In addition, exposure to PM also compromises the macrophage response to infectious agents, possibly via an oxygen-radical–mediated process, by decreasing the cell's ability to phagocytose bacteria (Becker and Soukup 1998; Yang et al. 1999; Mundandhara et al. 2006). These studies suggest that the functional capacity of macrophages is affected by exposure to PM in such a manner that host lung defenses and immune functions are decreased.

NEP AND ITS PHYSIOLOGIC IMPORTANCE

NEP (also known as neutral endopeptidase, EC 3.4.24.11, enkephalinase, and common acute lymphoblastic leukemia antigen) is a membrane-bound zinc-dependent endopeptidase. Structurally, NEP is a 90–110 kDa type II integral membrane glycoprotein consisting of a short N-terminal cytoplasmic domain, a transmembrane hydrophobic region, and a large extracellular domain that contains the catalytic site. The human *NEP* gene is located on chromosome 3 (q21–q27). NEP plays a central regulatory role in the maintenance of homeostasis and regulation of sensory reflexes including apnea, laryngeal narrowing, bronchoconstriction, sneezing, aspiration, and expiration reflexes (Martins et al. 1990; Nadel 1991). NEP is also involved in the pathogenesis of cardiopulmonary diseases, Alzheimer

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disease, and cancer (Bozic et al. 1996; El-Amouri et al. 2008; Iijima-Ando et al. 2008). The role of NEP in several organs has also been documented (Borson 1991; Joos 2000). The following is a brief summary of background information for the current study, culled from the large body of literature on NEP.

Cellular Distribution of NEP—Investigators have examined the cellular distribution of NEP by using specific antibodies, measuring its expression in different cell types. NEP is widely distributed in mammalian tissues. In the lung, NEP is abundantly expressed on airway or alveolar epithelial cells and is present in airway smooth muscle cells, submucosal gland cells, fibroblasts, postcapillary venules, and nerves (Painter et al. 1988; Baraniuk et al. 1995). Moreover, NEP is expressed on neutrophils and macrophages (Johnson et al. 1985a,b).

Physiologic Function of NEP—NEP is an enzyme with broad specificity. It effectively controls the bioavailability of peptide mediators. It maintains low levels of its substrates in the extracellular fluid under basal conditions. These substrates include tachykinins, endothelins, angiotensin II, bombesin, gastrin-releasing peptide, atrial natriuretic peptide, enkephalins, insulin B chain, and the chemotactic peptide N-formyl-met-leu-phe. Most of these substrates are neurotransmitters and proinflammatory mediators, which are released from sensory nerve terminals and activate immunoinflammatory cells such as neutrophils, eosinophils, lymphocytes, and macrophages (Nadel 1991). NEP substrates play important roles in numerous physiologic and pathophysiologic processes, including inflammatory processes (Lotz et al. 1988; Lilly et al. 1994; Di Maria et al. 1998), hyperresponsiveness (Dusser et al. 1989; Wu and Lee 1999), and carcinogenesis (Nanus 1998; Papandreou et al. 2000; Suzuki et al. 2001; Tomoda et al. 2003; Sumitomo et al. 2004). The NEP cytoplasmic tail plays a role in providing a scaffold for signaling proteins in the regulation of cell repair pathways and the organization of the membrane-associated cytoskeleton (Iwase et al. 2004; Sumitomo et al. 2004). The almost ubiquitous distribution of NEP, with its broad substrate specificity, suggests it has a role in the cleavage of different peptides involved in several functions. When NEP expression or activity is inhibited, its substrates are less rapidly inactivated and accumulate in tissue (Martins et al. 1990; Wong et al. 2004), thus contributing to an exaggerated response or increased susceptibility to environmental stressors.

Role of NEP in Lung Disease—NEP plays a key role in airway homeostasis and the development of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) (Wong et al. 2003), asthma (Lundberg et al. 1991; van Der Velden et al. 1999), chronic obstructive pulmonary disease (COPD) (Lotz et al. 1988), and lung cancer (D'Adamio et al. 1989; Shipp et al. 1991). In addition, several reviews (Borson 1991; Nadel 1991; Di Maria et al. 1998) of the role of NEP in lung airways suggest that upregulation of *NEP* gene expression may be one mechanism of the anti-inflammatory action of glucocorticoids.

NEP is a critical protective enzyme in limiting the activity of endogenously released substance P (SP), abnormal levels of which may be involved in the pathogenesis of ALI/ARDS. Loss of NEP activity clearly leads to a persistent increase in endogenous SP, which may in turn lead to exaggerated microvascular permeability, edema, and severe hypoxia. It

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is well known that SP, as a potent proinflammatory mediator, activates numerous signaling transduction pathways involving a complex network of chemokines, cytokines, reactive oxygen/nitrogen species, and other mediators. SP released in the lungs may signal immuno-inflammatory cells to generate these mediators through neurokinin (NK) receptors that may not be involved in the cellular responses to SP under normal physiologic conditions. The affected cell populations include neutrophils, eosinophils, lymphocytes, and macrophages, which express NK-1R on their cell surfaces. Once these cells are activated, an uncontrolled inflammatory cascade develops and progresses, with the involvement of multiple immuno-inflammatory cells and their mediators. This is similar to the early pathophysiologic processes of ALI/ARDS. A substantial disruption of NEP occurs, and high microvascular permeability and pulmonary edema may develop. This scenario is supported by previous experimental observations (Fine et al. 1989), which showed that removal of the airway epithelium cells or inhibition of NEP could induce airway hyperresponsiveness to SP.

Day and colleagues (2005) reported that NEP activity determines the severity of pancreatitis-associated lung injury. *Nep*-null and *Nep*-intact mice pretreated with the NEP antagonist phosphoramidon (10 mg/kg) had significant elevations of lung myeloperoxidase and worsened lung histology compared with *Nep*-intact mice given elastase to induce lung injury (Day et al. 2005). As observed in our animal models of ALI/ARDS–like injury, fire smoke inhalation induced a dose-dependent reduction in pulmonary NEP activity beginning as early as one hour after insult (Wong et al. 2004). The changes in NEP activity observed through immunohistochemistry were mainly attributable to damage of the epithelial lining, such as membrane disruption, necrosis, and sloughing of epithelial cells in the airways from the trachea to bronchioles of the smoke-exposed animals (Wong et al. 2004). Additionally, the causes of loss of NEP activity in this model could include inhibition of NEP activity by oxidants from combustion products such as nitrogen dioxide (NO_2), ozone (O_3), and particulates, but also from the thermal denaturing of plasma proteins and from inflammatory cell activation.

Bronchial asthma and COPD are conditions in which the tone of airway smooth muscle, airway secretions, bronchial circulation, and inflammatory and immune cells are affected. These responses are highly regulated by NEP, which is predominantly responsible for controlling levels of tachykinins in the lungs; uncontrolled or exaggerated responses due to decreased NEP show many similarities to the clinical symptoms of patients with bronchial asthma and COPD (Di Maria et al. 1998; Joos et al. 2000; Wick et al. 2011). Moreover, NEP may play a key role in the development of childhood asthma (Joos et al. 2000), not only because of the vulnerable nature of the children's lungs, but also because the airway sensory innervations of a neonate develop rapidly during early postnatal life in parallel with the developing lung (Hislop et al. 1990). Additionally, NEP expression in airway epithelium is age-dependent and implicated in the regulation of peptides associated with normal lung growth in the fetus (Sunday et al. 1992).

Cohen and colleagues (1996) showed that NEP mRNA was not expressed or was at a low level in most lung cancer cell lines. Protein expression and activity for NEP were also reduced or undetectable in most small-cell lung carcinoma (SCLC), adenocarcinoma tumors, and lung adenocarcinoma cell lines. Similarly, NEP mRNA was undetectable in SCLC,

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adenocarcinoma, squamous cell carcinoma, and carcinoid tumors of the lung (Cohen et al. 1996). Genetically targeted disruption of the *Nep* locus in mice resulted in an enhanced lethality to endotoxin, indicating an important protective role for NEP in septic shock (Lu et al. 1996, 1997).

DEP TOXICITY ON NEP IN THE AIRWAYS

Expression of NEP varies widely in normal human lung tissue taken from different individuals (Cohen et al. 1999). This variation could be attributed to either environmental or individual genetic factors. Studies have shown that a variety of environmental factors, including exposure to viruses, allergens, cigarette smoke, and respiratory toxins, are able to reduce NEP activity, thus enhancing the effects of neuropeptides within the airways (Turner et al. 1993; Lilly et al. 1994; Sun et al. 2004; Wong et al. 2004). Therefore, we initially hypothesized that DEP exposure in rats could downregulate pulmonary NEP. Our studies have indicated that NEP activity in rat lungs was significantly reduced by exposure to DEP (Wong et al. 2003; Witten et al. 2005). Because of the high density of NEP expression in airway epithelium and NEP's important regulatory role, it is not surprising that a reduction in NEP is accompanied by increases in bronchopulmonary plasma extravasation, vascular permeability, cytokine expression, and inflammatory mast cell infiltration, possibly evoked by abnormally high levels of peptides after DEP exposure. Reduced NEP activity may set up the airways to respond in an exaggerated fashion to irritation and other inflammatory mediators, thus producing a hyperresponsive state. Therefore, we speculate that a decrease in NEP activity after exposure to DEE may be a precursor in lungs for the ultimate loss of NEP expression. This may contribute to the DEE-related increase in health risk, including the risk of asthma, COPD, and lung cancer. This concept is strongly supported by numerous investigations reporting a decrease of NEP in first- and secondhand cigarette smokers and the loss of NEP due to lung cancer and in most cell lines derived from lung cancers (as well as many other human malignancies) (Shipp et al. 1988, 1991; Dusser et al. 1989; Cohen et al. 1996; Papandreou et al. 2000). Loss of NEP may be implicated in the broad, adverse effects observed in other organ diseases, such as cardiovascular diseases and Alzheimer disease. We believe that the inhalation of particles is an important environmental factor in the decrease or loss of NEP, which is directly or indirectly involved in the development of PM-related disorders.

Our pilot study (Wong et al. 2007 pilot study) sought to extend our earlier findings in rats (Wong et al. 2003; Witten et al. 2005). We exposed human airway epithelial cells to DEP (0–40 μg/mL, noncytotoxic) for 24 hours and observed the downregulation of both NEP expression and enzymatic activity in a concentration-dependent manner (Wong et al. 2007 pilot study). A substantial decrease (90%) in NEP mRNA expression occurred with exposure to 5 μg/mL DEP. A further stability test indicated that NEP downregulation by DEP occurs at the transcriptional level (Wong et al. 2007 pilot study). It is likely that the *NEP* gene promoter may have DEP responsive elements that are activated via signal transduction pathways after DEP challenge. Alternatively, the *NEP* gene may be directly affected by certain components of DEP. Using RNAi technique, we showed that the cell proliferation of NEP siRNA-infected cells was inhibited in a concentration-dependent manner with increased DEP concentrations ($P < 0.01$) (Wong et al. 2007 pilot study). The

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net proliferation inhibition rates at 5, 10, 20, and 40 μg/mL DEP were 1.4%, 7.6%, 10%, and 14.4%, respectively, when compared with their controls. This finding suggests that NEP per se is involved in cell proliferation in the presence of DEP. We interpreted our pilot study (Wong et al. 2007 pilot study) as indicating that DEP exposure resulted in the downregulation of NEP expression at the transcriptional level, which was associated with a change in cell proliferation, an important process in pathophysiology. However, the mechanisms of NEP downregulation by DEP and its relevance to these broad adverse health effects need to be investigated.

We believe that DEP is an important contributor to NEP downregulation in the airways. The decrease or loss of NEP expression and/or activity may cause a greater susceptibility to respiratory disorders due to dysregulated respiratory irritation, inflammatory response, and tissue repair or remodeling. To our knowledge, no previous cellular, animal, or human studies have been conducted on the effects of PM on airway epithelial NEP and its relevance to the development or exacerbation of airway disorders. Given that NEP is an important regulator of numerous pathophysiologic processes and is affected by inhaled particulates, the study of epithelial NEP is of considerable importance in the understanding of PM-induced adverse health effects.

SPECIFIC AIMS

In the current study, we had the following four specific aims:

1. To examine the role of NEP in DEP-induced inflammatory injury using *Nep*-intact and *Nep*-null mice.
2. To examine which components of DEP are associated with NEP downregulation in vitro.
3. To determine the molecular impact of DEP exposure and decreased NEP expression on airway epithelial cells' gene expression in vitro, using a combination of RNAi and microarray approaches.
4. To evaluate the effects on NEP activity of human exposure to DEE.

HYPOTHESIS

Based on our previous study (Wong et al. 2007 pilot study), we hypothesize that certain components of DEP downregulate the functional expression of airway epithelial NEP via a transcriptional mechanism. Consequently, the affected cells' metabolic, cellular, and regulatory functions in the maintenance of cellular homeostasis are compromised, leading to an exacerbated inflammatory response or increased susceptibility to injury.

There were five rationales for this hypothesis: First, NEP is abundantly expressed on airway epithelial cells and is directly targeted by DEP. Second, structurally NEP is a cell-surface metalloprotease with a large extracellular domain (700 amino acids), which contains six potential N-glycosylation sites and the pentapeptide consensus sequence (His-Glu-[Ile, Leu, Met]-X-His) of zinc-binding metalloproteases, in which the two histidines link up zinc and glutamic acid. NEP's large extracellular domain containing the catalytic sequence may

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explain not only its critical structural ability to rapidly cleave substrates, but also its tendency to be highly susceptible to toxic insults. Third, NEP plays a central regulatory role in the maintenance of homeostasis and in the development of pathophysiologic processes involved in ALI or ARDS, asthma, COPD, and lung cancer. Fourth, a significant decrease in NEP activity in lung tissue has been demonstrated in rats exposed to ambient and occupational levels of diesel exhaust (DE) (Wong et al. 2003). Fifth and finally, exposure of human airway epithelial cells to DEP (0–40 μg/mL, noncytotoxic) for 24 hours downregulates NEP expression, as well as its enzymatic activity, in a concentration-dependent manner (Wong et al. 2007 pilot study).

METHODS AND STUDY DESIGN

AIM 1: TO EXAMINE THE ROLE OF NEP IN DEP-INDUCED INFLAMMATORY INJURY USING *Nep*-INTACT AND *Nep*-NULL MICE

Experimental Design—Specific-pathogen–free wild-type and *Nep*-null mice (from Dr. Craig Gerard of Harvard Medical School) on a *C57BL/6* background (50/50 male/female, approximately 8 weeks old, weighing approximately 25 g) were used in this part of the study. The mice were bred and housed in the American Association of Animal Laboratory and Care (AAALAC)–approved animal facility at the University of Arizona Health Sciences Center. The mice were on a 12h:12h light–dark cycle and were given a standard mouse chow diet, tap water ad libitum, and filtered air. In order to simultaneously characterize the dose–response of DEP on respiratory NEP and examine the role of NEP in response to DEP exposure, we utilized a 2 × 3 factorial design (66 mice = 11 mice/group × 6 groups; wild-type vs. *Nep*-null mice exposed to control, low, and high doses of DEP). We examined the differences in DEP-induced pulmonary response between *Nep*-null and wild-type mice, as measured by BAL cell profile, cytokines, cell proliferation, and histopathologic evaluation. In addition, NEP protein expression in the lung tissues of wild-type mice was quantified by DuoSet (R&D Systems, Minneapolis, MN) enzyme-linked immunosorbent assay (ELISA) and localized by immunohistochemistry.

DEP Instillation—Considering that DEP composition is highly variable, depending on, among other factors, engine type and engine load (Madden et al. 2003), we utilized standard DEP (SRM 2975; National Institute of Standards and Technology, U.S. Department of Commerce) originally generated by a diesel-powered industrial forklift and whose physical aspects and chemical composition have been well characterized. SRM 2975 was instilled through an intratracheal cannula under anesthesia. This technique, although a non-physiologic method of administration, is useful for comparative studies in which collected samples cannot easily be used in inhalation exposures. The doses used in this study were 10 and 100 μg for the low and high exposure levels, respectively, of DEP instillation. These doses were chosen to simulate a continuous 7-day inhalation exposure of 50 and 500 μg/m^3, using the following equation:

$$\text{Dose}=50\mu\text{g/m}^3 \text{ or } 500\mu\text{g/m}^3 \times 1/2 \times 0.06\text{m}^3/\text{ day } \times 7 \text{ days},$$

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where 50 μg/m^3 and 500 μg/m^3 were the low and high levels of DEP exposure per day, respectively; 1/2 is the deposition coefficient of PM in the lung; and 0.06 m^3/day is the daily inhalation volume for a mouse.

Briefly, we first anesthetized mice with an intramuscular injection mixture of ketamine hydrochloride (80 mg/kg; Parke-Davis, Morris Plains, NJ), xylene (10 mg/kg; Mobay, Shawnee, KS), and acepromazine maleate (3 mg/kg; Fermenta, Kansas City, MO). These animals were then intubated by a nonsurgical technique. Using a bulb-headed cannula inserted approximately 10 mm into the trachea, we instilled a suspension containing either 10 or 100 μg DEP in 100 μL phosphate-buffered saline (PBS) that was free of endotoxins and Ca^{2+}/Mg^{2+}, followed by 100 μL air. The suspension of DEP was sonicated on ice for 1 minute before instillation, using a model 250 Sonifier (Branson Ultrasonic Corporation, Danbury, CT) at a moderate level of 20 W. Sham control animals received 100 μL PBS only, followed by 100 μL air. In our experience, the instillation of 100 μL PBS does not cause any measurable stress effects, such as the expression of cytokines. Animals were treated humanely and with regard for alleviation of suffering. Compliance with AAALAC-approved animal protocol (#04-104) ensures that these animals were cared for and treated according to NIH guidelines.

Bronchoalveolar Lavage and Cell Count—At 7 days after DEP or PBS instillation, we euthanized the anesthetized animals (N = 7) by exsanguination of the abdominal aorta, and removed their lungs and cannulated them with a Teflon intravenous catheter. The lungs were lavaged three times with sterile isotonic saline (Baxter, Deerfield, IL) at a volume of 1 mL for each wash. BAL fluid was centrifuged at 4°C for 15 minutes at 500*g*. We stored the supernatant and lung tissue at −75°C for cytokine assays and NEP expression. We determined the number of cells using a hemocytometer and performed differential cell counts by counting 300 cells per slide on a Diff-Quik–stained (Dade Diagnostics, Aguada, PR) cytocentrifuged slide.

Protein Expression Quantification Using ELISA—We determined the interleukin (IL)-1[3, IL-6, and IL-10 concentrations in BAL fluid using the commercially available Enzyme Immunoassay Kits (R&D Systems). We quantified each sample in triplicate and then averaged it to obtain the final value. We used a BioTek ELx808IU microplate reader (BioTek Instruments, Winooski, VT) for spectrophotometer analyses at a reading wavelength of 450 nm and a reference wavelength of 570 nm for each analysis.

We followed the protocol for ELISA kits provided by the manufacturer to quantify NEP protein in lung tissue extracts of wild-type mice (R&D Systems). We coated a clear 96-well microplate with 100 μL of goat anti-mouse NEP antibody (as the capture antibody) per well at a concentration of 0.8 μg/mL in PBS and incubated it overnight at room temperature. We then washed the wells three times with 400 μL of wash buffer and blocked them with 300 μL of Reagent Diluent at room temperature for a minimum of 1 hour. The plates were washed three times with 400 μL of wash buffer. We added various dilutions (0–6000 pg/mL) of NEP and the sample (total volume, 100 μL) to the wells and incubated them for 2 hours at room temperature. After washing, biotin-conjugated donkey antigoat IgG secondary antibody (as the detection antibody) diluted at 400 ng/mL in Reagent Diluent was added to each well and

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incubated for 2 hours at room temperature. We then washed the wells again and incubated them at room temperature for 20 minutes with 100 μL streptavidin conjugated to horseradish-peroxidase diluted in Reagent Diluent. After the wells were washed, we added 100 μL freshly prepared substrate solution (0.55 mg of 2,2'-azino-bis- 3-ethylbenzothiazoline-6-sulfonic acid [ABTS]/mL and 0.001% H_2O_2 in 0.1-M citrate buffer, pH 4.3) to each well and incubated them at room temperature in the dark. After 20 minutes, we stopped the reaction by adding 50 μL 2N sulfuric acid to the wells. We read the absorbance at 450 and 570 nm with a BioTek ELx808IU microplate reader.

Immunohistochemistry—To directly analyze epithelial cell proliferation, we administered 100 mg/kg 5-bromo-2'-deoxyuridine (5-BrdU; Sigma, St. Louis, MO) intraperitoneally to mice 24 hours before euthanasia. Fixed tissue sections (4 μm) were deparaffinized, hydrated, and pretreated with blocking solution to decrease nonspecific antibody binding. We used a biotinylated monoclonal mouse anti-BrdU antibody (Zymed, South San Francisco, CA) according to the manufacturer's protocol, followed by a streptavidin-peroxidase–conjugated secondary antibody and DAB substrate (Zymed). We counterstained sections with hematoxylin. We calculated a BrdU-labeling index for airways with epithelial cells incorporating BrdU by counting the number of BrdU-expressing nuclei relative to the total number of epithelial cells within 50 to 75 cell regions of the airways. We analyzed a total of 200 to 500 cells in each lung section.

We localized NEP protein in fixed lung tissue (N = 4/group) of wild-type mice using the protocol provided by Santa Cruz Biotechnology. Briefly, slides were deparaffinized three times using xylene for 5 minutes each and hydrated through graded ethanol solutions. We quenched the endogenous peroxidase activity on the slides by incubating them in 0.1% H_2O_2 for 5 minutes. We blocked non-specific binding with 10% horse or goat serum in PBS. After washing, we added goat antimouse NEP antibody (4 μg/mL in PBS) (R&D Systems) to each slide, and we incubated them for 2 hours at room temperature in a humidified chamber. The slides were rinsed in PBS and subsequently incubated with biotinconjugated donkey anti-goat IgG secondary antibody for 30 minutes. The slides were then incubated for 30 minutes in avidin-biotinylated horseradish peroxidase complex and substrate. After dehydration, we immediately mounted and observed the slides using light microscopy.

AIM 2: TO EXAMINE WHICH COMPONENTS OF DEP ARE ASSOCIATED WITH NEP DOWNREGULATION IN VITRO

Experimental Design—In order to clarify the contribution of major components of DEP in the downregulation of NEP, we compared the effects induced by (1) untreated DEP; (2) DEP treated with chelators to remove divalent cations, particularly transition metals (cDEP); and (3) DEP treated with dichloromethane to remove everything but the carbonaceous core, or "stripped DEP" (sDEP). We exposed BEAS-2B cells (described in the next section) to three noncytotoxic concentrations (0, 1, and 10 μg/cm^2) of DEP (SRM 2975), cDEP, and sDEP for 24 hours. We ran parallel cell cultures with no DEP vehicle (see "Preparation of DEP," below) as time-course controls. To determine whether the observed NEP downregulation was a specific response to DEP, we utilized standard urban dust (SRM

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1649a) over the same concentration range as that of DEP (called here, oDEP). In all cases, the cells were monitored visually for viability and collected for measurements of NEP mRNA expression by using real-time polymerase chain reaction (RT-PCR) analysis or particle uptake by transmission electron microscopy. (Details on the methods used in the particle uptake portion of the experiment supporting Aim 2 can be found in Appendix A, available on the HEI Web site: www.healtheffects.org.)

Cell Culture—In our in vitro study, we used the BEAS-2B cell line—an immortalized human bronchial epithelial cell line transformed by an adenovirus 12-SV40 hybrid virus (American Type Culture Collection #CRL-9609, Manassas, VA). This cell line exhibits genotypic and phenotypic characteristics of human bronchial epithelial cells and so is broadly used to study in vitro molecular or cellular effects of agents that affect the airways. We cultured the cells as recommended by the suppliers. Briefly, BEAS-2B cells were used at passages 4–8 and maintained in complete keratinocyte growth medium (KGM) consisting of keratinocyte basal medium (KBM; Clonetics, San Diego, CA) and supplemented with bovine pituitary extract, human epidermal growth factor (5 ng/mL), hydrocortisone (0.5 mg/mL), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), and insulin (5 mg/mL). We seeded cells on 6-well plates (2.5×10^4 cells/well) in 2 mL of complete KGM medium. Cells were grown to between 85% and 95% confluence in 6-well plates and then were treated with the different sets of particles described in the following section. We cultivated all cells under a humidified atmosphere of 5% carbon dioxide (CO_2) and 95% air at 37°C. Cell toxicity was determined using the trypan blue-exclusion assay.

Preparation of DEP—We prepared a stock solution (2 mg/mL) of DEP by sonicating 10 mg SRM 2975 in 5 mL of 0.0025% culture solution containing Tween 80 (Sigma-Aldrich, St. Louis, MO) at 50 W for 2 minutes using a model 250 Sonifier (Branson Ultrasonic Corporation, Danbury, CT). We obtained sDEP by extracting 10 mg SRM 2975 in dichloromethane in a Soxhlet extractor (Sigma-Aldrich). The collected particles were extracted a second time to remove additional organic compounds and then suspended in 5 mL of 0.0025% culture solution containing Tween 80. For the cDEP preparation, we used a published method (Zhou and Kobzik 2007) that utilizes Chelex 100 (Bio-Rad, Hercules, CA), which preferentially removes divalent cations, particularly transition metals. We suspended 10 mg SRM 2975 in 2% Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA) and centrifuged it at 8000 rpm for 5 minutes at 4°C. We recovered the supernatant (soluble fraction) and then added 50 mg/mL Chelex beads and mixed them on a rotating wheel for 4 hours at room temperature. After incubation, we centrifuged samples at 13,000 rpm for 5 minutes to convert the Chelex beads into pellets. The same amount of cDEP suspension as used with other DEP subtypes was mixed with culture solution. Finally, we added these resultant suspensions to cultured cells at a concentration of either 1 or 10 $\mu g/cm^2$, based on the original mass of particles before extraction. The DEP suspension and control were added at equal volume to the cells.

RNA Preparation and RT-PCR—Cells were incubated with DEP (SRM 2975), cDEP, sDEP, or urban dust (SRM 1649a) for 24 hours. Using the Aurum Total RNA Mini Kit according to the manufacturer's protocol (Bio-Rad), we isolated total RNA from BEAS-2B

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cells. We incubated all samples with ribonuclease-free deoxyribonuclease (DNase) (20 U/reaction) for 10 minutes at 37°C to eliminate DNA contamination. We quantified the RNA concentration by ultraviolet spectrophotometry at 260 nm and determined the purity by the 260-to-280-nm absorbance ratio (SpectraMax PLUS, Molecular Devices, Sunnyvale, CA). We verified the integrity of the RNA by electrophoresis on a 1.2% agarose gel containing form-aldehyde (2.2 mol/L) and ethidium bromide in 1 × 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (40 mmol/L MOPS [pH 7.0], 10 mmol/L sodium acetate, and 1 mmol/L ethylenediaminetetraacetic acid [pH 8.0]). One microgram of total RNA treated with DNase I was reverse-transcribed using the iScript complementary DNA (cDNA) synthesis kit (Bio-Rad), according to the manufacturer's protocol. Subsequently, polymerase chain reactions (PCRs) were set up in 96-well plates, each containing 0.3 μM of oligonucleotide primer, 1 × SYBR Green Supermix (Bio-Rad), and 1 μL of cDNA synthesis reaction (in a total volume of 20 μL). All primer sequences designed specifically for Specific Aims 2 and 3 are listed in Table 1. Reactions were run and analyzed on a Bio-Rad iCycler iQ Real-Time PCR detection system. We determined the cycling parameters and analyzed the resulting data according to Applied Biosystems' protocols. Briefly, data were analyzed using the comparative C_T method as a means of relative quantitation, normalized to an endogenous reference (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and 18S ribosomal RNA [18S rRNA]) and relative to a calibrator (a normalized C_T value obtained from vehicle-treated BEAS-2B cells) and expressed as 2^{-CT} according to Applied Biosystems' *User Bulletin 2: Rev. B: Relative Quantitation of Gene Expression*.

AIM 3: TO DETERMINE THE MOLECULAR IMPACT OF DEP EXPOSURE AND DECREASED NEP EXPRESSION ON AIRWAY EPITHELIAL CELLS' GENE EXPRESSION IN VITRO, USING A COMBINATION OF RNAi AND MICROARRAY APPROACHES

Experimental Design—A test based on a 2 × 3 factorial design (NEP siRNA-transfected cells vs. mock controls, and controls vs. low and high DEP exposure) was conducted. This involved NEP siRNA–transfected BEAS-2B cells or mock control cells being exposed to 0 (control), 10, and 40 μg/cm^2 DEP (SRM 2975) for 24 hours. We monitored the cells visually for cell viability and collected them for microarray analysis. With this experimental design, we aimed to dissect complex transcriptional responses mediated by (1) DEP exposure (concentration–effects) and (2) NEP downregulation.

NEP siRNA Assay—We seeded BEAS-2B cells in a 6-well tissue culture plate, at 3000 cells per square centimeter in 2 mL antibiotic-free normal KGM medium. We incubated the cells at 37°C in a CO_2 incubator until the cells were 60% to 80% confluent. The NEP-specific siRNA or a control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was transfected using Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly, for each 6-well transfection, 3.6 μL of 10-μM siRNA duplex was diluted into 40 μL siRNA Transfection Medium (Solution A), and 2.4 μL of siRNA Transfection Reagent was diluted into 40 μL siRNA Transfection Medium (Solution B). Solution A was added directly to Solution B using a pipette and then mixed and incubated for 20 minutes at room temperature. After incubation, we added 0.32 mL siRNA Transfection Medium to each tube containing the siRNA–siRNA Transfection Reagent complex and mixed gently. The mixture was overlaid onto the washed cells and incubated

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for 5 to 7 hours at 37°C in a CO_2 incubator. Then, we added 0.4 mL of normal growth medium containing 2 times the normal serum and antibiotics concentration (2 × normal growth medium) and incubated the cells for an additional 24 hours. Equal amounts of total protein (20 μg) from cultured plates were separated on 12% poly-acrylamide gels and transferred to a nitrocellulose membrane. We performed Western blotting (described below), using mouse monoclonal antibodies to human NEP (SN5c) and [3-actin, as a control (both from Santa Cruz Biotechnology), to confirm the knockdown efficiency of NEP siRNA.

Western Blotting—We harvested BEAS-2B cells after no treatment, treatment with control siRNA, and treatment with NEP siRNA and washed them with PBS. Then the cells were lysed with 200 μL of Mammalian Protein Extraction Reagent (M-PER) with Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL) according to the manufacturer's protocol. We performed protein quantification using the Coomassie Plus Kit (Pierce). Samples were resolved using electrophoresis on a 4% to 20% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose. We quantified the signal intensity of the blots using densitometry. We scanned the film on a GS-700 Imaging Densitometer (Bio-Rad) and quantified it using Bio-Rad Quantity One software, version 4.1.1. NEP protein expression levels were normalized to [3-actin.

Microarray Analysis—BEAS-2B cells were treated with 0 (control), 10, or 40 μg/cm^2 DEP (SRM 2975), for 24 hours. We then isolated total RNAs from these cultured cells using the Qiagen RNeasy minikit (Valencia, CA) according to the manufacturer's instructions. The isolated total RNAs were used to produce a labeled target, hybridized to Affymetrix human U133 Plus 2.0 GeneChips, and read using the Agilent/Affymetrix 2500A scanner (Santa Clara, CA) according to the manufacturer's protocol. We analyzed the raw data (CEL files) using the GC-RMA algorithm as implemented in GeneSpring software, version 7.0 (Silicon Genetics, Redwood City, CA) to produce a normalized transcript-level signal for further analysis. We loaded transcript-level signal data from GeneSpring into BRB-ArrayTools (linus.nci.nih.gov/BRB-ArrayTools.html) and filtered them based on the variance of each transcript across samples. This method of filtering provided an unbiased selection of transcripts, independent of the tissue classes. We compared the variance of the log ratios for each transcript to the median of all transcript variances and selected transcripts whose variance was significant ($P < 0.05$).

Five steps were used to identify transcripts of interest as potential NEP-related pathways. First, an analysis of variance (ANOVA) was performed to identify transcripts with significantly ($P < 0.05$) altered expression compared with background controls. Second, our power analysis showed that the number of samples analyzed in each class could reliably detect 5-fold differences in expression. Thus, transcripts with less than a 10-fold difference in expression between DEP exposure and controls were further filtered out of those that had already been identified by ANOVA. Third, we compared the transcript differences (a) after doses of 0 (control), 10, or 40 μg/cm^2 DEP; and (b) between NEP siRNA–transfected and mock control cells. These analyses not only identified transcriptional responses mediated by NEP and DEP exposure, but also ruled out nonspecific responses to siRNA expression (using NEP siRNA–transfected cells vs. mock controls without DEP exposure). Fourth, we

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analyzed the data with the GeneSpring GX 9 software package, including unbiased cluster analyses (hierarchical clustering, k-means clustering, and self-organizing map). For each cluster, we analyzed the expression patterns and assigned the major functional categories using gene ontology (www.geneontology.org). Data on individual expression changes were sorted into potential biologic pathways using GenMAPP (www.genmapp.org), a recently developed tool for visualizing expression data in the context of biologic pathways. Last, we confirmed the expression of some key genes by using RT-PCR as stated earlier.

AIM 4: TO EVALUATE THE EFFECTS ON NEP ACTIVITY OF HUMAN EXPOSURE TO DEE

Experimental Design—This phase of the current study was approved by the University of Arizona (UA) Institutional Review Board. The study methods are described in greater detail in a previous publication (Burgess et al. 2007). Informed consent was obtained from all subjects volunteering to participate in the study. Mining students undergoing undergraduate and graduate training in mining engineering at UA were eligible for participation if they were 18 years or older. Students who were current or previous smokers, had existing lung diseases, and/or were taking inhaled steroids were excluded. None of the subjects were current asthmatics, and no subjects reported taking anti-inflammatory medications. Eleven subjects — 10 males and 1 female, ranging in age from 19 to 33 years (mean 23.7 ± 4.3 yr)—completed the study (Table 2). Seven (64%) of the subjects described themselves as White, 2 (18%) as Hispanic, and 1 each as Asian and other. At baseline and before the exposure experiments, none of the subjects reported having had a cold, flu, allergies, or any respiratory symptoms within the previous 6 days. These subjects had no record of having been exposed to very high levels of emissions exhaust, room dust, drilling mist, or other particulate sources within 1 month before baseline or exposure.

The study was carried out at the San Xavier Underground Mining Laboratory, a research and training facility devoted to occupational health and safety in the mining and underground construction industries, operated under the auspices of the UA College of Engineering in collaboration with the UA College of Public Health. Mine access and ventilation are designed to simulate underground conditions found in an actual production facility. Respiratory protection was not worn during the exposures. Changes of cell numbers by type, protein, and NEP activity in the collected sputum of subjects were evaluated as the difference between baseline and after-exposure values.

Exposure Conditions—DEE exposure was characterized from a diesel-powered 1984 Jarvis Clark JS-220 load-haul-dump (LHD) vehicle (Mackwood Group, Elliot Lake, Ontario, Canada) with a 2-cubic-yard bucket and an 82-hp Deutz F6L-912W diesel engine fitted with a catalytic converter. Experiments using the LHD were conducted in a conventional 4 m × 4 m tunnel decline, and a 15-hp axial auxiliary fan provided ventilation during exposure. During baseline and the DEE exposure, two samples collected over a period of 66 to 68 minutes demonstrated background particulate matter (PM) concentrations of less than 10 $\mu g/m^3$. DEP was collected on precleaned 37-mm open-face quartz fiber filters (SKC, Eighty Four, PA) with Escort ELF personal sampling pumps (Mine Safety Appliances, Pittsburgh, PA) and analyzed for elemental carbon according to National Institute for Occupational Safety and Health method 5040 by the Wisconsin State

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Laboratory of Hygiene (Madison, WI). Personal exposure to DEE, as measured by elemental carbon (N = 11), averaged 575 ± 512 μg/m^3 (range, 91– 1800) (Table 2). Exposure times averaged 90 minutes (range, 56–134). NO_2 and carbon monoxide (CO) concentrations were assessed with an MSA multi-gas detector (Mine Safety Appliances) during a single mucking shift (without concomitant rock removal operations) using the diesel-powered LHD. For a single experimental shift monitored for 60 minutes, peak concentrations for NO_2 and CO were 1.5 ppm and 22 ppm, respectively. All subjects underwent two evaluations: a baseline evaluation on a nonexposure day and an evaluation after DEE exposure. These exposures were at least 1 week apart. Sputum induction, a health history, and an exposure questionnaire were completed on nonexposure days. On exposure days, groups of 1 to 3 subjects first completed an interim health history and then were exposed for a 1- to 2-hour period, depending on their individual class schedules. One hour after cessation of exposure, the subjects completed sputum induction. A 1-hour postexposure test time was chosen in order to take into account the timelines of both the acute response of the subjects' airways and any possible change in soluble NEP activity.

Sputum Induction and Exposure—Induced sputum (based on methods in Djukanović et al. 2002) was collected using DeVilbiss Ultra-Neb 99HD ultra-sonic nebulizers (Somerset, PA) filled with 3% saline and set on maximum output. Sputum samples were diluted with 10% Sputolysin (Calbiochem, San Diego, CA) in PBS with penicillin–streptomycin and 0.5% bovine serum albumin (BSA). Supernatant was removed by centrifugation and frozen at −80°C for later analysis of NEP activity. The cellular pellet was reconstituted in 1 mL PBS in order to perform total cell counts with the use of a hemocytometer and trypan blue stain (Sigma Chemical Co.). A portion of the cell pellet was cytocentrifuged using a Shandon Cytospin (Thermo-Shandon, Pittsburgh, PA) onto a microscope slide and stained with Diff-Quik for cell number analysis. The protein concentration was determined using a Coomassie Plus Protein Assay (Pierce) with BSA as the standard.

Enzyme Activity Measurement—We measured cell-free NEP activity in sputum spectrophotometrically by a coupled assay, as described previously (Wong et al. 2004). Briefly, 5 μL of cell-free extract was incubated with 1 mM succinyl-Ala-Ala-Phe-*p*-nitroanilide (Suc-Ala-Ala-Phe-pNA) (Bachem Bioscience, King of Prussia, PA) as a substrate in 0.1 M of Tris-HCl (pH, 7.6) and 1 μL (0.14 units/μL) of porcine kidney aminopeptidase N (Sigma). We performed the reaction in duplicate in a 96-well microtiter plate. In this coupled activity assay, NEP cleaves Suc-Ala-Ala-Phe-pNA between Ala and Phe, yielding Phe-pNA. Aminopeptidase N (APN) then cleaves Phe-pNA, generating pNA as the final product. We determined the increase in specific absorbance (the accumulation of free pNA) at 405 nm using a plate reader (BioTek Instruments) after a 30-minute incubation at 37°C. We ran cell-free (substrate alone or substrate with APN) and substrate-free blanks in parallel. We determined the protein concentration by using a Coomassie Plus Protein Assay (Pierce) with BSA as the standard.

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STATISTICAL METHODS AND DATA ANALYSES

We double-entered the laboratory and clinical data into a database and checked for miscoding of variables. Initially, standard descriptive statistics were run on the data to evaluate distributions, determine any needed transformations, and assess potential outliers or discrepancies in the data. First, we tested the data for homogeneity of variance using the Bartlett test and normalized them as appropriate, following a Gaussian distribution. Depending on the experimental designs and the size of the sample number (*N*), we used either paired or unpaired Student *t* tests or ANOVA for comparisons of mean concentrations between groups. Data were expressed as mean ± standard error of the mean (SEM), and we considered $P < 0.05$ to indicate significance. We performed statistical analyses using SPSS, version 17 (Chicago, IL). Duane L. Sherrill, professor of biostatistics and Associate Dean for Research at the University of Arizona College of Public Health, assisted us in all of the statistical analyses. The detailed statistical methods are specifically described for each aim as follows.

Aim 1

This aim examined the difference in DEP-induced inflammatory injury between *Nep*-null and wild-type mice, as measured by cell profiles and cytokines in BAL fluid, and epithelial proliferation. For this purpose, we normalized the data using $\log_{10}$ because they did not follow a Gaussian distribution. We used a factorial ANOVA for multiple comparisons of means in a 2 × 3 factorial design for each transformed measurement. Since the measures are independent variables, we evaluated mean changes for all groups using post hoc linear contrasts with adjustment for multiple comparisons, which were made using Bonferroni-corrected significance levels. Additionally, we used ANOVA for normalized measure comparisons for mRNA and protein expression of NEP in wild-type mice, comparing groups of controls and low and high DEP exposure. We calculated Pearson correlation coefficients to evaluate the strength of the linear relations for all of the dose–effect measurements in *Nep*-null mice versus wild-type mice.

Aim 2

For this aim, we assessed the concentration-dependent downregulatory effects of NEP induced by DEP and its contents (cDEP and sDEP); or (2) another type of particle. For this purpose, we normalized the data using $\log_{10}$ because they did not follow a Gaussian distribution. We used one-way factorial ANOVA for multiple comparisons of means, and we evaluated all mean changes using post hoc linear contrasts, with adjustments for multiple comparisons using Bonferroni-corrected significance levels. We performed additional statistical analyses to evaluate the strength of the linear relations for all of the concentration–effect measurements in the DEP, sDEP, and cDEP groups. We calculated the Pearson correlation coefficients.

Aim 3

First, we assessed the effects of *NEP* gene knockdown by RNAi using one-way ANOVA. Then, we compared the difference in transcripts in a 2 × 3 factorial design including (1) control versus 10 or 40 $\mu g/cm^2$ DEP in NEP siRNA-transfected cells; (2) control versus 10

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or 40 μg/cm^2 DEP in mock controls; and (3) NEP siRNA-transfected cells versus mock controls in 0, 10, and 40 μg/cm^2 DEP. We performed one-way factorial ANOVA using GeneSpring GX 9 software. We used the global error model (multisample interpretation), based on replicates of the samples, to estimate the variability in gene expressions within — and between — sample measurements. Correction for multiple testing was performed using the Benjamini-Hochberg-Yekutieli False Discovery Rate, set to 0.05% (i.e., a baseline of 5% of the genes identified as significant being false positives). The Tukey post hoc test was used to evaluate where statistically significant differences lay between the sample classes. We performed a power analysis, as described in Dobbin and Simon (2005), for single-label microarrays. We calculated within-dose variance using all data. We used the 90th percentile as a sample size estimate valid for 90% of the transcripts measured. Alpha was set at 0.05, and the power was set to 0.90.

Aim 4

We assessed how DEE exposure affects NEP activity and cell numbers in sputum. For this purpose, we first normalized the data using $\log_{10}$ because they did not follow a Gaussian distribution. Then, we used the paired sample *t* tests to compare NEP activity between pre- and post-exposure sputum levels. We used one-way ANOVA for normalized measure comparisons of sputum levels of cells and total protein. Additionally, we calculated the Pearson correlation coefficients to identify linear relations between (1) NEP activity and the product of exposure concentration × exposure time; and (2) NEP activity and the number of total cells, macrophages, neutrophils, and epithelial cells in sputum.

RESULTS

AIM 1: TO EXAMINE THE ROLE OF NEP IN DEP-INDUCED INFLAMMATORY INJURY USING *Nep*-INTACT AND *Nep*-NULL MICE

Increased BAL Fluid Cell Numbers in *Nep*-Null Mice in Response to DEP— Total inflammatory cell numbers increased, depending on dose, in wild-type and *Nep*-null mice 7 days after DEP exposure (Table 3). After the low and high levels of DEP doses, the inflammatory cell numbers increased approximately 1.7- and 2.4-fold, respectively, in wild-type mice, and approximately 2.8- and 3.3-fold, respectively, in *Nep*-null mice. There were significant differences in total inflammatory cells between wild-type and *Nep*-null mice in response to both doses of DEP challenge. The changes in macrophage count followed the same trend as that of total inflammatory cells except in wild-type mice treated with the low DEP, where differences were not statistically significant. After the high dose of DEP, we observed significant increases in granulocytes and lymphocytes in *Nep*-null mice, but not in wild-type mice.

Epithelial cell numbers in *Nep*-null mice treated with the high dose of DEP were significantly higher than those in wild-type mice treated with the high dose (Table 3).

Increased BAL Fluid Cytokine Response in *Nep*-Null Mice When Exposed to DEP—To determine whether the cytokine response induced by DEP was associated with NEP expression, we performed ELISA assays for IL-1[3, IL-6, and IL-10 with BAL fluid

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from wild-type and *Nep*-null mice 7 days after DEP exposure (Figure 1). In wild-type mice, the protein content of IL-6 was significantly elevated at the high dose, but not at the low dose, of DEP instillation. There were trends toward increased levels of IL-1[3 and IL-10 after both doses of DEP, but they were not statistically significant. In the *Nep*-null mice, the protein content for IL-6 was significantly elevated at both DEP dose levels and displayed a dose-dependent response to DEP. The levels of IL-1[3 and IL-10 proteins were significantly elevated at the high dose, but not at the low dose, in the *Nep*-null mice. Between wild-type and *Nep*-null mice, there was a statistically significant difference in IL-6 protein levels in response to both doses of DEP.

Downregulation of NEP in Wild-Type Mice by DEP—Our previous study (Wong et al. 2007 pilot study) showed that exposure of human airway epithelial cells to DEP (0– 40 μg/mL) for 24 hours downregulated NEP expression as well as its enzymatic activity in a concentration-dependent manner. We noted in that study that a substantial decrease (90%) in NEP mRNA expression occurred at 5 μg/mL DEP. To confirm these in vitro findings, we quantified the concentration of NEP protein in lung tissue in the current study. Exposure of wild-type mice to DEP significantly decreased NEP protein levels (Figure 2) in a dose-dependent manner. Both levels of DEP exposure resulted in significant reductions in NEP protein when compared with that of controls. The protein levels were reduced by 10.4% and 50.0% of the control at the low and high DEP doses, respectively, suggesting that the changes may be attributable to DEP exposure. Using immunohistochemistry, we observed NEP expression on epithelial cells, alveolar type II cells, and macrophages (Figure 3A). Low and high levels of DEP (Figures 3B and 3C, respectively) appeared to reduce NEP protein expression in the epithelial cells of small airways.

Effects of DEP on Epithelial Proliferation in Wild-Type and *Nep*-Null Mice—We monitored the incorporation of labeled BrdU into airway epithelial cell DNA as a marker of epithelial proliferation. In wild-type mice, we observed no significant changes in airway epithelial proliferation after the high dose of DEP when compared with its control (2.67 ± 0.58 positive cells/10^2 cells vs. 2.00 ± 1.41 positive cells/10^2 cells; $P = 0.976$). Also, we did not observe any significant changes in airway epithelial proliferation in *Nep*-null mice treated with the high dose of DEP when compared with control (2.04 ± 1.00 positive cells/10^2 cells vs. 2.00 ± 0.71 positive cells/10^2 cells, $P = 0.627$). DEP exposure did not result in significant changes in epithelial proliferation between wild-type and *Nep*-null mice ($P = 0.667$). Either there was no detectable wound-repair process in the acute phase of epithelial inflammatory response to DEP exposure 7 days after exposure or the sample size was not large enough to detect the presumably mild changes resulting from the current exposure conditions.

AIM 2: TO EXAMINE WHICH COMPONENTS OF DEP ARE ASSOCIATED WITH NEP DOWNREGULATION IN VITRO

Our previous study indicated that NEP downregulation by DEP occurs at the transcriptional level (Wong et al. 2007 pilot study). To clarify the contribution of the major components of DEP in the downregulation of NEP, we compared the effects induced by (1) DEP; (2) cDEP; and (3) sDEP. We also examined another type of particle— oDEP, or standard urban dust

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(SRM 1649a) — to test whether NEP downregulation is a nonspecific response to PM. We conducted parallel cell cultures with the same concentration–response designs (using concentrations of 0, 1, and 10 μg/cm^2) in the same period of time (24 hr). The changes in NEP were not associated with cell proliferation because the cells stopped growing in the culture wells before exposure to the different levels of DEP. The results showed that BEAS-2B cells cultured with DEP statistically significantly downregulated NEP mRNA expression at 1 μg/cm^2 (50%) and 10 μg/cm^2 (45%), as measured by RT-PCR (Figure 4). Cells treated with 1 and 10 μg/cm^2 cDEP expressed approximately 80% and 55% NEP mRNA, respectively, of control cells. When compared with controls, the decrease in NEP mRNA expression was significant at the higher, but not the lower, level of cDEP, suggesting that cDEP has less effect than DEP due to removal of the divalent cations, particularly transition metals, by chelators. Moreover, cells treated with sDEP were not significantly affected at either concentration, indicating that DEP, after organic stripping, lost its ability to downregulate NEP mRNA expression. Collectively, these findings suggest that downregulation of NEP by DEP was mostly attributable to DEP-adsorbed organic compounds, whereas the carbonaceous core of NEP has little or no effect on NEP mRNA expression under the current experimental conditions.

NEP mRNA expression in cells treated with oDEP followed the same pattern as that of DEP (Figure 4). This suggests that the observed NEP downregulation is a nonspecific response to PM, because the two kinds of particles (DEP and standard urban dust) have different chemical characteristics (Table 4). SRM 1649a contains additional trace metals, pesticides, and a number of components from multiple sources of air pollutants in addition to DEP.

AIM 3: TO DETERMINE THE MOLECULAR IMPACT OF DEP EXPOSURE AND DECREASED NEP EXPRESSION ON AIRWAY EPITHELIAL CELLS' GENE EXPRESSION IN VITRO, USING A COMBINATION OF RNAi AND MICROARRAY APPROACHES

In Vitro Model of *NEP* Knockdown—To determine the impact of NEP downregulation due to DEP exposure, we knocked down *NEP* gene expression in BEAS-2B cells using the RNAi technique. The controls for our experiments were untreated (uninfected) cells and cells infected with a vector carrying siRNA against *lacZ* (mock), which has little or no homology to any human gene. The effects of NEP siRNA transfection, compared with controls, were measured 30 hours later by Western blot analysis of NEP protein expression with anti-NEP antibody. We used [3-actin levels as internal controls. The results of densitometric quantification of NEP/[3-actin ratios are shown in Figure 5. Efficient reduction (63.5% when compared with mock control) of protein levels was confirmed. There was no difference between untreated and mock transfected controls.

Gene Expression Profile—Twenty-four hours after transfection with siRNA, we treated the *NEP*-knockdown cells and mock controls with zero, low, and high concentrations of DEP for an additional 24 hours for the microarray study. Figure 6 illustrates that hybridization signal intensities varied statistically significantly as a function of *NEP* knockdown, DEP exposure, and a combination of both. Neither NEP siRNA transfection nor DEP exposure induced statistically significant changes in cell viability. Labeled complementary RNA (cRNA) from mock or NEP siRNA–transfected cells after incubation

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with 0, 10, and 40 μg/cm^2 DEP was evaluated on two different Affymetrix U133 Plus human genome GeneChips. Overall, about 2158 probe sets, out of a total of 54,675, were detected above background and shown to be statistically different ($P < 0.01$) in the NEP siRNA experimental group when compared with the mock controls.

Genes Associated with *NEP* Knockdown—In order to identify genes that were specifically upregulated or downregulated and associated with decreased NEP expression in BEAS-2B cells, we employed hierarchical clustering (a functional annotation tool that is part of the GeneSpring GX 9 software, which measures the relations among the annotation terms based on the degree of co-association of genes into the group) in order to evaluate the 121 genes identified in the statistical analysis. Table 5 lists 31 genes with a high fold change when compared with mock controls; the normalized (to MOCKp0) fluorescent-spot intensity values are shown in Figure 7. Initial analysis suggests that the depletion of NEP expression (by siRNA) in epithelial cells induced upregulation (17 genes) or downregulation (14 genes) of genes involved in DNA/protein binding, calcium channel activities, and the intracellular signaling cascade of cytokines. The identified genes included *IL6*, *IL8*, and epidermal growth factor receptor (*EGFR*). The depletion of NEP expression also disturbed the transcription of genes involved in cellular monooxygenase, guanosine triphosphatase (GTPase), and protein kinase activities. These data suggest that NEP is involved in a complex molecular cascade in different subcellular compartments, possibly influencing normal cellular functions and inflammatory conditions.

Using hierarchical clustering, we consistently observed five genes in the top three ranking pathways delineated by the GeneSpring GX 9 software — “The Role of Cytokines in Mediating Communication between Immune Cells,” “IL-17 Signaling,” and “Colorectal Cancer Metastasis Signaling” — and in the top twelve pathways, including “IL-8 Signaling,” “IL-6 Signaling,” and “HGF Signaling.” These five genes (*IL6*, *IL8*, *EGFR*, prostaglandin-endoperoxide synthase 2 [*PTGS2*], and *BCL2L11*) were chosen due to their known association with inflammation. Increased expression of IL-6, IL-8, and PTGS2 was confirmed by RT-PCR to be associated with *NEP* knockdown (Figure 8).

RT-PCR assays for IL-6, IL-8, and PTGS2 were performed with cDNAs generated from the microarray study (Figure 8). A comparison analysis showed that mRNA expression of *IL6*, *IL8*, and *PTGS2* in the siRNAp0 group was approximately 6.1-fold greater than that of the MOCKp0 group, suggesting the knockdown of *NEP* significantly enhanced the expression of these genes. However, the degree of increased expression of the measured genes became less with DEP exposures of 10 μg/cm^2 (4.8-fold, siRNAp10 vs. MOCKp10) and 40 μg/cm^2 (1.3-fold, siRNAp40 vs. MOCKp40), suggesting that DEP exposure covers the effect of *NEP* knockdown on gene expression. In both siRNA and MOCK groups, moreover, the expression of *IL6*, *IL8*, and *PTGS2* mRNA was significantly elevated in a dose-dependent manner in response to DEP exposure. Thus these data further confirmed the findings observed in the microarray and in vivo studies.

Genes Associated with DEP Exposure—Tables 6 and 7 represent the up- and downregulated genes, respectively, after exposure of BEAS-2B cells to DEP. The normalized fluorescent-spot intensity values are shown in Figure 9. There were 151

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upregulated and 59 downregulated genes with in vitro exposure to DEP. Of the up- or downregulated genes, 89.5% and 46.6%, respectively, were changed in a DEP concentration-dependent manner (data not shown). The results showed that DEP incubation increased the mRNA levels of metabolic enzymes cytochrome P450 CYP1A1 and CYP1B1, cytokines IL-6 and IL-8, PTGS2, and chemokine (C-X-C motif) ligand 1 and 2 (*CCL1* and *CCL2*), death inducer-obliterator 1 (*DIDO1*), heat shock 70kDa protein 1B (*HSPA1B*), aquaporin 3 (*AQP3*), early growth response 2 and 3 (*EGR2* and *EGR3*), among others. In contrast, DEP decreased the expression of many enzymes in BEAS-2B cells including phosphoinositide-3-kinase (*PIK3C2A*), phosphoribosylglycinamide formyl-transferase (*GART*), and threonyl-tRNA synthetase (*TARS*), indicating that DEP may potentially influence a multitude of biologic processes.

Utilizing the DAVID bioinformatics database (david.abcc.ncifcrf.gov) — which annotates, visualizes, and integrates lists of genes — we analyzed 243 preselected genes based on dose–response changes in expression as a result of DEP exposure. We obtained DAVID identification for 229 genes. Our annotative analysis indicated that 74% of the genes map to biologic processes listed as Gene Ontology (GO) terms in the DAVID database, 73% map to a cellular component, and 78% map to a molecular function. Of the cellular components, 118 genes map to intracellular; 84, to intracellular membrane-bound; 92, to intracellular organelle; 71, to the cytoplasm; and 65, to the nucleus. Among the genes distributed to GO terms that were associated with molecular function, 149 were to binding terms: 107, to protein binding; 47, to nucleic acid binding; 38, to nucleotide binding; and 13, to protein kinase activity. Interpro (part of the DAVID system) identified 83% of the genes and indicated that 11 genes were associated with a protein kinase core. The Self-Monitoring, Analysis, and Reporting Technology system identified 52% of the genes and indicated that 8 genes were associated with basic-leucine zipper transcription factors, and 9 genes were associated with the serine or threonine-specific kinase subfamily. The Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa Laboratories, Kyoto, Japan) software mapped 9 genes to focal adhesion and 9 genes to MAPK signaling pathways. BioCarta (also part of the DAVID system) mapped 3 genes to IL-6 signaling pathways and 3 genes to thrombospondin-1 (TSP-1)–induced apoptosis in microvascular endothelial cell pathways. A clustering analysis at medium stringency produced 27 significant clusters, the most frequently occurring categories of which are listed in Figure 10.

AIM 4: TO EVALUATE THE EFFECTS ON NEP ACTIVITY OF HUMAN EXPOSURE TO DEE

Cell Profile—The exposure of subjects to DEE induced an increase of inflammatory cells (Table 8), as shown by a statistically significant increase in the total number of cells in sputum. The concentration of macrophages— but not neutrophils and lymphocytes — increased after DEE exposure. The data suggest that DEE exposure resulted in a macrophage-related proinflammatory or inflammatory response in the airways.

The increase (4.44×) in sputum epithelial cell counts after exposure of subjects to DEE was statistically significant (Table 8), indicating that the exposure resulted in acute airway epithelial cell shedding.

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Total Protein and NEP Activity: We found that DEE exposure (0.88 ± 0.08) did not induce a statistically significant change in total sputum protein when compared with baseline control (0.82 μg/μL ± 0.09) (Table 9). We conducted an analysis of soluble NEP activity in sputum for all 11 healthy nonsmoking volunteers. The results showed a range of baseline values from 0.115 to 0.445 nmol/μg protein/min, averaging 0.303 ± 0.036 (mean ± SEM). We observed individual increases in NEP activity in 9 out of 11 subjects after DEE exposure (Table 9). When compared with baseline control, the average net increase after DEE exposure was 31%, with a maximum change of 0.26 nmol/μg protein/min. The change indicates that an increase of NEP activity, observed in sputum, occurred after the acute exposure of subjects to approximately 575 $\mu g/m^3$ of elemental carbon (DEE). A Pearson correlation analysis indicated that NEP activity in individual sputum after DEE exposure was slightly associated with the product of exposure concentration × exposure time (Figure 11). DEE exposure was not corrected for pre-exposure levels of NEP activity (data not shown).

Associations of Sputum NEP Activity with Cell Types: To further evaluate the association between changes in sputum cell type and NEP activity, we conducted Pearson correlation analyses. Sputum NEP activity was not significantly associated with the changes in total cell number or with the changes in macrophages, neutrophils, or epithelial cells (data not shown).

DISCUSSION AND CONCLUSIONS

Using a number of approaches (in vitro, animal, and controlled human exposure), we examined the role of NEP in DEE-induced airway inflammatory response, taking the following steps:

1. To simultaneously characterize the dose–response of DEP on respiratory NEP and the role of NEP in the inflammatory response to DEP exposure, we conducted an animal study with a 2 × 3 factorial design. We observed that pulmonary NEP protein expression in wild-type mice at 7 days after exposure to DEP via intratracheal installation was significantly downregulated in a concentration-dependent manner, decreasing by 10.4% and 50.0% at the low and high levels of DEP exposure compared with control. The changes were accompanied by increases in macrophages, epithelial cells, and the proinflammatory cytokine IL-6 (but not IL-1[3 or IL-10) in BAL fluid. *Nep*-null mice displayed enhanced increases in these markers of the inflammatory response when compared with wild-type mice and showed increases in IL-1[3 and IL-10 in BAL fluid, especially in response to the high dose of DEP. These in vivo findings suggest that loss of NEP in mice could cause an increased susceptibility to injury or exacerbate the inflammatory response to DEP exposure by allowing the release of higher levels of specific cytokines from the lungs.
2. To clarify the contribution of major components of DEP (SRM 2975) in the downregulation of NEP in BEAS-2B cells, we compared the effects induced by DEP, cDEP, and sDEP. We also examined oDEP (or standard urban dust [SRM 1649a]), another type of particle, to test whether NEP downregulation was a

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specific response to DEP. Parallel BEAS-2B cell cultures were conducted with 0, 1, and 10 μg/cm^2 DEP for 24 hours. As measured by RT-PCR, cells treated with 1 and 10 μg/cm^2 DEP had approximately 50% and 45% NEP mRNA expression, respectively, of control. Cells treated with cDEP at 10 μg/cm^2, but not at 1 μg/cm^2, also downregulated NEP mRNA expression by about 45% compared with control, suggesting that cDEP has less effect than DEP due to removal of chelated divalent cations, particularly transition metals. Moreover, NEP mRNA expression was not affected in cells exposed to sDEP, indicating that DEP after organic stripping lost its downregulation effect. Collectively, these findings suggest that downregulation of NEP by DEP was mostly attributable to DEP-adsorbed organic compounds, whereas the carbonaceous core and transition metal components of DEP had little or no effect on NEP mRNA expression at the current experimental condition. This NEP downregulation in vitro was not a specific response to DEP or its contents because the change also occurred after urban dust exposure, which has a different composition.

To further understand the molecular impact of NEP loss in response to DEP and the effect of DEP on epithelial NEP in vitro, we collected transcriptome profiles of the concentration–effects of SRM 2975 in cultured BEAS-2B cells through a 2 × 3 factorial design. Microarray data showed that there were 151 upregulated and 59 downregulated genes in response to DEP exposure. Of the up- or downregulated genes, 89.5% and 46.6%, respectively, changed in a concentration-dependent manner. These genes included metabolic enzymes *CYP1A1* and *CYP1B1*, *IL6*, *IL8*, *CCL1* and *CCL2*, *DIDO1*, *HSPA1B*, *AQP3*, and *EGR2* and *EGR3*. Utilizing the DAVID bioinformatics database, we analyzed 243 preselected genes based on concentration-dependent changes in expression as a result of DEP exposure. The KEGG pathways mapped 9 genes to focal adhesion and 9 genes to MAPK signaling pathways. BioCarta mapped 3 genes to IL-6 signaling pathways and 3 genes to TSP-1–induced apoptosis in the microvascular endothelial cell pathway. Cells depleted of NEP expression (by siRNA) showed numerous up-regulated (17) or downregulated (14) genes, involving DNA/protein binding, calcium channel activities, and intracellular signaling cascade of cytokines. Genes identified included *IL6*, *IL8*, and *EGFR*. Knockdown of *NEP* expression also disturbed the transcriptions of many genes relevant to cellular monooxygenase, GTPase, and protein kinase activities. Increased expression of *IL6*, *IL8*, and *PTGS2* was confirmed by RT-PCR to be associated with both DEP exposure and *NEP* knockdown. The data generated from a combined RNAi–microarray approach revealed that there is a complex molecular cascade mediated by NEP in different subcellular compartments, possibly influencing inflammatory response.

3. In the controlled human exposure study, DEE induced significant increases in the number of macrophage, neutrophil, and airway epithelial cells. Moreover, we observed statistically significant increases in soluble NEP activity in sputum after DEE exposure (0.40 ± 0.03 nmol/μg protein/min; $P = 0.035$) when compared with the baseline control (0.30 ± 0.04 nmol/μg protein/min), with a 31% average net increase. Based on this, we speculate that the change in NEP activity in sputum

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may possibly be a new endpoint to measure the effects of DEE exposure. Change in NEP activity may also provide insight into the mechanism of airway effects after DEE exposure, if it can be confirmed in human investigation with a larger sample size and at ambient exposure levels.

We discuss these summarized results in detail along with our previous findings in the pilot study (Wong et al. 2007 pilot study) in the following sections.

LOSS OF NEP ENZYMATIC ACTIVITY IN RAT AND HUMAN LUNG FOLLOWING DEE EXPOSURE

Our previous in vivo study showed that NEP activity in rat lung was significantly reduced when rats were exposed to DEP (35 μg/m^3) (Wong et al. 2003; Witten et al. 2005). The change in NEP activity was accompanied by increases in bronchopulmonary plasma extravasation, vascular permeability, and cytokine expression, as well as inflammatory/mast cell infiltration, possibly evoked by abnormally high levels of peptides after DEP exposure. Reduced NEP activity could induce the airways to respond in an exaggerated fashion to peptide substrates, thus producing a hyperresponsive or abnormal response state. The fact that diesel components enhance the vasoconstrictive effects of endothelin-1 and reduce the dilatory response to sodium nitroprusside (Campen et al. 2005) indirectly suggests the diesel-mediated loss of NEP activity in the response to endothelin-1 in vivo. Thus, this reduction in NEP activity may affect the bioavailability of many peptide mediators (such as neurokinins, endothelins, and bombesin) released from sensory nerve terminals or immunoinflammatory cells (Joos et al. 2000). To evaluate loss or decrease in human airway NEP activity in Aim 4, we took advantage of a collected sputum sample from an earlier controlled exposure study (Burgess et al. 2007). The results showed that there was a rapid increase in soluble NEP activity in sputum shortly after the exposure of human volunteers to DEE. Increased NEP activity in sputum may reflect a local inflammatory response, with subsequent shedding of membrane-bound enzymes in response to lung injury (Soleilhac et al. 1996). We also found a similar change in sputum NEP activity after exposure of these subjects to mining dust particulates, suggesting that the change was not a specific response to DEE exposure (data not shown). These results were consistent with the findings from exposure to viruses, fire smoke, and cigarette smoke (Lilly et al. 1994; Wong et al. 2004). We believe that the current investigation provides additional environmental evidence for the finding that NEP expression varies widely in human lung tissue from different individuals (Cohen et al. 1999). Although this effect remains to be confirmed in a large population, our data suggest that DEE-induced effects may be linked mechanistically to changes in NEP activity and/or expression.

The cellular origin of soluble NEP activity in the sputum of humans exposed to DEE remains to be characterized. The results of this study suggest that airway epithelial cells could be one of the major sources of soluble NEP activity found in sputum due to exposure-induced epithelial membrane damage. In addition, inflammatory cells, especially airway macrophages, might be another major source of soluble NEP activity in sputum as a result of DEE exposure. Our speculation is based on our findings that both epithelial and macrophage cells, which abundantly express NEP, were affected by DEP exposure in this study. Since, structurally, NEP is a cell-surface metalloprotease with an extracellular domain of 700

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amino acids, its large extracellular domain containing the catalytic sequence may explain its critical ability to rapidly cleave substrates. However, this structure is fully exposed to an extracellular environment that is possibly susceptible to toxic insults. Therefore, the likely mechanism to explain the increase of NEP activity in sputum is the shedding of affected cells into the airways after DEE exposure, with membrane-bound proteins being released with portions of plasma membrane or as proteolipid aggregates.

We note that the dose (575 μg/m^3 of elemental carbon) used in the human exposure part of our study was too high to reflect a possible change in NEP activity after exposure to an ambient level of DEP. Future studies with large sample sizes are needed to test whether exposure to real-world levels of DEP and other particulates causes a change in NEP activity with functional consequences for airway biology. However, we hope that soluble NEP activity in sputum could serve as a potential early endpoint in identifying DEP-mediated health risks if our results are confirmed by animal investigations and by studies using a larger human population at an ambient exposure level.

DOWNREGULATION OF NEP EXPRESSION BY DEP IN HUMAN EPITHELIAL CELL AND MOUSE MODELS

Our previous in vitro experiments have shown that DEP could downregulate NEP at the levels of gene and protein expression (Wong et al. 2007 pilot study). In human airway epithelial cells (BEAS-2B), we observed that exposure to DEP for 24 hours significantly repressed NEP expression, as well as its enzymatic activity, in a concentration-dependent manner. Furthermore, experiments with DEP in primary human airway epithelial cells confirmed the results in BEAS-2B cells; an approximate 90% decrease in NEP mRNA expression at 5 μg/mL DEP concentration was observed in our 2007 pilot study when compared with controls. Therefore, downregulation of NEP expression after DEP exposure was not a cell-line–specific effect. These findings were confirmed in the current in vitro study. Moreover, in the current study, we also demonstrated the down-regulation of NEP expression in mouse lung after acute exposure to DEP. The mechanisms underlying downregulation of NEP expression by DEP remain to be investigated.

DOWNREGULATION OF NEP BY DEP-ADSORBED COMPOUNDS IN VITRO

DEP is composed of a carbonaceous core with adsorbed transition metals and various organic substances including polycyclic aromatic hydrocarbons (PAHs), nitro-aromatic hydrocarbons, quinones, and acids (McClellan 1987). To examine the contribution of the major classes of DEP components in downregulation of NEP expression, we compared the effects induced by whole DEP; sDEP, with everything removed but the carbonaceous core; and cDEP, without transition metals. Our data showed that the organic compounds attached to particles might play a major role in any downregulation effects of NEP. It has been reported that the treatment of diesel exhaust that diminished the adsorbed organic compounds could reduce the DEP-induced inflammatory response and in vivo toxicity (Boland et al. 1999). Therefore, the current data provide additional evidence that DEP may pose a particularly high risk of adverse effects, possibly because of the high fraction of potentially toxic substances found on the relatively large surface of the particles (Rudell et al. 1999; Cohen et al. 2002; Kulkarni et al. 2006).

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Downregulation of NEP mRNA expression may not be a specific effect of DEP. In the same culture conditions, epithelial cells exposed to urban dust (SRM 1649a) had reduced NEP expression that was similar to the reduction after exposure to SRM 2975. Certified analyses have indicated that the two types of particles have certain common or similar organic compounds (NIST Certificate of Analysis 2000, 2001). A comparison between the two particles showed that the concentrations of many nitro-PAHs in SRM 2975 were an order of magnitude higher than concentrations measured in SRM 1649a, due to dilution of DEP by other non-diesel particles in the urban air sample (Bamford et al. 2003). 1-Nitropyrene was pointed out to be the dominant nitro-PAH present in the diesel particulate samples, while 2-nitrofluoranthene was the highest nitro-PAH concentration measured in the urban air sample (Bamford et al. 2003). However, additional in vitro experiments are needed to further demonstrate that organic compounds of SRM 1649a play a major role in the downregulation of NEP expression.

IMPACT OF NEP DOWNREGULATION IN DEP-INDUCED INFLAMMATORY INJURY IN VIVO AND IN VITRO

The current study showed that the DEP-induced inflammatory response in wild-type mice was dramatically enhanced in *Nep*-null mice, as measured by BAL fluid cell profiles, proinflammatory cytokines, and alterations identified histopathologically. In addition, in vitro experiments showed that human airway epithelial cells depleted of NEP expression displayed higher expression of proinflammatory cytokines IL-6 and IL-8 than those of control cells. These findings suggest that these inflammatory responses to DEP exposure were, at least in part, associated with either NEP expression or NEP activity or both. These findings also suggest new insights into the mechanisms underlying the DEP-induced inflammatory response. Since IL-6 and IL-8 are not substrates for NEP, the exacerbated inflammation and injury responses were likely mediated by diminished degradation of NEP substrates and/or nonenzymatic functional loss, such as injury repair, of NEP protein, or both.

In addition, our previous in vitro study (Wong et al. 2007 pilot study) suggested that NEP expression, rather than its catalytic activity, may possibly affect the regulatory machinery involved in cell proliferation. Other studies have also shown that NEP is involved in cell growth (Ganju et al. 1994). In our pilot study, *NEP* knockdown by RNAi in airway epithelial cells resulted in a concentration-dependent reduction of epithelial proliferation in response to DEP exposure. The change in epithelial proliferation may possibly be attributable to the nonenzymatic interaction of NEP protein through its cytoplasmic tail with several intracellular proteins. For example, it is known that the NEP cytoplasmic domain contains putative sites for phosphorylation by casein kinase II (Ganju et al. 1996). The casein kinase II-mediated phosphorylation of specific cellular proteins has been linked with proliferation (Ganju et al. 1996). Also, NEP can associate with ezrin/radixin/moesin (ERM) proteins and inhibit the association of ERM proteins with CD44, and may thus play a role in restricting CD44-mediated cell motility (Iwase et al. 2004). In addition, NEP directly associates with and stabilizes PTEN tumor suppressor, leading to dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate to its less active form. This results in regulation of downstream cell growth and cell survival pathways such as those regulated by Akt/PKB

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kinase (Sumitomo et al. 2004). It is likely that these mechanisms participate in epithelial repair in vitro through NEP-mediated signal transduction pathways.

However, in the current study, we were unable to detect the effects of DEP on epithelial proliferation in wild-type or *Nep*-null mice, possibly because there were limited numbers of BrdU-positive cells observed in slides of dissected mouse lung tissue. This suggests that the extent of the injury was too mild to be observed using the immunohistochemical quantification of incorporated BrdU as a measure of epithelial proliferation. It is possible that the degree of cell injury caused by DEP did not result in cell death, which would ordinarily result in synthesis of new cells. It is known that there are two cell populations that proliferate in response to airway injury (Rawlins et al. 2007): basal cells in the trachea and primary bronchi, and Clara cells in the more distal bronchi, bronchioles, and bronchoalveolar duct junction. Under normal conditions, epithelial cell turnover in the lung is relatively low. Nevertheless, if these cells are extensively damaged, the lung can replace lost cells quickly. In the current study, however, histopathologic examination showed that instillation of DEP at a level of 10 or 100 μg resulted in mild lung injury, which did not destroy the majority of the Clara cells in either the proximal or distal conducting airways. It may be necessary to design additional studies with mice exposed to the higher dose of DEP in order to determine whether NEP has a role in epithelial proliferation and repair.

NEP-MEDIATED MOLECULAR MECHANISM IN DEP-INDUCED INFLAMMATORY INJURY

With three techniques (RNAi, microarrays, and RT-PCR), we dissected the complicated molecular networks mediated by NEP. A comparison of expression files between normal and *NEP*-knockdown cells reveals highly NEP-connected pathways. The analysis showed that genes associated with the induction of apoptosis (*BCL2L11*), signal transduction (*EGFR*, *SLC6A19*), and inflammatory processes (*IL6*, *IL8*, *PTGS2*, *PTGER4*) were significantly over-represented. To our knowledge, this is the first report of array-based "genetic unmasking" in combination with *NEP* knockdown, and it reveals the potential of this strategy in identifying particular signaling transduction pathways affected by NEP. For example, previous in vivo, in vitro, and human studies showed that proinflammatory cytokines IL-6 and IL-8 frequently increase after exposure to DEP, leading to immuno-inflammatory injury (Diaz-Sanchez et al. 1994; Bayram et al. 1998; Nightingale et al. 2000; Li et al. 2002; Gong et al. 2003). The changes in cytokine expression are mediated in part through the activation of many signaling transduction pathways, involving transcription factors such as NFKB2, AP-1, and STAT3 (Takizawa et al. 2003; Zhang et al. 2004; Cao et al. 2007), MAPKs (Fahy et al. 2000; Hashimoto et al. 2000) via reactive oxygen species–dependent mechanisms (Baulig et al. 2003). Our study suggested that the abnormal response pattern of a cascade of cellular and molecular events brought on by a depletion of NEP induced by DEP contributed not only to inflammatory processes, but also to apoptosis and abnormal signal transduction.

In addition, our study showed that depletion of NEP expression (by siRNA) in epithelial cells was associated with many pathways, including DNA/protein binding, calcium channel activities, and intracellular signaling cascades. The proteins identified included cellular monooxygenase, GTPase, PTGS2, and protein kinase. It is noted that PTGS2, also known as

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cyclooxygenase, is the key enzyme in prostaglandin biosynthesis and acts both as a dioxygenase and as a peroxidase. PTGS2 is regulated by specific stimulatory events and is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis. Collectively, our data from the microarray experiments suggest that there is a complex molecular cascade mediated by NEP in different subcellular compartments in response to DEP challenge, possibly influencing the metabolic, cellular, and regulatory functions of epithelial cells.

We conclude that (1) exposure to DEP may downregulate NEP expression in a concentration-dependent manner, and we attribute this effect mostly to DEP-associated organic compounds; (2) *Nep*-null mouse and cellular models with *NEP* knockdown helped us discover that NEP plays an important role in the inflammatory response to DEP exposure in vivo and in vitro; (3) there exists a complex molecular cascade mediated by NEP in different subcellular compartments, further influencing normal cellular response to DEP; and (4) exposure of human subjects to DEE resulted in changes in airway NEP activity. We speculate that downregulation of airway NEP may be one of the mechanisms that links DEE exposure to airway inflammatory injury or airway disease susceptibility.

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This Investigators' Report is one part of Health Effects Institute Research Report 159, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project.

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APPENDIX AVAILABLE ON THE WEB

The following material may be obtained from HEI's Web site, www.healtheffects.org:

Appendix A

Uptake of DEP into Human BEAS-2B Cells Observed Using Transmission Electron Microscopy.

CRITIQUE Health Review Committee

Research Report 159, *Role of Neprilysin in Airway Inflammation Induced by Diesel Exhaust Emissions*, S.S. Wong et al.

INTRODUCTION

Diesel engines emit less carbon dioxide (CO_2*) and carbon monoxide (CO) than gasoline engines, but until recently, they have released higher amounts of particulate matter (PM) and nitrogen oxides (NO_x). Concerns about the health effects of diesel exhaust emissions (DEE) and the particulate component of these emissions (diesel exhaust particles, or DEP) have resulted in the funding of numerous epidemiologic and toxicologic studies (reviewed in HEI 2007).

HEI periodically issues a request for preliminary applications (RFPA) for novel research on the health effects of air pollutants derived from motor vehicle emissions. In response to RFPA 05–3, "Health Effects of Air Pollution," issued in 2005, Dr. Simon Wong of the University of Arizona and colleagues submitted an application for a study titled "The Molecular Effects of Diesel Exhaust Particulates on Respiratory Neutral Endopeptidase." Dr. Wong proposed to study whether the exposure of human airways (in vivo and in vitro) to diesel exhaust affects the expression or activity of neprilysin (also known as neutral endopeptidase, or NEP), an enzyme that degrades multiple vasoactive peptides in the airways (discussed in more detail in the next section). Dr. Wong hypothesized that components of diesel exhaust downregulate the function or expression of NEP in the airways and that this may lead to disorders in airway function; he proposed to evaluate the effects of DEP on airway epithelial cells in vitro and of DEE in human volunteers.

Previously, HEI had funded a one-year pilot study by Dr. Wong and colleagues, in which they had obtained preliminary data on the effects of DEP on NEP expression and activity in

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

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airway epithelial cells in vitro (Wong et al. 2007 pilot study). The HEI Research Committee considered the proposed study a logical extension of the pilot study and invited Dr. Wong to submit a full application. After reviewing the application, the Committee suggested that Dr. Wong include in vivo studies in an animal model to complement the proposed human studies. Dr. Wong submitted a revised application that included experiments in mice, and the Research Committee recommended the study for funding.

SCIENTIFIC BACKGROUND

NEPRILYSIN AND ITS FUNCTION

NEP is a widely distributed endopeptidase expressed on the outer surface of several cell types in a variety of tissues and organs (Ronco et al. 1988). For example, in the lung it is expressed on the surface of cells including epithelium, endothelium, nerve cells, macrophages, and neutrophils. In the airways, the extracellular portion of NEP cleaves a wide range of physiologically important vasoactive peptides that include substance P (SP), bradykinin, and endothelin-1, -2, and -3 (Koehne et al. 1998). NEP substrates such as bombesin and endothelin-1 also induce cell migration (Sumitomo et al. 2000). Inside the cell, NEP is involved in many signaling pathways through interactions with the tumor suppressor gene *PTEN*, the activation of several kinases (focal adhesion kinase, phosphatidylinositol-3, and Rho), and degradation of phosphokinase C delta.

Because of NEP's ability to catabolize vasoactive peptides that affect airway function, there have been a number of studies investigating changes in NEP expression in several airway conditions in humans as well as animal models. Older studies suggested that NEP levels are elevated in the serum of patients with adult respiratory distress syndrome and cardiogenic pulmonary edema, and in a subset of patients with chronic obstructive pulmonary disease (COPD) (Johnson et al. 1985). However, NEP levels were not different in the serum or bronchoalveolar lavage (BAL) fluid of asthma patients compared with those of healthy individuals (van der Velden et al. 1999). At the time the current study was funded, the role of NEP in human inflammatory lung conditions such as COPD and asthma was uncertain (Barnes 2001).

In animal studies, pharmacologic inhibition of NEP activity or genetic deletion of *Nep* resulted in enhanced injury in an elastase-induced mouse lung injury model (Day et al. 2005). Decreases in NEP expression or activity have also been noted in a variety of laboratory animals exposed to factors such as cigarette smoke, allergens, and respiratory irritants (Di Maria et al. 1998). In a rat hypoxia model, animals deprived of oxygen had significantly reduced NEP expression in the lungs and increased vascular leak (Carpenter and Stenmark 2001).

Before the current study, Wong and colleagues had conducted several studies of the effects of exposure to different types of air pollutants on NEP in animals. In one study, they exposed rats via nose-only inhalation to DEE (specifically, to either 669 or 35.3 $\mu g/m^3$ of PM) for 4 hr/d, 5 d/wk, for 3 weeks. They found that exposed rats had an approximately 30% decrease in NEP activity in lung tissue, as well as airway inflammatory responses and changes in SP levels and SP signaling (Wong et al. 2003; Witten et al. 2005). In another

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study (Wong et al. 2004), rats were exposed through the nose only to fire smoke (1.24 ppm CO_2, 2.0 ppm NO_x, and 99.5 mg/L PM) for 20 minutes as a model of acute respiratory distress. Wong and colleagues reported increases in endogenous SP levels and decreases in NEP activity and expression in lungs of rats from 1 to 24 hours after the exposure.

It is also of note that NEP expression is altered in many neoplasias — downregulated in some (e.g., prostate and small-cell lung tumors [Cohen et al. 1996; Sumitomo et al. 2004]), but upregulated in others (e.g., the progression of colorectal tumors and diffuse large B-cell lymphomas [Bai et al. 2003, Fujimoto et al. 2005]).

In summary, these results indicate that changes in NEP activity or expression are found in a variety of lung and airway conditions, especially those involving injury, but that these changes do not appear to be specific or causal.

EFFECTS OF CONTROLLED EXPOSURES TO DEE AND ITS COMPONENTS

Emissions from diesel engines are important components of urban air pollution and contribute significantly to traffic-derived ambient PM concentrations (HEI Panel on the Health Effects of Traffic-Related Air Pollution 2010). Increasingly stringent emissions standards, as well as advances in engine technology, have resulted in decreasing concentrations of PM and gaseous pollutants in the emissions from diesel engines. However, PM and NO_x generated from older engines still in use (both on-road and off-road) remain a public health concern. Additionally, engine type and age affect the levels of PM and other pollutants emitted, with older engines contributing larger amounts of PM and NO_x than newer engines.

Diesel emissions are a complex mixture of particulate and gaseous components. Diesel exhaust particles are composed of a carbon core to which nitrate, sulfate, metals, and organic compounds adhere. Thus, DEP is often characterized by the level of elemental carbon (EC) and the presence of multiple polycyclic aromatic hydrocarbons. The principal gaseous components are CO_2, nitrogen and sulfur compounds (including nitrogen dioxide [NO_2]), and low-molecular-weight hydrocarbons.

Some studies in humans have suggested that administration of DEP enhances features characteristic of an allergic pattern of immune response. For example, administering 300 μg of DEP into the noses of healthy and asthmatic individuals enhanced the total number of immunoglobulin E–secreting B cells in the upper airways (Diaz-Sanchez et al. 1994), and administering DEP in combination with ragweed allergen enhanced the production of messenger RNA (mRNA) specific for interleukin (IL)-4 and IL-13 — cytokines associated with the development of an allergic response (Diaz-Sanchez et al. 1997). Studies in rodents (e.g., Fujimaki et al. 1997; Takano et al. 1997) have reported that the administration of DEP with allergens such as ovalbumin enhanced key features of the allergic response.

However, the effects on airway responses and pulmonary function resulting from short-term controlled exposure to DEE of either healthy or asthmatic subjects have been less clear-cut. Salvi and colleagues (1999, 2000) reported some changes in immunologic and inflammatory endpoints in healthy and asthmatic volunteers exposed to DEE (300 $\mu g/m^3$ PM); lung

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function in healthy volunteers was not affected, however (Salvi et al. 1999; Nightingale et al. 2000). Nordenhäll and colleagues (2001) found no changes in airway inflammatory markers, except in cytokine IL-6 (a molecule that is "proinflammatory" — i.e., activates the inflammatory response), in 14 asthmatic human subjects exposed to DEE (300 μg/m^3 PM) in an exposure chamber for 1 hour. They found changes in some measures of airway and pulmonary function (specifically, increased airway hyperresponsiveness and airway resistance) but no change in forced vital capacity (FVC) or forced expiratory volume in one second (FEV_1).

Zhang and colleagues evaluated asthmatic volunteers who walked for 1 to 2 hours at two sites in Central London: a street on which a high proportion of diesel vehicles travelled (Oxford Street) and a control site less than a mile away with far less traffic (Hyde Park). Compared with walking in Hyde Park, walking on Oxford Street resulted in mild changes in some markers of airway inflammation and respiratory parameters (a 3–4% decrease in FEV_1 and FVC), but no changes in self-reported symptoms (McCreanor et al. 2007; Zhang et al. 2009).

In summary, although the administration of DEP — either to the upper airways of humans or via instillation in rodents — enhances features of the allergic-type immune response, controlled exposures to DEE in healthy and asthmatic humans appear to have some effects on markers of inflammation in the airways, but little effect on pulmonary function. Because Wong and colleagues obtained preliminary data to suggest that exposure to DEP decreased NEP mRNA levels in BEAS-2B cells (a transformed human airway epithelial cell line) (Wong et al. 2007 pilot study), they proposed to investigate further the connection between exposure to diesel exhaust and changes in NEP expression and activity in airway cells.

TECHNICAL EVALUATION

SPECIFIC AIMS

The goals of Dr. Wong's study were to evaluate (a) whether exposure to diesel exhaust components in vivo in mice and humans and in vitro in a human cell line affected the activity or expression of NEP, and (b) whether a decrease in NEP expression affected responses induced by diesel exhaust. Wong and colleagues hypothesized that exposure to diesel exhaust would decrease the activity or expression of NEP in airway cells, and that decreased expression of NEP would enhance responses induced by diesel exhaust.

Their specific aims and general approaches were as follows:

1. *To examine the role of NEP in DEP-induced inflammatory injury using* Nep-*intact and* Nep-*null mice*. The investigators evaluated whether airway inflammatory responses after the instillation of DEP were greater in mice genetically deficient in *Nep* (*Nep*-null mice) than responses in wild-type, control mice.
2. *To examine which components of DEP are associated with NEP downregulation in vitro.* Wong and colleagues evaluated whether untreated DEP, DEP treated to remove certain components, or a control particle affected the expression of NEP in the transformed human airway epithelial cell line BEAS-2B.

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3. *To determine the molecular impact of DEP exposure and decreased NEP expression on airway epithelial cells' gene expression in vitro, using a combination of RNA interference (RNAi) and microarray approaches.* The investigators assessed the effects of exposure to DEP on gene expression in two sets of BEAS-2B cells: control cells, which express NEP, and cells in which NEP expression had been decreased — "knocked down" — by treating BEAS-2B cells with an siRNA specific for NEP.
4. *To evaluate the effects on NEP activity of human exposure to DEE.* Wong and colleagues measured NEP activity and markers of inflammation in the airways of volunteers who had been exposed to DEE in a controlled mining scenario.

STUDY DESIGN AND METHODS

Key features of the study design are summarized in Critique Table 1 and in the following sections.

Diesel Exhaust, Control Particles, Emissions, and Exposures

DEP: In Aims 1, 2, and 3 the investigators evaluated the effects of resuspended DEP in the form of SRM 2975 particles from the National Institute of Standards and Technology (NIST), a set of particles originally generated by a diesel-powered industrial forklift. Physical and chemical characteristics of SRM 2975, including particle size distribution and concentration of selected polycyclic aromatic hydrocarbons, are shown in the Investigators' Report (IR) Table 4.

In Aim 2, the investigators treated DEP with the chelator Chelex-100, which preferentially removes divalent cations, particularly transition metals ("cDEP"), or dichloromethane to strip all components except for the carbonaceous core ("sDEP").

Control Particles: In Aim 2, the investigators also evaluated the effects of a second particle, SRM 1649a, obtained from NIST, as a control for the DEP (SRM 2975). The physical and chemical characteristics of SRM 1649a, described as a "standard urban dust," are also shown in IR Table 4.

Emissions: In Aim 4, DEE were generated from a 1984 Jarvis Clark JS-220 load-haul-dump vehicle commonly used in mining. The vehicle was equipped with a 2-cubicyard bucket and an 82-hp Deutz F6L-912W diesel engine fitted with a catalytic converter.

Exposures to DEP or DEE: In Aim 1, resuspended particles were instilled intratracheally in mice. In Aims 2 and 3 resuspended particles were incubated with the cell line BEAS-2B. In Aim 2, uptake of SRM 2975 particles into BEAS-2B cells was assessed by transmission electron microscopy. In reviewing the study, HEI's Review Committee thought these uptake studies were ancillary to the main part of the study on biologic effects; thus these experiments are described in IR Appendix A (available on the Web at www.healtheffects.org).

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Critique Table 1

Study Design[a]

Specific Aim	Test System	Exposures: Concentration and Duration	Endpoints (Timing of Post-Exposure Measurement)
1	*NEP* knockout and control (wild-type) mice	Intratracheally instilled with 0, 10, or 100 μg resuspended DEP (SRM 2975)	NEP and cytokine levels in BAL fluid, NEP expression in lung (in wild-type only), and airway epithelial cell proliferation (day 7)
2	BEAS-2B (transformed epithelial human cell line)	Treated for 24 hr with 0, 1, or 10 μg/cm^2 particles: DEP (SRM 2975), cDEP, sDEP, control (SRM 1649a)	NEP mRNA levels (24 hr)
3	BEAS-2B human cells: untreated or treated with NEP-specific siRNA to knock down NEP expression	Treated for 24 hr with 0, 10, or 40 μg/cm^2 DEP (SRM 2975)	RNA expression, measured by microarray and RT-PCR, of multiple genes (and molecular pathways) affected by either DEP treatment or *NEP* knockdown (24 hr)
4	11 Healthy human volunteers (ages 19–33 yr)	Single inhalation exposure to DEE. Individual exposure concentrations ranged from 0.09 to 1.80 mg/m^3 EC and 56 to 134 min duration	NEP activity, and differential cell count and protein content in induced sputum (baseline pre-exposure and 1 hr post-exposure [at least 1 wk after baseline])

[a] cDEP indicates SRM 2975 treated with the chelator Chelex-100; sDEP indicates SRM 2975 treated with dichloromethane; RT-PCR indicates real-time polymerase chain reaction.

In Aim 4, 11 human volunteers were exposed to DEE at the University of Arizona's San Xavier Underground Mining Laboratory, an underground facility in which the effects of mining can be studied (www.mge.arizona.edu/templates/standard/index.php?ID=31). The original exposure study (Burgess et al. 2007) was led by a collaborator on the current study, Dr. Jefferey Burgess. The investigators expressed the exposure concentration in terms of EC, which had been collected on a quartz filter during the exposure. Individual exposures to DEE ranged in concentration from 0.09 to 1.80 mg/m^3 of EC and in duration from 56 to 134 minutes.

Biologic Endpoints

Inflammatory Markers: In the experiment supporting Aim 1, at 7 days after instillation of DEP or control, the investigators measured levels of the cytokines IL-1[3, IL-6, and IL-10 in BAL fluid, and BAL total and differential cell counts. The DEP effects on the proliferation of airway epithelial cells were assessed by evaluating the number of cells that had incorporated 5-bromo-2'-deoxyuridine (5-BrdU) injected intraperitoneally 24 hours before euthanasia.

In Aim 4, Wong and colleagues measured changes in differential cell count and protein content in the induced sputum of 11 healthy individuals (see IR Table 2 for details) exposed to DEE. Each subject underwent two sputum inductions: the first on a nonexposure day to establish a baseline, and the second— at least 1 week later — 1 hour after the DEE exposure.

NEP Expression: Levels and Activity of Protein in Tissue, and Levels of mRNA: In Aim 1, the investigators assessed levels of NEP protein in the lung tissue extracts of wild-type

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mice by enzyme-linked immunosorbent assay (ELISA). Expression of NEP protein in the lungs of these mice was localized by immunohistochemistry using light microscopy. In Aim 2, NEP mRNA levels in BEAS-2B cells were measured after a 24-hour incubation period with the different types of particles. In Aim 4, cell-free NEP activity in induced sputum of human volunteers was measured spectrophotometrically.

To assess gene expression changes by microarray analysis in Aim 3, the investigators extracted total RNA from the following:

1. *BEAS-2B cells exposed for 24 hours to DEP (0, 10, or 40 μg/cm^2 SRM 2975)*. This experiment was to determine which genes were affected by exposure to DEP.
2. *BEAS-2B cells transfected with an siRNA specific for NEP and subsequently exposed to DEP (0, 10, or 40 μg/cm^2 SRM 2975)*. This experiment was to determine which genes were affected by exposure to DEP in a cell with decreased expression of NEP. The investigators established that NEP siRNA transfection decreased expression of the NEP protein by about 63% compared with untreated cells or cells transfected with a control siRNA (IR Figure 5).

The investigators organized gene expression changes into major functional categories and potential biologic pathways, using different types of software described in the IR. To determine whether changes in expression in the gene microarray assay corresponded to changes in RNA levels, the investigators used an alternative technique — real-time polymerase chain reaction (RT-PCR) — to evaluate some genes whose expression was found to differ from control cells by microarray.

RESULTS

Aim 1: Effects of DEP in Mice

Airway Inflammatory Responses: Instillation of either 10 or 100 μg of DEP (SRM 2975) resulted in increases in several markers of the inflammatory response in the BAL fluid and cells of both *Nep*-null and wild-type mice measured 7 days after the treatment (IR Figure 1 and Table 3). At the same doses of DEP, increases in total, macrophage, and epithelial cell counts, as well as IL-6 levels, were greater in the *Nep*-null than in the wild-type mice. The higher, but not the lower, dose of DEP increased IL-1[3 and IL-10 levels in *Nep*-null mice but not in wild-type mice.

NEP Protein Level: Instillation of DEP reduced NEP protein level in lung tissue by approximately 10% at 10 μg and 50% at 100 μg (IR Figure 2). The investigators showed that DEP instillation reduced NEP protein expression in lung sections, concluding that the reduction in NEP expression was localized to small airway epithelial cells (IR Figure 3). In the absence of DEP, NEP was expressed on macrophages and alveolar type II cells, as well as on epithelial cells.

Epithelial Cell Proliferation: Exposure to high-dose DEP did not result in increased epithelial cell proliferation in either wild-type or *Nep*-null mice.

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Aim 2: DEP Effects on NEP mRNA Expression in BEAS-2B Cells In Vitro—DEP (SRM 2975 particles) at either 1 or 10 μg/cm^2 decreased expression of NEP mRNA in BEAS-2B cells by approximately 45% compared with control cells after a 24-hour culture period (IR Figure 4). Similarly, cDEP (DEP with transition metals removed) decreased expression of NEP mRNA by 45% at 10 μg/cm^2 (but not at 1 μg/cm^2). On the other hand, sDEP (DEP stripped down to the carbonaceous core) did not affect NEP mRNA levels significantly at doses of either 1 or 10 μg/cm^2. As a comparison, standard urban dust (SRM 1649a) decreased NEP mRNA expression by approximately 60% at both levels (1 and 10 μg/cm^2).

Aim 3: Effects of DEP Exposure on Gene Expression in Control BEAS-2B Cells and BEAS-2B Cells with Decreased NEP Expression In Vitro

Genes Associated with DEP Exposure: Exposure to DEP upregulated the expression of 151 genes and downregulated the expression of 59 genes (IR Tables 6 and 7) in multiple intracellular pathways (IR Figure 10). Upregulated genes included the metabolic enzymes cytochrome P450 *CYP1A1* and *CYP1B1*, cytokines *IL6* and *IL8*, chemokine ligand 1 and 2 (*CCL1* and *CCL2)*, death inducer-obliterator 1 (*DIDO1*), heat shock 70kDa protein 1B (*HSPA1B*), aquaporin 3 (*AQP3*), and early growth response 2 and 3 (*EGR2* and *EGR3*). Downregulated genes included phosphoinositide-3-kinase (*PIK3C2A*), phosphoribosylglycinamide formyltransferase (*GART*), and threonyl-tRNA synthetase (*TARS*).

Genes Associated with *NEP* Knockdown: The investigators showed that transfecting a NEP-specific siRNA into BEAS-2B cells decreased expression of NEP protein approximately 63% compared with control cells. In BEAS-2B cells with reduced NEP expression, the investigators identified 31 genes with the greatest changes in expression (17 upregulated and 14 downregulated) compared with control, mock-infected cells (IR Table 5 and Figure 7). The genes included *IL6*, *IL8*, and epidermal growth factor receptor, and pathways that involved DNA/protein binding, calcium channel activity, cytokine signaling, cellular monooxygenase, guanosine triphosphatase (GTPase), and protein kinases.

Of the 5 genes whose expression was upregulated in the microarray and selected for further investigation of expression by RT-PCR, only 3 (specifically, *IL6*, *IL8*, and *PTGS2*) showed increased expression by the RT-PCR technique (IR Figure 8).

Aim 4: DEE Effects on Airway Endpoints in Human Volunteers—In induced sputum evaluated 1 hour after exposure to DEE, the total number of cells increased approximately 2.5-fold (mostly macrophages, but neutrophil numbers also increased concomitantly), and epithelial cell numbers increased approximately 4.5-fold (IR Table 8). Total protein level was not affected.

The group mean of the NEP activity in induced sputum after DEE exposure increased 31% over baseline (IR Table 9). Exposure to DEE — calculated as the product of the EC concentration during the exposure and the duration of exposure — was highly correlated with NEP activity in sputum after the exposure period (IR Figure 11).

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HEI REVIEW COMMITTEE EVALUATION OF THE STUDY

In its independent evaluation of the study, the HEI Review Committee considered that Wong and colleagues had made a comprehensive attempt to explore the role of NEP in response to exposure to components of DEE, using an appropriate animal model (an *Nep*-null mouse), human studies, and in vitro models employing a human cell line. The Committee thought that the study design and statistical analyses for the diverse sets of experiments were appropriate. The human exposure study, however, had the limitation that the investigators used samples and data that had already been collected in an earlier study, and so only a limited number of assays could be performed.

Key results were that airway inflammatory effects in response to DEP instillation (i.e., increases in BAL fluid of the numbers of macrophage and epithelial cells and the levels of cytokines that these cells synthesize) were greater in mice genetically deficient in *Nep* than in wild-type mice that express *Nep*. In addition, the fact that DEP instillation (at 100 μg) into wild-type mice resulted in a decrease in NEP protein expression both in lung tissue extracts and in airway epithelial cells and macrophages suggests at first glance that exposure to DEP reduces NEP expression in the lung. Other important findings were that exposure of human volunteers to a high concentration of DEE via inhalation resulted in airway inflammatory effects measured in induced sputum — in particular increased numbers of macrophages and epithelial cells and, in some participants at least, *increased* NEP activity. However, this finding is at odds with the investigators' hypothesis for the study, that exposure to DEE would *decrease* NEP levels in the airways.

The Committee noted that responses in the mouse lung were measured 7 days postexposure to DEP, whereas responses in the exposed human volunteers were measured at 1 hour postexposure. Thus, making comparisons between the human responses to DEE and mouse responses to DEP is challenging, since the investigators did not establish an optimal time for measuring effects. Nonetheless, the Committee thought that the study's human and mouse findings could be interpreted in a consistent fashion— that is, that the observed diesel-exhaust–induced decreases in NEP levels in lung tissue are the result of an injury response in the airways. As a consequence, airway epithelial cells are shed and macrophage numbers are increased, and hence both changes are measurable in either induced sputum (human) or BAL fluid (mouse). Since both epithelial cells and macrophages express NEP, this would explain the increase in NEP activity in the induced sputum of some of the human volunteers. The investigators did not measure NEP levels in the BAL fluid of DEP-exposed, NEP-expressing (i.e., wild-type) mice, so they do not know whether the decrease in NEP expression in the mouse lung is accompanied by an increase in NEP expression or activity in the BAL fluid.

The finding that mice genetically lacking *Nep* showed greater inflammatory response than wild-type mice after exposure to DEP suggests that in this model NEP plays some role in damping down inflammatory responses to agents introduced into the airways. This supports one of the hypotheses of the study, namely, that decreased expression of NEP would enhance responses induced by diesel exhaust. Because the *Nep*-null mice exposed to DEP synthesized more IL-6, as seen in BAL fluid, than did wild-type mice, the investigators not

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unreasonably interpret these findings as suggesting that the loss of NEP resulted in enhanced production of this proinflammatory cytokine. This enhancement of IL-6 synthesis was also supported by the results of the gene expression studies supporting Aims 2 and 3. How NEP expression and IL-6 synthesis may be linked was not part of the current study. It is also likely that, in the absence of NEP, levels of neuropeptides such as SP may also increase, which may also play a role in enhancing inflammatory effects, as Wong and colleagues have previously suggested (Wong et al. 2003; Witten et al. 2005).

Wong and colleagues' findings of heightened effects in *Nep*-null mice after challenges to the airways are consistent with other findings in these mice. For example, Dempsey and colleagues (2009) found that, after the induction of chronic hypoxia, *Nep*-null mice showed increases in pulmonary hypertension, proliferation of smooth muscle cells from pulmonary arteries, and muscularization of vessels compared with wild-type, control mice. Dempsey and colleagues (2009) also found that in hypoxic wild-type mice, NEP expression was decreased early in distal pulmonary arteries, the site of prominent vascular remodeling. In follow-up studies, these investigators found that COPD patients had decreased NEP expression in the alveolar walls and distal vessels of their lungs and that pulmonary arterial smooth muscle cells exposed to cigarette smoke extract or hypoxia showed decreased expression of NEP protein and mRNA levels (Wick et al. 2011).

In this report Wong and colleagues speculate that changes in NEP in sputum may be a useful early marker of diesel-exhaust–associated responses in humans. The Review Committee noted that the DEP and DEE exposures used in the current study were unlikely to reflect the properties of emissions generated by the current fleet of diesel-powered, on- or off-road vehicles, but recognized that the diesel exhaust exposures used were intended to offer a proof of principle. Nonetheless, the Committee held that changes in NEP activity or levels are unlikely to be useful human biomarkers of exposure to diesel exhaust for two reasons. First, in the limited sample of 11 volunteers, only 5 showed changes greater than 15% in NEP activity in induced sputum in response to DEE, and only after exposure to a high level of DEE (PM in the milligram/m^3 range). Second, the observed effects on NEP activity are not specific to diesel exhaust exposures: The results of Aim 2 in the current study show that standard urban dust particles, which contain lower levels of EC and thus presumably lower levels of DEE components, decrease NEP mRNA levels in BEAS-2B cells as effectively as DEP. In addition, Wong and colleagues have published elsewhere that the same volunteers who were exposed to DEE in the current study were also exposed separately to high levels of particles in "overshot mucking"— a blasting process in mining (Wong et al. 2010). Overshot mucking exposures contain little or no diesel exhaust components, but the correlation between exposure (concentration × duration) to overshot mucking particles and NEP activity was very similar to the correlation between exposure to DEE and NEP activity (Wong et al. 2010).

Regarding the correlation between DEE exposure and NEP activity in sputum that the investigators reported in the current study (IR Figure 11) and their recent paper (Wong et al. 2010, Figure 1), the Committee noted that it was with NEP activity measured *after* the exposure period (i.e., not corrected for an individual's baseline NEP activity). The Committee thought it would have been more convincing to show a correlation between DEE

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exposure and the *difference* in NEP activity before and after the exposure period (i.e., show the change in NEP activity resulting from the DEE exposure). The investigators have indicated that they had evaluated that correlation but had not found it to be significant (S. Wong, personal communication, October 2010).

The investigators' in vitro studies indicated that organic components of particles, rather than metals, were likely to be important in the downregulation of NEP expression in BEAS-2B cells. Because both standard urban dust particles and DEP contain organic components such as polycyclic aromatic hydrocarbons, this may explain why both sets of particles were equally effective at decreasing NEP mRNA levels in these cells.

Based on preliminary in vitro data from their pilot study, Wong and colleagues hypothesized that exposure to DEP would affect the proliferation of airway epithelial cells in mice. No effects were found, however, in either wild-type or *Nep*-null mice. Thus, the investigators could not find evidence to support a role for DEP in affecting the proliferation of airway epithelial cells that either did or did not express NEP.

The investigators provided some useful information about changes in gene expression after exposure to DEP in a transformed airway epithelial cell line. The Committee thought that the investigators' use of *NEP* knockdown by a specific siRNA was an innovative way to approach the role that *NEP* may play in gene expression changes in response to DEP. The studies also provided information about which genes' expression in a transformed airway epithelial cell line may be affected by exposure to DEP; the results may be helpful in trying to determine what pathways are involved in the lungs' response to DEP and how NEP may play a role.

These findings will need to be confirmed or refuted in future studies. However, the Committee noted that some questions remain about the interpretation of the gene expression studies (supporting Aims 2 and 3). First, BEAS-2B cells exposed to DEP did not show significant changes in NEP mRNA levels by microarray (see last line of IR Table 7), but did in a different assay (RT-PCR; see IR Figure 4). Second, of the 5 genes the investigators selected to submit to RT-PCR testing in order to confirm the microarray findings, confirmatory results were shown for only 3 (IR Figure 8). If extended to a broader set of genes, this would suggest that 2 out of 5 genes (i.e., 40%) identified by the microarray method may be false positives.

SUMMARY AND CONCLUSIONS

Wong and colleagues explored the role of NEP in response to DEE exposure, using an appropriate animal model (an *Nep*-null mouse), a human study, and in vitro models. The investigators hypothesized that (a) DEE exposure would decrease NEP expression in airway cells and that (b) decreased expression of NEP would enhance responses induced by diesel exhaust. Key findings were that (1) DEP instillation into the airways of mice resulted in stronger airway inflammatory effects (i.e., increases in macrophage and epithelial cell numbers and in levels of cytokines that these cells synthesize, as observed in BAL fluid) in mice genetically deficient in *Nep* than in mice expressing *Nep*; and (2) DEP instillation into

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wild-type mice resulted in a 50% decrease in NEP expression in lung tissue and particularly in airway epithelial cells. The HEI Review Committee agreed with the investigators that these findings suggest that expression of NEP in some way damps down inflammatory responses in the airways, supporting one of the hypotheses for the study.

Other important findings were that exposure of human volunteers to a high concentration of DEE via inhalation resulted in airway inflammatory effects measured in the induced sputum, particularly increased numbers of macrophages, neutrophils, and epithelial cells and, in some participants at least, *increased* NEP activity. However, the Committee thought that this last result was at odds with the investigators' other hypothesis for the study— that exposure to DEE would *decrease* NEP levels in the airways.

The Review Committee noted that it was challenging to compare the responses in mouse airways with those in human airways because the exposures were of different levels, durations, and routes of exposure, and the effects were measured at different times after the exposures. Nonetheless, the Committee thought that the study's human and mouse diesel-exhaust–associated findings could be interpreted in a consistent fashion — that is, that the observed changes in NEP levels result from a response to injury in the airways, measured as the shedding of airway epithelial cells and as an increase in macrophage numbers in the induced sputum (human) or BAL fluid (mouse). Since both epithelial cells and macrophages express NEP, this could explain the increase in NEP activity found in the induced sputum of some of the human volunteers.

The in vitro studies in the transformed human airway epithelial cell line also provided some useful information about DEP constituents that affect NEP expression — namely, that removing metals did not change NEP expression but removing organic components did. The studies also provided information about which genes' expression in a transformed airway epithelial cell line may be affected by exposure to DEP. These data may help in determining the pathways involved in the airway response to DEP and any possible NEP role in that response.

The investigators speculated in the report that changes in NEP in sputum might be a useful early marker of DEE-induced injury in humans. However, the Committee thought that changes in NEP activity or levels in the airways are unlikely to be useful biomarkers of exposure to diesel components, because the observed effects in airway cells were not specific to diesel exposures and because baseline levels of airway NEP activity differed markedly in different people. Thus, although changes in NEP function and activity have been noted in airway conditions, particularly after injury, the role of NEP is still not resolved.

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Duane L. Sherrill is a professor of biostatistics and associate dean for research at the Mel and Enid Zuckerman College of Public Health, University of Arizona. He received a B.S. in mathematics at the Metropolitan State College in 1976 and an M.S. and Ph.D. in biometrics at the University of Colorado Health Science Center in 1982 and 1987, respectively. His research interests include the development of measurement and analytical techniques for estimating the spatial relationships between lung function and diseases.

Craig J. Gerard is a professor of pediatrics, chief and director of research at the Division of Respiratory Diseases, Children's Hospital, Harvard Medical School, Boston, Massachusetts. His research focuses on the molecular mechanisms, including tachykinins and chemokines, of leukocyte trafficking in host defense and inflammatory responses. He has used both mouse genetic models (*Nep*-null mice) and in vitro transfection and expression analyses to understand the complex balance between defense and untoward inflammation associated with these mediators and receptors. He received a B.S. at the University of Connecticut in 1976, an M.S. and Ph.D. in chemistry at the University of California, San Diego, California, in 1981, and an M.D. at the Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina, in 1985. He received postdoctoral training at the Harvard Medical School, Boston, Massachusetts, in 1988. He has published more than 154 journal articles.

Jefferey L. Burgess is a professor and director of the Community, Environment and Policy Division at the University of Arizona Mel and Enid Zuckerman College of Public Health in Tucson, Arizona. His research includes respiratory toxicology in firefighters and smoke inhalation victims; reduction of mining-related injuries and exposures; environmental arsenic exposure; and hazardous materials exposures, including in methamphetamine

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laboratories. He is the principal investigator at the Arizona Center for Public Health Preparedness, which is funded by the Centers for Disease Control and Prevention, as well as for multiple research projects evaluating fire smoke, mining injury surveillance, and arsenic exposure. He received his M.D. at the University of Washington in 1988, an M.S. in toxicology/industrial hygiene at the University of Arizona in 1993, and an M.P.H. at the University of Washington in 1996.

ABBREVIATIONS AND OTHER TERMS

18S rRNA	18S ribosomal RNA
AAALAC	American Association for Animal Laboratory and Care
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
ALI	acute lung injury
ANOVA	analysis of variance
AP-1	activator protein-1
AQP3	aquaporin 3
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
cDEP	DEP with divalent cations, particularly transition metals removed using a chelator
cDNA	complementary DNA
CO	carbon monoxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
cRNA	complementary RNA
CYP	cytochrome P450
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAVID	Database for Annotation, Visualization and Integrated Discovery (U.S. NIAID)
DEE	diesel exhaust emissions
DEP	diesel exhaust particles
DIDO	death inducer-obliterator
DNase	deoxyribonuclease
EC	elemental carbon

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EGFR	epidermal growth factor receptor
EGR2&3	early growth response 2&3
ELISA	enzyme-linked immunosorbent assay
ERM	ezrin/radixin/moesin
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTPase	guanosine triphosphatase
GO	Gene Ontology (DAVID database)
H_2O_2	hydrogen peroxide
hp	horsepower
IL	interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGM	keratinocyte growth medium
LHD	load-haul-dump
MAPK	mitogen-activated protein kinase
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
NEP	neprilysin, or neutral endopeptidase
NFKB2	nuclear factor-kappa B
NIST	National Institute of Standards and Technology
NK	neurokinin
NO_2	nitrogen dioxide
NO_x	nitrogen oxides
O_3	ozone
oDEP	standard urban dust (another type of diesel exhaust particle)
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PM	particulate matter
$PM_{2.5}$	PM ::: 2.5 μm in aerodynamic diameter
PTGS2	prostaglandin-endoperoxide synthase 2

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RNAi	RNA interference
RT-PCR	real-time polymerase chain reaction
SAEC	small airway epithelial cell
SCLC	small-cell lung carcinoma
sDEP	“stripped DEP” carbonaceous core of DEP
SEM	standard error of the mean
siRNA	small interfering RNA
SP	substance P
SRM	standard reference material
TSP-1	thrombospondin-1
UA	University of Arizona

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HEI STATEMENT Synopsis of Research Report 159

Role of Neprilysin in Airway Inflammation Induced by Diesel Exhaust Emissions

BACKGROUND

Neprilysin — also known as neutral endopeptidase (NEP) — is an enzyme that degrades multiple peptides that affect airway blood vessels. The expression of NEP on the surface of cells varies in a number of airway injury conditions and in several types of cancers. HEI periodically issues a Request for Preliminary Applications for novel research on the health effects of air pollutants derived from motor vehicle emissions. In response to Request for Preliminary Applications 05-3, "Health Effects of Air Pollution," issued in 2005, Dr. Simon Wong at the University of Arizona and colleagues submitted an application to study whether exposure to diesel exhaust affected the airway expression or function of NEP. Some prior studies had shown that exposure of humans and laboratory animals to diesel exhaust particles (DEP) or whole diesel exhaust emissions (DEE) affected the airways. Dr. Wong and colleagues hypothesized that components of diesel exhaust downregulate the function or expression of NEP in the airways and that this may lead to disorders in airway function. They also hypothesized that in conditions in which NEP expression was decreased, responses to diesel exhaust would be increased.

APPROACH

Wong and colleagues evaluated airway inflammatory responses and NEP expression and activity in the fluid and cells obtained from bronchoalveolar lavage and the lung tissue of mice genetically deficient in NEP (*Nep*-knockout mice) or wild-type mice (control). The mice were instilled with 10 or 100 μg resuspended DEP, and their BAL fluid was analyzed 7 days later; the investigators used National Institute of Standards and Technology SRM 2975 particles, which were originally generated by a diesel-powered industrial forklift. The investigators also measured airway inflammatory responses and NEP activity in the induced sputum of 11 healthy human volunteers (ages 19–33 yr) 1 hour after exposure to DEE in a staged mining environment. Individual exposure concentrations ranged from 0.09 to 1.80 mg/m^3 elemental carbon (a component of DEE), and duration ranged from 56 to 134 minutes. A baseline measurement of NEP activity was taken at least 1 week before volunteers were exposed to DEE.

In addition, the investigators used a transformed human airway epithelial cell line, BEAS-2B, to evaluate whether exposure to DEP decreased the expression of NEP messenger RNA (mRNA). They used 1 or 10 $\mu g/cm^2$ untreated DEP (SRM 2975); DEP treated with chelating agents to remove divalent cations, particularly transition metals; DEP treated with dichloromethane to remove all but the carbonaceous core; or a control particle (standard urban dust SRM 1649a). Using microarray and real-time polymerase chain reaction approaches on extracts of these cells, the investigators also evaluated the expression of genes affected by exposure to DEP. Similar gene expression studies were conducted in DEP-exposed BEAS-2B cells with depleted levels of NEP by incubating the cells with a small interfering RNA specific for NEP.

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RESULTS

The investigators found airway inflammatory effects in response to DEP instillation— specifically, increases, found in bronchoalveolar lavage fluid, of the numbers of macrophage and epithelial cells and levels of cytokines that these cells synthesize— that were greater in *Nep*-knockout mice than in wild-type mice that express NEP. They also found a 50% decrease in NEP protein expression in lung tissue and decreased NEP protein expression in airway epithelial cells and macrophages after DEP instillation into wild-type mice.

In human volunteers, the investigators observed that baseline levels of NEP activity in induced sputum varied considerably among the volunteers. Exposure to a high concentration of DEE via inhalation resulted in airway inflammatory effects, measured as increased numbers of macrophages and epithelial cells in the induced sputum, and, in some participants at least, increased NEP activity.

In addition, the investigators observed changes (either up or down) in the expression of several genes in the airway epithelial cell line in response to DEP in normal control cells as well as in cells in which NEP expression was decreased.

NEP mRNA expression in BEAS-2B cells was decreased by approximately 45% after exposure to untreated DEP or to DEP with transition metals removed and by approximately 60% after exposure to the control standard urban dust particle. DEP that was stripped down to its carbonaceous core did not affect NEP mRNA levels significantly.

CONCLUSIONS

In its independent review of the study, the HEI Health Review Committee considered that Wong and colleagues had made a comprehensive attempt to explore the role of NEP in response to exposure to diesel exhaust components, using an appropriate animal model (an *Nep*-knockout mouse), human studies, and in vitro models using a human cell line. For most of the experiments performed, the study design was strong and the statistical analyses appropriate. The human exposure study, however, had the limitation that the investigators used samples and data that had already been collected in an earlier study, and so only a limited number of assays could be performed.

The investigators found that DEP instillation into the airways of mice resulted in stronger airway inflammatory effects in mice genetically deficient in *Nep* than in mice expressing *Nep* and was accompanied by a significant decrease in NEP expression in lung tissue. The Committee concluded that these findings plausibly suggested that expression of NEP in some way damps down inflammatory responses in the airways — supporting one of the hypotheses for the study — but noted that the mechanism by which this might occur was not addressed in this study. The Committee thought that other important study findings were that exposure of human volunteers to a high concentration of DEE via inhalation resulted in airway inflammatory effects and, in some participants at least, *increased* NEP activity. However, this was at odds with the investigators' other hypothesis for the study, that exposure to DEP would *decrease* NEP levels in the airways.

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The Committee noted that it was challenging to compare the responses in the mouse and human airways because the exposures were of different levels, durations, and routes, and the effects were measured at different times after the exposures. Nonetheless, the Committee thought that the study's human and mouse diesel-exhaust–associated findings could be interpreted in a consistent fashion — that is, that the observed changes in NEP levels result from a response to injury in the airways, measured as the shedding of airway epithelial cells and an increase in macrophage numbers in induced sputum (human) or bronchoalveolar lavage fluid (mouse). Since both epithelial cells and macrophages express NEP, this could explain the increase in NEP activity found in the induced sputum of some of the human volunteers.

The in vitro studies in the transformed human airway epithelial cell line provided some useful information about DEP constituents that affect NEP expression — namely, removing metals did not change NEP expression, but removing organic components did. The studies also provided information about which genes' expression in a transformed airway epithelial cell line may be affected by exposure to DEP. These data may help in determining the pathways involved in airway response to DEP and any possible NEP role in that response.

The investigators speculated in the report that changes in NEP in sputum might be a useful early marker of DEE-induced injury in humans. However, the Committee thought that changes in NEP activity or levels in the airways are unlikely to be useful biomarkers of exposure to diesel components, because the observed effects in airway cells were not specific to diesel exposures, and because baseline levels of airway NEP activity differed markedly in different people. Thus, although changes in NEP function and activity have been noted in airway conditions, particularly after injury, the role of NEP is still not resolved.

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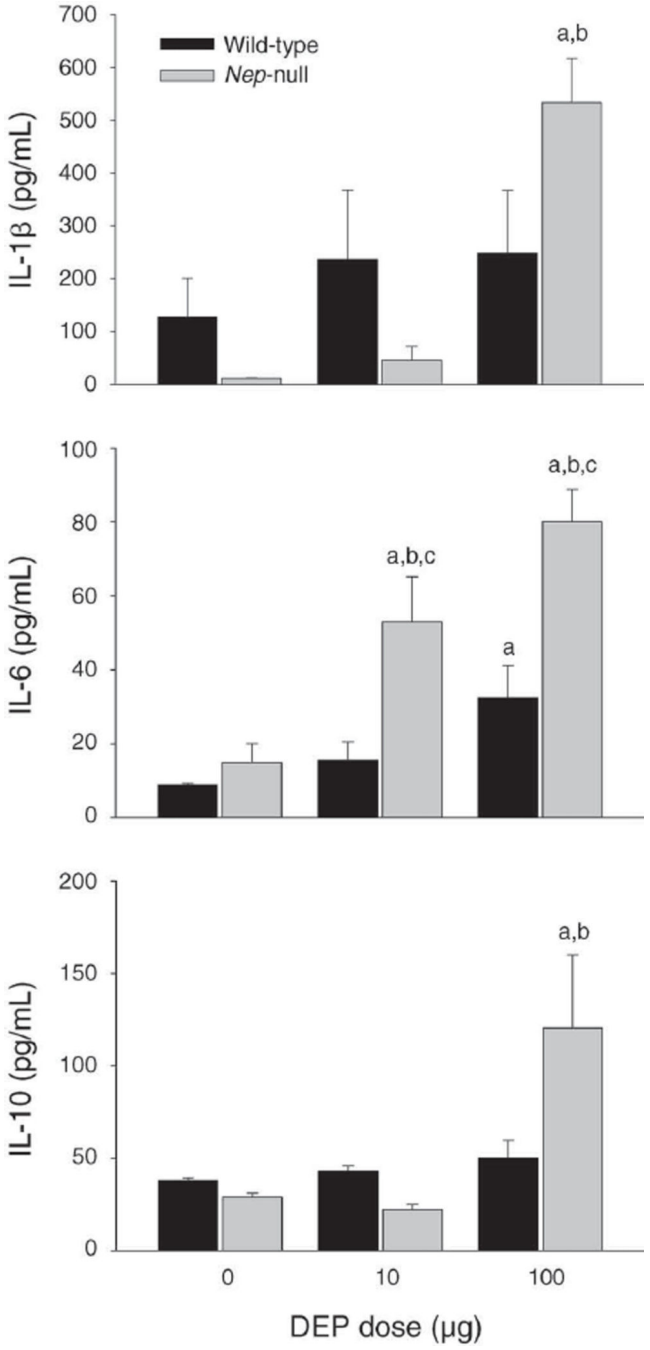

Figure 1. Differences in the amounts of cytokines IL-1(3, IL-6, and IL-10 in BAL fluid of *C57BL/6* wild-type mice and *Nep*-null mice following DEP instillation

Data expressed as mean ± SEM ($N = 7$). Letter "a" indicates significantly higher compared with the wild-type control group; "b" indicates significantly higher compared with *Nep*-null control group; and "c" indicates significant difference between wild-type and *Nep*-null mice with the same dose of DEP ($P < 0.05$). The DEP exposure was meant to approximate an accumulated dose of 7 days.

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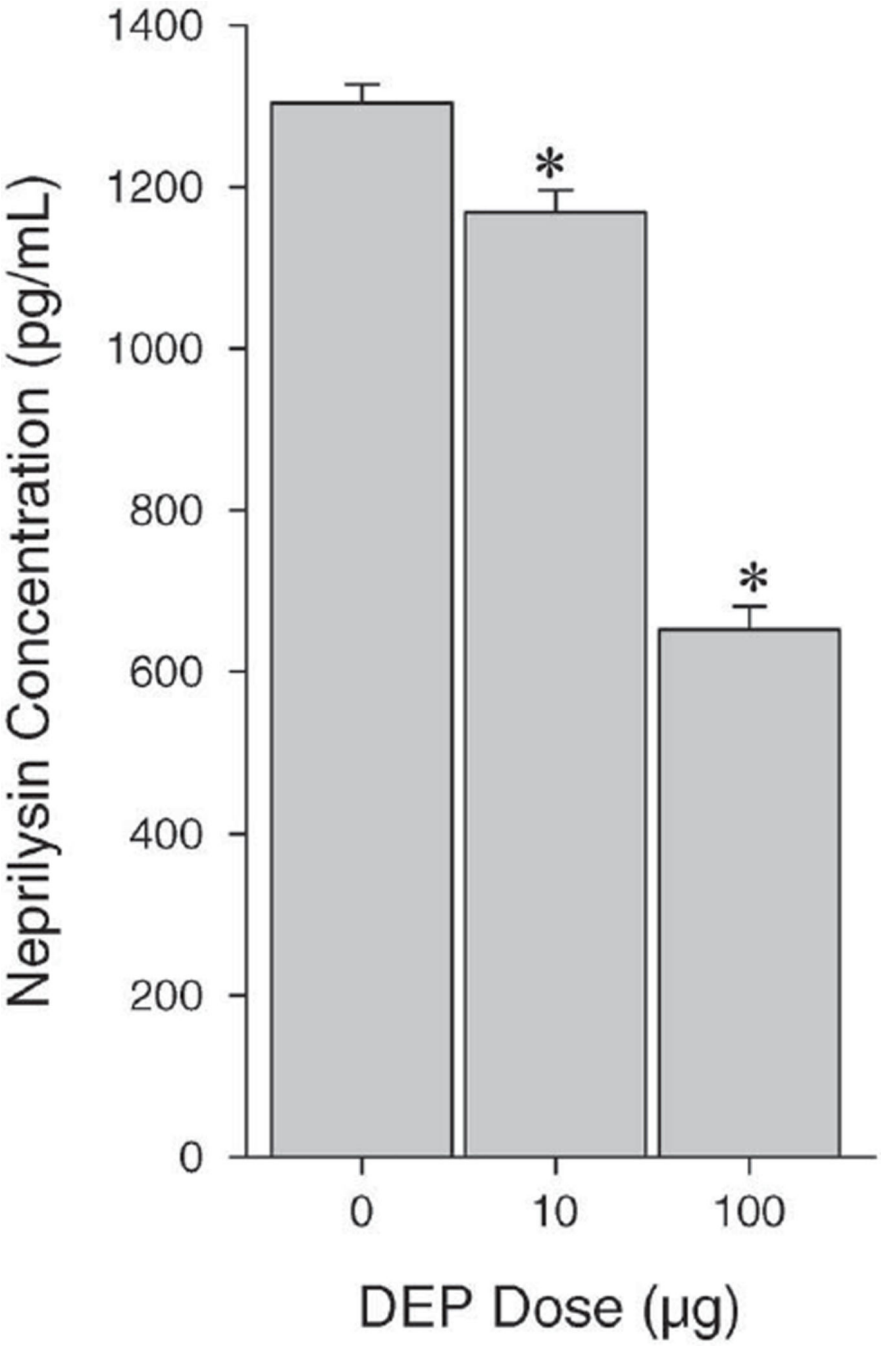

Figure 2. Reduced expression of NEP protein in lung tissue following DEP instillation

Wild-type mice received 0.85% saline vehicle, or 10 or 100 μg DEP in saline. Lung tissue was harvested 7 days after instillation. Data expressed as mean ± SEM ($N = 7$). Asterisk (*) indicates statistically significantly higher compared with control group ($P < 0.05$).

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Figure 3. Immunohistochemical micrographs of lung tissue showing localization and reduction of NEP protein expression after DEP instillation of 0 μg (control); (B) 10 μg; and (C) 100 μg

NEP stain (brown color) was mostly detected in airway epithelial cells, alveolar type II cells (Al), and macrophages. NEP stain density appeared to be reduced in small airway epithelial cells (arrows) after exposure to DEP. Magnification: ×40.

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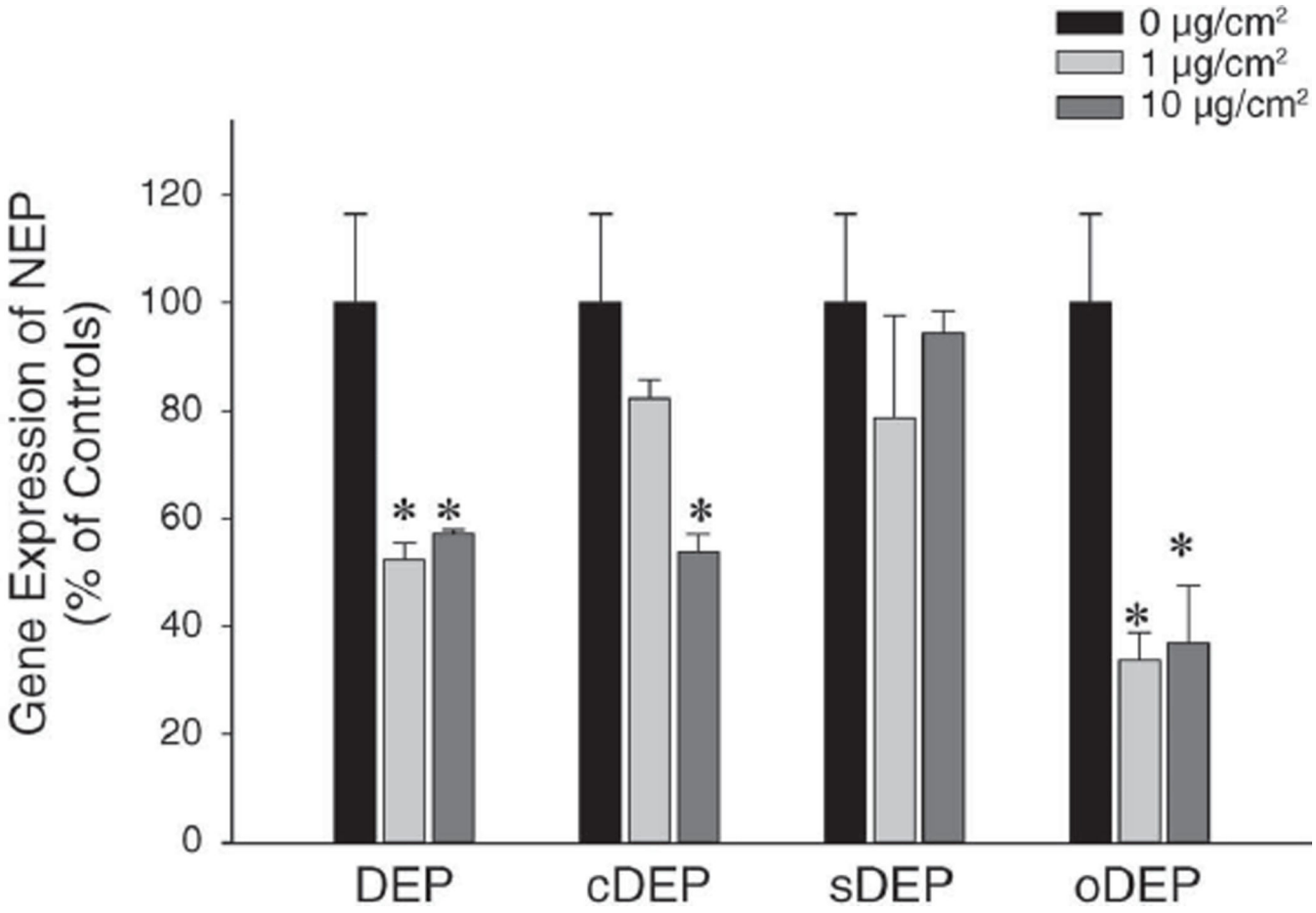

Figure 4. Comparisons of downregulated NEP mRNA expression by DEP and its components in cultured BEAS-2B cells

Cells were treated with (1) DEP (SRM 2975); (2) DEP without transition metals, using chelators (cDEP); (3) carbonaceous core, represented by stripped DEP (sDEP); and (4) another type of particle, oDEP, or standard urban dust (SRM 1649a), at 0, 1, and 10 μg/cm^2 for 24 hours. The mRNA levels were determined by RT-PCR and normalized to endogenous references (GAPDH and 18S rRNA). Values represent the means ± SEM (% of controls) of 3 independent experiments (*P < 0.05).

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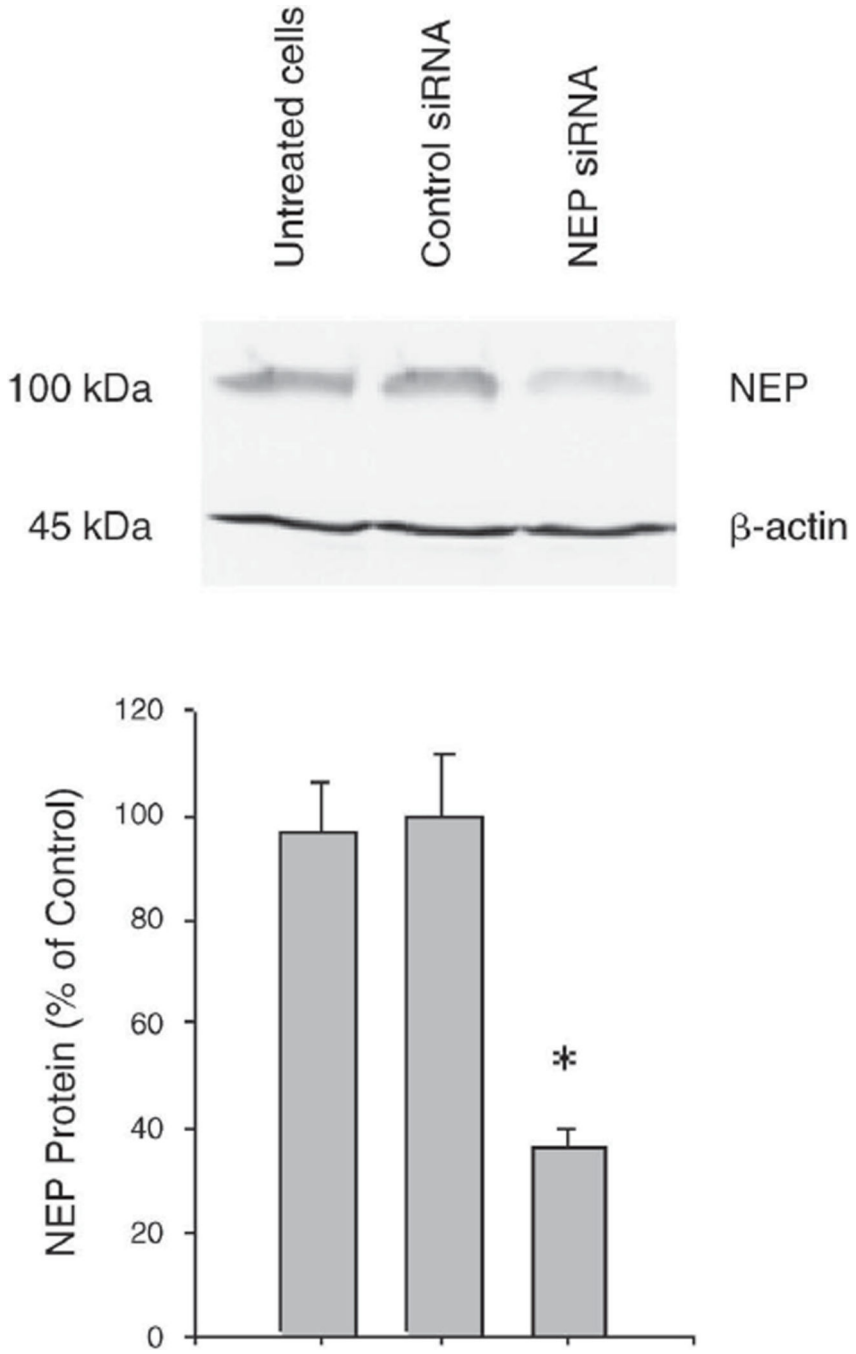

Figure 5. *NEP* transcript knockdown in BEAS-2B cells with *NEP*-specific siRNA

Cells (at 3000 cells/cm^2) were transfected with control siRNA (middle column) or NEP siRNA (right column). Cells were harvested 30 hours post-transfection, and the effect of siRNA transfection was determined by Western blotting. NEP protein expression levels were normalized to [3-actin (*$P < 0.05$).

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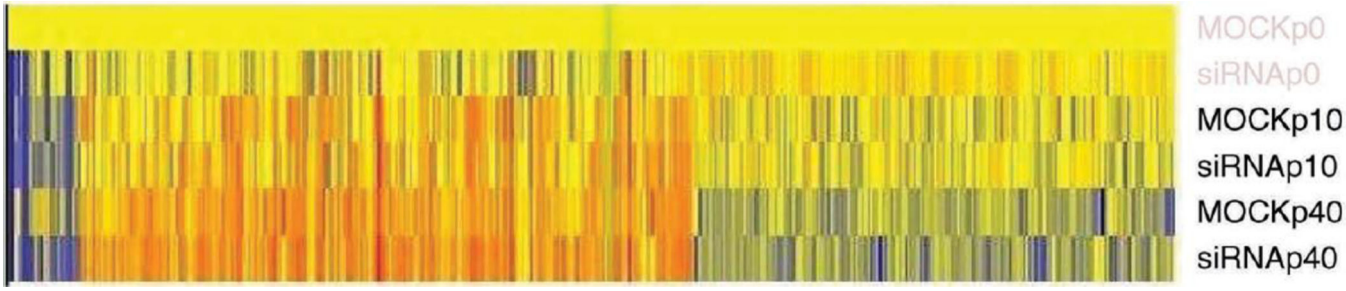

Figure 6. Hybridization signal intensities, which indicate the changes in gene expression in both mock-control and NEP siRNA-transfected cells after DEP exposure

Intensities varied statistically significantly as a function of *NEP* knockdown and DEP exposure. Labeled cRNA from mock or NEP siRNA-transfected cells was tested using two different Affymetrix U133 Plus human genome GeneChips, following 0, 10, 40 μg/cm^2 DEP incubation. About 2158 probe sets, out of a total of 54,675, were detected above background and shown to be statistically different ($P < 0.01$) in the NEP siRNA experimental group when compared with mock controls.

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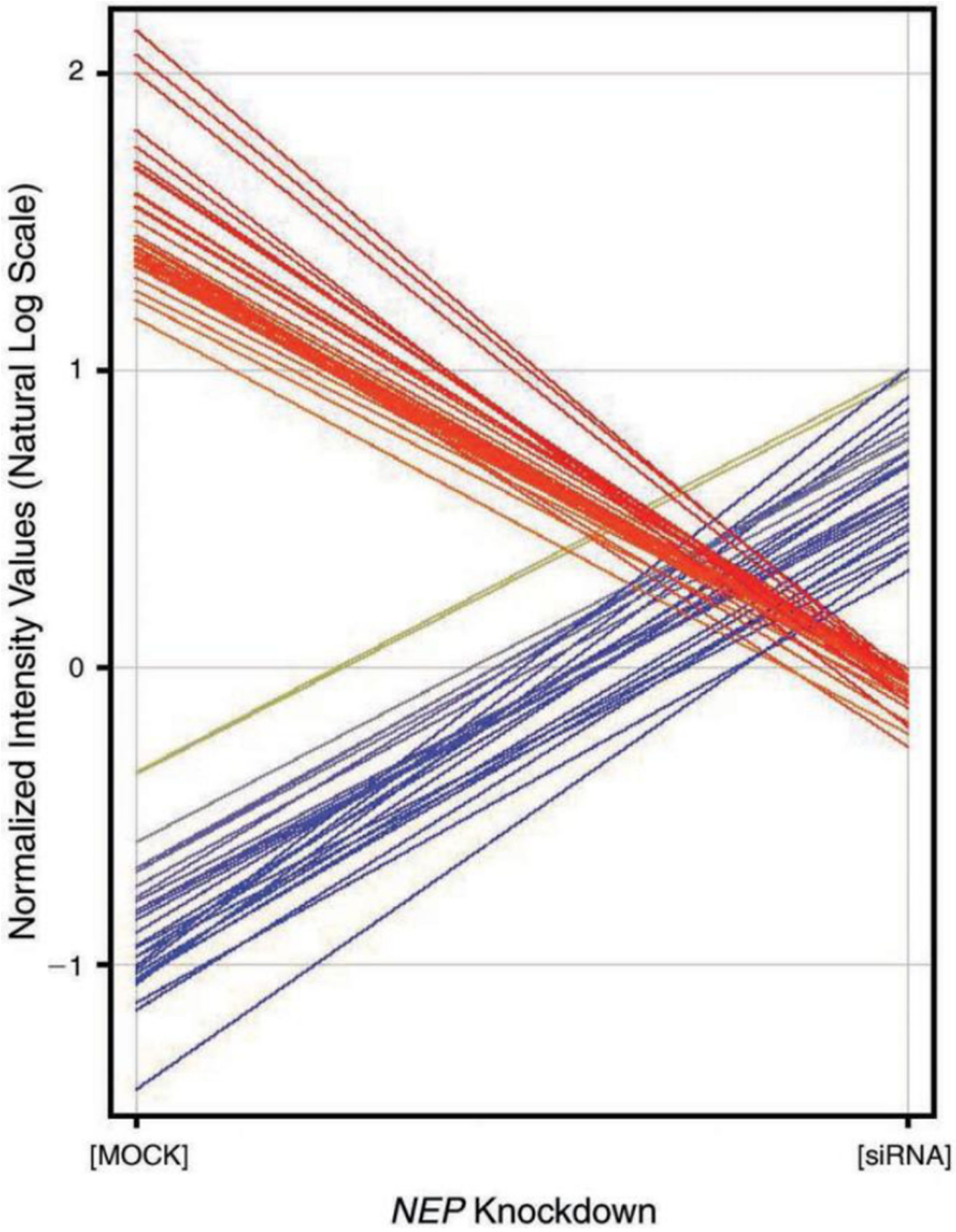

Figure 7. The changes in fluorescent-spot intensity values between *NEP* knockdown and controls

NEP knockdown resulted in 17 upregulated and 14 downregulated genes in in vitro cultures of human airway epithelial BEAS-2B cells.

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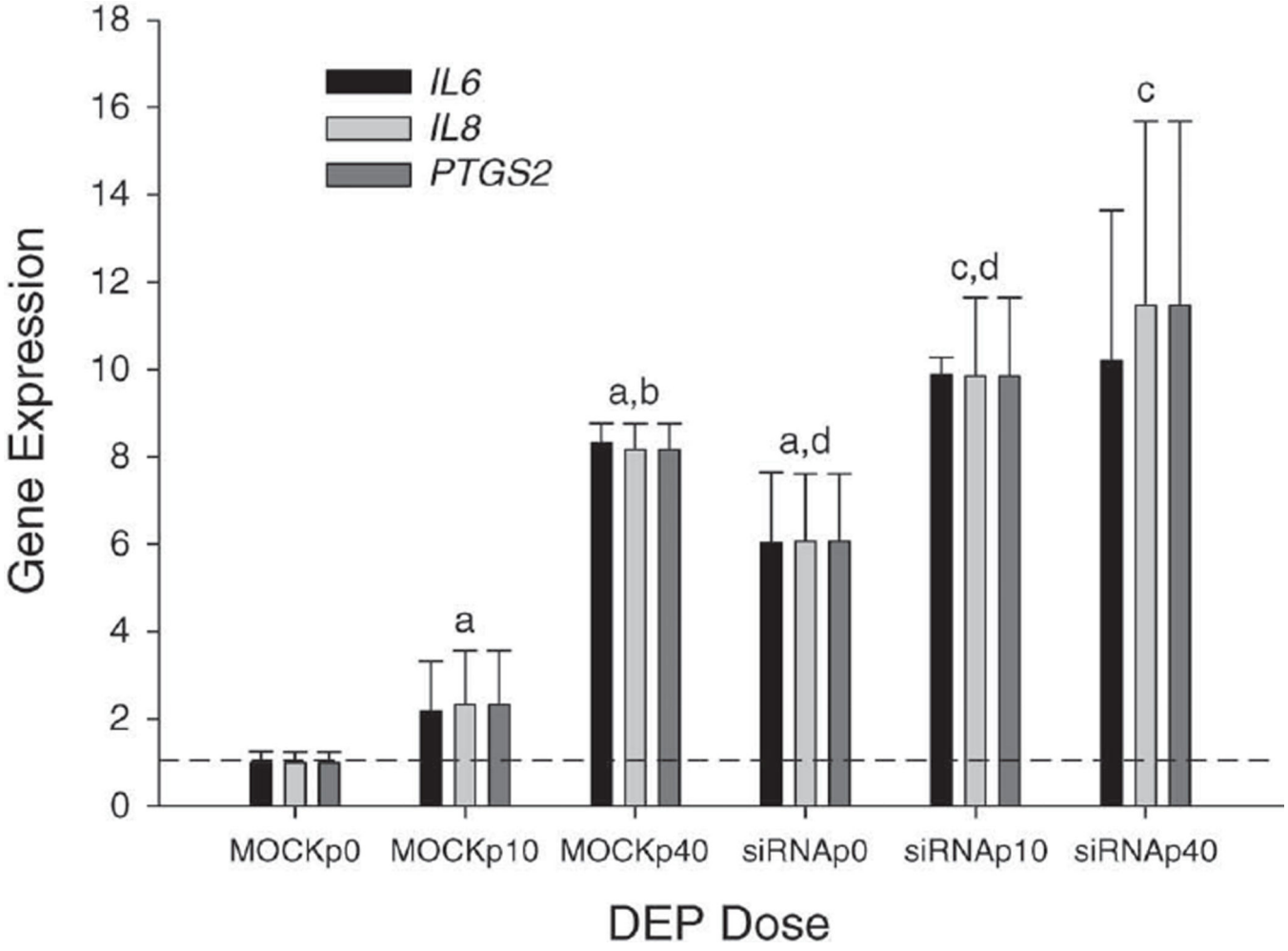

Figure 8. Confirmation of selected upregulated genes (normalized to MOCKp0) either by DEP exposure (0, 10, or 40 μg/cm^2) or by *NEP* knockdown using RT-PCR

Total RNAs isolated from BEAS-2B cells were the same as those of the microarray analyses. Comparisons are as follows: a and b indicate significantly higher compared with the MOCKp0 and MOCKp10 groups, respectively; c indicates significantly higher compared with the siRNAp0 group; and d indicates a significant difference between siRNA and MOCK groups with the same level of DEP ($P < 0.05$).

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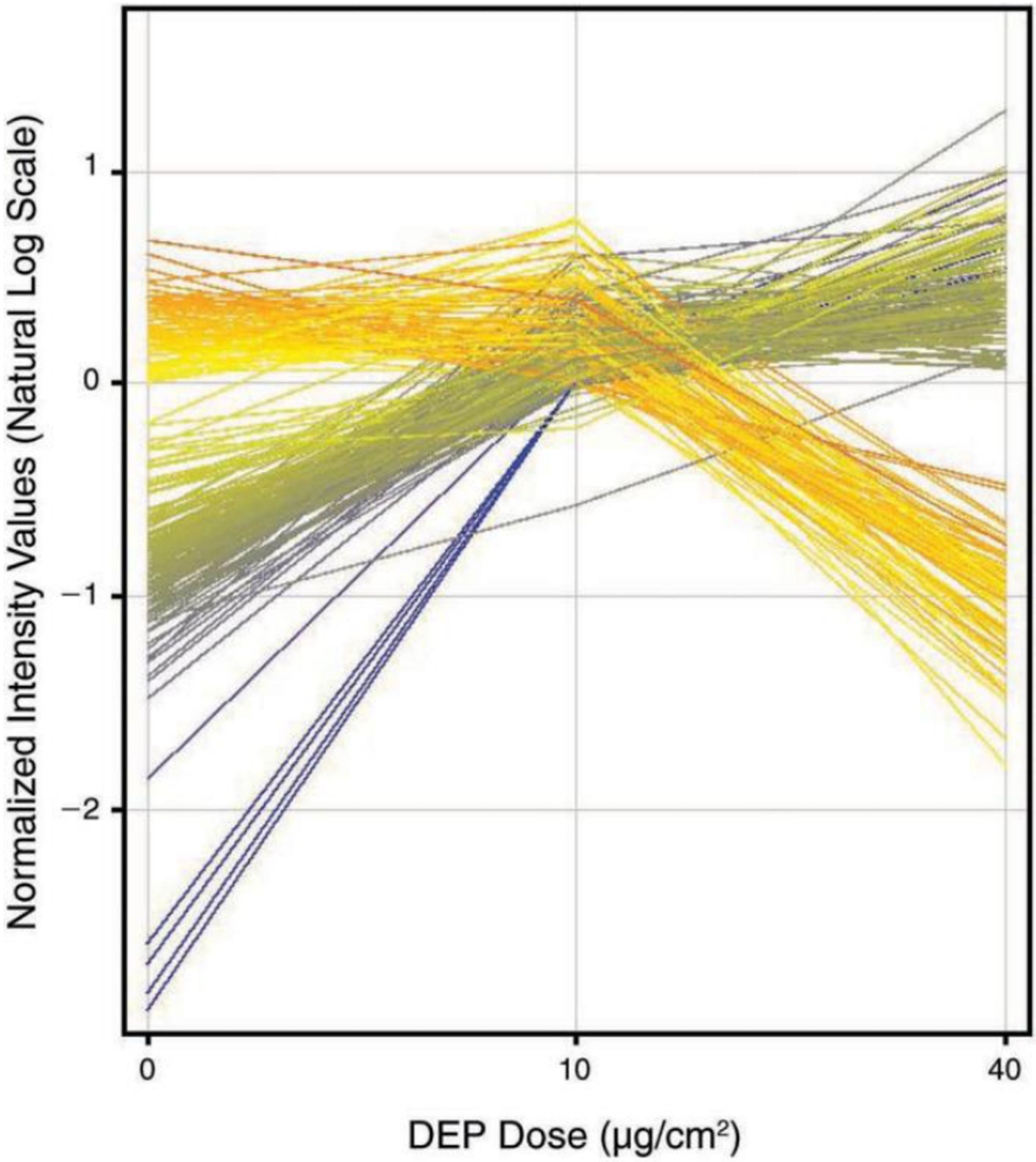

Figure 9. The changes in fluorescent-spot intensity values for genes after DEP exposure

Exposure to either 10 or 40 μg/cm^2 DEP for 24 hours resulted in 151 upregulated and 59 downregulated genes in in vitro cultures of human airway epithelial BEAS-2B cells.

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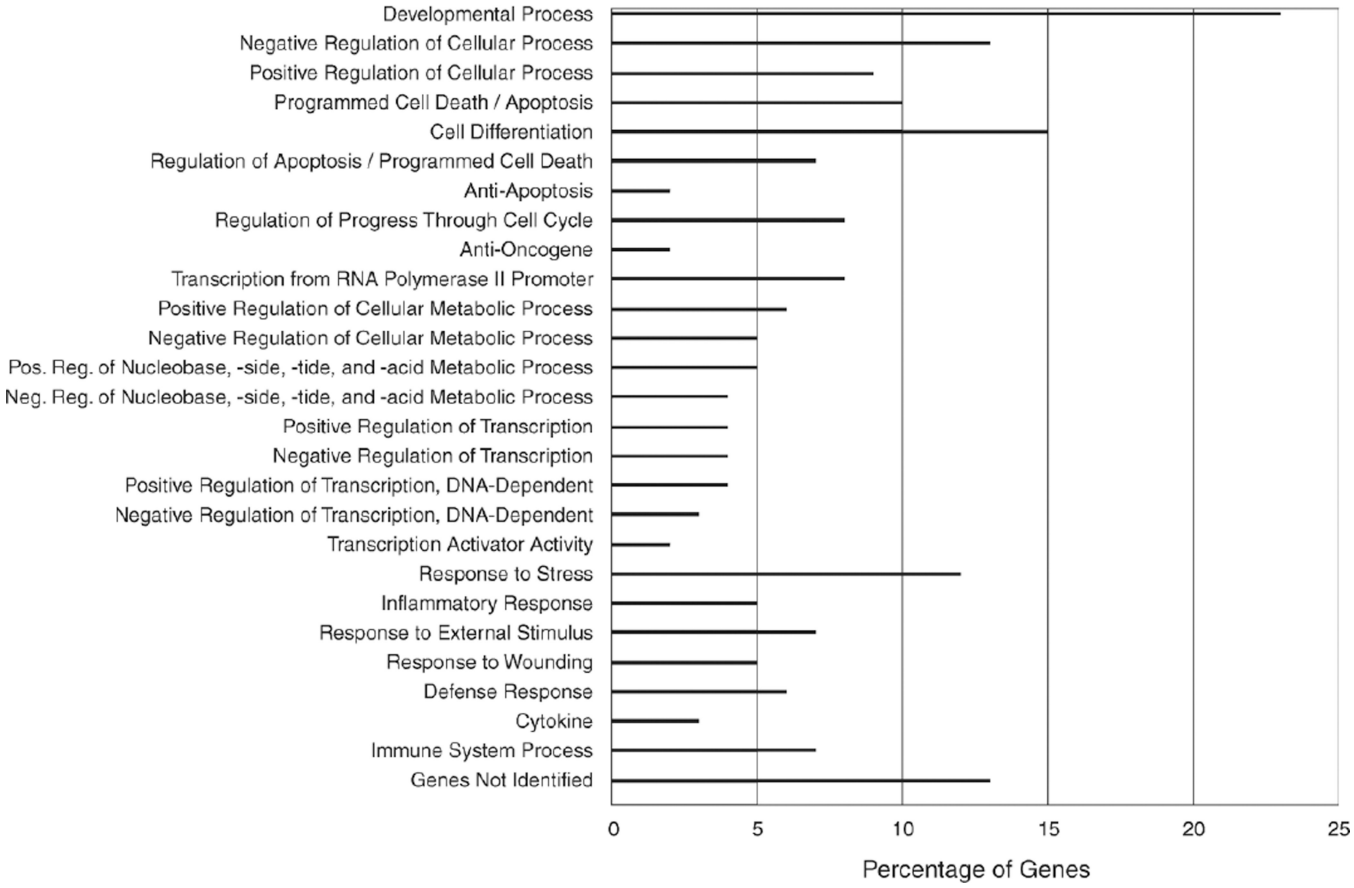

Figure 10. Functional annotation clustering of changed genes in BEAS-2B cells following DEP exposure

Cells were treated with 0, 10, 40 μg/cm^2 DEP for 24 hours. Categories of mode of action are represented on the *y*-axis according to the GO category of cell function (DAVID database). The bars in the histogram indicate the percentage of genes in each category that were up- or downregulated in a dose-dependent manner.

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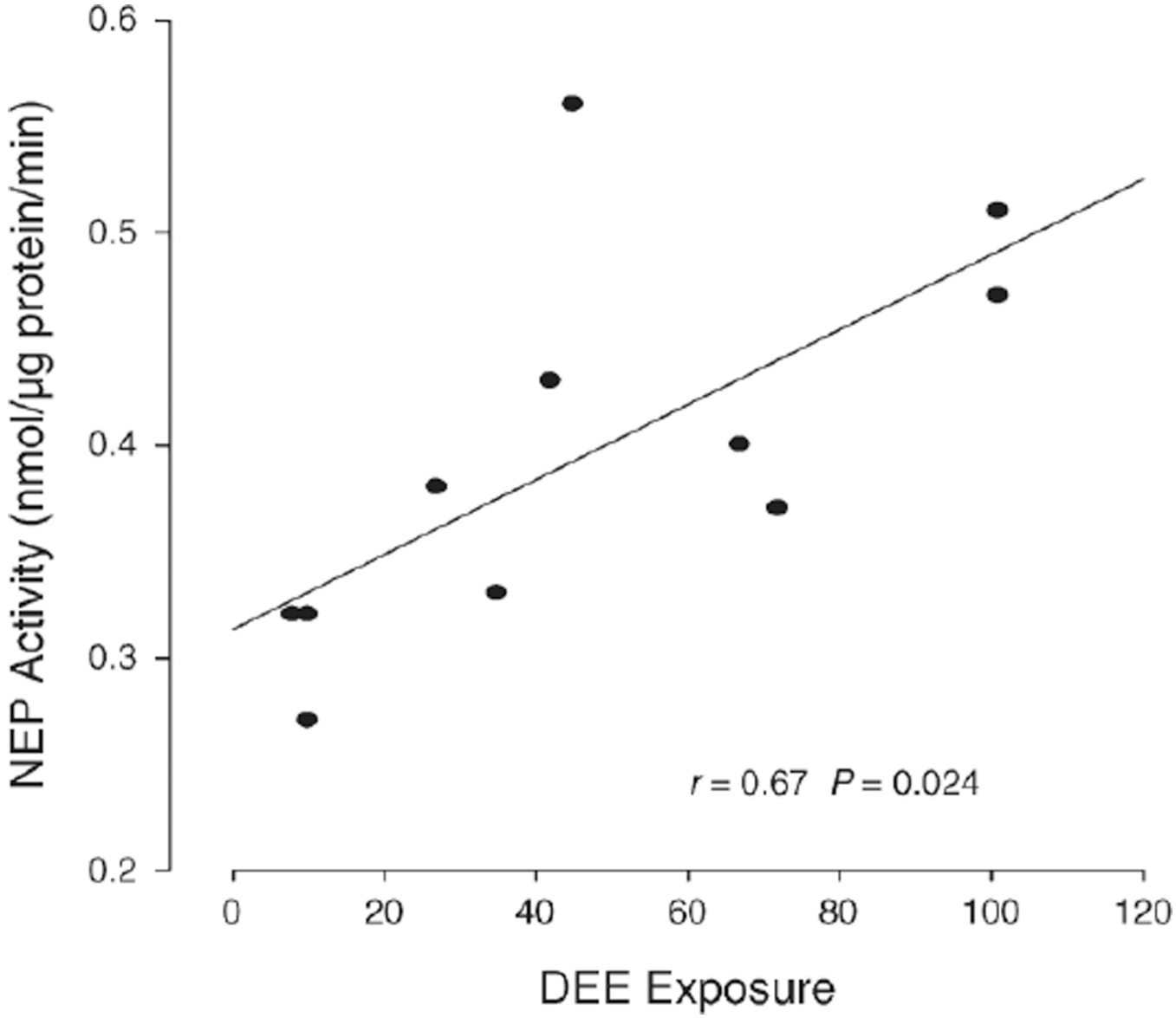

Figure 11. Statistically significant correlations of soluble NEP activity in subject sputum (*N* = 11) with DEE exposure (concentration [mg/m^3] × time [min])

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Table 1

Primer Sequence Sets

Target Gene	Primer / Probe	Sequence (5'→3')	Length (mer)
NEP	Forward	CAGCCGAACCTACAAGGAGTC	21
	Reverse	TGCAATCAAATCCTCGACCAC	21
IL6	Forward	CAATCTGGATTCAATGAGGAGAC	23
	Reverse	CTCTGGCTTGTTCCTCACTACTC	23
IL8	Forward	GAACTGAGAGTGATTGAGAGTGGA	24
	Reverse	CTCTTCAAAAACTTCTCCACAACC	24
EGFR	Forward	GGAGAACTGCCAGAAACTGACC	22
	Reverse	GCCTGCAGCACACTGGTTG	19
PTGS2	Forward	GGAACACAACAGAGTATGCG	20
	Reverse	AAGGGGATGCCAGTGATAGA	20
BCL2L11	Forward	ATCCCCGCTTTTCATCTTTA	20
	Reverse	AGGACTTGGGGTTTGTGTTG	20
18S rRNA	Forward	ACGGACAGGATTGACAGATT	20
	Reverse	GCCAGAGTCTCGTTCGTTAT	20
GAPDH	Forward	ATCCCTCCAAAATCAAGTGG	20
	Reverse	CAGAGATGATGACCCTTTTGG	20

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Table 2

Characteristics of Subjects and Exposure Conditions

Subject Identification	Age (years)	Sex	Race[a]	Exposure Conditions: DEE (EC, mg/m^3)	Exposure Conditions: Time (min)
01	26	M	1	1.20	60
02	33	M	4	0.64	66
03	19	M	1	1.80	56
04	21	M	1	0.75	134
05	22	M	1	0.50	134
06	23	M	1	0.38	119
07	21	F	1	0.32	85
08	19	M	1	0.12	85
09	30	M	2	0.38	93
10	26	M	3	0.15	66
11	22	M	2	0.09	90

[a]Race codes: 1, White; 2, Hispanic; 3, Asian; 4, Other.

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Table 3

Cell Counts by Types (× 10^4 cells/mL) in BAL Fluid of Mice at 7 Days Following DEP Treatment

	0 μg DEP		10 μg DEP		100 μg DEP	
	Wild-type	***Nep*-null**	**Wild-type**	***Nep*-null**	**Wild-type**	***Nep*-null**
Inflammatory cells						
Total cells	41.17 ± 2.93	45.83 ± 3.66	70.29 ± 11.69[a]	130.50 ± 17.09[a,b,c]	100.00 ± 9.21[a]	153.43 ± 10.61[a,b,c]
Macrophages	37.86 ± 2.67	43.16 ± 3.57	65.43 ± 12.21	120.07 ± 18.47[a,b,c]	88.90 ± 10.35[a]	135.18 ± 5.6[a,b,c]
Granulocytes	1.40 ± 0.33	1.21 ± 0.32	2.51 ± 0.44	4.80 ± 0.94	4.07 ± 0.67	6.24 ± 1.06[b]
Lymphocytes	1.91 ± 0.22	1.47 ± 0.29	3.61 ± 0.87	5.63 ± 1.64	7.03 ± 1.79	10.09 ± 2.95[b]
Epithelial cells	1.77 ± 0.65	1.95 ± 0.69	13.01 ± 2.34[a]	19.50 ± 4.43[a,b]	19.35 ± 3.98[a]	37.71 ± 6.42[a,b,c]

[a] $P < 0.05$ compared to wild-type mice at 0 μg DEP exposure.

[b] $P < 0.05$ compared to *Nep*-null mice at 0 μg DEP exposure.

[c] $P < 0.05$ compared to wild-type mice given the same DEP dose.

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Table 4

The Key Characteristics of DEP and Standard Urban Dust

	SRM 2975	SRM 1649a
Sources	DEP (from industrial forklift)	Urban dust
Particle diameter (μm)	31.9	20.8
Particle distribution (μm)		
90%	70	48.6
10%	5.3	2.2
Elemental carbon	+++[a]	+
Trace metals	+	+++
Pesticides	[not available]	Heptachlor, levoglucosan, dibenzo-*p*-dioxin, dibenzofuran congeners
Selected PAHs[b]		
Phenanthrene	17.0 ± 2.8	4.14 ± 0.37
Anthracene	0.038 ± 0.008[c]	0.432 ± 0.082
Fluoranthene	26.6 ± 5.1	6.45 ± 0.18
Pyrene	0.90 ± 0.24	5.29 ± 0.25
Benz[*a*]anthracene	0.317 ± 0.066	2.208 ± 0.073
Chrysene	4.56 ± 0.16	3.049 ± 0.060
Triphenylene	5.22 ± 0.20	1.357 ± 0.054
Benzo[*b*]fluoranthene	11.5 ± 3.6[c]	6.45 ± 0.64
Benzo[*k*]fluoranthene	0.678 ± 0.076	1.913 ± 0.031
Benzo[*a*]fluoranthene	0.06 ± 0.02[c]	0.409 ± 0.035
Benzo[*e*]pyrene	1.11 ± 0.10	3.09 ± 0.19
Benzo[*a*]pyrene	0.0522 ± 0.0053	2.509 ± 0.087
Perylene	0.054 ± 0.009[c]	0.646 ± 0.075
Anthanthrene	0.038 ± 0.008[c]	0.450 ± 0.067
Benzo[*g,h,i*]perylene	0.498 ± 0.044	4.01 ± 0.91
Indeno[1,2,3-*cd*]pyrene	1.4 ± 0.2[c]	3.18 ± 0.72
Dibenz[*a,j*]anthracene	0.37 ± 0.07[c]	0.310 ± 0.034
Dibenz[*a,c/a,h*]anthracene	0.52 ± 0.08[c]	0.488 ± 0.025
Pentaphene	0.038 ± 0.007[c]	0.151 ± 0.035
Benzo[*b*]chrysene	0.08 ± 0.03[c]	0.315 ± 0.013
Picene	1.0 ± 0.2[c]	0.426 ± 0.022
Dibenzo[*b,k*]fluoranthene	2.7	0.724 ± 0.076
Dibenzo[*a,e*]pyrene	0.57	0.565 ± 0.060

[a]Plus signs (+) indicate levels relative to each other.

[b]Values are mass fractions (expressed in mg/kg) of a mean of means from two or more analytical methods ± expanded uncertainty.

[c]Referenced concentrations for selected PAHs.

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Table 5

Up- and Downregulated Genes in *NEP*-Knockdown Human Airway Epithelial Cells

Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log) ([siRNA]/[MOCK])
231578_at	Hs.62661	*GBP1*	Guanylate binding protein 1	1.27
1554997_a_at	Hs.196384	*PTGS2*	Prostaglandin-endoperoxide synthase 2	1.28
204897_at	Hs.199248	*PTGER4*	Prostaglandin e receptor 4 (subtype ep4)	1.81
202393_s_at	Hs.435001	*KLF10*	Kruppel-like factor 10	1.48
219995_s_at	Hs.653124	*ZNF750*	Zinc finger protein 750	1.44
229450_at	Hs.47338	*IFIT3*	Interferon-induced protein with tetratricopeptide repeats 3	1.52
211506_s_at	Hs.624	*IL8*	Interleukin 8	1.89
205207_at	Hs.654458	*IL6*	Interleukin 6	1.73
201565_s_at	Hs.180919	*ID2*	Inhibitor of dna binding 2	1.43
202672_s_at	Hs.460	*ATF3*	Activating transcription factor 3	1.64
202241_at	Hs.444947	*TRIB1*	Tribbles homolog 1 (drosophila)	1.75
238688_at	Hs.133892	*TPM1*	Tropomyosin 1 (alpha)	1.66
1555372_at	Hs.469658	*BCL2L11*	Bcl2-like 11 (apoptosis facilitator)	1.67
222662_at	Hs.458513	*PPP1R3B*	Protein phosphatase 1	1.74
222162_s_at	Hs.643357	*ADAMTS1*	Adam metallopeptidase with thrombospondin type 1 motif	2.12
234608_at	Hs.436367	*LAMA3*	Laminin 3	2.27
206115_at	Hs.534313	*EGR3*	Early growth response 3	3.81
238177_at	Hs.585128	*SLC6A19*	Solute carrier family 6 (neutral amino acid transporter)	−1.09
1558814_s_at	Hs.482873	*TMED5*	Transmembrane emp24 protein transport domain containing 5	−1.04
1565484_x_at	Hs.488293	*EGFR*	Epidermal growth factor receptor	−1.06
1560878_at	Hs.696346	*SYT15*	Synaptotagmin xv	−1.03
236033_at	Hs.56281	*ASB12*	Ankyrin repeat and socs box-containing 12	−1.02
1565537_at	Hs.526396	*NKX1-1*	Nk1 homeobox 1	−1.04
228691_at	Hs.505202	*BICD1*	Bicaudal d homolog 1 (drosophila)	−1.04
1553657_at	Hs.10697	*VWA3A*	von Willebrand factor A domain containing 3A	−1.03
243534_at	Hs.652240	*CC2D2B*	Coiled-coil and C2 domain containing 2B	−1.05
1565483_at	Hs.488293	*EGFR*	Epidermal growth factor receptor	−1.03
1554400_at	Hs.584808	*TCTE3*	T-complex-associated-testis-expressed 3	−1.03
220421_at	Hs.189109	*BTNL8*	Butyrophilin-like 8	−1.06
1568513_x_at	Hs.654496	*IL23A*	Interleukin 23	−1.03
214414_x_at	Hs.654744	*HBA2*	Hemoglobin	−1.01

[a]Per Avadis software platform (Strand Life Sciences, San Francisco, CA).

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Table 6

Gene Upregulation After DEP Exposure in Cultured Human Airway Epithelial Cells

Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
202437_s_at	Hs.154654	*CYP1B1*	Cytochrome P450	1.00	1.22
202435_s_at	Hs.154654	*CYP1B1*	Cytochrome P450	1.00	1.19
205749_at	Hs.72912	*CYP1A1*	Cytochrome P450	1.00	1.29
202436_s_at	Hs.154654	*CYP1B1*	Cytochrome P450	1.00	1.21
202393_s_at	Hs.435001	*KLF10*	Kruppel-like factor 10	1.05	1.51
208937_s_at	Hs.504609	*ID1*	Inhibitor of DNA binding 1	1.08	1.47
208892_s_at	Hs.298654	*DUSP6*	Dual specificity phosphatase 6	1.15	1.29
202241_at	Hs.444947	*TRIB1*	Tribbles homolog 1 (Drosophila)	1.19	1.56
201631_s_at	Hs.591785	*IER3*	Immediate early response 3	1.13	1.30
222168_at	Hs.675520		10q21 mRNA sequence	1.46	1.59
208891_at	Hs.298654	*DUSP6*	Dual specificity phosphatase 6	1.18	1.41
225133_at	Hs.298658	*KLF3*	Kruppel-like factor 3 (basic)	1.25	1.38
234608_at	Hs.436367	*LAMA3*	Laminin	1.36	1.85
202859_x_at	Hs.624	*IL8*	Interleukin 8	1.12	1.42
233413_at	Hs.210390		CDNA FLJ13457 fis	1.06	1.38
231578_at	Hs.62661	*GBP1*	Guanylate binding protein 1	0.48	1.13
213848_at	Hs.595184		MRNA; cDNA DKFZp586F2224	1.09	1.27
227335_at	Hs.517172	*DIDO1*	Death inducer-obliterator 1	1.00	1.73
238909_at	Hs.143873	*S100A10*	S100 calcium binding protein A10	1.00	1.29
235803_at	Hs.658293		Transcribed locus	1.22	1.40
208893_s_at	Hs.298654	*DUSP6*	Dual specificity phosphatase 6	1.24	1.32
232947_at	Hs.661499		CDNA FLJ11476 fis	1.05	1.49
202768_at	Hs.590958	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	0.98	2.20
202581_at	Hs.274402	*HSPA1B*	Heat shock 70kDa protein 1B	1.00	1.58
228280_at	Hs.512833	*ZC3HAV1L*	Zinc finger CCCH-type	1.21	1.43
203394_s_at	Hs.250666	*HES1*	Hairy and enhancer of split 1	0.99	1.50
213680_at		*KRT6B*	Keratin 6B	1.12	1.57

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
204014_at	Hs.417962	*DUSP4*	Dual specificity phosphatase 4	1.44	1.35
232164_s_at	Hs.200412	*EPPK1*	Epiplakin 1	1.38	1.46
212665_at	Hs.12813	*TIPARP*	TCDD-inducible poly(ADP-ribose) polymerase	1.21	1.19
214696_at	Hs.597755	*C17orf91*	Chromosome 17 open reading frame 91	1.07	1.71
1558220_at	Hs.599259	*MUC20*	Mucin 20	1.12	1.32
213765_at	Hs.512842	*MFAP5*	Microfibrillar associated protein 5	1.23	1.22
205207_at	Hs.654458	*IL6*	Interleukin 6 (interferon)	1.19	1.40
227280_s_at	Hs.471234	*CCNYL1*	Cyclin Y-like 1	1.22	1.33
212099_at	Hs.502876	*RHOB*	Ras homolog gene family	1.00	1.72
235629_at	Hs.658355		Transcribed locus	1.36	1.15
200769_s_at	Hs.516157	*MAT2A*	Methionine adenosyltransferase II	1.00	1.35
227697_at	Hs.527973	*SOCS3*	Suppressor of cytokine signaling 3	1.29	1.10
227949_at	Hs.473218	*PHACTR3*	Phosphatase and actin regulator 3	1.03	1.38
203395_s_at	Hs.250666	*HES1*	Hairy and enhancer of split 1	1.02	1.51
242471_at	Hs.191475		Clone HLS_IMAGE_238756 mRNA sequence	1.21	1.13
232174_at	Hs.655763		CDNA: FLJ21635 fis	1.43	1.12
218559_s_at	Hs.702085	*MAFB*	v-maf Musculoaponeurotic fibrosarcoma oncogene	1.06	1.82
205476_at	Hs.75498	*CCL20*	Chemokine (C-C motif) ligand 20	1.49	1.12
228955_at	Hs.280387		Transcribed locus	1.28	1.40
216997_x_at	Hs.444213	*TLE4*	Transducin-like enhancer of split 4	1.18	1.07
211506_s_at	Hs.624	*IL8*	Interleukin 8	1.31	1.51
204614_at	Hs.594481	*SERPINB2*	Serpin peptidase inhibitor	1.58	1.28
226734_at	Hs.292026	*EIF4E2*	Eukaryotic translation initiation factor 4E-2	1.00	1.46
223441_at	Hs.597422	*SLC17A5*	Solute carrier family 17 (anion/sugar transporter)	1.15	1.09
209774_x_at	Hs.590921	*CXCL2*	Chemokine (C-X-C motif) ligand 2	1.40	1.43
1557094_at	Hs.463110	*LOC653110*	Hypothetical LOC653110	1.00	1.73
232181_at	Hs.483816	*LOC153346*	Hypothetical protein LOC153346	1.32	1.32
209684_at	Hs.472270	*RIN2*	Ras and Rab interactor 2	1.08	1.45
212993_at	Hs.531457		MRNA; cDNA DKFZp667B1718	1.00	1.77
221840_at	Hs.127022	*PTPRE*	Protein tyrosine phosphatase	1.16	1.35

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
205064_at	Hs.1076	*SPRR1B*	Small proline-rich protein 1B (cornifin)	1.02	1.16
234594_at	Hs.612888	*C14orf85*	Chromosome 14 open reading frame 85	1.24	1.28
212660_at	Hs.483419	*PHF15*	PHD finger protein 15	1.00	1.65
208712_at	Hs.523852	*CCND1*	Cyclin D1	1.00	1.51
212200_at	Hs.654628	*KIAA0692*	KIAA0692	1.26	1.23
226982_at	Hs.592742	*ELL2*	Elongation factor	1.39	1.17
232165_at	Hs.200412	*EPPK1*	Epiplakin 1	1.40	1.55
201466_s_at	Hs.525704	*JUN*	Jun oncogene	1.22	1.63
226860_at	Hs.688627	*TMEM19*	Transmembrane protein 19	1.16	1.46
244354_at	Hs.128434		ELISC-1	1.12	1.30
241950_at	Hs.680156		Transcribed locus	1.07	1.24
230653_at	Hs.671710	*LOC728555*	Hypothetical protein LOC728555	1.13	1.48
244447_at	Hs.666767		Transcribed locus	1.13	1.24
205463_s_at	Hs.705381	*PDGFA*	Platelet-derived growth factor alpha polypeptide	1.00	1.54
39248_at	Hs.234642	*AQP3*	Aquaporin 3 (Gill blood group)	1.16	1.57
243431_at	Hs.674461		Transcribed locus	1.13	1.54
219016_at		*FASTKD5*	FAST kinase domains 5	1.01	1.69
232541_at	Hs.664233		CDNA FLJ20099 fis	1.18	1.24
202936_s_at	Hs.700579	*SOX9*	SRY (sex determining region Y)-box 9	0.96	1.46
238477_at	Hs.634167		CDNA clone IMAGE:4830091	1.00	1.52
209189_at	Hs.25647	*FOS*	v-fos FBJ murine osteosarcoma viral oncogene homolog	1.00	1.58
227140_at	Hs.28792		CDNA FLJ11041 fis	1.73	1.50
202016_at	Hs.270978	*MEST*	Mesoderm specific transcript homolog (mouse)	1.19	1.58
229126_at	Hs.688627	*TMEM19*	Transmembrane protein 19	1.10	1.30
221768_at	Hs.355934	*SFPQ*	Splicing factor proline/glutamine-rich	1.13	1.27
226974_at	Hs.594057		cDNA clone CS0DF038YD07 of Fetal brain	1.36	1.39
213051_at	Hs.133512	*ZC3HAV1*	Zinc finger CCCH-type	1.06	1.42
212239_at	Hs.132225	*PIK3R1*	Phosphoinositide-3-kinase	1.15	1.24
227034_at	Hs.355455	*ANKRD57*	Ankyrin repeat domain 57	1.15	1.47
212168_at		*RBM12*	RNA binding motif protein 12	1.00	1.49

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
200768_s_at	Hs.516157	*MAT2A*	Methionine adenosyltransferase II	1.00	1.37
232235_at	Hs.124673	*DSEL*	Dermatan sulfate epimerase-like	1.18	1.59
223159_s_at	Hs.197071	*NEK6*	NIMA (never in mitosis gene a)-related kinase 6	1.01	1.35
229549_at	Hs.592258		Transcribed locus	1.06	1.34
225262_at	Hs.220971	*FOSL2*	FOS-like antigen 2	1.00	1.55
208691_at	Hs.529618	*TFRC*	Transferrin receptor (p90)	1.01	1.57
204326_x_at	Hs.374950	*MT1X*	Metallothionein 1X	1.00	1.32
1554250_s_at	Hs.661254	*TRIM73*	Tripartite motif-containing 73	1.22	1.62
221215_s_at	Hs.517310	*RIPK4*	Receptor-interacting serine-threonine kinase 4	0.84	1.79
227964_at	Hs.578433	*FRMD8*	FERM domain containing 8	1.20	1.38
226360_at	Hs.655242	*ZNRF3*	Zinc and ring finger 3	1.28	1.49
204470_at	Hs.789	*CXCL1*	Chemokine (C-X-C motif) ligand 1	1.45	1.40
223474_at	Hs.179260	*C14orf4*	Chromosome 14 open reading frame 4	1.00	1.49
211538_s_at	Hs.432648	*HSPA2*	Heat shock 70kDa protein 2	1.00	1.95
223218_s_at	Hs.319171	*NFKBIZ*	Nuclear factor of kappa light polypeptide gene enhancer	1.43	1.31
219476_at	Hs.32417	*C1orf116*	Chromosome 1 open reading frame 116	1.21	1.88
225601_at	Hs.693708	*HMGB3*	High-mobility group box 3	1.19	1.37
213139_at	Hs.360174	*SNAI2*	Snail homolog 2 (Drosophila)	1.17	1.38
229830_at	Hs.535898		Transcribed locus	1.00	1.44
238431_at	Hs.593044		Transcribed locus	1.29	1.39
225634_at	Hs.133512	*ZC3HAV1*	Zinc finger CCCH-type	1.00	1.81
202935_s_at	Hs.700579	*SOX9*	SRY (sex determining region Y)-box 9	0.94	1.38
201465_s_at	Hs.525704	*JUN*	Jun oncogene	1.14	1.64
242434_at	Hs.529514		CDNA FLJ31093 fis	1.08	1.51
239448_at	Hs.658524		Transcribed locus	1.33	1.81
209446_s_at		*C7orf44*	Chromosome 7 open reading frame 44	1.04	1.40
222243_s_at	Hs.474978	*TOB2*	Transducer of ERBB2	1.21	1.39
200799_at	Hs.520028	*HSPA1A*	Heat shock 70kDa protein 1A	1.00	2.02
229492_at	Hs.515130	*VANGL1*	Vang-like 1 (van gogh)	1.04	1.57
211985_s_at	Hs.282410	*CALM1*	Calmodulin 1 (phosphorylase kinase)	1.00	1.86

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
228977_at	Hs.130652	*LOC729680*	Hypothetical protein LOC729680	1.11	1.44
208161_s_at	Hs.463421	*ABCC3*	ATP-binding cassette	1.00	1.44
209360_s_at	Hs.149261	*RUNX1*	Runt-related transcription factor 1	1.01	1.55
218051_s_at	Hs.84753	*NT5DC2*	5'-Nucleotidase domain containing 2	1.00	2.04
223217_s_at	Hs.319171	*NFKBIZ*	Nuclear factor of kappa light polypeptide gene enhancer	1.38	1.49
233506_at	Hs.102941		Full length insert cDNA clone ZB81B12	1.17	1.53
226632_at	Hs.95120	*CYGB*	Cytoglobin	1.00	1.67
222450_at	Hs.517155	*TMEPAI*	Transmembrane	1.00	2.58
209260_at	Hs.523718	*SFN*	Stratifin	1.00	1.69
235086_at	Hs.164226	*THBS1*	Thrombospondin 1	1.47	2.40
205027_s_at	Hs.432453	*MAP3K8*	Mitogen-activated protein kinase kinase kinase 8	1.68	1.85
201150_s_at	Hs.701968	*TIMP3*	TIMP metallopeptidase inhibitor 3	1.00	1.76
200880_at	Hs.445203	*DNAJA1*	DnaJ (Hsp40) homolog	1.00	1.66
234730_s_at	Hs.517310	*RIPK4*	Receptor-interacting serine-threonine kinase 4	1.13	2.04
219371_s_at	Hs.107740	*KLF2*	Kruppel-like factor 2 (lung)	0.76	1.77
214702_at	Hs.203717	*FN1*	Fibronectin 1	1.74	1.64
242539_at	Hs.657550	*LOC730069*	Nuclear receptor binding factor 2 pseudogene	1.06	2.26
226574_at	Hs.213198	*PSPC1*	Paraspeckle component 1	1.00	1.88
202434_s_at	Hs.154654	*CYP1B1*	Cytochrome P450	1.00	1.87
208711_s_at	Hs.523852	*CCND1*	Cyclin D1	1.00	2.07
229978_at	Hs.130661	*LOC440338*	Hypothetical gene supported by AJ002784	1.00	2.28
208153_s_at	Hs.591255	*FAT2*	FAT tumor suppressor homolog 2 (Drosophila)	1.00	2.29
215462_at	Hs.632415	*PLK3*	Polo-like kinase 3 (Drosophila)	1.00	2.08
1557049_at	Hs.700947	*LOC149478*	Hypothetical protein LOC149478	1.40	2.32
207332_s_at	Hs.529618	*TFRC*	Transferrin receptor (p90)	1.00	2.12
211984_at	Hs.282410	*CALM1*	Calmodulin 1 (phosphorylase kinase)	1.00	2.33
200800_s_at	Hs.520028	*HSPA1*	Heat shock 70kDa protein 1A	1.00	2.80
222853_at	Hs.41296	*FLRT3*	Fibronectin leucine rich transmembrane protein 3	1.00	3.14
205249_at	Hs.1395	*EGR2*	Early growth response 2 (Krox-20 homolog)	1.64	2.78
222895_s_at	Hs.699440	*BCL11B*	B-cell CLL/lymphoma 11B (zinc finger protein)	1.00	3.69

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
1555847_a_at	Hs.436426	*LOC284454*	Hypothetical protein LOC284454	0.33	3.34
206393_at	Hs.523403	*TNNI2*	Troponin I type 2 (skeletal)	1.00	3.64
206115_at	Hs.534313	*EGR3*	Early growth response 3	2.52	3.70
228523_at	Hs.591918	*NANOS1*	Nanos homolog 1 (Drosophila)	1.00	5.08

[a]Per Avadis software platform (Strand Life Sciences, San Francisco, CA).

[b]Compared with control (0 μg/cm^2 DEP).

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Table 7

Gene Downregulation After DEP Exposure in Cultured Human Airway Epithelial Cells

Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
219270_at	Hs.155569	*CHAC1*	ChaC	−0.11	−23.60
206085_s_at	Hs.19904	*CTH*	Cystathionase (cystathionine gamma-lyase)	3.12	−18.20
1555788_a_at	Hs.516826	*TRIB3*	Tribbles homolog 3 (Drosophila)	1.78	−16.33
217168_s_at	Hs.146393	*HERPUD1*	Homocysteine-inducible	2.05	−14.18
238760_at	Hs.213264	*YARS*	Tyrosyl-tRNA synthetase	0.65	−13.95
223062_s_at	Hs.494261	*PSAT1*	Phosphoserine aminotransferase 1	1.41	−11.09
220892_s_at	Hs.494261	*PSAT1*	Phosphoserine aminotransferase 1	0.22	−13.13
209383_at	Hs.505777	*DDIT3*	DNA-damage-inducible transcript 3	0.49	−11.42
241905_at	Hs.175343	*PIK3C2A*	Phosphoinositide-3-kinase	1.16	−8.36
213672_at	Hs.632707	*MARS*	Methionyl-tRNA synthetase	2.43	−7.78
1554008_at	Hs.120658	*OSMR*	Oncostatin M receptor	0.22	−7.09
205047_s_at	Hs.489207	*ASNS*	Asparagine synthetase	0.14	−10.87
223195_s_at	Hs.469543	*SESN2*	Sestrin 2	0.78	−6.36
1554933_at	Hs.658434	*PSIP1*	PC4 and SFRS1 interacting protein 1	0.00	−5.86
243299_at	Hs.666703		Transcribed locus	1.71	−5.78
202388_at	Hs.78944	*RGS2*	Regulator of G-protein signaling 2	−1.00	−7.41
239451_at	Hs.658060		CDNA FLJ26407 fis	0.06	−6.45
222763_s_at	Hs.620490	*WDR33*	WD repeat domain 33	−0.40	−5.31
220195_at	Hs.458312	*MBD5*	Methyl-CpG binding domain protein 5	0.18	−5.04
238311_at	Hs.668598		Transcribed locus	1.19	−5.11
204203_at	Hs.429666	*CEBPG*	CCAAT/enhancer binding protein (C/EBP)	0.03	−5.48
232017_at	Hs.50382	*TJP2*	Tight junction protein 2 (zona occludens 2)	−0.54	−5.95
206884_s_at	Hs.534699	*SCEL*	Sciellin	0.77	−4.23
244822_at	Hs.473648	*GART*	Phosphoribosylglycinamide formyltransferase	0.24	−5.74
243543_at	Hs.655159		Transcribed locus	−0.49	−4.88
1556602_at	Hs.663643		CDNA FLJ36293 fis	0.91	−3.93
233406_at	Hs.667905		CDNA FLJ12038 fis	1.43	−6.13

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b] 10 μg/cm²	40 μg/cm²
218943_s_at	Hs.190622	*DDX58*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	−0.03	−4.83
203438_at	Hs.233160	*STC2*	Stanniocalcin 2	1.94	−4.76
230097_at	Hs.473648	*GART*	Phosphoribosylglycinamide formyltransferase	−0.30	−6.62
244075_at	Hs.380900		Transcribed locus	−0.15	−4.79
244803_at	Hs.658791		Transcribed locus	0.74	−4.64
229999_at	Hs.432355		Full length insert cDNA clone ZE12A08	−0.84	−3.77
238875_at	Hs.656795		Clone HLS_IMAGE_731119 mRNA sequence	0.48	−6.35
243332_at	Hs.670186		Transcribed locus	0.82	−4.46
236244_at	Hs.166463	*HNRNPU*	Heterogeneous nuclear ribonucleoprotein U	−1.00	−3.59
215470_at			General transcription factor IIH	−0.98	−3.67
240206_at	Hs.481860	*TARS*	Threonyl-tRNA synthetase	−0.78	−3.39
236149_at	Hs.677583		Transcribed locus	0.77	−2.96
242784_at	Hs.665271		Transcribed locus	−0.37	−3.31
241433_at	Hs.696152	*RCOR3*	REST corepressor 3	−1.00	−3.32
221577_x_at	Hs.616962	*GDF15*	Growth differentiation factor 15	−0.30	−4.77
206411_s_at	Hs.159472	*ABL2*	v-abl Abelson murine leukemia viral oncogene homolog 2	−0.75	−3.41
218986_s_at	Hs.591710	*FLJ20035*	Hypothetical protein FLJ20035	−0.66	−3.33
233436_at	Hs.657656	*MTBP*	Mdm2	−0.16	−2.88
242712_x_at	Hs.652430	*RANBP2*	RAN binding protein 2	−0.30	−3.59
243631_at	Hs.659894	*LOC642333*	Similar to M-phase phosphoprotein	−0.06	−3.84
237839_at	Hs.672300		Transcribed locus	0.02	−3.39
238348_x_at	Hs.667738		Transcribed locus	−0.67	−4.14
230375_at	Hs.520287	*SFRS18*	Splicing factor	0.21	−4.04
238357_at	Hs.282901	*RBM39*	RNA binding motif protein 39	−1.00	−2.82
230516_at	Hs.87385	*C7orf30*	Chromosome 7 open reading frame 30	0.01	−3.04
238666_at	Hs.659573		Transcribed locus	−0.37	−3.75
239358_at	Hs.655048		Transcribed locus	−0.65	−3.06
236696_at	Hs.596572	*SR140*	U2-associated SR140 protein	0.38	−2.36
237379_at	Hs.325838	*KIAA1542*	CTD-binding SR-like protein rA9	−1.00	−1.93
217616_at	Hs.613488		Transcribed locus	−1.00	−1.78

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Probe Set ID	Unigene ID[a]	Gene Symbol	Gene Title	Fold (log)[b]	
				10 μg/cm^2	40 μg/cm^2
1559691_at	Hs.684424		CDNA clone IMAGE:3869664	−0.41	−2.12
203434_s_at	Hs.307734	*MME (NEP)*	Membrane metallo-endopeptidase (neprilysin)	−0.53	−0.76

[a]Per Avadis software platform (Strand Life Sciences, San Francisco, CA).

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Table 8

Sputum Cell Profiles in Human Subjects Exposed to DEE

Cell Type	Cell Number[a]	
	Baseline	After DEE Exposure
Inflammatory cells (× 10^6/mL)		
Total	7.98 ± 1.68	20.29 ± 7.65[b]
Macrophages	6.53 ± 1.32	16.10 ± 5.57[b]
Neutrophils	1.29 ± 0.49	4.05 ± 2.32
Lymphocytes	0.15 ± 0.05	0.21 ± 0.08
Epithelial cells (× 10^3/mL)	2.80 ± 0.82	12.43 ± 7.98[b]

[a]Average for 11 subjects ± SEM.

[b]$P < 0.05$ when compared with baseline ($N = 11$).

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Table 9

Induced Sputum Protein Level and NEP Activity in Human Subjects Before and After Exposure to DEE

	Total protein (μg/μL)		NEP (nmol/μ protein/min)	
	Baseline	**After DEE Exposure**	**Baseline**	**After DEE Exposure**
	1.05	1.07	0.364	0.369
	0.66	0.99	0.445	0.431
	1.00	0.97	0.436	0.507
	1.27	1.21	0.258	0.465
	0.41	0.84	0.395	0.399
	0.56	0.43	0.294	0.558
	0.82	1.13	0.413	0.272
	1.16	0.79	0.159	0.382
	0.74	0.92	0.289	0.332
	0.94	1.00	0.164	0.324
	0.41	0.32	0.115	0.320
Mean	0.82	0.88	0.303	0.396
SEM	0.09	0.08	0.036	0.026
P value[a]		0.42		0.035

[a] *P* values based on a two-tailed, paired sample *t* test ($N = 11$).