

Pro-inflammatory responses by diesel exhaust particles in epithelial lung cells:

Importance of Toll-like receptor 3 priming and
role of soluble organic components.

Nicolai Skoglund Bach



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Dedicated to Kirsten Bach.

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Nicolai Skoglund Bach

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Abstract

Ambient air pollution in urban areas may contain a considerable proportion of diesel exhaust particles (DEP) which have been implicated in adverse pulmonary health effects. People with preexisting pulmonary diseases may be at increased risk towards DEP-induced negative health outcomes where inflammation seems to be a key factor. In this study we have investigated cytokine and chemokine production, and the underlying cellular mechanisms, in immortalized human bronchial epithelial cells (BEAS-2B) exposed to DEP, DEP-extracts or fractionated DEP-extracts. The Toll-like receptor (TLR)3 agonist polyinosinic:polycytidylic (Poly I:C) which mimics viral RNA, was used to prime BEAS-2B cells before exposure. DEP appeared to induced stronger interleukin (IL)-6/IL-8 responses in primed cells compared to unprimed cells, but at the same time also suppressed RANTES responses in TLR-3-primed cells. The increased IL-6/IL-8 responses by DEP in TLR3-primed cells were possibly due to poly I:C induced activation of signaling pathways required for optimal IL-6/IL-8 responses (NF- κ B and p38). Moreover, DEP suppressed certain poly I:C induced signaling pathways (ERK and JNK), possibly resulting in the reduced RANTES responses in TLR3-primed cells. Non-polar soluble DEP-components appeared to induce both IL-6 and IL-8 in BEAS-2B cells, whereas high-polar soluble components only appeared to induce IL-6. Knock-down of the aryl hydrocarbon receptor (AhR), a main cellular sensor of aromatic hydrocarbons, did not reduce, but rather enhanced DEP-induced IL-6/IL-8 responses, suggesting that “classical” AhR-activating PAHs may not be the main drivers of DEP-induced inflammation. However, knock-down of protease activated receptor (PAR)-2 suppressed DEP-induced IL-6. Our results show altered cytokine/chemokine responses in TLR3-primed versus unprimed cells, which imply that “sick” cells may respond differently to DEP than “healthy” cells. Furthermore, different compounds in DEP, partly separated by polarity, seemed to differentially induce cytokine/chemokine responses, indicating that polarity may be an important parameter when evaluating the toxicity of DEP.

Abbreviations

AD, aerodynamic diameter; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; B[a]P, benzo[a]pyrene; BEAS-2B, immortalized human bronchial epithelial cells; BEC, bronchial epithelial cells; COPD, chronic obstructive pulmonary disease; CYP, cytochrome P450; CCL5/RANTES, Regulated on Activation, Normal T cell Expressed and Secreted; CXCL8/IL-8, interleukin-8; DE, diesel exhaust; DEP, diesel exhaust particles; ELISA, *Enzyme-Linked ImmunoSorbent Assay*; ERK, extra-cellular regulated kinase; HRP, horseradish peroxidase; I κ B, inhibitor of κ B; IL-6, interleukin-6; JNK, c-jun N-terminal kinase; LM, lipid mediators; LPS, lipopolysaccharide; MAPK, Mitogen-Activated Protein Kinase; NEMO, NF- κ B essential modulator; NO₂, nitrogen dioxide; NF- κ B, nuclear factor-kappa B; O₃, ozone; PAH, polycyclic aromatic hydrocarbons; PAMP, pathogen associated molecular patten; PAR, proteinase activated receptor; PI, propidium iodide; PM, particulate matter; Poly I:C, polyinosinic:polycytidylic acid; PRR, pathogen recognition receptors; real-time RT-PCR, real-time reverse transcriptase polymerase chain reaction; PI, propidium iodide; PM, particulate matter; ROS, reactive oxygen species; siRNA, small interfering RNA; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SO₂, sulfur dioxide; TLR, Toll-like receptors; TMB, tetramethylbenzidine; TP, toxic proteases; XRE, xenobiotic response elements.

Background

Introduction

Ambient air pollution is composed of a complex mixture of particles, gases and aerosols, which varies depending on the location, source and/or climate. Humans have always been exposed to ambient air pollutants and have therefore adapted cellular defense mechanisms to protect against agents that may cause disease. Natural sources like volcanos, forest fires and debris from deserts and oceans contribute to a certain extent to air pollution. However, previously dominated by burning of coal, the composition of ambient air pollutants have changed dramatically the last centuries, due to the industrial revolution and greater reliance on fossil fuel. Emissions from anthropogenic sources, especially the industry, increased rapidly during the 1900s with several incidents linking excessive anthropogenic air pollution to increased morbidity and mortality. For example, the air pollution disaster in 1932 in Meuse Valley, Belgium, over a thousand people were affected with adverse respiratory symptoms and the incidence caused around 60 deaths over a period of four days (Nemery et al. 2001). Moreover, in 1952, the city of London was covered by a dense smog resulting in several thousand deaths over a period of a few months (Bell & Davis 2001). These and other events led to a higher focus on the possible adverse effects caused by air pollution. Therefore, there was a need for regulation, and the government of USA formed *The Clean Air Act* (CAA) in 1970, which was followed by the establishment of the *Environmental Protection Agency* (EPA). A higher focus on regulation of air pollution was also developing in Europe during the 1980s. As a result, levels of air pollution in industrialized countries have decreased drastically the last decades. Interestingly however, air pollution still reaches levels that may cause adverse health effects, such as excess mortality (Madsen et al. 2012). Moreover, as developing countries are getting more industrialized, they seem to face the same challenges (Klaassen 2007). Currently, ambient air pollution is ranked by WHO as the second leading cause of adverse health effects in humans due to environmental chemicals (Prüss-Ustün et al. 2011). Therefore, air pollution will probably still play an important role for human health the following decades.

While the sources of pollutants in ambient air were mainly dominated by wood fire and industry until the mid 1900s, motor vehicles have become a major contributor in the later years. Notably, diesel motor vehicles are increasingly popular, especially in Europe, partly due to higher fuel efficiency, longer durability and lower fuel cost (Wichmann 2007; Weisenberger 1984). In addition,

the governments of several European countries, including Norway, have indirectly favored diesel engine motor vehicles over gasoline by reducing emission taxes, to bring down CO₂-emissions (Tanaka et al. 2012). Despite lower levels of air pollution overall, the composition of air pollution has changed. Diesel exhaust (DE), characterized by a large number of small particles, is a major contributor to the air pollution in urban areas. These particles appear to significantly contribute to adverse pulmonary health effects (Wichmann 2007).

Particulate matter

PM, along with SO₂, NO₂ and O₃, may account for the greatest part of toxic effects of air pollution (Prüss-Ustün et al. 2011). PM often derives from combustion or abrasion processes, especially in urban areas. Typical sources are vehicles, roads or woodstoves (Schwarze et al. 2006). The effects of PM may affect the pulmonary and/or the cardiovascular system and is associated with increased morbidity and mortality and may lead to acute deaths (Pope & Dockery 2006; Madsen et al. 2012). The biological responses of PM may be assigned to the particle's size, surface properties and attached components (Schwarze et al. 2006). Smaller particles seem to be more toxic than larger particles of similar type (Donaldson et al. 2001). Moreover, epidemiological studies correlate exposure to PM and adverse health effects, especially in patients with preexisting diseases (Kappos et al. 2004; Dockery et al. 1993; Dockery & Pope 1994; Samet et al. 2000; Goeminne et al. 2012; NCEA 2002). Therefore, several illnesses such as asthma, cystic fibrosis, chronic obstructive pulmonary disease (COPD) and diabetes may increase susceptibility to PM (Sacks et al. 2011). In addition, PM may contain mutagenic and carcinogenic substances and have been linked to the development of cancer (Pope et al. 2002).

Particle size

The size of PM is defined as aerodynamic diameter (AD), which is the diameter of a hypothetical spherical particle with density equal to 1 g/cm³ falling with the same velocity in gas as the irregular-shaped particle of interest. Due to the small size, these particles have a large surface area to mass ratio, which gives them excellent properties as a vehicle for toxic components. Fig. 1 illustrates size fractions used: PM₁₀ (AD < 10 µm), PM_{2.5} (AD < 2.5 µm), PM₁ (AD < 1 µm) and PM_{0.1} (AD < 0.1 µm). PM is usually divided in three categories: Coarse fraction (PM_{10-2.5}), fine fraction (PM_{2.5}) and ultrafine fraction (PM_{0.1}).

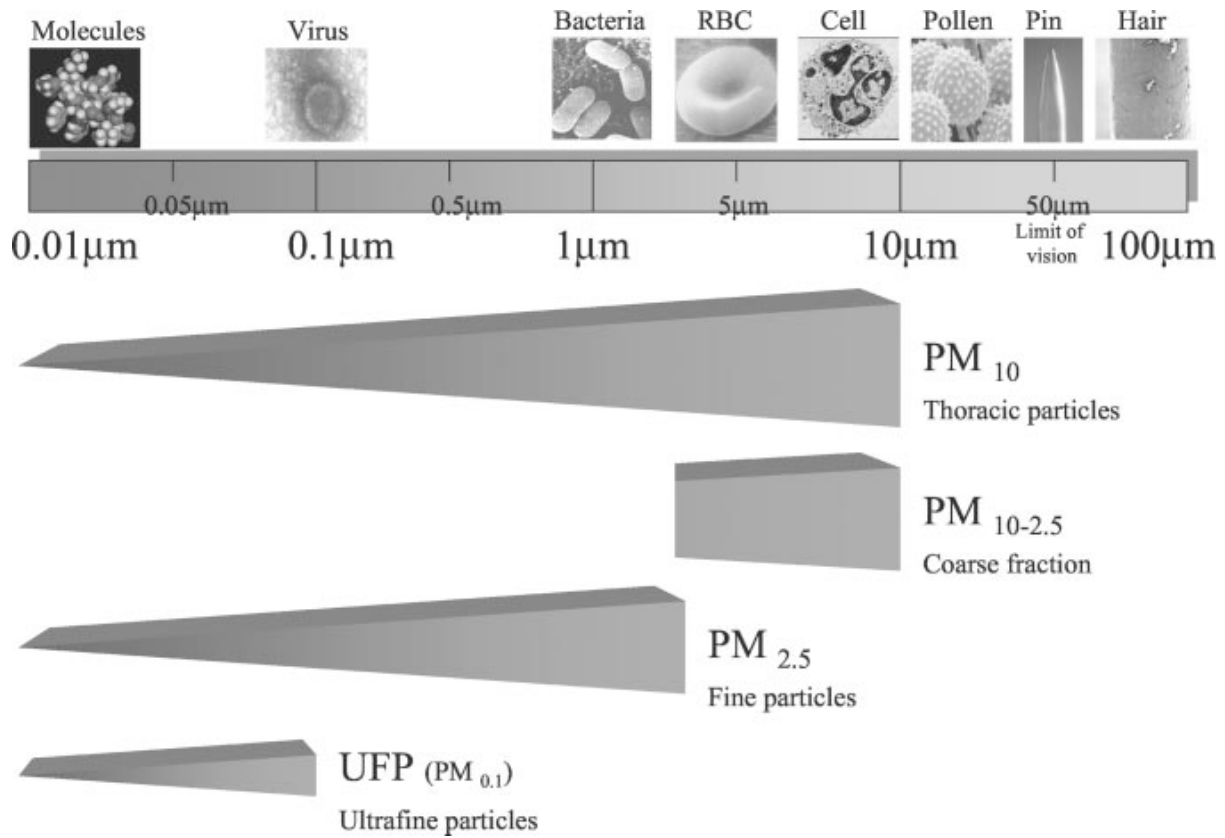


Fig. 1. Particle size fractions. Graphic representation of particle sizes in relation to hair, a pin, pollen, a cell, a red blood cell, bacteria, virus and molecules (from Brook, 2004).

Particle deposition

Besides several other factors, the size may determine where inhaled particles deposit (Fleming et al. 1996). In general, larger particles mainly deposit in the nasopharyngeal region while smaller particles tend to deposit in the peripheral alveolar region. However, this is not straight forward. As Fig. 2 shows, particles less than 10 μm normally deposit in the upper airways, such as the nose and throat, but may also reach further down the respiratory tract and reach the alveoli and the surrounding vasculature (Salvi & Holgate 1999; Wichmann 2007). Smaller particles tend to deposit more efficiently in the lungs, whereas the smallest particles normally deposit in the nose/throat. Particles may also accumulate in certain *hot spots*, deposited unevenly. This leads to a particle exposure of higher concentrations in certain areas within the lung (Phalen et al. 2010). Moreover, patients with COPD usually have an elevated particle deposition (C. S. Kim & Kang 1997), implying that these people may be exposed to higher concentrations than healthy people.

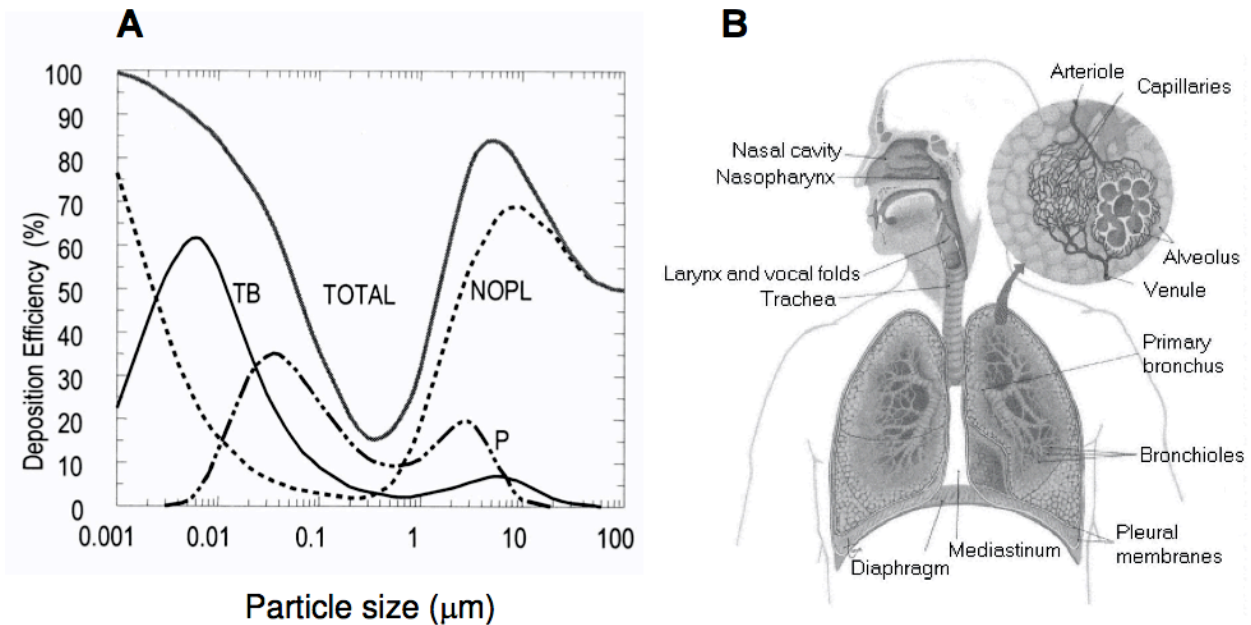


Fig 2. Pulmonary particle deposition. Figure A shows pulmonary particle distribution differing by region and size. NOPL; nasopharyngeal region (nose, mouth and throat), TB; tracheobronchial region (trachea and bronchi), P; peripheral region (alveoli) (From Phalen, 2002). Figure B shows a schematic figure of the human respiratory tract (From Scanlon and Sanders, 1995).

Diesel exhaust

A considerable proportion of urban air pollution comes from diesel engines used by on-road and off-road vehicles (Salvi & Holgate 1999; D'Amato et al. 2005). Due to advances in technology, the type of diesel emissions has also changed and will probably do so in the future thus making it difficult to assess potential risks associated with diesel exhaust (DE) emissions. However, the components of DE that are of health concern are elemental carbon (EC) of the particle core, (in)organic compounds adsorbed to the particle surface, organic compounds in gas phase, as well as gaseous oxides of carbon, nitrogen and sulfur (Wichmann 2007).

Diesel exhaust particles

Diesel exhaust particles (DEP) typically consist of EC with adsorbed polycyclic aromatic hydrocarbons (PAH), PAH-derivatives, benzene, alkenes, alkanes, aldehydes and traces of metallic ashes, illustrated by Fig. 3. A major fraction of the particles is in the fine particular range ($< 2.5 \mu\text{m AD}$), of which most are $\approx 0.1 \mu\text{m AD}$ (Kerminen et al. 1997). DEP are classified as a group 1 carcinogens (IARC 2012), and have the potential to induce inflammatory responses (Garshick et al. 2004; Nordenhäll et al. 2000). Which components of DEP that are responsible for health effects is an important question. Several studies indicate a role of the soluble organic extract of the particle surface to account for the toxicity of DEP (Yang et al. 1997; Takano et al. 2007; Bonvallot et al. 2001; Totlandsdal et al. 2012). Organic extracts may contain much of the PAH and PAH-derivatives found in DEP (Totlandsdal 2012., submitted). Several PAH, such as benzo[a]pyrene (B[a]P), have been linked to DNA-damage and development of cancer (IARC 1989), but the role in inflammatory responses is less clear. Therefore, other components may also be suggested as potential candidates for the inflammatory effects. To complicate things further, the composition of DEP varies depending on engine type, engine load, fuel type and weather (Wichmann 2007). For example, Tal et al. studied the effects of DEP with organic content (OC) ranging from low to high *in vitro*, and reported that the OC is an important determinant for pro-inflammatory responses in airway epithelial cells (Tal et al. 2010). Due to the importance of inflammation in adverse pulmonary health effects induced by PM, it is important to elucidate the inflammatory components in DEP.

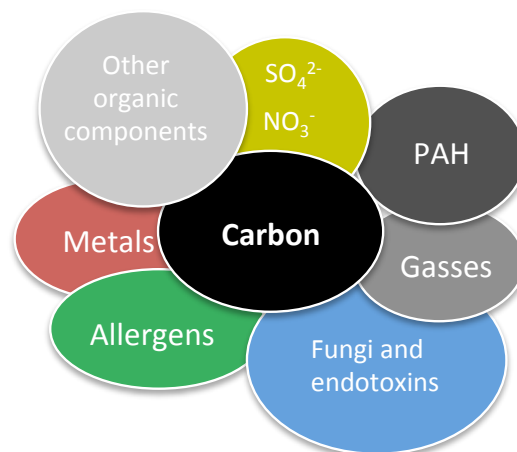


Fig. 3. A typical combustion particle. The figure illustrates a simplified model of a combustion particle consisting of a carbonaceous core with adsorbed (in)organic compounds and gasses. (modified figure from the Norwegian Institute of Public Health, 2004).

Inflammation

An acute inflammatory response is a natural and important reaction of the body to fight invasions of pathogens. Inflammation is characterized by vasodilation, pain, redness and heat. Production of pro-inflammatory mediators, including cytokines and chemoattractant cytokines (chemokines), is characteristic for an inflammatory response. Cytokine-induced up-regulation of cell adhesion molecules on endothelial cells will attract leukocytes from the bloodstream in a multistep adhesion cascade (Murphy et al. 2011). This optimizes recruitment to fight and remove the invaders by phagocytosis and production of cytokines, reactive oxygen species (ROS), lipid mediators (LM) and toxic proteases (TP) (Springer 1994; Ley et al. 2007; Folkerts et al. 2001). Normally this is a transient event and an effective way of killing and removing possible pathogens that can do harm to the host.

Pulmonary epithelial cells: role in inflammation

The pulmonary epithelium has a large surface area and is exposed to more than 10.000L of air each day (Salvi & Holgate 1999). It consists of a single polarized cell layer and functions as a protective barrier against inhaled pathogens and particles. On the apical side (outer), epithelial cells are involved in protection against pathogens by secretion of mucus that lines the epithelium. In addition, ciliary beat activity moves pollutants upwards to the pharynx where they are coughed up or swallowed. On the basolateral side, cytokines and chemokines may be secreted to recruit immune cells and to up-regulate endothelial adhesion molecules (K. C. Kim 2012; Davies & Holgate 2002). Phagocytosis of particles by macrophages and removal by the pulmonary ciliary escalator is usually a non-inflammatory response. However, interaction with particles and the epithelial surface may result in an inflammatory response (Seagrave 2008; Donaldson & Tran 2002; Bonvallot et al. 2002; Dybdahl et al. 2004). Contrary to pathogen-induced inflammation, PM-induced inflammation is unwanted. Moreover, if the burden is too strong and/or long-lasting, the inflammatory response may continue into a vicious circle, resulting in chronic inflammation (Fig. 4). The recruited cells will continue to produce ROS, LM and TP that eventually may outdo the intrinsic anti-oxidant levels (Kelly 2003), and damage neighboring cells. Such secondary effect may further develop the inflammatory response. In addition, inflammation may have an important part in cancer development (Coussens & Werb 2002; Hussain & Harris 2007).

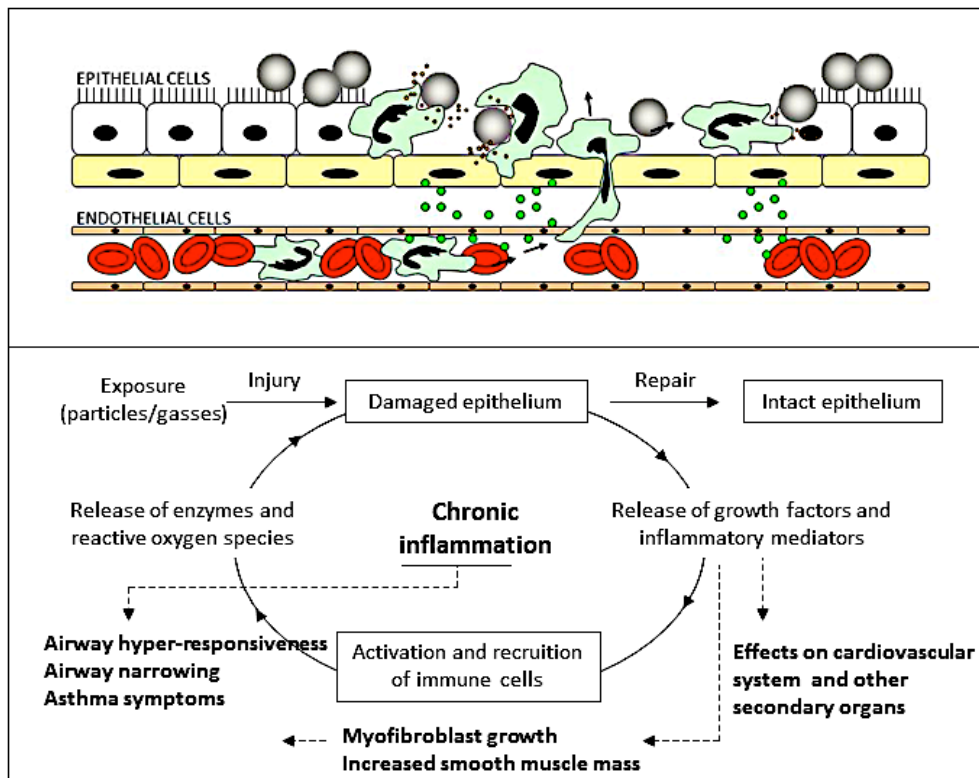


Fig 4. Mechanisms of pulmonary inflammation. Inhaled particulate matter interact with epithelial cells which respond by secretion pro-inflammatory proteins, including chemokines. Chemokines attract immune cells from the blood stream to initiate an immune response. The release of reactive oxygen species, toxic proteases and lipid mediators by immune cells may result in further tissue damage and increased production of pro-inflammatory proteins by the epithelial cells. This vicious circle is what characterizes chronic inflammation (The figure is reproduced by courtesy of J. Øvrevik, Norwegian Inst. of Public Health).

Cytokines and chemokines

Cytokines and chemoattractant cytokines (chemokines) are small proteins/glycoproteins that cells secrete to communicate to itself (autocrine) or each other (paracrine, endocrine), and attract other cells (chemotaxis). During an inflammatory response, different pro-inflammatory cytokines and chemokines are produced to recruit help, to warn other cells, or to down-regulate a response. Binding of cytokines to specific receptors in the membrane mediates activation of signal cascades resulting in specific gene expressions. Cytokines are divided in families based on their composition and function. Examples are interferons, interleukins, tumor-necrosis family, colony stimulating factors and chemokines (Murphy et al. 2011). Chemokines are divided into two large subfamilies, based on conserved amino acids near the N-termini. The CXC subfamily consists of two cysteine residues separated by a single amino acid. The CC chemokines have two adjacent cysteine residues (Rollins 1997). A role of chemokines is to attract other immune cells by chemotaxis, such as neutrophils (Baggiolini et al. 1989). Exposure to DEP has been shown to induce pro-inflammatory

mediators, such as the cytokine interleukin (IL)-6, and the chemokines IL-8 (Steerenberg et al. 1998) and RANTES (regulated on activation, normal T-expressed and secreted) (Li et al. 2011).

Interleukin-6

IL-6 is a pleiotropic cytokine and is expressed in several types of cells, including epithelial cells (Quay et al. 1998). It is involved in inflammation by enhancing expression of adhesion molecules which promotes transmigration of neutrophils (Thacker 2006; Mihara et al. 2012). By also reducing levels of other pro-inflammatory mediators, IL-6 is regarded to have both pro- and anti-inflammatory properties (Aderka et al. 1989; Schindler et al. 1990). The cytokine enhances levels of acute phase proteins (Gauldie et al. 1987), and controls production of other pro-inflammatory cytokines (Xing et al. 1998). It also has a role in the transition from acute to chronic inflammation and may also be involved in the pathogenesis of cancer (Kaplanski et al. 2003) (Mihara et al. 2012).

Interleukin-8

IL-8, also known as CXCL8, is a chemokine expressed ubiquitously, including in epithelial cells (Rollins 1997). Various agents such as bacteria, viruses, cigarette smoke and cytokines stimulate its production and secretion (Harada et al. 1994; Castro et al. 2008; Sha et al. 2004). Its main role is to up-regulate adhesion molecules, and attract and activate neutrophils to the infected area (Baggiolini et al. 1989). Neutrophils function as a host-defense mechanism stimulating mucus production and initiates phagocytosis to kill the invaders. An excessive secretion with corresponding neutrophil influx characterizes symptoms in patients with chronic airway diseases such as COPD (S. Kim & Nadel 2004). In addition to attracting neutrophils and inflammatory responses, the chemokine may also possess mitogenic, angiogenic and motogenic properties (Xie 2001).

CCL5/RANTES

The chemokine RANTES (regulated on activation, normal T cell expressed and secreted), is an eosinophil attractant involved in allergic inflammation and asthma (Lim et al. 1996), and is expressed in epithelial cells (Wang et al. 1996).

Signaling pathways

To transfer a signal from the outside to the inside of a cell, cells have many kinds of receptors on the cell surface and intracellularly. These receptors respond to extracellular stimuli that can initiate a signaling pathway that eventually result in activation of specific genes and production of corresponding proteins. To protect and react against environmental threats, many cells are provided with pattern recognition receptors (PRR) which are found in many types of cells, including epithelial cells (Lafferty et al. 2010). PRR recognize evolutionary conserved motifs on pathogens, also known as pathogen-associated molecular patterns (PAMP), from viruses and bacteria (Bianchi 2007). Several classes have been described, including the Toll-like receptors (TLR) and the non-classical PRR proteinase activated receptors (Nhu et al. 2010; Akira 2001). Triggering receptors is followed by activation of intracellular signaling cascades and often results in an inflammatory response. To transfer signals from the receptors to the genes, the cells contain a complex network of signaling pathways of which nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPK) are often involved in the regulation of pro-inflammatory cytokines (Dong et al. 2002).

Toll-like receptors

The most known PRR-family is the Toll-like receptors (TLR) which consists of 11 extra- or intracellular receptors. TLR are mainly expressed in myeloid cells (Medzhitov 2001), but also in the airway epithelium (Sha et al. 2004). Activation of TLR results in production of pro-inflammatory cytokines and chemokines that initiate an inflammatory response, as well as triggering adaptive immune responses (Lafferty et al. 2010). For instance, TLR2 and 4 are activated by various bacterial components such as lipopolysaccharide (LPS) found in Gram-negative bacteria (Hoshino et al. 1999; Takeuchi et al. 1999), while double-stranded viral RNA activates TLR3 (Alexopoulou et al. 2001). PM may contain TLR agonists, such as LPS (Becker et al. 2002). Furthermore, several *in vitro* studies indicate that activating cells with TLR agonists, alters the cytokine/chemokine response upon PM-exposure (Inoue et al. 2006; Imrich et al. 1999).

Proteinase activated receptors

Proteinase activated receptors (PAR) are seven transmembrane G-coupled receptors activated by different stimuli, including proteases (Steinhoff et al. 2005), and have been shown to be involved in cytokine regulation (Asokanathan et al. 2002). In addition, DEP has been shown to induce PAR-2 mediated release of matrix metalloproteinases, which is associated with inflammation (Li et al. 2011).

NF- κ B transcription factor

The dimeric nuclear factor- κ B (NF- κ B) is one of the best described pro-inflammatory transcription factors. NF- κ B is expressed ubiquitously and is activated by UV-light, oxidative stress, infections/pathogens or cytokines. An activation results in expression of pro-inflammatory proteins, such as cytokines and chemokines, in addition to many other responses (Baker et al. 2011; Hayden & Ghosh 2008). The classical NF- κ B pathway consist of the sub-units p65 (Rel A) bound to p50, sequestered in the cytoplasm in a resting cell by inhibitor of κ B (I κ B- α), and the up-stream I κ B kinase (IKK), consisting of IKK- α , IKK- β and IKK- γ or NEMO (NF- κ B essential modulator) (Häcker & Karin 2006). Initiation of the classical pathway is characterized by phosphorylation of I κ B- α by IKK- β and NEMO (Oeckinghaus et al. 2011). Upon stimulation, phosphorylation of I κ B by IKK leads to a separation of I κ B from NF- κ B and subsequent ubiquitination and degradation of the inhibitor. NF- κ B translocates then to the nucleus, binds to κ B-sites in the promoters of target genes and initiates transcription of pro-inflammatory molecules (Fig. 5) (Iwai 2012). NF- κ B may regulate production of IL-6 and/or IL-8, which are central in pulmonary inflammation (Libermann & Baltimore 1990; Kunsch & Rosen 1993).

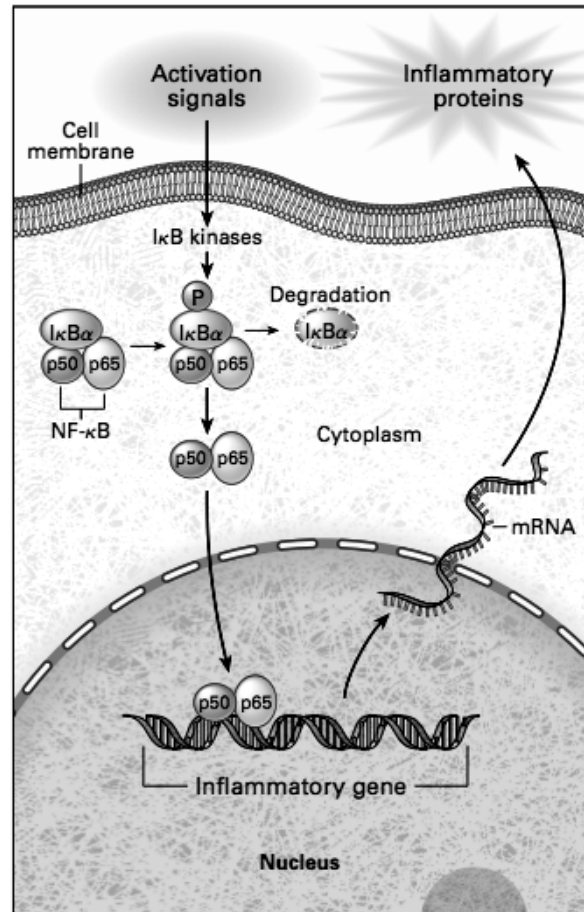


Fig 5. Classical NF-κB signaling pathway. Phosphorylation of IκB by IKK leads to separation of IκB from the p65/p50 heterodimer. IκB is directed to the proteasome for degradation. The p65/p50 heterodimer translocates to the nucleus to initiate transcription of inflammatory genes (From Barnes, 1997).

MAPK signaling pathway

Mitogen activated protein kinases (MAPK) constitute evolutionary conserved signaling pathways expressed ubiquitously, including in airway epithelial cells (Puddicombe & Davies 2000). MAPK include several protein kinases, such as the well characterized extra-cellular protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (Fig. 6). ERK is mainly activated by growth factors, whereas JNK and p38 MAPK are activated upon cellular stress and cytokines (Lewis et al. 1998). In general, the MAPK are phosphorylated by MAPK kinases (MKK). MAPK kinase kinases (MKKK) phosphorylate MKK completing the phospho-relay system, converting a signal to a cellular response (Johnson & Lapadat 2002). The MAPK pathways are involved in cytokine production, cell proliferation and differentiation, environmental stress adaption and apoptosis (Widmann et al. 1999), as well as regulation of NF-κB (Mercurio & Manning 1999). Whereas ERK

is involved in cell proliferation, JNK and p38 MAPK typically have a role in inflammation, cell survival and apoptosis (Puddicombe & Davies 2000). MAPK are central to PM-induced cytokine and chemokine responses. For example, it has been reported that PM may induce JNK phosphorylation (Timblin et al. 1998), and that this MAPK may result in IL-8 production upon exposure to DE (Pourazar et al. 2005). Moreover, DEP-induced phosphorylation of p38 MAPK in airway epithelial cells has also been involved in IL-8 production (Totlandsdal et al. 2010; Hashimoto et al. 2000), supposedly by an mRNA stabilization mechanism (Hoffmann et al. 2002).

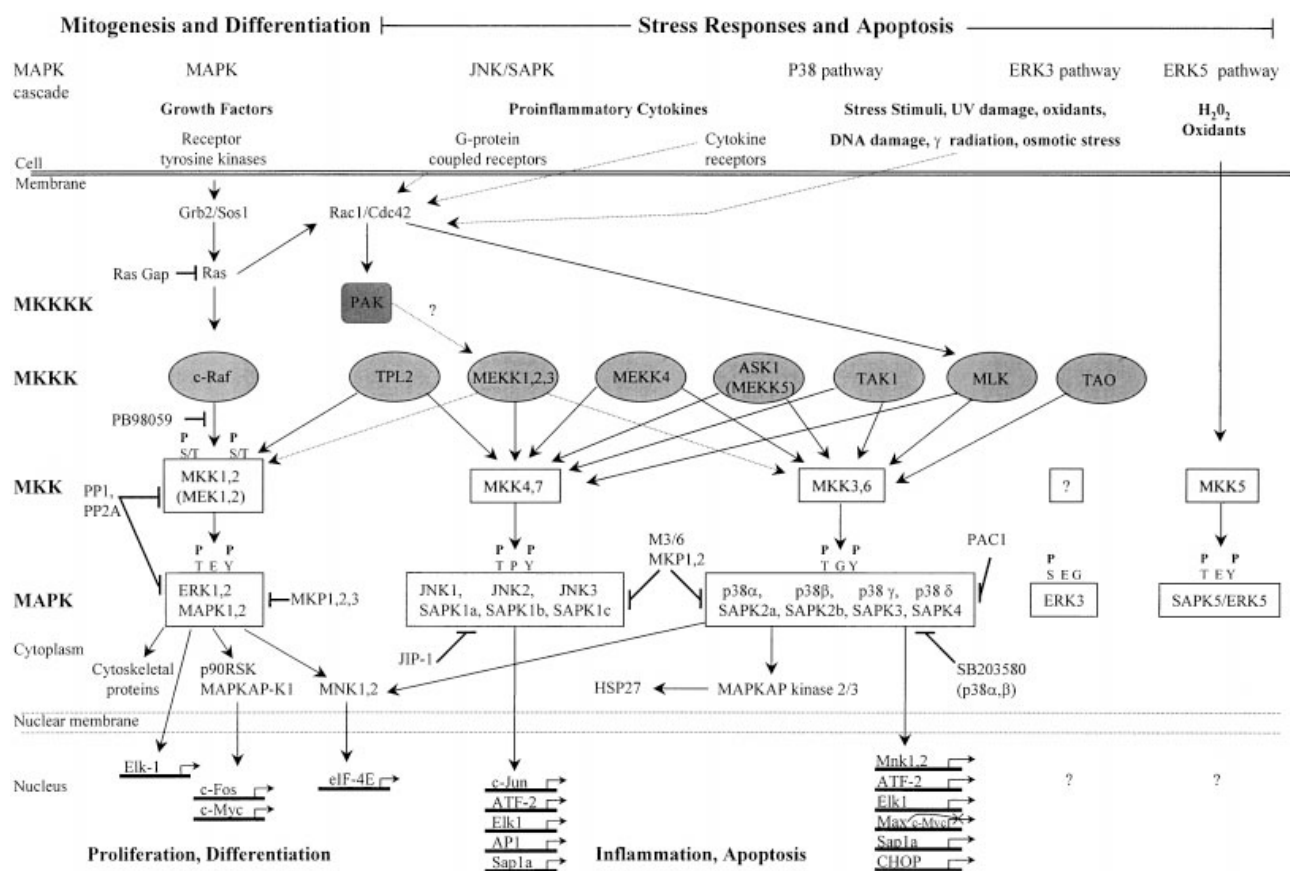


Fig 6. MAPK signaling pathway. The figure shows several MAPK signaling pathways including ERK, JNK and p38 (From Puddicombe, 2000).

AhR/ARNT

The aryl hydrocarbon receptor (AhR) is a cytosolic xenosensor inducing production of cytochrome P450 (CYP) enzymes upon activation by xenobiotics, including PAH and DEP (Totlandsdal et al. 2010; Whitlock 1999). The activated AhR dissociates from heat-shock protein 90 (hsp90) and translocates to the nucleus, where it associates with AhR nuclear translocator (ARNT). This complex functions as a transcription factor by binding to xenobiotic response elements (XRE) promoting CYP-expression, including CYP1A1 (Whitlock 1999). CYP-enzymes often metabolize hydrophobic compounds into more hydrophilic compounds and represent an important defense mechanism against harmful substances. However, CYP may also increase the toxicity of compounds through formation of reactive electrophile intermediates that can interact with DNA, RNA and proteins, in addition to ROS-formation, which can activate redox-sensitive pathways (Namazi 2009). Furthermore, AhR has been shown to interact with the NF- κ B pathway (Tian et al. 1999). Concerning the wide range of different ingredients in PM from combustion processes, activation of both the AhR and/or NF- κ B pathways may result in an interaction between the two, thus complicating the understanding of biological mechanisms upon PM-exposure.

Cytotoxicity

There are two main types of cell death. Apoptosis has characteristic morphological features like cell shrinkage, nuclear fragmentation and chromatin condensation. Whereas necrosis is characterized by a swelling and plasma membrane rupture followed by loss of intracellular contents. As a result necrotic cells release harmful contents affecting surrounding cells, thus contribute to development of inflammatory reactions. Apoptosis does not induce inflammation and is considered as a controlled mode of cell death, while necrosis is a pathological and passive process (Nicotera et al. 1999; Fadeel & Orrenius 2005).

Aims of study

The main objectives of the present study were to explore the effects of diesel exhaust particles (DEP) on cytokine production in lung epithelial cells, clarify which fraction of DEP-organic components (DEP-OC) that contribute most to the responses, investigate interactions between DEP/DEP-OC and the TLR3 ligand polyinosinic:polycytidylic acid (poly I:C, a synthetic double-stranded RNA) as a model for viral infections and examine cellular mechanisms responsible for the effects.

Since PM may exacerbate airway infections, we hypothesized that TLR3-priming would sensitize the cells against DEP-induced pro-inflammatory effects. Moreover, as DEP consist of a wide variety of different chemicals, we also hypothesized that different fractions of soluble DEP-OC may contribute differentially to DEP-induced cytokine responses.

To explore this, the following specific aims were pursued:

- Study DEP-induced cytokine responses in poly I:C primed and unprimed BEAS-2B cells (Paper 1).
- Study effects of DEP on pathways involved in cytokine regulation, with focus on MAPK and NF- κ B signaling in poly I:C primed and unprimed cells (Paper 1).
- Study effects of DEP with high or low organic content on cytokine responses in BEAS-2B cells (Paper 2).
- Study effects of fractionated DEP- organic extracts with different polarities on cytokine responses in poly I:C primed and unprimed BEAS-2B cells (Paper 2).
- Examine involvement of PAR-2 and AhR-receptors in DEP-induced cytokine responses (Paper 2).

Experimental considerations

Cell model

In this study, BEAS-2B, an SV40 adenovirus-transformed bronchial epithelial cell line from normal human lungs was used. Ambient air particles may deposit at the branching area in the bronchi (Schlesinger & Lippmann 1978), and the BEAS-2B cell line has the greatest homology in gene expression to primary cells, compared to several other cell lines (Courcot et al., 2012). Therefore, the BEAS-2B cell line is a common and relevant model for studying the toxic effects of ambient air pollutants. There are several advantages using a transformed cell line. Cell lines have a relatively low cost, are easily reproducible, can be cultured indefinitely, as well as the exposure conditions are controllable. Since cell lines are easier to maintain and culture, they are advantageously for mechanistic studies, compared to complex models like primary cells. However, it is important to remember that cell lines do not necessarily represent *in vivo* conditions correctly. Moreover, interactions between different cell types or genetic variability are not accounted for. If important findings are found, experiments with cell lines may be supplemented with primary cells. An advantage of primary cells is that such cells to a greater extent represent *in vivo*, as they to a lesser degree are adapted to *in vitro* culture conditions. However, primary cells have a short life-span, are more expensive, demand more work and are not as easily accessible as cell lines. In addition, greater genetic variability may account for less reproducible responses. *In vivo* experiments usually involve animals and sometimes humans. These experiments further give increased confidence interpreting the results and may represent real life responses. However, high cost and strict regulation due to ethical questions impede such experiments.

Priming with poly I:C

In this study, we used poly I:C which is a synthetic analog of double-stranded viral RNA. It can be used in cell culture experiments to mimic viral infections. It activates TLR3 and can therefore be used to determine if a viral infection may be a confounding factor of responses towards exposure to agents (Alexopoulou et al. 2001).

RNA interference

Transfection with siRNA (small interfering RNA) is used to down-regulate expression of specific genes. SiRNA or RNAi (RNA interference) are small double stranded RNA molecules that either block RNA translation or degrade the mRNA of interest. For the latter, the RNA is cleaved by the enzyme DICER, and further incorporated in to a protein complex called RNA-induced silencing complex (RISC). This complex recognizes target mRNA by an anti-sense strand (Semizarov et al. 2003). To obtain the best results, it is important to optimize the amount of siRNA, the ratio of transfection reagent to siRNA and the cell density at transfection. It is also important to use a non-targeting siRNA control.

ELISA

ELISA (Enzyme-Linked ImmunoSorbent Assay) is a well known quantitative method for measuring protein levels in serum, or other biological fluids, and is widely utilized clinically as well as in biomedical research. It provides an accurate and sensitive way of detecting a protein of interest by using antigen-antibody complexes to determine cytokine concentrations in the supernatant (Leng et al. 2008). “Sandwich”-ELISA is one of three types ELISA and is the most specific by using two different antibodies against two different epitopes on one antigen. The antibodies are usually conjugated with isotopes, enzymes or fluorescent compounds or compounds that illuminate by luminescence. A great advantage of this method is the quantitative possibility. By comparing the accumulated proteins of interest with a standard curve, one can determine almost the exact concentration of the protein. Notably, protein concentrations may be affected by reduced cell growth, cytotoxicity, or can be re-uptaken by the cells. In this study, “Sandwich”-ELISA with horse-raddish peroxidase (HRP) and tetramethylbenzidine (TMB) were applied to measure IL-6, IL-8 and RANTES protein concentrations. However, in preliminary experiments with media from DEP-exposed cells analyzed by ELISA, we discovered great variability in our results, as exemplified by Fig. 7. Other studies have shown a potential binding of IL-8 to DEP (Seagrave et al. 2004; Kocbach et al. 2008; Akhtar et al. 2010), possibly causing an underestimation of responses. Based on our results and these studies, we chose to analyze mRNA-expression by real-time RT-PCR.

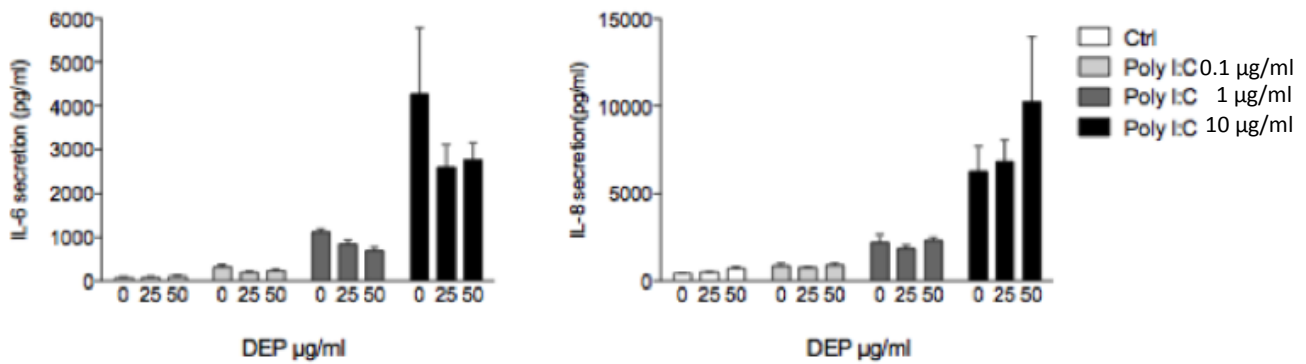


Fig 7. Cells were exposed to 0, 25 and 50 µg/ml DEP in combination with different concentrations of the TLR3 ligand poly I:C. Protein release of IL-6 and IL-8 was measured after 24 h and analyzed by ELISA as described under “materials and methods” (Paper 2).

Real-time RT-PCR

Real-time RT-PCR (real-time Reverse Transcriptase Polymerase Chain Reaction) is a method to measure expression of genes by isolating mRNA, reverse transcribe the mRNA to cDNA and run the cDNA through a polymerase chain reaction (PCR) to amplify the genes of interest. Briefly, cells were lysed with 200 µl lysis buffer, RNA was isolated and reverse transcribed to cDNA following a real-time PCR amplification. By comparing with a house keeping control gene (18S), relative measurements of IL-6, IL-8, CYP1A1, RANTES and PAR-2 were analyzed. In contrast to the ELISA method, real-time RT-PCR results represent a snapshot of the cellular responses at a particular time point, as mRNA is rapidly degraded. For example, exposing cells for 4h or 8h may show different results (Totlandsdal et al. 2012). For cytokine/chemokine protein levels in a supernatant, short-term variations in release are less important due to greater stability and longer half-life of the accumulating proteins. Therefore, one should optimize experiments by time course- and concentration curve screening when using real-time RT-PCR analysis. However, a great advantage is the amplification of small amounts of nucleic acids. Moreover, a problem of potential protein-binding to particles in supernatant is avoided.

Western blotting

Western blotting (or immunoblotting) is a semi-quantitative method used for separation and identification of expression levels of proteins. In Western blotting, equal amounts of cellular proteins are separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and transferred electrophoretically from the gel to a nitrocellulose support membrane (used in our study). Primary monoclonal or polyclonal antibodies are added to interact with a specific antigen epitope, which is presented by the target protein attached to the support membrane. Furthermore, a secondary antibody, conjugated with HRP (horse raddish peroxidase) is added and binds to the primary antibody-antigen complex. Chemiluminescent (luminol and hydrogen peroxide) substrates are then used to visualize the bound components. The HRP, with hydrogen peroxide, will convert the luminol to chemiluminescence. With this method, it is possible to detect activation or degradation of specific proteins relative to total protein levels. Identification of proteins, together with the specific antibody, is possible by using a standard protein ladder (Precision Plus Protein® WesternC® Standards). In this study AhR, phosphorylation of MAPK (ERK1/2, JNK1/2, p38) and p65, and degradation of I κ B, were measured. Advantages of Western blotting are that it can detect proteins of very low concentrations. Moreover, the method is specific due to the separation of proteins, as well as the use of specific antibodies. However, expression levels of proteins are only semi-quantitative as they are compared to specific house-keeping proteins. Exact concentrations of the protein of interest are therefore not possible to measure.

Fluorescence microscopy

Many methods are available for characterizing different kinds of cell death. Fluorescence microscopic examinations of cells stained with Hoechst 33342/PI were utilized in this study. With this method it is possible to determine if a cell is apoptotic, necrotic or viable, based on color and nuclear morphology. However for an untrained eye, it may be time consuming and sometimes difficult to discover apoptotic cells with little nuclear condensation and fragmentation. Both compounds can stain DNA, but only Hoechst 33342 will penetrate an intact plasma membrane as found in viable and apoptotic cells. Cells were classified as apoptotic, necrotic or viable. Cells with distinct condensed nuclei, segregated nuclei and apoptotic bodies were counted as apoptotic (PI-negative cells), and the fraction compared to the total number of cells was determined. Non-apoptotic cells, excluding PI, were categorized as viable cells. PI-stained cells with a round

morphology and homogeneously stained nucleus due to the loss of plasma membrane integrity were termed necrotic (PI-positive).

Statistical analysis

Using statistics, one can analyze data and draw general conclusions based on a limited set of data. ANOVA is a generalization of the t-test, which makes it possible to compare means of a variable in three or more groups at the same time using post-tests, including Bonferroni or Dunnett's. Several requirements are needed and are as follows: independent observations, Gaussian distribution and the same scatter of data in each sample. If these requirements are not fulfilled, it will reduce the strength of the test. Moreover, *in vitro* experiments are often performed at a relatively low number of replicates (usually $n = 3$) and may therefore reduce confidence.

Summary of findings

The results of the present study are presented as two separate manuscripts: Paper 1 and Paper 2. The main findings are summarized below.

Paper 1:

- Poly I:C priming alters cytokine responses in BEAS-2B cells. DEP induced enhanced IL-6 and IL-8 expression in poly I:C primed BEAS-2B cells, compared to unprimed cells. Moreover, the elevated levels of IL-6 and IL-8 expression in poly I:C primed cells seems to be due to interactive effects between DEP and Poly I:C. DEP-exposure alone did not induce RANTES expression in BEAS-2B cells, but attenuated the poly I:C induced RANTES expression in poly I:C primed cells.
- DEP-exposure alone did not enhance phosphorylation of the MAPK signaling pathway or the classical NF- κ B pathway. DEP did not enhance poly I:C induced phosphorylation of MAPK or the classical NF- κ B signaling pathway. Exposure to DEP reduced poly I:C induced phosphorylation of ERK and JNK, and slightly increased poly I:C induced phosphorylation of p38 in BEAS-2B cells.

Paper 2:

- DEP with different organic content induce IL-6 and IL-8 expression differently. Low OC did not induce IL-6 or IL-8 expression, whereas DEP with high OC induced both IL-6 and IL-8 expression.
- Components of different polarities in DEP induce IL-6 and IL-8 differently in poly I:C primed and unprimed BEAS-2B cells. Polar fractions mainly induced IL-6 release in unprimed cells, but did not induce any IL-6 or IL-8 release in primed cells. Non-polar fractions induced both IL-6 and IL-8 release in primed cells. However, a non-polar heptane extract induced both IL-6 and IL-8 release in unprimed cells.
- The AhR has an anti-inflammatory role in the DEP-induced pro-inflammatory responses in BEAS-2B cells. Transfection of AhR (siAhR) did not attenuate the DEP-induced IL-6 and IL-8 expressions. The expression was rather increased.
- PAR-2 is involved in DEP-induced IL-6 responses in BEAS-2B cells. Transfection of PAR-2 (siPAR-2) reduced IL-6 expression, but not IL-8 expression.

Conclusions

The results of the present work show that priming cells with the TLR-3 ligand poly I:C, sensitized the cells against DEP by increased IL-6 and IL-8 expression. However, exposure to DEP also attenuated poly I:C induced RANTES. By showing that exposure to DEP in poly I:C primed BEAS-2B not only enhances, but also alters the effects, we therefore partly confirm that poly I:C priming sensitizes the cells upon DEP-exposure. Poly I:C activated signaling pathways (NF- κ B and p38), possibly required for optimal DEP-induced IL-6 and IL-8 responses. Furthermore, the DEP suppression of certain poly I:C-induced signaling pathways (ERK and JNK), may have caused the reduction of RANTES responses in TLR3-primed cells. These results may imply that people with preexisting infections respond differently to DEP than healthy people.

In accordance with previous studies, the results show that the pro-inflammatory compounds of DEP are mainly the soluble organic compounds. Moreover, we confirm that different fractions of soluble DEP organic extracts contribute differentially, by polarity, in the DEP-induced cytokine responses. Moreover, the partly increase of IL-6 and IL-8 responses in the absence of AhR, indicate that it is not the classical PAH that is important for DEP-induced pro-inflammatory responses.

Future work

The following suggested experiments would strengthen the results and conclusions of the presented work:

Paper 1

- Test if MAPK and/or NF- κ B signaling pathways are involved in the DEP-induced pro-inflammatory effects in poly I:C primed cells, by the use of pharmacological inhibitors and/or siRNA transfection.
- Investigate other possible mechanisms involved in DEP-induced cytokine/chemokine production, such as AP-1 (activator protein-1).

Paper 2

- Test for additive or synergistic IL-6 and IL-8 responses by exposing poly I:C primed and unprimed cells to a combination of all the fractions.
- Investigate effects by fractionated methanol DEP organic extracts in combination with the use of siRNA transfection against PAR-2, to clarify a possible involvement of PAR-2 and cytokine/chemokine production induced by polar compounds.
- Clarify the lack of DEP-induced IL-6 expression in cells transfected with non-targeting control (siNT) by exposing siNT-transfected cells and non-transfected cells to DEP.

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Paper 1

Title:

Toll like receptor-3 priming alters diesel exhaust particle-induced cytokine responses in human bronchial epithelial cells

Preliminary author list:

Nicolai Bach^{1,2}, Marit Låg¹, Jørn A. Holme¹ and Johan Øvrevik¹

Affiliation:

¹Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, N-0403 Oslo, Norway

²Department of Biology, Faculty of Mathematics and Natural Sciences, University of Oslo, P.O. Box 1066 Blindern, N-0316 Oslo, Norway

Corresponding author:

Johan Øvrevik

Department of Air Pollution and Noise

Division of Environmental Medicine

Norwegian Institute of Public Health

P.O.Box 4404 Nydalen

N-0403 Oslo, Norway

E-mail: johan.ovrevik@fhi.no

Phone: +47 21076408

Fax: +47 21076686

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Abstract

Background: Ambient air is composed of a wide range of pollutants, including particulate matter (PM). Patients with a preexisting pulmonary disease may be more susceptible to adverse health effects upon exposure to PM where inflammation seems to play a key role. Diesel exhaust particles (DEP) often constitute a large proportion of PM, especially in urban areas. However, the mechanisms behind are not well understood.

Objective: To investigate changes in susceptibility towards exposure to DEP in a Toll-like receptor 3 (TLR3) activated immortalized human bronchial epithelial cell line (BEAS-2B) and study the underlying mechanisms.

Methods: Polyinosinic:polycytidylic acid (Poly I:C) primed BEAS-2B were exposed to DEP. Interleukin (IL)-6, IL-8 and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) gene expression were measured by real-time RT-PCR. MAPK and NF- κ B protein phosphorylation were analyzed by Western blotting.

Results: DEP-exposed poly I:C primed BEAS-2B cells respond differently by enhanced IL-6 and IL-8 expression and attenuated RANTES expression. DEP did not enhance poly I:C induced MAPK or NF- κ B activation.

Conclusion: DEP-exposed poly I:C primed BEAS-2B cells show quantitatively and qualitatively different responses, compared to unprimed cells, suggesting an altered level of susceptibility in infected cells towards DEP.

Introduction

Inhalation of particulate matter (PM) is associated adverse pulmonary diseases (Pope et al. 1991; Ghio et al. 2000). Studies show that preexisting inflammatory lung diseases, such as chronic obstructive pulmonary disease (COPD), asthma or influenza infections, may be a susceptibility factor upon exposure to PM (Wong et al. 2010; Schwartz et al. 1993; Atkinson et al. 1999; Dockery et al. 1993; Naess et al. 2007). Inflammation is believed to play a major role in the pathogenesis of PM-induced adverse health outcomes (Donaldson et al. 2005; Lazarus 1986; Chauhan & Johnston 2003; Rabinovitch et al. 2006; Salvi & Holgate 1999). However, the cellular mechanisms underlying enhanced susceptibility towards PM remains poorly understood.

Notably, diesel exhaust particles (DEP) are known to constitute a large proportion of PM, especially in urban areas (Robinson et al. 2010; Kittelson 1998; Salvi, Frew, et al. 1999b). Furthermore, DEP mainly consist of fine and ultra-fine particles, known to deposit in *hot spots* in the respiratory tract, such as the bronchial airways (Balashazy et al. 2003; Phalen et al. 2010; Kerminen et al. 1997). The pulmonary epithelium is a physical barrier to the outside environment, and is therefore a primary target of toxic pollutants in ambient air. Previous studies from our group and others have shown that DEP induced increased expression of pro-inflammatory cytokines and chemokines, including interleukin (IL)-6 and IL-8 in BEAS-2B, a human bronchial epithelial cell line. (Totlandsdal et al. 2010; Steerenberg et al. 1998). IL-6 is a pleiotropic cytokine, associated with the acute phase response, and may be important for the transition from acute to chronic inflammation (Gauldie et al. 1987; Gabay 2006). The chemokine IL-8 is a potent neutrophil-attractant of which elevated levels are found in respiratory diseases including cystic fibrosis, COPD and asthma (Gibson et al. 2001; Bonfield et al. 1995; Yamamoto et al. 1997). Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) attracts eosinophils and is involved in allergic inflammation and asthma (Lim et al. 1996).

Cellular expression of IL-6 and IL-8 induced by DEP or PM may involve redox-sensitive signaling transduction pathways such as mitogen-activated protein kinases (MAPK) (Hashimoto et al. 2000; Totlandsdal et al. 2010), and/or nuclear factor- κ B (NF- κ B) (Takizawa et al. 1999; Quay et al. 1998; Silbajoris et al. 2011) (Baulig et al. 2003). MAPK is a family of highly conserved protein kinases activated by stress, growth factors and cytokines, and is involved in key cellular processes, including inflammatory responses (Widmann et al. 1999). The three main subfamilies of MAPK are

the extracellular-regulated kinase (ERK)1 and -2, the c-jun N-terminal kinase, (JNK)1 and -2, and p38. ERK is known to be involved in regulation in cell proliferation as well as in inflammatory responses, including production of IL-6 (Thalhamer et al. 2008). Often activated by stress, JNK and p38 may regulate cytokine gene expression (Ip & Davis 1998; Johnson & Lapadat 2002). P38 may also function as a post-transcriptional mRNA stabilizing protein (Bhattacharyya et al. 2011). NF- κ B is a family of transcription factors which is central in regulation of a variety of pro-inflammatory genes, including various cytokines/chemokines (Barnes 1997). In its inactive state of the classical NF- κ B pathway, p65 (Rel A)/p50 is bound to the inhibitor of κ B (I κ B) in cytosol. Upon activation, I κ B is phosphorylated, ubiquitinated and degraded, releasing p65/p50 which translocates to the nucleus and binds to κ B-sites in the promoters of target genes (Iwai 2012).

Activation of Toll-like receptors (TLR) is a key feature in recognizing pathogens for initiation of immune responses, such as secretion of pro-inflammatory cytokines and chemokines (Medzhitov 2001). While bacterial components such as lipopolysaccharide (LPS) activates TLR2/4 (Takeuchi et al. 1999), several *in vitro* and *in vivo* studies indicate that activated TLR2/4 enhances responses towards exposure to DEP, DEP organic extract or cigarette smoke condensate (CDC) (Takano 2002; Inoue et al. 2006; Castro et al. 2008). Therefore, priming cells with cytokines or TLR ligands may interfere with, or enhance, the pro-inflammatory responses upon exposure to PM or DEP. TLR3 recognizes viral double-stranded RNA (dsRNA) (Alexopoulou et al. 2001). We hypothesized that priming cells with the TLR3-ligand polyinosinic:polycytidylic acid (poly I:C), a synthetic analog of viral double stranded RNA (dsRNA), may sensitize the cells upon DEP-exposure. We also investigated a possible role of the MAPK and NF- κ B pathways in DEP-exposed poly I:C primed BEAS-2B cells.

Materials and methods

Reagents

LHC-9 cell culture medium was from Invitrogen (Carlsbad, CA, USA) and PureCol™ collagen from Inamed Biomaterials (Fremont, CA, USA). Polyinosinic:polycytidylic acid (Poly I:C), Ponceau S, phenylmethylsulfonyl fluoride (PMSF), aprotinin, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and polyoxyethylene octyl phenyl ether (Triton X-100) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All real-time RT-PCR reagents and TaqMan probes/primers were purchased from Applied Biosystems (Foster City, CA, USA). Bio-Rad DC protein assay was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Pepstatin A were from Calbiochem (Cambridge, MA, CA, USA). Leupeptin was from Amersham Biosciences (Uppsala, Sweden). Antibodies against phospho- and total ERK1/2, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against IκB, phospho- and total p65, p38, JNK1/2, were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against β-actin, as well as secondary antibodies horseradish peroxidase-conjugated goat-anti-rabbit IgG were from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Horseradish peroxidase-conjugated rabbit anti-mouse IgG was from Dako (Glostrup, Denmark) was applied. Mild antibody stripping solution® was from Chemicon International (Termeclula, CA, USA). All other chemicals used were purchased from commercial sources at the highest purity available.

Culture of cells

BEAS-2B cells, an immortalized SV40-adenovirus-hybrid (Ad12SV40) transformed human bronchial epithelial cell line was from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were grown at 37°C in a humidified incubator with a 5% CO₂ atmosphere, where they were passaged twice per week. Cells were cultured in serum-free LHC-9 medium on collagen (PureCol™)-coated culture dishes and flasks. Prior to exposure, cells were plated in 6-well culture dishes, grown to near confluence in serum free LHC-9 medium and exposed as described below.

Particles

Edinburgh-DEP were generated by an unloaded diesel engine (Deutz, 4 cylinder, 2.2 L, 500 rpm) using gas oil as described elsewhere (Totlandsdal et al. 2010). For each experiment, particles were suspended in fresh LHC-9 cell exposure medium (2 mg/ml) and stirred overnight in room temperature before exposure.

Exposure of cells

In all experiments, fresh medium was added the day after seeding and right before exposure and, depending on the experiments, the cells were exposed to 50 or 100 µg/ml DEP for 2 and 4 h (immunoblotting) or 4 h (mRNA expression). The controls were added medium that had been subjected to the same stirring procedure as the particle suspensions. In experiments with primed cells, poly I:C (10 µg/ml) was added 30 min prior exposure to DEP, previously optimized in our laboratory.

Gene expression analysis by real-time RT-PCR

Total RNA was isolated using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) and reverse transcribed to cDNA on a PCR System 2400 (PerkinElmer, Waltham, MA, USA) using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using pre-designed TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix and run on ABI 7500 fast (Applied Biosystems). Gene expression of IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1) and RANTES (Hs00174575_m1) were normalized against 18S rRNA (Hs99999901_s1), and expressed as fold change compared to untreated control as calculated by the $\Delta\Delta\text{Ct}$ method ($\Delta\text{Ct} = \text{Ct}[\text{Gene of Interest}] - \text{Ct}[18\text{S}]$; $\Delta\Delta\text{Ct} = \Delta\text{Ct}[\text{Treated}] - \Delta\text{Ct}[\text{Control}]$; Fold change = $2^{[-\Delta\Delta\text{Ct}]}$).

Examination of protein levels by Western blotting

DEP-induced phosphorylation of MAPK (ERK1/2, JNK1/2, p38) and p65, and degradation of IκB, were measured by Western blot analysis. After exposure, cell culture medium was removed and the dishes were immediately rinsed with ice-cold PBS, and stored at -70°C until further processing.

Frozen cells were thawed, harvested and sonicated in lysis buffer (20 mM Tris-HCL, pH = 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.4 mM Na-pyrophosphate, 1.0 mM orthovanadate, 1 mM NaF, 21 μ M leupeptin, 1.5 μ M aprotinin, 15 μ M pepstatin A, 1 mM PMFS and 1% Triton-X) prior to protein determination using the BioRad DC Protein Assay (BioRad Life Science, CA, USA). Subsequently glycerol, β -mercaptoethanol and SDS were added to all samples, whereas final sample protein concentrations were adjusted by adding more lysis buffer. Proteins (10-20 μ g/well) from whole-cell lysates were separated by 10-15% SDS-PAGE and blotted onto nitrocellulose membranes. To ensure that the protein levels of each well were equal, Ponceau-staining was used for loading control. The membranes were then probed with antibodies for the respective phosphorylated kinases (p-ERK1/2, p-JNK1/2, p-p38), antibodies for I κ B or antibodies for phosphorylated p65, prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed using the Super-Signal® West Dura chemiluminescence system (Pierce, Perbio Science, Sweden) according to the manufacturer's instructions. Finally, the membranes were stripped by incubation for 15 min at room temperature with mild antibody stripping solution, and re-probed with β -actin, or with the total amount of the respective kinases or p65. Optical quantification of the protein bands were performed by using Image Lab Analysis Software (BioRad).

Statistical analysis

If not stated otherwise, statistical analysis was performed by two-way ANOVA with Bonferroni post-test. Results are expressed as means \pm SEM. All calculations were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Results

DEP-induced cytokine and chemokine expression

To explore the time-course of DEP-induced IL-6 and IL-8 gene expression, we exposed the cells for 2, 4 and 6 h (100 µg/ml). Fig. 1 shows significant up-regulation of mRNA levels of IL-6 after 6 h, and IL-8 at all time points, after exposure to DEP. These results confirm the DEP-induction of IL-6 and IL-8 expression in BEAS-2B cells, as previously reported (Totlandsdal et al. 2010).

Cytokine gene expression in DEP-exposed cells primed with Poly I:C

Next, we investigated if DEP induced expression of IL-6, IL-8 and RANTES in poly I:C primed cells. As shown by Fig. 2, expression of IL-6 was significantly induced by DEP alone at 100 µg/ml. Expression of IL-8 was significantly induced by DEP alone at 50 and 100 µg/ml, in a concentration dependent manner. DEP alone had no effect on RANTES. Poly I:C alone induced all mediators giving a ~ 10, 4 and 290-fold significant induction of IL-6, IL-8 and RANTES, respectively. Exposure of DEP to poly I:C primed cells appeared to yield an increased concentration-dependent IL-6 and IL-8 expression, compared to unprimed cells. This was most prominent for IL-6 at 100 µg/ml and IL-8 at 50 µg/ml. In contrast, exposure to DEP significantly down-regulated RANTES expression in poly I:C primed cells. This suggests a quantitative and qualitative alteration of response in production of cytokines and chemokines in poly I:C primed versus unprimed cells exposed to DEP.

Role of the MAPK pathway

To characterize the intracellular mechanisms of DEP-enhanced IL-6 and IL-8 expression in primed cells, we investigated the role of the MAPK signaling after 2 and 4 h exposure. Poly I:C increased phosphorylation of ERK, p38 and JNK at both time points, except for ERK after 4 h exposure. DEP alone did not induce phosphorylation of any of the MAPKs, but rather decreased ERK after 4 h. However, a slight increase in phosphorylation of p38 induced by DEP alone was observed at both time points. No enhanced phosphorylation of any of the MAPK was observed in DEP-exposed poly I:C primed cells compared to poly I:C primed cells alone. However, although not significant, a small increase was observed for p38 after 4 h. DEP reduced phosphorylated levels of ERK and JNK in poly I:C primed cells (Fig. 3). In general, the enhanced DEP-induced IL-6 and IL-8 responses

seen in poly I:C primed BEAS-2B cells did not involved an enhanced phosphorylation of any of the MAPK.

Role of the NF- κ B signaling pathway

We also investigated the involvement of the NF- κ B pathway. After 2 and 4 h, DEP alone did not induce p65 phosphorylation, but I κ B degradation was observed after 4 h. Poly I:C priming alone appeared to induce increased levels of p65 phosphorylation, as well as increased I κ B degradation, at both time points. DEP-exposure to poly I:C primed cells did not increase phosphorylation of p65, but a possible enhanced degradation of I κ B after 4 h (Fig. 4). In general, DEP did not enhance poly I:C induced NF- κ B activation BEAS-2B cells.

Discussion

Several studies have shown pulmonary inflammation in healthy volunteers exposed to PM (Salvi, Blomberg, et al. 1999a; Ghio et al. 2000). Moreover, individuals with preexisting pulmonary infections or chronic diseases, such as asthma and COPD, appear to respond more adversely to PM (Dockery et al. 1993; Dockery & Pope 1994; Samet et al. 2000; Anderson et al. 2012; Naess et al. 2007; Ostro et al. 1993; Peters et al. 1997). However, the mechanisms behind these effects are still poorly understood.

In the present study, we have shown that exposure to diesel exhaust particles (DEP) induce IL-6 and IL-8 expression in BEAS-2B cells, which is in accordance with previous studies from our group and others (Steerenberg et al. 1998; Totlandsdal et al. 2010). Moreover, our results show that priming BEAS-2B cells with the TLR3 ligand poly I:C prior to DEP-exposure, sensitizes the cells. IL-6 and IL-8 responses were most notably enhanced, compared to effects in unprimed DEP-exposed cells. Activation of TLR3 by poly I:C mimics viral infections, which initiates innate immune responses, including production of cytokines (Alexopoulou et al. 2001). Thus, virus-infected cells may be more prone to DEP and react stronger than uninfected, normal cells. In line with this, an epidemiological study carried out by Wong and co-workers (Wong et al. 2010) suggest that influenza infections may be a susceptibility factor against exposure to air pollution. Other studies have shown that increased responses to air pollution may not be limited to preexisting viral infections per se. For example, increased sensitivity to DEP is found in primary cells from asthmatic subjects by enhanced IL-8 responses (Devalia et al. 1999). Moreover, exposing lung epithelial cells to tumor necrosis factor- α (TNF- α) significantly enhanced PM-induced IL-8 responses (Ning et al. 2004).

As PM itself may cause pulmonary inflammation (Salvi, Blomberg, et al. 1999a), exposure to ambient air particles may increase susceptibility to infections. Ciencewicki et al. (Ciencewicki et al. 2006) hypothesized that exposure to an aqueous solution of diesel exhaust would increase susceptibility to poly I:C exposure. The study revealed enhanced induction of IL-6 responses in A549 cells, indicating a sensitizing effect by diesel exhaust. Furthermore, the same author also found enhanced pulmonary IL-6 responses in diesel exhaust exposed mice/*in vivo* upon exposure to influenza virus (Ciencewicki et al. 2007). The results given suggest that PM, and especially DEP,

exacerbate or sensitize pulmonary inflammation by exaggerating innate immune responses causing further tissue injury.

In contrast to the enhanced DEP-induced IL-6 and IL-8 responses in poly I:C primed cells, RANTES, a potent eosinophil attractant, was only induced in poly I:C primed cells and attenuated upon exposure to DEP. Similarly, Devalia and colleagues found that RANTES expression in cells from asthmatic patients, was attenuated upon DEP-exposure (Devalia et al. 1999). The enhancement of IL-6 and IL-8 expression, and reduction of RANTES expression in poly I:C primed DEP-exposed BEAS-2B cells, indicate that DEP-exposure may favor an immune response dominated by neutrophils and not by eosinophils. Thus, DEP-exposure may promote both an enhancement and a shift in the immune response in patients with preexisting pulmonary inflammation.

Both DEP and poly I:C have been shown to induce expression of cytokines and chemokines through up-regulation of MAPK signaling pathway and/or NF- κ B (Hashimoto et al. 2000; Takizawa et al. 1999; Totlandsdal et al. 2010; Alexopoulou et al. 2001). Here we show that there was little effect on phosphorylation of p38 and that DEP rather decreased phosphorylation-levels of ERK and JNK, compared to poly I:C primed cells alone. Of notice, up-regulated phosphorylation of p38 has been shown in DEP-exposed BEAS-2B and BET-1A cells. (Hashimoto et al. 2000; Totlandsdal et al. 2010), which may indicate a role of p38 in the DEP-enhanced IL-6 and IL-8 expression in poly I:C primed cells. Furthermore, an increased degradation of I κ B in DEP-exposed poly I:C primed cells may indicate an increased translocation of p65 to the nucleus without affecting phosphorylation-levels of p65. Of notice, these results show that DEP may induce IL-6 and IL-8 without enhancing phosphorylation of MAPK and NF- κ B pathways. NF- κ B and MAPK are regarded as important for activation of IL-6 and IL-8 (Hoffmann et al. 2002). Thus, these pathways may be rate-limiting factors for the DEP-induced pro-inflammatory responses. As DEP itself may not be a strong regulator of MAPK or NF- κ B, one can argue that the poly I:C-enhanced phosphorylation of MAPK and NF- κ B may be exploited by DEP, directly or indirectly, to optimize DEP-induced IL-6 and IL-8 responses in poly I:C primed cells.

Surprisingly, phosphorylation of ERK and JNK was down-regulated in the DEP-exposed poly I:C primed cells. It is therefore tempting to speculate that any of these down-regulated MAPK account for the decreased RANTES expression. Notably, down-regulation of poly I:C induced RANTES

and p-JNK production has also been found in BEAS-2B exposed to cigarette smoke extract (CSE) (Eddleston et al. 2011). Other pathways may also have a role for the enhanced cytokine and chemokine expression in DEP-exposed poly I:C primed cells. Both viral infections and diesel exhaust are shown to activate IRF-3 (interferon regulatory factor) (Yoneyama et al. 1998; Cienciewicki et al. 2006). Moreover, DEP with high organic content have been shown to induce IL-8 through AP-1 (activator protein-1) in an NF- κ B independent manner, in airway epithelial cells (Tal et al. 2010). For future investigations, we postulate that these pathways may be involved in the DEP-induced cytokine and chemokine responses.

The present study suggests that DEP-exposed immortalized human bronchial epithelial cells respond differently in the presence of a TLR3 agonist. More specifically, DEP enhanced IL-6 and IL-8 expression, and attenuated RANTES expression, in poly I:C primed cells compared to unprimed cells. Thus, DEP-exposure may not only enhance, but also alter pro-inflammatory responses in infected cells. This may imply that preexisting infections may be a susceptibility factor for PM-induced adverse health effects. However, as the present results were obtained in an immortalized cell line, these findings should be interpreted with caution.

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Figures

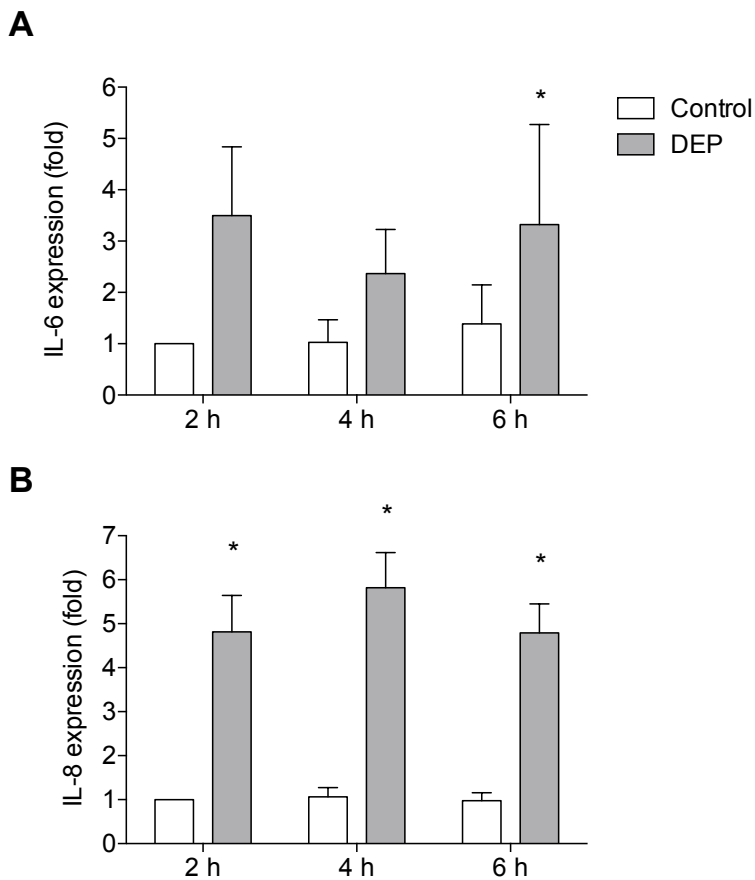


FIGURE 1. DEP induced a time-dependent IL-6 and IL-8 expression in BEAS-2B cells. Cells were exposed to DEP (100 $\mu\text{g/ml}$) for 2, 4 and 6 h (A and B). IL-6 and IL-8 expression was measured by real-time RT-PCR. Bars represent means \pm SEM of fold increase relative to unexposed cells (n=3-4). * $p < 0.05$; exposed vs non-exposed cells. Statistic analysis was based on normalized data.

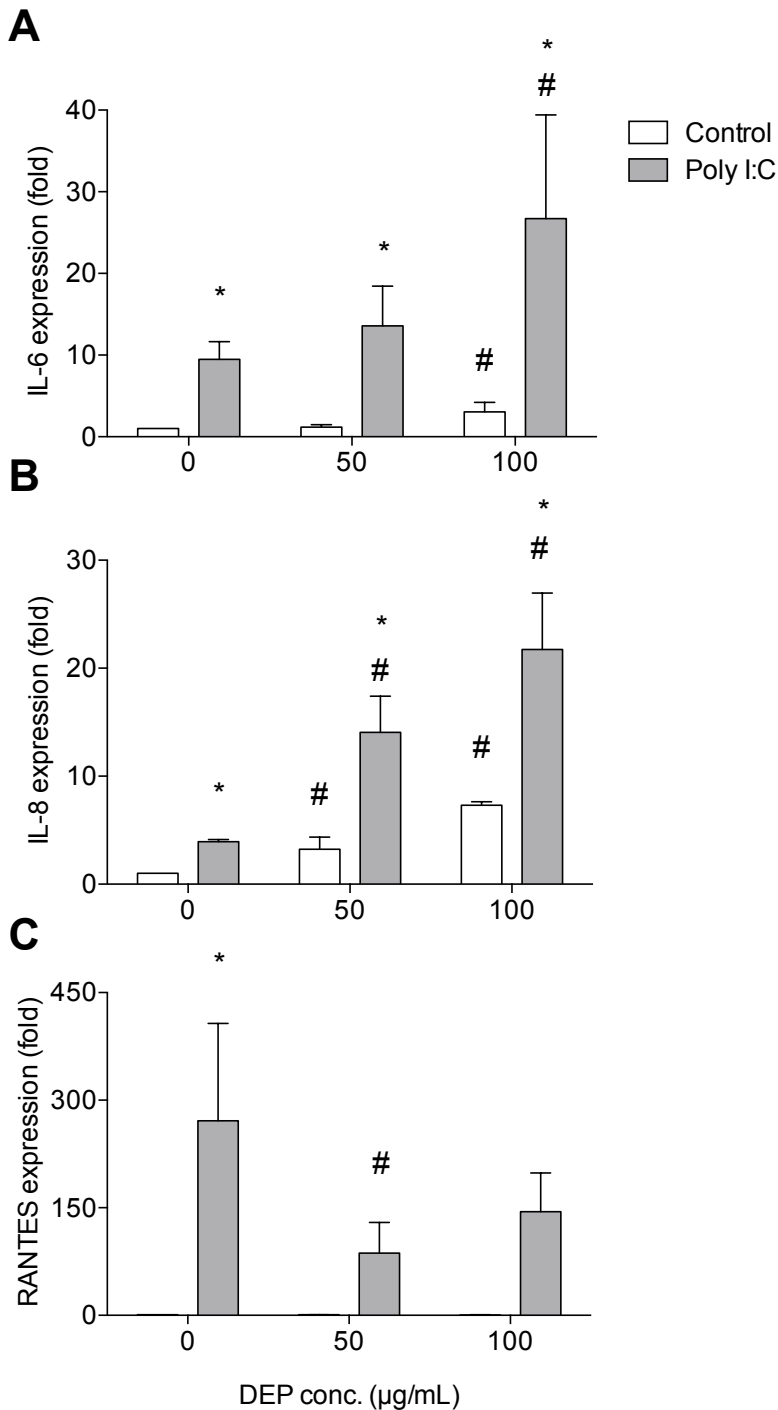


FIGURE 2. DEP enhanced IL-6 and IL-8 expression but suppressed RANTES expression in Poly I:C primed BEAS-2B cells. Cells were primed with Poly I:C (10 µg/ml) for 30 min prior exposure to DEP (0, 50 and 100 µg/ml). IL-6 (A), IL-8 (B) and RANTES (C) expression was measured after 4 hours using real-time RT-PCR. Bars represent means \pm SEM of fold increase relative to unexposed cells. (n=3). * $p < 0.05$; primed vs non-primed cells. # $p < 0.05$; exposed vs non-exposed cells. Statistic analysis was based on log-transformed data.

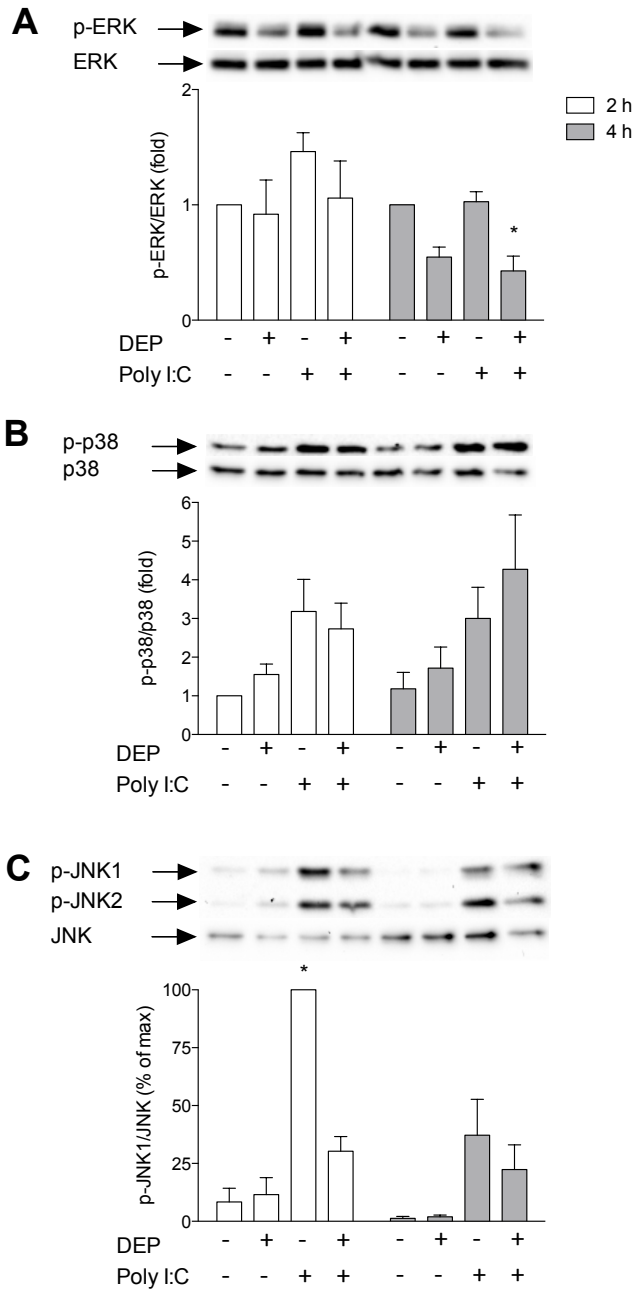


FIGURE 3. DEP did not enhance MAPK-signaling in Poly I:C primed BEAS-2B cells. Cells were primed with Poly I:C (10 μ g/ml) for 30 min prior exposure to DEP (100 μ g/ml) for 2 and 4 h. Intracellular protein levels of total and phosphorylated of ERK, p38, JNK were detected by western blotting as described under “Materials and methods”. The figure displays representative blots of ERK(A), p38(B) and JNK(C). The graphs depict relative changes quantified by densitometric analysis of the Western blots (Image Lab, BioRad). Bars represent means \pm SEM of fold increase relative to unexposed cells. (n=3).

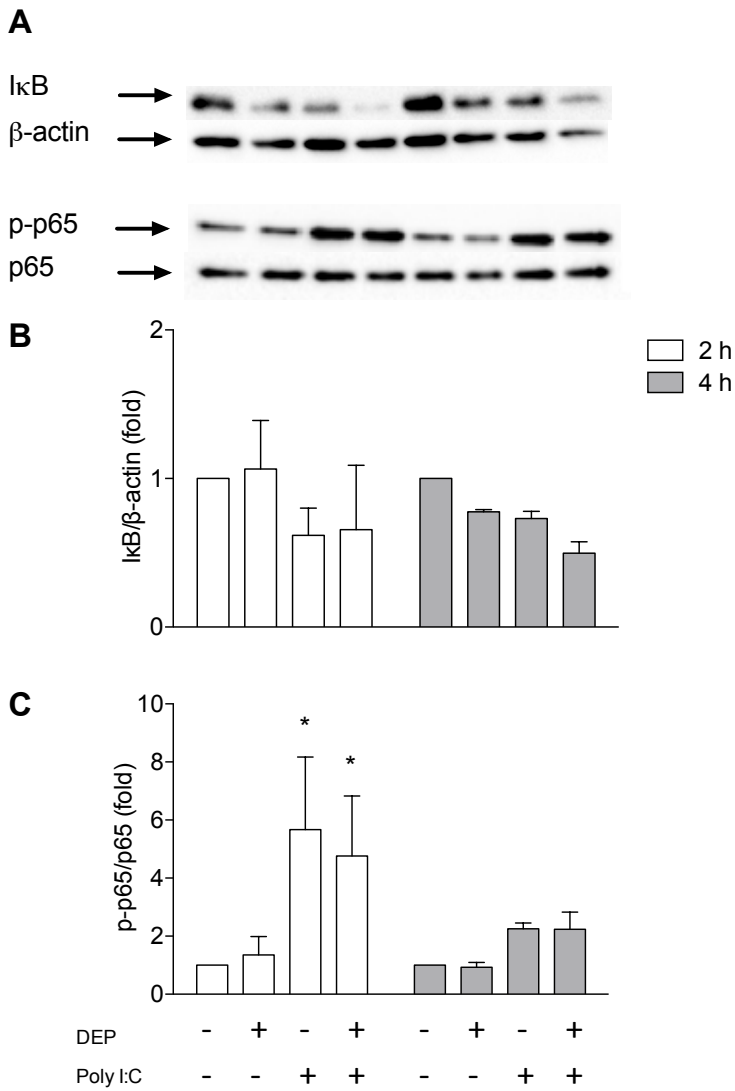


FIGURE 4. DEP did not enhance NF- κ B-signaling in Poly I:C primed BEAS-2B cells. Cells were primed with Poly I:C (10 μ g/ml) for 30 min prior exposure to DEP (100 μ g/ml) 2 and 4 h. Intracellular protein levels of total and phosphorylated p65, as well as I κ B and β -actin were detected by Western blotting as described under “Materials and methods”. The figure displays representative blots of I κ B and p-p65 (A). The graphs (B and C) depict relative changes in I κ B and p-p65 quantified by densitometric analysis of the Western blots (Image Lab, BioRad). Bars represent means \pm SEM of fold increase relative to unexposed cells. (n=3).

Paper 2

Title:

Polar and non-polar soluble organic chemicals contribute differentially to diesel exhaust particle-induced cytokine responses in human bronchial epithelial cells

Preliminary author list:

**Nicolai Bach^{1,2}, Anette Kocbach Bølling¹, Annike I. Totlandsdal¹, Jørn A. Holme¹, Marit Låg¹
and Johan Øvrevik¹**

Affiliation:

¹Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, N-0403 Oslo, Norway

²Department of Biology, Faculty of Mathematics and Natural Sciences, University of Oslo, P.O. Box 1066 Blindern, N-0316 Oslo, Norway

Corresponding author:

Johan Øvrevik

Department of Air Pollution and Noise

Division of Environmental Medicine

Norwegian Institute of Public Health

P.O.Box 4404 Nydalen

N-0403 Oslo, Norway

E-mail: johan.ovrevik@fhi.no

Phone: +47 21076408

Fax: +47 21076686

KEY WORDS: Inflammation, DEP, organic extract.

Abstract

Background: Diesel exhaust particles (DEP) are a considerable constituent of particulate matter (PM), especially in urban ambient air. DEP have been associated with adverse cardiopulmonary health effects, such as exacerbation of chronic obstructive pulmonary diseases (COPD) and asthma, of which inflammation seems to play a key role. However, the components of DEP responsible for inflammatory responses in healthy and diseased airways, and the mechanisms behind, are not well known.

Objective: To characterize pro-inflammatory components of DEP in human bronchial epithelial cells (BEAS-2B), and BEAS-2B cells primed with the Toll-like receptor (TLR)3 agonist polyinosinic:polycytidylic acid (poly I:C). Moreover, we wanted to elucidate an involvement of initiation mechanisms in DEP-induced IL-6 and IL-8 expression.

Methods: BEAS-2B cells were exposed to DEP or DEP-extracts. Poly I:C primed and unprimed cells were exposed to fractionated DEP-extracts. Expression of interleukin (IL)-6 and IL-8 was analyzed by real-time RT-PCR. Release of IL-6, IL-8 and RANTES was analyzed by ELISA. A possible involvement of proteinase activated receptor-2 (PAR-2) and aryl hydrocarbon receptor (AhR) was tested using siRNA transfection in DEP-induced IL-6 and IL-8 expression.

Results: We found that DEP with varying organic content induced different levels of IL-6 and IL-8 expression in BEAS-2B cells. Furthermore, polar fractionated methanol DEP-extracts induced IL-6 release in unprimed cells but did not induce any cytokine release in poly I:C primed cells, above respective controls. In contrast, non-polar methanol DEP-fractions did not induce cytokine release unprimed cells but induced IL-6 and IL-8 cytokine release in poly I:C primed cells. RANTES was only induced by poly I:C and attenuated by exposure to fractions of methanol DEP-extracts. However, non-polar heptane DEP-extract induced IL-6 and IL-8 release in unprimed cells. DEP-induced IL-6 and IL-8 expression was rather up-regulated in siAhR transfected cells. DEP-induced IL-6 expression was down-regulated in siPAR-2 transfected cells.

Conclusion: Compounds of different polarities differentially induced IL-6 and IL-8 responses in DEP-exposed BEAS-2B cells. Infected cells responded differently than uninfected cells. Presence of AhR seemed to be anti-inflammatory in DEP-induced IL-6 and IL-8 expression, while PAR-2 appeared to be involved in DEP-induced IL-6 expression airway epithelial cells.

Introduction

Diesel exhaust particles (DEP) are a major constituent of particulate matter (PM) in urban areas (Kittelson 1998; Salvi et al. 1999), and are associated with negative health effects, such as increased pulmonary inflammation and cancer (Garshick et al. 2004; Hart et al. 2006). Moreover, PM-induced inflammation may play a key role in onset and exacerbation of cardiopulmonary diseases, such as asthma or atherosclerosis (Brook et al. 2004; Chauhan & Johnston 2003; Donaldson et al. 2005). DEP are composed of a carbonaceous core with a variable and complex mixture of adsorbed organic and inorganic components, including polycyclic aromatic hydrocarbons (PAH) and PAH-derivatives (Wichmann 2007). Identifying the components accounting for negative health effects may help reduce the DEP-toxicity in the future.

Both *in vivo* and *in vitro* studies of DEP-exposure suggest that pro-inflammatory responses are caused by the soluble organic compounds (Yang et al. 1997; Bonvallot et al. 2001; Li et al. 2011; Fuentes-Mattei et al. 2010). In addition, induction of interleukin (IL)-6 and IL-8 expression by PAH-rich methanol extractable organic compounds of DEP have been reported by our group (Totlandsdal et al. 2012). Although certain PAH and PAH-derivatives may induce pro-inflammatory proteins at high concentrations in BEAS-2B cells (Ovrevik et al. 2009; Ovrevik et al. 2010), recent results from our lab suggest that PAH may not necessarily be the prime drivers of the DEP-induced cytokine responses (Totlandsdal, submitted). More specifically, it was found that exposure to the more polar fraction of the methanol DEP-extract appeared to be responsible for the cytotoxicity and IL-6 responses. The mid-polar fraction, containing the majority of PAH, nitro- and oxy-PAH, and the non-polar fraction, did not induce any IL-6 or IL-8 cytokine production. Thus, other components may be responsible for DEP-toxicity.

Environmental pollutants, such as PAH, may bind to the aryl hydrocarbon receptor (AhR) in cytosol. In its inactive state, AhR is sequestered in cytosol. When activated, the AhR translocates to the nucleus and binds to AhR nuclear translocator (ARNT). The AhR:ARNT transcription factor heterodimer binds to xenobiotic response elements (XRE) at the promoter of specific genes, including cytochrome P450 (CYP)-genes. CYP-enzymes may be involved in the metabolic activation of several xenobiotics, leading to formation of electrophilic metabolites and/or reactive oxygen species (ROS) (Namazi 2009), which can cause damage to DNA and/or vital proteins. ROS may also activate redox-sensitive pathways resulting in various inflammatory responses (Gelboin

1980; Whitlock 1999; Baulig et al. 2003). However, while levels of AhR is associated with lung carcinomas (Lin et al. 2003), the contribution of AhR in DEP-induced inflammation responses is less understood.

Proteinase activated receptor (PAR)-2 is a seven transmembrane G-coupled receptor activated upon cleavage of the N-termini by proteinases, exposing a tethered ligand that can bind to another site on the same receptor (Nystedt et al. 1995), sensing extracellular proteinases generated during infections and suggested to play a role in the inflammatory response. PAR-2 is expressed ubiquitously, including epithelial cells (Steinhoff et al. 2005). Recently, Li and colleagues (Li et al. 2011) suggested a novel role of PAR-2 in DEP-induced matrix metalloproteinase-1 (MMP-1) secretion. It was shown that exposure to DEP organic extract activated PAR-2 leading to Ca^{2+} -influx mediated by the Transient Receptor Potential vanilloid, family member 4 (TRPV4). However, any possible role of PAR-2 in DEP-induced cytokine and chemokine responses still needs further verification.

In the present study we focused on the pro-inflammatory properties of various DEP-fractions. We compared the ability of different fractionated methanol DEP organic extracts, separated by polarity, to induce cytokine IL-6, IL-8 and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) production. IL-6 is a cytokine known to be involved in the transition of acute to chronic inflammation (Kaplanski et al. 2003). IL-8 is chemokine and a potent neutrophil attractant (Baggiolini et al. 1989). The chemokine RANTES attracts eosinophils and is expressed in epithelial cells (Kwon et al. 1995; Rot et al. 1992). Polyinosinic:polycytidylic acid (Poly I:C) is a double stranded viral RNA analog and is shown to activate Toll-like receptor (TLR)3, mimicking viral infections (Alexopoulou et al. 2001). TLR is a family of pathogen recognition receptors (PRR), expressed in a wide variety of cells, which recognize conserved motifs on pathogens, which induce pro-inflammatory responses (Jiang 2004). Previous studies from our lab indicate altered pro-inflammatory responses in DEP-exposed BEAS-2B cells primed with poly I:C, compared to unprimed cells (Bach et al., unpublished results). Therefore, we also investigated the responses in these cells to the fractionated DEP-extracts. Finally, we studied the role of PAR-2 and AhR in the DEP-induced expression of IL-6 and IL-8 in human bronchial epithelial cells.

Materials and methods

Reagents

LHC-9 cell culture medium was from Invitrogen (Carlsbad, CA, USA) and PureCol™ collagen from Inamed Biomaterials (Fremont, CA, USA). Polyinosinic:polycytidylic acid (Poly I:C), Ponceau S, phenylmethylsulfonyl fluoride (PMSF), dimethyl sulfoxide (DMSO), propidium iodide (PI), Hoechst 33342, aprotinin, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and polyoxyethylene octyl phenyl ether (Triton X-100) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bio-Rad DC protein assay was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Pepstatin A were from Calbiochem (Cambridge, MA, CA, USA). Leupeptin was from Amersham Biosciences (Uppsala, Sweden). Antibodies against AhR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against β -actin, as well as secondary antibodies horseradish peroxidase-conjugated goat-anti-rabbit IgG were from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Horseradish peroxidase-conjugated rabbit anti-mouse IgG was from Dako (Glostrup, Denmark). Mild antibody stripping solution® was from Chemicon International (Termeclula, CA, USA). All real-time RT-PCR reagents and TaqMan probes/primers were purchased from Applied Biosystems (Foster City, CA, USA). Cytokine ELISA assays for IL-6 (Human IL-6 Cytoset) and CXCL8 (Human IL-8 Cytoset) were purchased from Biosource International (Camarillo, CA, USA). Cytokine ELISA assay for CCL5/RANTES (Human DuoSet) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Small interfering RNA (siRNA) against AhR (sc-29654), PAR-2 (sc-36188) and non-targeting control siRNA (sc-37007) were purchased Santa Cruz Biotechnology (Santa Cruz, CA, USA). HiPerFect® Transfection Reagent from QIAGEN (Hilden, Germany). All other chemicals used were purchased from commercial sources at the highest purity available.

Culture of cells

BEAS-2B cells, an immortalized SV40-adenovirus-hybrid (Ad12SV40) transformed human bronchial epithelial cell line was from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were grown at 37°C in a humidified incubator with a 5% CO₂ atmosphere, where they were passaged twice per week. Cells were cultured in serum-free LHC-9 medium on collagen (PureCol™)-coated culture dishes and flasks. Prior to exposure, cells were plated in 6-well

(immunoblotting or mRNA) or 12-well (protein release and cell death) culture dishes, grown to near confluence in serum free LHC-9 medium and exposed as described below.

Particles

Edinburgh-DEP were generated by an unloaded diesel engine (Deutz, 4 cylinder, 2.2 l, 500 rpm) using gas oil and chemically characterized as described elsewhere (Totlandsdal et al. 2010). Standardized DEP, Standard Reference Material (SRM 2975), were obtained from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA. For each experiment, particles were suspended in fresh LHC-9 cell exposure medium (2 mg/ml) and stirred overnight in room temperature before exposure.

Particle extracts

As previously described (Totlandsdal et al. 2012)(Totlandsdal, submitted), organic methanol extract from Edinburgh-DEP was fractionated using solid phase extraction (SPE) with an amino propyl SPE cartridge (Sep-Pak, Waters, Milford, MA, USA). The SPE was preconditioned with 6 ml of DCM followed by 6 ml of n-hexane. Three fractions were eluted sequentially with 5 ml n-hexane (non-polar), 5 ml 20% dichloromethane (DCM) in n-hexane (mid-polar), and 5 ml methanol (MeOH) (high-polar). To prepare the extracts for in vitro experiments, the fractionated SPE-extracts and the soxhlet extracts (MeOH extraction of washed particles) were dried under nitrogen gas and stored at -20°C until further processing. Later, these extracts were thawed, re-suspended in dimethyl sulfoxide (DMSO), and aliquoted in glass tubes before storage at -20°C until the day of exposure. On the day of exposure, aliquots of each extract fraction were further suspended in cell culture medium at a concentration corresponding to 2 mg/ml of the native particles.

In addition, non-polar constituents were eluted directly from Edinburgh-DEP by heptane. Heptane DEP-extracts and corresponding residual DEP-particles were prepared by sonication in heptane, subsequent separation of particles from the extract by centrifugation, and dried under nitrogen gas, before storage at -20°C until use. When used for exposure of cells, samples were re-suspended in DMSO and subsequently in cell exposure medium (2 mg/ml). The final concentrations of residual particles and extracts in the cell culture wells corresponded to 0, 10, 25, 50, 100 and 200 µg/ml of native particles.

Exposure of cells

In all experiments fresh medium was added the day after seeding and right before exposure and, depending on the experiments, the cells were exposed to various concentrations of DEP or DEP-extracts for 4 h (immunoblotting), 24 h (protein release and cell death). In all experiments, medium that had been subjected to the same stirring procedure as the particle suspensions, were added to the controls. In experiments where compounds were dissolved in DMSO, control cultures were treated with vehicle (DMSO) only. The final concentration of DMSO was $\leq 0.5\%$ (v/v). Effects induced by the extracts and residual particles were compared with effects induced by native particles undergoing equivalent extraction treatment, but without separation with centrifugation. In experiments with poly I:C primed cells, poly I:C (10 $\mu\text{g/ml}$) was added 30 min prior exposure to DEP, previously optimized in our laboratory.

Cytokine measurements by ELISA

After exposure, the medium was harvested and centrifuged at 1000 x g for 2 min to remove particles. The final supernatants were stored at -70°C . Protein levels of IL-6, IL-8 and RANTES were determined by ELISA (Enzyme-Linked ImmunoSorbent Assay) according to the manufacturer's guidelines. Absorbance was measured and quantified using a plate reader (TECAN sunrise, Phoenix Research Products, Hayward, CA, USA), complete with software (Magellan V 1.10; Phoenix Research Products).

Gene expression analysis by real-time RT-PCR

Total RNA was isolated using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) and reverse transcribed to cDNA on a PCR System 2400 (PerkinElmer, Waltham, MA, USA) using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using pre-designed TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix and run on ABI 7500 fast (Applied Biosystems). Gene expression of IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), CYP1A1 (Hs00153120_m1) and PAR-2 (Hs00608346_m1) were normalized against 18S rRNA (Hs99999901_s1), and expressed as fold change compared to untreated control as calculated by the $\Delta\Delta\text{Ct}$ method ($\Delta\text{Ct} = \text{Ct}[\text{Gene of Interest}] - \text{Ct}[18\text{S}]$; $\Delta\Delta\text{Ct} = \Delta\text{Ct}[\text{Treated}] - \Delta\text{Ct}[\text{Control}]$; Fold change = $2^{[-\Delta\Delta\text{Ct}]}$).

Gene silencing by siRNA

BEAS-2B cells were transfected with the respective siRNA, using HiPerFect transfection reagent according to the Fast-Forward protocol for adherent cells recommended by Qiagen (Germany). In brief, siRNA against AhR, PAR-2 and HiPerFect were mixed by vortexing in LHC-9 medium and incubated for 5-10 min at room temperature to form transfection complexes. A non-targeting siRNA was used as control. Immediately after seeding, the transfection complexes (100 μ l/well) were added drop-wise to the cell cultures to give a final siRNA concentration of 10 nM and 4 μ l of HiPerFect in a total of 1.5 ml growth medium. Medium was changed (refreshed) after 24 h, and on the day of exposure (48 h after transfection). The effectiveness of gene silencing was monitored by measuring gene expression by real-time RT-PCR or protein levels by Western blotting.

Examination of protein levels by Western blotting

AhR protein levels in siRNA transfected cells were measured by Western blot analysis. After exposure, cell culture medium was removed and the wells were immediately rinsed with ice-cold PBS, and stored at -70°C until further processing. Frozen cells were thawed, harvested and sonicated in lysis buffer (20 mM Tris-HCL, pH = 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.4 mM Na-pyrophosphate, 1.0 mM orthovanadate, 1 mM NaF, 21 μ M leupeptin, 1.5 μ M aprotinin, 15 μ M pepstatin A, 1mM PMFS and 1% Triton-X) prior to protein determination using the BioRad DC Protein Assay (BioRad Life Science, CA, USA). Subsequently glycerol, β -mercaptoethanol and SDS were added to all samples, whereas final sample protein concentrations were adjusted by adding more lysis buffer. Proteins (10-20 μ g/well) from whole-cell lysates were separated by 10-15% SDS-PAGE and blotted onto nitrocellulose membranes. To ensure that the protein levels of each well were equal, Ponceau-staining was used for loading control. The membranes were then probed with antibodies for AhR before incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed using the Super-Signal[®] West Dura chemiluminescence system (Pierce, Perbio Science, Sweden) according to the manufacturer's instructions. Finally, the membranes were stripped by incubation for 15 min at room temperature with mild antibody stripping solution, and re-probed with β -actin. Optical quantification of the protein bands were performed by using Image Lab Analysis Software (BioRad).

Cytotoxicity by fluorescence microscopy

Plasma membrane-damage and changes in nuclear morphology associated with apoptosis and necrosis were determined by fluorescence microscopy (Nikon Eclipse E 400 fluorescence microscope). Cells were exposed to various concentrations of heptane extracts for 24 h. Following exposure, the cells were trypsinated and stained with Hoechst 33342 (5 µg/ml) and propidium iodide (PI) (10 µg/ml) as described previously (Solhaug et al. 2004). PI-positive necrotic cells were counted as the fraction of the total number of cells. A minimum of 300 cells per slide were counted.

Statistical analysis

If not stated otherwise, statistical analysis was performed by two-way ANOVA with Bonferroni post-test. All calculations were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Results

DEP-induced cytokine and chemokine expression

We investigated IL-6 and IL-8 responses in BEAS-2B cells of two different DEP with different organic content. The cells were exposed to the commercially available SRM2975-DEP, and Edinburgh-DEP (from here on referred to as DEP). Chemical analysis show that the particles contain ~ 1.5% and 60% extractable organic content, respectively (Stevens et al. 2009; Totlandsdal 2012., submitted). DEP induced increased mRNA levels of IL-6 and IL-8 already at 25 µg/ml with a steady increase to 200 µg/ml. SRM2975 did not significantly induce IL-6 or IL-8 expression at any concentrations (Fig. 1). Therefore, DEP with the high organic content appeared to be the most inflammogenic in BEAS-2B cells.

Cytokine secretion after exposure to fractionated methanol DEP-extracts

We have previously observed that cytokine responses BEAS-2B cells primed with the TLR3 ligand poly I:C are stronger and/or differently upon exposure to DEP or PAH (Ovrevik et al. 2012, submitted, Bach et al., unpublished results). We therefore investigated cellular release of IL-6 and IL-8 after exposure to fractionated methanol DEP-extracts in unprimed and poly I:C primed cells. Cells were exposed to three fractions (A: non-polar, B: mid-polar, C: polar) or a Soxhlet-extract at a concentration corresponding 100 µg/ml DEP. None of the fractions induced release of IL-8 or chemokine RANTES in unprimed cells. Although not statistically significant, the highly polar C-fraction seemed to induce a slight increase in IL-6 release. In contrast, the non-polar A-fraction induced cytokines in poly I:C primed cells. A statistically significant induction of IL-8 was observed, whereas IL-6 seemed to be induced, but this effect was not statistically significant. All the fractions inhibited poly I:C induced RANTES responses (Fig. 2). Differential effects were observed between poly I:C primed and unprimed BEAS-2B cells.

Cytokine secretion after exposure to heptane DEP-extracts

Recent studies have shown that DEP and methanol DEP-extracts induced IL-8 expression, whereas single fractions of methanol DEP-extracts, separated by polarity, did not induce IL-8 release (Totlandsdal et al. 2012; Totlandsdal 2012., submitted). Shown by Fig. 2, the non-polar fraction may contain compounds that enhance IL-6 and IL-8 responses in poly I:C primed cells, but are not

sufficient for IL-6 or IL-8 responses in unprimed cells. Notably, fractionation of the methanol DEP-extracts was shown to result in a loss of organic content by 50%. Therefore, to further investigate the lack of responses in unprimed cells exposed to fractionated methanol DEP-extracts, we extracted organic compounds of DEP directly using the non-polar solvent heptane. Most interestingly, heptane DEP-extract was found to significantly induce both IL-6 and IL-8 release. We observed that the particles and extracts induced low cytotoxicity (Fig 3). These results show that non-polar compounds in DEP are able to induce IL-6 and IL-8 responses in unprimed BEAS-2B cells. Furthermore, increased release of IL-6 by heptane insoluble particles is in accordance with the above findings, suggesting a role of polar compounds in IL-6 release.

Role of AhR in the IL-6 and IL-8 gene expression

We next investigated if AhR was involved in the DEP-induced IL-6 and IL-8 responses by siRNA transfection as the AhR and/or CYP-enzymes may be involved in pro-inflammatory signaling pathways. Our results show that CYP1A1 gene expression was increased ~ 10-fold after exposure to 10 µg/ml DEP, followed by a possible concentration-dependent decrease after exposure to 50 and 100 µg/ml DEP. Transfection against AhR (siAhR) reduced CYP1A1 gene expression to baseline, as expected. DEP induced IL-8 in a concentration-dependent manner reaching maxima at 100 µg/ml DEP. DEP alone did not induce IL-6 expression in siNT (non-targeting control siRNA) cells. Whether this could be due to unspecific effects of the siRNA transfections, remains unclear. More importantly, in siAhR transfected cells, gene expression of IL-6 and IL-8 appeared to increase in all concentrations compared to control. Down-regulation of AhR protein levels was confirmed by Western analysis (Fig. 4). Therefore, the results rather indicate an increased sensitivity in AhR-depleted cells.

Role of PAR-2 in the IL-6 and IL-8 gene expression

It has previously been shown an involvement of PAR-2 in MMP-1 secretion induced by organic extracts of DEP in BEAS-2B cells (Li et al. 2011). Therefore, we investigated a possible interaction with PAR-2 in the DEP-induced IL-6 and IL-8 expression in BEAS-2B cells by siRNA transfection. DEP-induced IL-6 expression was significantly down-regulated in siPAR-2 transfected cells. In contrast, the level of IL-8 expression was not significantly reduced. The mRNA levels of PAR-2 appeared to be down-regulated, as expected (Fig. 5). This suggests that PAR-2 may be involved in the activation of IL-6 gene expression upon DEP-exposure.

Discussion

DEP contain a complex mixture of components which may cause adverse cardiopulmonary health effects in humans. Identifying the inflammogenic compounds of DEP is important to elucidate the mechanisms of DEP-toxicity. In the present study, we show that levels of organic content (OC) in DEP may affect the pro-inflammatory responses in BEAS-2B cells. Moreover, a differentially induction of IL-6 and IL-8 expression based on polarities of the fractions was observed. More specifically, polar fraction mainly accounted for IL-6 release, whereas the non-polar fractions induced both IL-6 and IL-8 release. In line with previous studies from our group (Ovrevik et al, submitted), the presence of AhR in DEP-exposed BEAS-2B cells seemed to be anti-inflammatory. The present study indicates that the pro-inflammatory properties were potentially highest in DEP with high OC. Interestingly, Tal and co-workers reported that SRM2975-DEP, with low OC, induced IL-8 expression in BEAS-2B cells (Tal et al. 2010). The lack of effects by the SRM2975-DEP with low OC in our model, may be explained by differences in exposure conditions, as reported elsewhere (Veranth et al. 2008).

We further tested various fractions of the methanol DEP-extract. We show that the DEP-induced IL-6 release may be assigned to polar compounds in unprimed cells. This is in line with other studies suggesting an IL-6 release by polar PBS DEP-extracts and polar acetone PM_{2.5} organic extract (Totlandsdal et al., unpublished results, Fuentes-Mattei et al. 2010). Interestingly, our results indicate that certain components in the non-polar fraction may enhance both IL-6 and IL-8 in TLR3-primed cells, but are not able to initiate this response in unprimed cells. Previous studies have shown that TLR3 priming alters the responses in cells exposed to DEP or DEP-derivatives (Bach et al., unpublished results, Ovrevik et al. 2012., submitted). Thus, polarity may be an important parameter to identify compounds accounting for DEP-toxicity in both healthy and diseased cells. However, exposure of unprimed cells to a non-polar heptane DEP-extract induced both IL-6 and IL-8 release. Thus, the lack of IL-6 and IL-8 responses of the non-polar fraction of the methanol DEP-extract, is most probably due to loss of components during the fractionation process.

To characterize the mechanisms behind DEP-induced pro-inflammatory IL-6 and IL-8 expression, we investigated a role of AhR by siRNA transfection. We found that the absence of AhR rather increased the IL-6 and IL-8 expression. In a recent study, we showed that benzo[a]pyrene, with a

high affinity for AhR, did not induce any of the pro-inflammatory responses in BEAS-2B cells (Ovrevik et al. 2010). Thus, together with our results, this indicates that it is not the classical PAH, with high affinity for the AhR, that are responsible for the inflammatory effect, suggesting a possible anti-inflammatory role of AhR. Previously, a mutual repression between AhR and NF- κ B has been proposed, such that activated AhR may interact with activated NF- κ B and thereby inhibiting IL-6 and IL-8 responses (Tian et al. 1999). However, here we could not observe an NF- κ B-dependent DEP-induction of IL-8 in airway epithelial cells (Bach et al., unpublished results), as also published by others (Tal et al. 2010). One might therefore speculate if AhR may work as an anti-inflammatory regulator for other pro-inflammatory pathways than NF- κ B. Of notice, levels of AhR varies in an individual and between individuals. Thus, a possible anti-inflammatory effect of AhR suggests that AhR could be an important factor in determining the susceptibility of individuals to DEP-induced inflammation.

Investigating a possible involvement of PAR-2, we found that PAR-2 may be involved in DEP-induced IL-6 expression. PAR-2 has been reported to mediate induction of matrix metalloproteinase-1 (MMP-1) in BEAS-2B cells exposed to a polar acetone DEP-extract (Li et al. 2011). Furthermore, as IL-6 release is associated with exposure to polar extracts, it is therefore tempting to speculate that PAR-2 is activated, directly or indirectly, by one or more of the polar compounds in DEP.

The present study demonstrates that variable DEP-induced pro-inflammatory effects may be explained by factors such as organic content, polarity of compounds, as well as the state of the cells. As inflammation is being considered to account for several adverse pulmonary health effects induced by PM, identifying compounds responsible for DEP-toxicity could be an important part of future strategies to reduce the damaging health effects of urban air pollution. Our results support the notion that particles with high organic content were more inflammatory than particles with low organic content. Moreover, we observed a differential response in BEAS-2B cells to fractionated methanol DEP-extracts of which the polar fraction induced IL-6 release and the non-polar fraction induced both IL-6 and IL-8 release. The differential response in poly I:C primed BEAS-2B cells, indicate that non-polar compounds may enhance the pro-inflammatory response during infections. Finally, AhR may be anti-inflammatory, and PAR-2 may be involved in DEP-induced IL-6 production.

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Figures

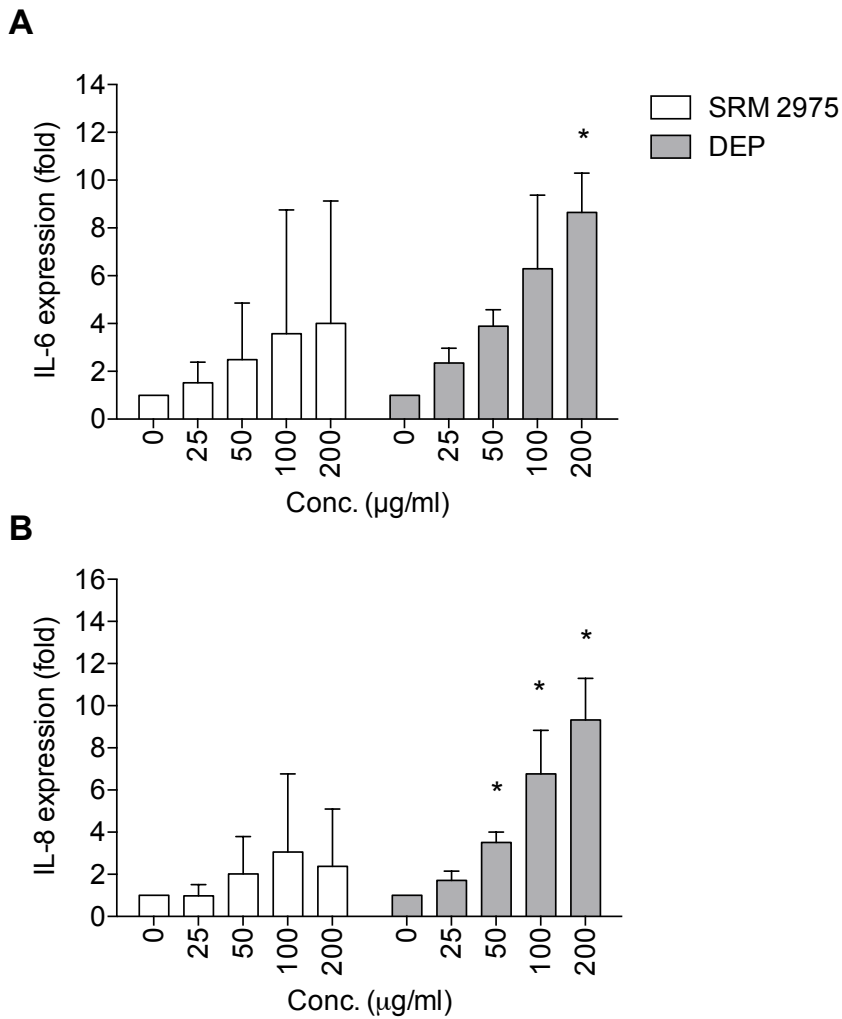


FIGURE 1. Edinburg-DEP induced a concentration dependent IL-6 and IL-8 expression in BEAS-2B cells. Cells were exposed to Edinburg-DEP or SRM2975 (0, 25, 50, 100 and 200 µg/ml) for 4 h (A and B). IL-6 and IL-8 expression was measured by real-time RT-PCR. Bars represent means \pm SEM of fold increase relative to unexposed cells. (n=3-4). * $p < 0.05$; exposed vs non-exposed cells. Statistic analysis was performed based on log-transformed data.

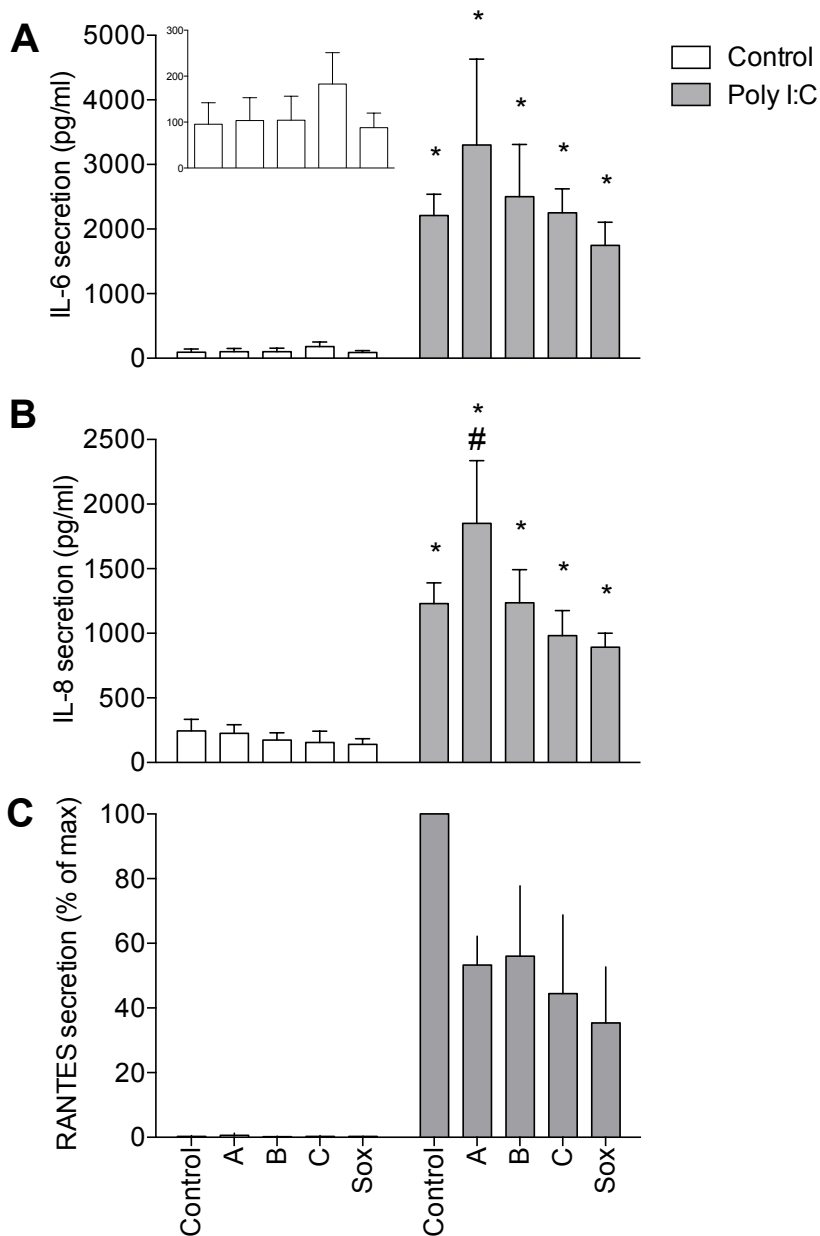


FIGURE 2. Fractionated methanol DEP-extracts differentially induce IL-6, IL-8 and RANTES in poly I:C-primed and unprimed BEAS-2B cells. Cells were primed with Poly I:C (10 μ g/ml) for 30 min prior exposure to methanol DEP-extracts equivalent of 100 μ g/ml DEP. Vehicle DMSO was used as control < 0.5% (v/v). Protein release of IL-6 (A), IL-8 (B) and RANTES (C) was measured after 24 h and analyzed by ELISA as described under “Materials and methods”. For A and B: Bars represent means \pm SEM of separate experiments (n=3). For C: Bars represent means \pm range of two separate experiments. * p<0.05; primed vs non-primed cells. # p<0.05; exposed vs unexposed.

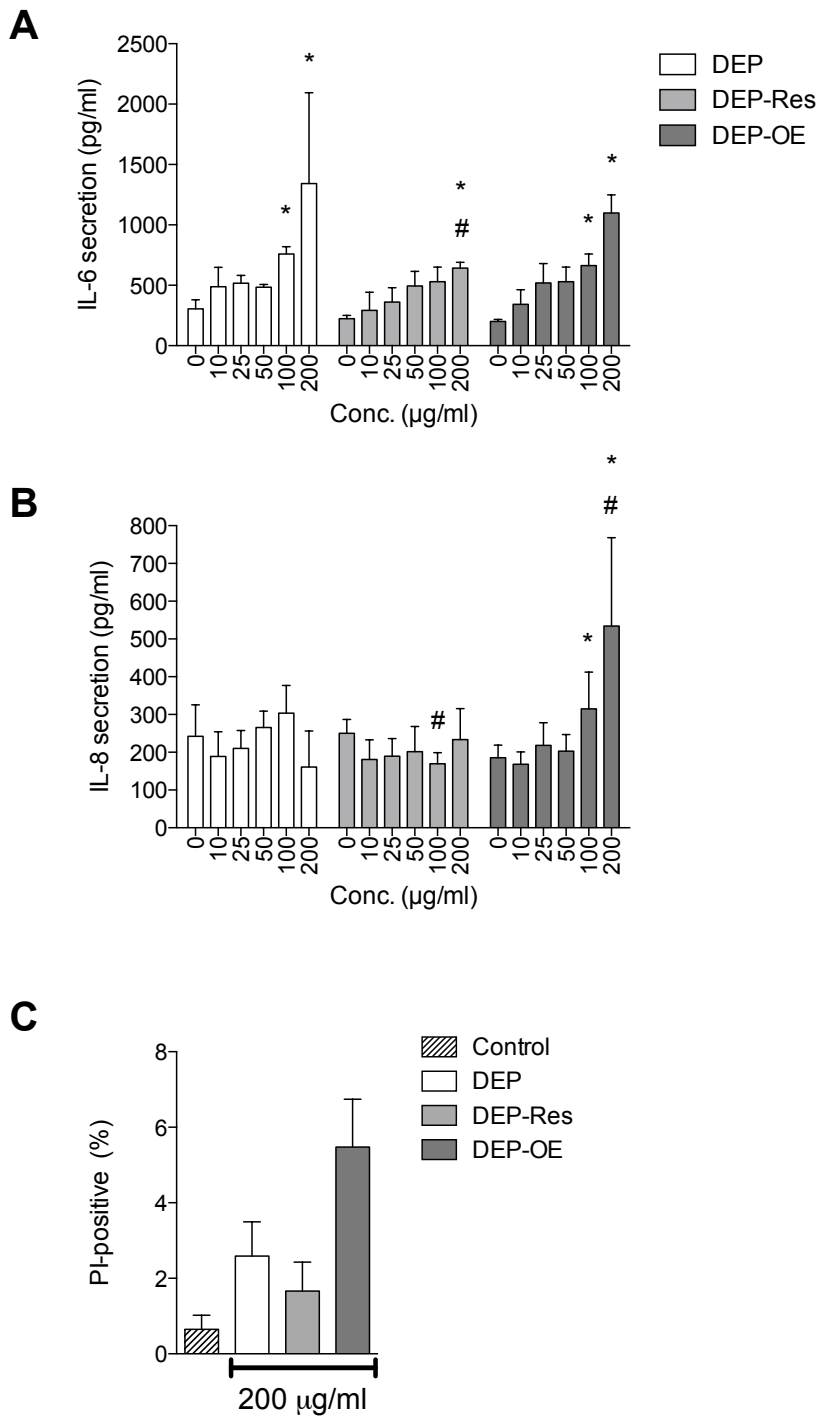


FIGURE 3. Heptane DEP-extracts induce IL-6 and IL-8 responses in BEAS-2B cells. Cells were exposed to DEP and heptane extracts equivalent of 0, 10, 25, 50, 100 and 200 µg/ml DEP. Vehicle DMSO was used as control (A and B). Protein release was measured after 24 h and analyzed by ELISA as described under “Materials and methods”. Bars represent means ± SEM of separate experiments (n=3). * p<0.05; exposed vs control. # p<0.05; DEP-Res or DEP-OE-exposed vs DEP-exposed. Cytotoxicity was evaluated as described under “Materials and methods” (C).

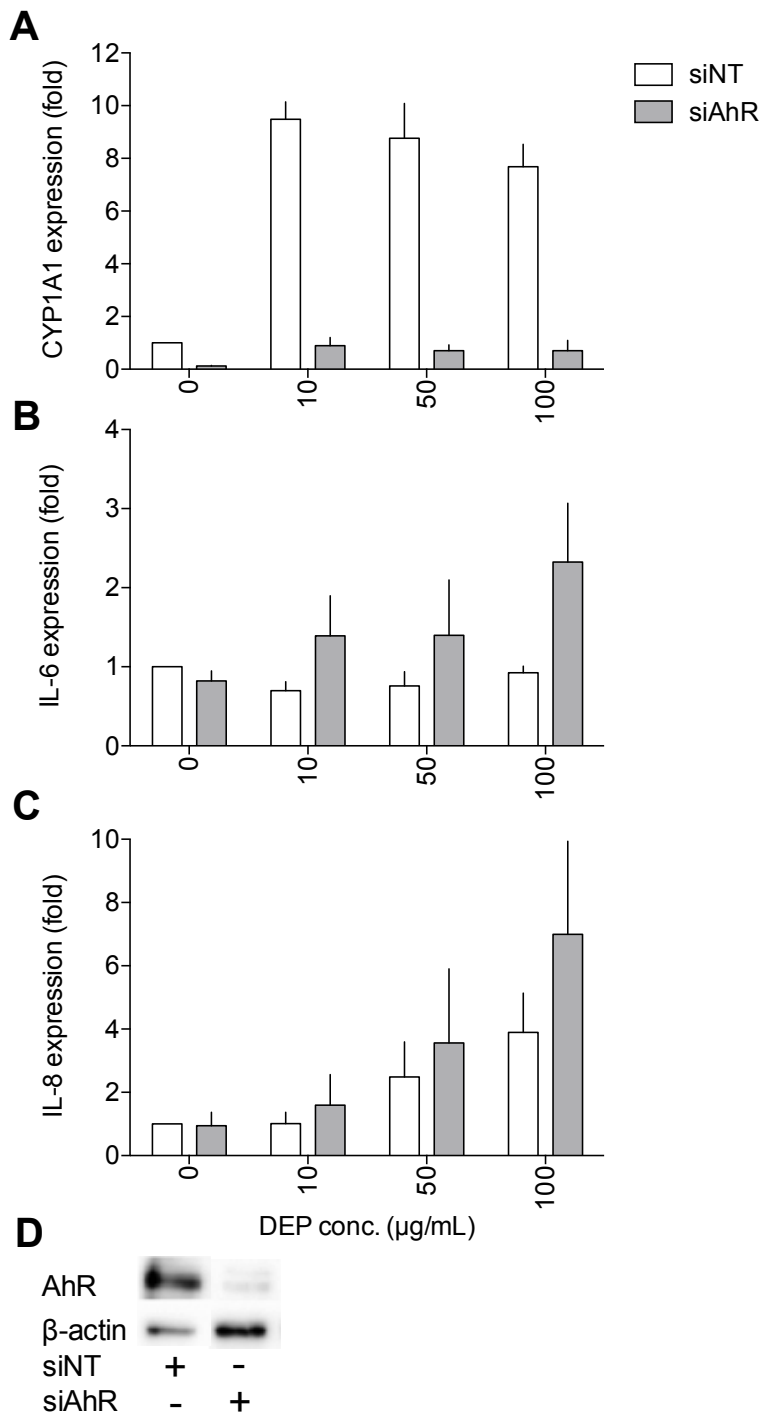


FIGURE 4. AhR suppress DEP-induced IL-6 and IL-8 expression in BEAS-2B cells. Cells were transfected with siRNA against AhR (siAhR) or non-targeting RNA (siNT). Transfected cells were exposed to DEP (0, 10, 50 and 100 µg/ml). CYP1A1 (A), IL-6 (B) and IL-8 (C) gene expression were measured after 4 h by real-time RT-PCR. The blots show knock-down of AhR in siAhR-transfected cells (D). Bars represent means \pm range of fold increase relative to unexposed cells (n=2).

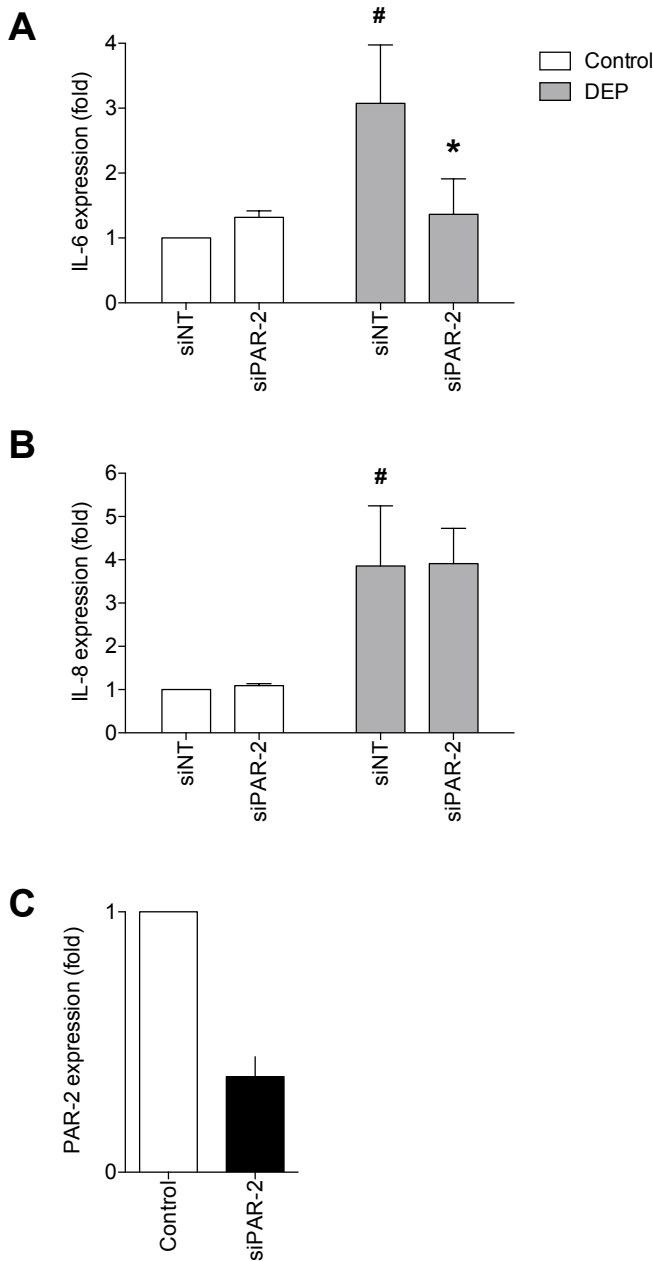


FIGURE 5. PAR-2 regulate DEP induced IL-6 expression but not IL-8 in BEAS-2B cells. Cells were transfected with siRNA against PAR-2 (siPAR-2) or non-targeting control siRNA (siNT). Transfected cells were exposed to DEP (100 μ g/ml). IL-6 (A), IL-8 (B) and PAR-2 (C) gene expression were measured after 4 h by real-time RT-PCR. For A and B, bars represent means \pm SEM of fold increase relative to control (n=3). For C, bars represent mean \pm range of fold increase relative to control (n=2). * $p < 0.05$; DEP-exposed siPAR-2-transfected versus DEP-exposed-siNT transfected cells. # $p < 0.05$; DEP-exposed-siNT transfected cells vs non-exposed-siNT transfected cells. Statistic analysis was performed using one-way ANOVA.