UMEÅ UNIVERSITY MEDICAL DISSERTATION
New series No 1033 ISBN 91-7264-102-9 ISSN 0346-6612

From the Department of Public Health and Clinical Medicine,
Respiratory Medicine and Allergy
Umeå University, Sweden

Activation of Epithelial Signal Transduction Pathways, Cytokine Production and Airway Inflammation Following Diesel Exhaust Exposure

Jamshid Pourazar

Umeå 2006

Copyright © 2006 by Jamshid Pourazar

ISSN 0346-6612, ISBN 91-7264-102-9

Printed by NRA Umeå, 2006

Cover: Schematic and photo:
Particular matter interaction in the airways
and possible pathways involved in the
DE induced inflammation.

Department of Public Health and Clinical Medicine,
Respiratory Medicine and Allergy
Umeå University
SE-901 85 Umeå, Sweden

To Susan, Elham and Sam

CONTENTS

ABSTRACT **7**
SVENSK SAMMANFATTNING **9**
SELECTED ABBREVIATIONS **11**
LIST OF PAPERS **13**
INTRODUCTION **15**
PARTICULATE MATTER AND DIESEL EXHAUST *15*
PROPERTIES OF DIESEL EXHAUST *16*
PREVIOUS DIESEL EXPOSURE STUDIES *16*
CYTOKINES *20*
ADHESION MOLECULES *20*
NEUTROPHIL CHEMOATTRACTANTS *21*
Th_2 CYTOKINES *21*
TRANSCRIPTION FACTORS *22*
MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) *22*
EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) *23*
AIMS **25**
MATERIAL AND METHODS **27**
SUBJECTS 27
STUDY DESIGN 27
STUDY I *27*
STUDY II *28*
STUDY III *28*
STUDY IV *28*
STUDY V *28*
DIESEL EXHAUST EXPOSURE 28
BRONCHOSCOPIES 29
BIOPSY PROCESSING 29
IMMUNOHISTOCHEMISTRY *30*
QUANTIFICATION OF IMMUNOSTAINING *30*
RT-PCR ELISA *31*
STATISTICAL EVALUATION 31
MAIN RESULTS **33**
STUDY I 33
STUDY II 33
STUDY III 33
STUDY IV 34
STUDY V 34
DISCUSSION **35**
DISCUSSION OF METHODS 35
DISCUSSION OF MAIN RESULTS 36
EFFECT OF DIESEL EXHAUST ON PRO-INFLAMMATORY CYTOKINES *36*
EFFECTS OF DIESEL EXHAUST ON Th_2 CYTOKINES *37*
EFFECTS OF DIESEL EXHAUST ON TRANSCRIPTION FACTORS AND MAPKs *38*
EFFECTS OF DIESEL EXHAUST ON EGFR EXPRESSION AND ACTIVATION *39*
EFFECTS OF DIESEL EXHAUST GENERATED AT EUROPEAN TRANSIENT CYCLE RUNNING CONDITION ON HUMAN AIRWAYS *40*
FINAL COMMENTS **41**
CONCLUSIONS **43**
ACKNOWLEDGEMENTS **45**
REFERENCES **47**

ABSTRACT

Adverse health effects of ambient air pollution are well recognised and include increased morbidity and mortality in respiratory and cardiovascular diseases. Diesel engines are major contributors to ambient particulate matter pollution and diesel particles have been shown to have strong toxicological and oxidative properties.

Mechanistic aspects of diesel engine exhaust exposure have been investigated in bronchial mucosal biopsies sampled during bronchoscopy of human subjects exposed in a validated experimental exposure set-up. Two exposure series were performed. Two separate groups of 15 healthy subjects each were exposed to filtered air and diesel exhaust during 1 hour in random order. The first exposure series was performed with the engine at idling with a PM_{10} concentration of 300μg/m^3 and the second was carried out during urban cycle (European Transient Cycle) running conditions with 270 μg particles/m^3. Bronchoscopies with sampling of bronchial mucosal biopsies were performed 6 hours after exposure. Biopsies fixed in acetone were bedded in glycolmethacrylate (GMA) resin and were stained for immunohistochemistry. Readings were done with light microscopy as well as image analyser with digital stainings processing of.

Diesel exhaust enhanced the expression of the cytokines IL-8 and GRO-α in the bronchial epithelium suggesting that the epithelium plays a major role in mediating the neutrophil-dominated airway mucosal inflammation. The bronchial expression of Th_1 and Th_2 cytokines was evaluated, addressing the hypothesis that diesel exhaust would induce a Th_2 airway response. Diesel exhaust enhanced the expression of Th_2 related cytokine IL-13 whereas the expression of Th_1 cytokines was unaffected.

The investigation of epithelial signal transduction pathways, by means of newly developed and validated cytoplasmic and nuclear stainings for key transcription factors and kinases, demonstrated that exposure to diesel exhaust increased the nuclear translocation of redox sensitive signal transduction components including phosphorylated (p)-p38-MAPK, p-JNK, p-c-jun (AP-1) and p65 (NFκB). These findings indicate novel mechanistic aspects to be involved in the airway response to particulate air pollution.

The expression of epidermal growth factor receptor (EGFR) as well as phosphorylated C-terminal Tyr 1173 increased significantly following DE exposure. The findings are consistent with the upregulation of p38 and JNK MAPkinases as well as increased NFκB expression. The MEK-ERK pathway was not affected and Src related phosphorylation was absent.

Diesel exposure at urban European transient cycle running conditions resulted in upregulation of the vascular adhesion molecule expression in the bronchial mucosa as signs of an early inflammatory response, while infiltration of inflammatory cells had not yet occurred. Differences in organic composition and particle concentration in the exhaust compared to idling situation may have influenced the outcome.

This thesis has added a mechanistic basis for the diesel exhaust induced airway inflammation in-vivo in humans. It is concluded that activation of epithelial signal transduction pathways, cytokine production and increased endothelial adhesion molecule expression play important roles in the airway inflammatory response to diesel exhaust.

SVENSK SAMMANFATTNING

Luftföroreningar såsom dieselavgaser från motorfordon är en betydelsefull källa till partikulära luftföroreningar. Dieselavgaser är toxiska och har oxiderande egenskaper. Epidemiologiska studier har visat en rad negativa hälsoeffekter av partikulära luftföroreningar framför allt associerade till försämrat hälsotillstånd hos känsliga grupper såsom individer med astma, KOL samt hjärt- och kärlsjukdomar. Kunskapen om de bakomliggande biologiska mekanismerna till dessa negativa hälsoeffekter är dock begränsad.

Denna avhandling syftar därför till att öka kunskapen kring de biologiska mekanismerna bakom luftvägseffekter av dieselavgaser hos friska individer. Studierna bygger på kammarexponeringar, där försökspersoner exponerades för dieselavgaser och luft vid två olika tillfällen i randomiserad ordning. Vid exponeringarna för dieselavgaser genererades avgaserna på två olika sätt; tomgångskörning och så kallad city körning. Effekterna av dieselavgaser jämfördes med effekter efter luftexponering med hjälp av bronkoskopi med biopsier och bronksköljningar.

I den första exponeringsserien (studie I-IV) genererades dieselavgaserna under tomgångskörning. Femton friska försökspersoner exponerades under en timme för dieselavgaser med en partikelkoncentration (PM_{10}) på 300 $\mu g/m^3$ och luft. Bronkoskopi med slemhinnebiopsier genomfördes sex timmar efter avslutad exponering.

Studie I avsåg att undersöka cytokineffekter i bronkslemhinnan efter exponering för dieselavgaser. Studien visade att den neutrofildominerade luftvägsinflammation, som tidigare beskrivits efter exponering för dieselavgaser, var associerad till en ökning av de neutrofilattraherande cytokinerna IL-8 och Gro-α.

I studie II undersöktes effekterna på Th_1- och Th_2-relaterade cytokiner i luftvägsepitelet. Vi konstaterade att exponering för dieselavgaser inducerade ett ökat uttryck av IL-13 i luftvägsepitelet. Detta skulle potentiellt kunna innebära en ökad risk för allergisk sensibilisering hos icke-atopiska individer och därigenom utgöra en förklaringsmodell till ökad känslighet för partikulära luftföroreningar hos allergiska astmatiker.

Studie III syftade till att studera de molekylära mekanismer som är involverade i signalöverföring i bronkepitelet efter exponering för dieselavgaser. Resultaten visade att dieselavgaser inducerade fosforylering av p38 och JNK MAPkinaser samt uppreglering av transkriptionsfaktorer såsom NFκB och AP-1 i cytoplasman med nukleär translokation som följd. Uppregleringen av dessa signalvägar förefaller vara medierad genom oxidativ stress utlöst av dieselexponering och leda till produktion av pro-inflammatoriska mediatorer och efterföljande inflammationsutveckling.

Studie IV avsåg att undersöka huruvida epidermal tillväxtfaktorreceptor (EGFR) var av betydelse i signalvägar för mediering av dieselavgaseffekter. Studien visade att dieselexponering inducerade ökat uttryck av EGFR samt fosforylering av tyrosin 1173 i bronkepitelet som ett tecken på aktivering eller transaktivering av EGFR.

I studie V exponerades femton friska försökspersoner för luft och dieselavgaser (partikelkoncentration 270 $\mu g/m^3$) genererade under så kallad city körning (European

Transient Cycle), vilket innebär att dieselmotorn accelereras och bromsas enligt ett förutbestämt protokoll för att imitera dieselavgaser genererade i storstadstrafik. Bronkoskopi utfördes sex timmar efter avslutad exponering.

Syftet med denna studie var att jämföra luftvägseffekter av dieselavgaser genererade i ”storstadstrafik” med tomgångssituationen. Man fann uppreglering av adhesionmolekylerna P-selectin och VCAM-1 i blodkärlen i bronkslemhinnan samt ökade eosinofiler i de alveolära luftvägarna, som tecken på ett tidigt inflammationssvar. Ytterligare studier behövs för att jämföra verkningsmekanismer och skillnader i effekter mellan dieselavgaser genererade vid tomgångskörning respektive i storstadstrafik.

Sammanfattningsvis har studierna i denna avhandling bidragit till att öka kunskapen kring hur den dieselinducerade luftvägsinflammationen medieras. Studierna beskriver uppreglering av cytokiner, MAPkinaser, transkriptionsfaktorer och EGFR i bronkslemhinnan som viktiga komponenter i regleringen av luftvägsinflammationen efter exponering för dieselavgaser, samt att dieselavgaser även synes inducera ett Th_2-svar i luftvägsslemhinnan. Vidare har exponering för dieselavgaser genererade under citykörning indikerat både likheter och skillnader i verkningsmekanismer och inflammatoriska effekter jämfört med tomgångskörning. Med resultaten från denna avhandling som bas är det nu av stor betydelse att genomföra motsvarande undersökningar på individer med luftvägssjukdomar såsom astma och KOL. Detta för att söka förstå de bakomliggande mekanismer till den ökade känslighet för partikulära luftföroreningar som noterats hos dessa grupper.

SELECTED ABBREVIATIONS

APRT	Adenine phosphoribosyl transferase
AP-1	Activator protein-1
AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
BW	Bronchial wash
DAG	Diacylglycerol
DE	Diesel exhaust
DEP	Diesel exhaust particle
EGFR	Epidermal growth factor receptor
ELF	Epithelial lining fluid
ERK	Extracellular regulated kinase
ETC	European Transient Cycle
GMA	Glycolmethacrylate
GPCR	G protein coupled receptor
Grb_2	Growth factor receptor-binding protein 2
GM-CSF	Granulocyte - macrophage colony-stimulating factor
Gro-α	Growth-regulated oncogen alfa
IL-	Interleukin-
ICAM-1	Intracellular adhesion molecule-1
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear factor-kappa B
PM	Particulate matter
PI3-K	Phosphatidylinositol 3 – kinase
PLC-γ	Phospholipase C-gamma
PAHs	Polycyclic aromatic hydrocarbons
PKC	Protein kinase C
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
RANTES	Regulated upon activation, normal T cell expressed and secreted
RTLF	Respiratory tract lining fluid
RT-PCR	Reverse transcriptase polymerase chain reaction
Shc	Src homology and collagen protein
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor alpha
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule-1

LIST OF PAPERS

I Salvi SS, Nordenhäll C, Blomberg A, Rudell B, Pourazar J, Kelly FJ, Wilson S, Sandström T, Holgate ST, Frew AJ.
Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways.
Am J Respir Crit Care Med 2000; 161:550-7.

II Pourazar J, Frew AJ, Blomberg A, Helleday R, Kelly FJ, Wilson S, Sandström T.
Diesel exhaust exposure enhances the expression of IL -13 in the bronchial epithelium of healthy subjects.
Respir Med 2004; 98:821-5.

III Pourazar J, Mudway IS, Samet JM, Helleday R, Blomberg A, Wilson SJ, Frew AJ. Kelly FJ, Sandström T.
Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways.
Am J Physiol Lung Cell Mol Physiol. 2005; 289:L724-30.

IV Pourazar J, Blomberg A, Sandström T.
Diesel exhaust increases EGFR and phosphorylated C-terminal Tyr 1173 in the bronchial epithelium.
Manuscript

V Pourazar J, Sehlstedt M, Behndig A, Blomberg A, Sandström T.
Airway inflammatory responses to diesel exhaust generated at European transient cycle running conditions.
Manuscript

INTRODUCTION

The adverse health effects associated with air pollution have been described in over a hundred epidemiological studies world wide (156). Clear associations have been demonstrated for nitrogen dioxide (NO_2), ozone (O_3) and particulate matter (PM). The adverse effects on health include increased airway symptoms, medication need, emergency room treatment and hospitalization related to increased respiratory and cardiovascular morbidity as well as mortality. Subjects with pre-existing respiratory and cardiovascular diseases have been indicated to be especially sensitive (102, 103, 118). Furthermore associations between reduction of lung function and ambient air pollution has been demonstrated including adverse effects on the lung development and growth in children (57, 58, 99). Pollutants generated by motor vehicles, including diesel exhaust (DE) have been identified as the major problem in many cities, and many studies have shown the relationship between adverse health effects and traffic related air pollution (27, 68, 143). There is now evidence suggesting, relationships between air pollution and exacerbation of pre-existing asthma, enhancement of allergic reactions and a range of manifestations of cardio-respiratory diseases. Still, the mechanism whereby air pollution is promoting these effects is not clear. Therefore, apart from the many epidemiological studies, a range of experimental in vitro and in vivo studies have been performed in order to determine the biological mechanisms contributing to adverse effects.

PARTICULATE MATTER AND DIESEL EXHAUST

Particulate matter air pollution and its relationship to traffic has often been suggested to be the major driving factor behind adverse health effects. Not only are subjects with pre-existing cardiovascular and respiratory diseases sensitive, but also healthy subjects may experience symptoms (5, 49, 50, 102, 114, 119, 120, 144). An increase of PM_{10} concentration by 10 $\mu g/m^3$ has been reported to be associated with 2-3% increase in asthma exacerbation. Regarding the association between mortality and particulate air pollution concentration, it has been shown that each 10$\mu g/m^3$ increase in PM_{10} is associated with increased respiratory and cardiovascular mortality by 3.4% and1.4% respectively (1).

Particulate matter with ability to enter airways can be grouped in three size categories according to the aerodynamic diameter; coarse (10-2.5μm), fine (2.5-0.1μm) and ultra fine (<0.1μm) particles. The properties of these particles differ not only by physical size, but also by shape, porosity and chemical composition (151). Diesel exhaust is a major component of PM pollution especially where particle numbers are concerned since diesel engines produce large amounts of nano-meter sized particles. These engines may produce up to 10-100 times more particles compared to petrol engines. Due to the sturdiness, low running costs, high efficiency and ability to use the less refined and often cheap diesel fuels, diesel engines are very widely used. Since they last very long, there is world wide a much slower turn-over of diesel engines than their gasoline counterparts (160). Pollutants generated by motor vehicles, including DE has been identified as the major problem in many cities, and many studies have shown a relationship between adverse health effects and traffic related air pollution

PROPERTIES OF DIESEL EXHAUST

Exhaust produced during the combustion of diesel fuel consists of a complex mixture of both gases and particles. The main gases are carbon monoxide (CO), nitric oxides (NO, NO_2), sulphur oxide (SO_2) and hydrocarbons including aromatic and polycyclic aromatic hydrocarbons (PAHs) (116). The majority of diesel exhaust particles containing various surface components including organic compounds such as PAHs, have a diameter of less than 0.1 μm and are capable of entering and depositing in the airways (123). It can be noted that the smaller particles (fine and ultrafine) have greater surface area in relationship to the mass, and therefore, greater bioavailability and biologic effects (95). Diesel exhaust particle (DEP) with a size <0.1 μm, may have a larger surface area on its carbonaceous core to which components such as polycyclic aromatic hydrocarbon (PAHs) and aldehyds, acids and transient metals are adsorbed, in comparison to particles with larger size, as for example >2.5 μm (117). The ability of particles or their adsorbed component to produce adverse effects is dependent on their physical and chemical characteristics which play important roles in the interaction with biological substances and compartments (54, 139). Deposition of inhaled particles in airways and alveoli and the subsequent interaction with the lung tissue has been shown to be dependent on both particle size and pattern of breathing (25). Deposition of particles increase both by particle number and mass concentration, as well as during exercise, compared to spontaneous breathing at rest (33). Particles deposited in the airways interact with various chemical entities including proteins and lipid substances in the liquid media of the airways (respiratory tract lining fluid, RTLF). Some of the particle bound components that have weak bindings primarily interact with molecules in the liquid media and the remaining components bound on the core surface may interact with cells such as macrophages and bronchial epithelial cells. Some smaller sized particles might even enter circulation. The association of adverse health effects with traffic related pollutants and increased sensitization to pollen have been shown to be associated with the number of trucks in traffic indicating that DE potentially could be an important contributor to these health outcomes (68, 143). DE have been studied with focus on the role of DEP in exacerbations of allergic airway diseases and enhancement of the allergic response (44-46, 127). Many studies have suggested that the DE-induced health effects depend on the its oxidative stress inducing properties and likely by its adsorbed organic components, such as PAHs, and quinones and interaction of these components with various components in the airways (11, 77, 82, 111). However the issue of air pollution and allergy is complex and the overall contribution of diesel exhaust is not resolved.

PREVIOUS DIESEL EXPOSURE STUDIES

Human exposure studies

A series of experimental human exposure studies using a validated chamber exposure system with controlled challenges with dilute diesel exhaust has been carried out to evaluate the diesel induced health effects (92, 110, 115). Exposure to high ambient levels of DE was associated with increases in symptoms and increased airway resistance (110). In-vitro tests with alveolar macrophages harvested during brochoscopy after diesel and air challenges in

healthy subjects demonstrated that DE exposure adversely affect the phagocytic function of these important immune regulatory cells (109). Short term exposure to DE (PM_{10}, 300 $\mu g/m^3$) was shown to induce a pronounced inflammatory response, including neutrophils, lymphocytes and mast cells in the bronchial mucosa (112). The inflammatory response was accompanied by a migration of lymphocyte subpopulations into the airways and activation of mast cells with histamine secretion. Furthermore, up-regulation of adhesion molecules, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in the vascular endothelium was observed (112). An increased neutrophil percentage together with increased IL-6 and methyl histamine was found in sputum performed 6 and 24 hours after high dose DE exposure (PM_{10}, 300 $\mu g/m^3$) (93). In the study performed by Nightingale, exposure to DE (200 $\mu g/m^3$ for 2 hours) caused increased neutrophil percentage and myeloperoxidase concentration in induced sputum at 4 h after DEP exposure (92). In asthmatic subjects treated with inhaled corticosteroids (800-1200 $\mu g/d$), DE induced a increase in airway responsiveness to methacholine at 24 hours after DE exposure (94). Even exposure to a concentration lower than in the studies mentioned above, (108$\mu g/m^3$ for 2h) was found to cause increased airway resistance in both healthy and asthmatic individuals. Bronchoscopy sampling at 6 hours post exposure showed a neutrophilic response in healthy subjects without any neutrophilic or eosinophilc response in asthmatics. In contrast, the investigators demonstrated a five fold increase in interleukin-10 (IL-10) expression in the bronchial epithelium in the asthmatics as compared with a reduction by half in the healthy subjects (127). In a recent study performed by Behndig et al, healthy subjects were challenged with a low concentration of DE (100 μg / m^3 for 2 h), and sampling at 18 h post-exposure showed an inflammatory response in the bronchial compartment (16). An increase in neutrophil numbers and in IL-8 concentration in the bronchial lavage, as well as increased neutrophil and mast cell numbers in bronchial mucosa was observed by the authors.

A series of studies by Diaz-Sanchez and co-workers have shown that instillations of diesel exhaust particle in combination with allergen induced increased IL-4 and other Th_2 type cytokines, suggesting that DEP can interact with allergen to promote allergic disease. The studies have shown that DEPs induce isotype switching to IgE and enhance allergen-specific IgE production, and that DEPs in combination with allergen caused a significant increase in the expression of Th_2-type cytokines (48, 56).

Animal studies

Several animal studies have been performed to evaluate biological effects of DE including pro-allergic effects of DEP. An acute instillation of DEPs in mice (111) has been shown to cause severe lung injury and high mortality, which were prevented by pretreatment with superoxide dismutase. This suggested that the toxicity of DEP was connected to the production of the radical superoxide (O_2^-). The effect of DEP on the production of IgE antibodies and its adjuvant activity in mice was also demonstrated. IgE levels were higher in animals treated with mixed allergen and DEP than those immunized with allergen alone (91). DE combined with antigen challenge in sensitized mice induced airway hyperresponsiveness and increased number of eosinophil and mast cells in the lung tissue (88, 89). The mechanisms that contribute to association between DEP exposure and asthma have been in the focus in animal studies. It has been suggested that DEP may

promote a Th2 response by production of inflammatory cytokines and simultaneously inhibiting production of interferon- γ (IFN-γ) (55). Quinone components can induce expression of IL-5, eotaxin, and increased inflammatory cells including eosinophils, in bronchoalveolar lavage fluid in mice challenged with combined allergen and quinone component. Quinones components of DEP may also be involved in the DEP toxicity and enhance the allergic responses (65, 66).

In-vitro studies

DEP has been suggested to modulate and increase susceptibility of the host to respiratory infections. In vitro studies have been reported to reduce the phagocytosis capacity of macrophages and decrease its antimicrobial ability (152). DEP and its organic extracts, such as phenanthrene, has been shown to enhance in vitro production of IgE from IgE-secreting B lymphocytes (134, 141). Airway epithelial cells are today known to have a prominent role in the pathogenesis of respiratory disease. Exposure of epithelial cells to DEP results in increased synthesis and release of pro-inflammatory cytokines and mediators such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and adhesion molecules (43). Bronchial epithelial and macrophage cell lines, as well as primary cultures of bronchial epithelial cells, can release a variety of chemokines and cytokines when treated with DE particles or their organic extracts (7, 11, 12, 74, 84). Much attention has been focused on the capacity of DEP to elicit oxidative stress in the airways and trigger regulating molecules, especially the redox sensitive transcription factors nuclear factor-κB (NFκB) and activator protein-1 (AP-1) (62, 135). The capacity of DEP to cause oxidative reactions in vivo, has been attributed to their content of metals, polycyclic aromatic hydrocarbons (PAHs), and quinones (11, 22, 23, 90, 125, 129). These components are suggested to be critical in the up-regulation of oxidative-stress-sensitive signalling pathways. The receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) are the primary mediators of many of stimuli and incoming signals (**Figure 1**). Notably, metals and polycyclic aromatic hydrocarbons, have been shown to be capable of activating or transactivation of EGFR pathway (11, 29, 63). In addition, DEP can up-regulate the expression of amphiregulin, an EGFR ligand in cultured human bronchial epithelial cells (18). The amphiregulin secretion was blocked by inhibiting the EGFR tyrosine kinase and treatment with the antioxidant N-acetyl cysteine, but inhibited EGFR had no effect in amphiregulin secretion, study suggested that oxidative stress may be involved in the trans-activation of EGFR.

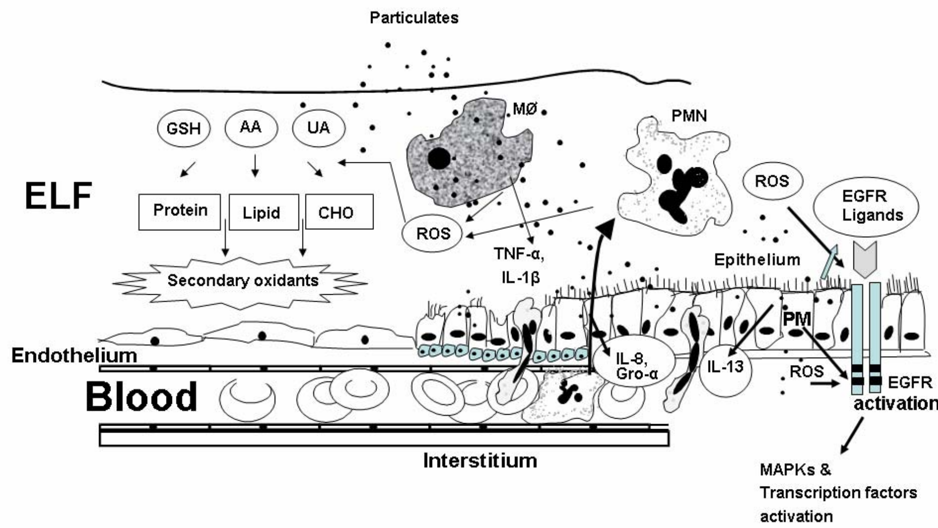

Figure 1: Schematic picture of particle interaction between the epithelial lining fluid (ELF) and epithelial cells, and subsequent responses [modified picture, original picture Mudway IS, Kelly FJ. Ozone and lung: a sensitive issue. Mol Aspects Med 2000]

Antioxidant components in the ELF scavenge external oxidant gases and particles with oxidative properties, as well as endogenously produced oxidant species released by macrophages and neutrophils. When antioxidant defences overwhelmed by the oxidative burden, oxidation of macromolecules in the ELF to production of secondary oxidants. Together with PM, cytokines and ROS produced by macrophages and neutrophils may increase the production of EGFR ligands which may directly activate EGFR, while oxidants may indirectly lead to transactivation of EGFR. Activation of EGFR results in phosphorylation of downstream kinases, activation of transcription factors and cytokine production.

CYTOKINES

Cytokines are a group of proteins that mediate and are involved in the development of inflammatory responses. These mediators are secreted by various cells involved in the regulation of different events in the inflammatory processes such as cell migration, growth, differentiation, repair, apoptosis and fibrosis. Cytokine production by cells is dependent on the properties of the incoming stimulus, and once cytokines are produced and secreted, they exert their effects on target cells. Cytokines stimulate target cells through cell surface receptors to produce mediators, to control and regulate inflammatory responses (71). Cytokines such as tumour necrosis factor-α (TNF-α), IL-6, IL-1, GM-CSF, interferon-γ (IFN-γ) and IL-12 are critical in the induction of the inflammatory response. All of these mentioned cytokines are pro-inflammatory. Some of the cytokines however, have down-regulating effects on inflammation such as, transforming growth factor-β (TGF- β), IL-10, IL-4, IL-13 and are known as anti-inflammatory cytokines (71). Furthermore, a number of cytokines have the ability to promote migration of leukocytes into inflammatory sites, and are described as chemokines. Examples of these are IL-8, growth regulated oncogene-α (Gro-α). The major sources for cytokine production are macrophages, T-lymphocytes and mast cells, but other cells such fibroblasts and epithelial cells are also capable of producing cytokines. The secreted cytokines bind to their own receptors and exert their effect on target cells. The majority of the receptors use intermediators, such as Janus-kinases (JAKs) to transduce signals. The bronchial epithelial and macrophage cell lines have been shown to release a variety of chemokines and cytokines when treated with particulate matter and DEP or its organic extracts (3, 20, 44, 74, 106).

ADHESION MOLECULES

Further mechanisms which are involved and regulate the recruitment and migration of leukocytes, such as neutrophils from circulation to the inflammatory sites are adhesion molecules. There are three families of adhesion molecules and these include the integrins, selectins and immunoglobulin superfamily. The integrins contain heterodimeric αβ subunits and are known as ligands to members of the immunoglobulin superfamily and extracellular matrix such as fibrinogen and fibronectin. The integrins, LFA-1 (CD11a/CD18), Mac-1(CD11b/CD18) on leukocytes, and VLA-4 (α4β1) on eosinophils, lymphocytes monocytes play a key role in transmigration of these cells through binding to their ligand counterreceptor on endothelial cells, intracellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1) (31, 40, 41). The selectins, E-selectin, P-selectin and L-selectin are important in cell-cell interaction and play key roles in the initial binding of leukocyte to endothelial cells in inflammatory responses (40, 100). P-selectin is constitutively synthesized and stored in weibel-palade bodies of endothelial cells and following stimulation of the endothelium, translocated to the plasma membrane within seconds, and reinternalized within 1h. E-selectin is activated by pro-inflammatory cytokines such TNF-α and IL-1 and expression on endothelial cells occurs within 1 hr following stimulation, with maximal expression between 4-8 hr.

NEUTROPHIL CHEMOATTRACTANTS

Recruitment of leukocytes including neutrophils from the circulation to the inflammatory site is a complex process and highly regulated by a number of molecules including chemokines (8). The effects of DE on chemokine production by macrophage and epithelial cells have been studied in rat and many *in vitro* studies (13, 96, 126). These studies demonstrated that DEP can induce the production of inflammatory mediators such as neutrophil attractants which are suggested to be involved in the recruitment of inflammatory cells following DE exposure. IL-8 and Gro-α are CXC chemokines which have a number of biological effects. The major role is to attract and activate leukocytes for example neutrophils. Other members of CXC chemokines are epithelial cell-derived neutrophil activating peptide-78 (ENA-78) and neutrophil-activating peptide-2 (NAP-2) which also have neutrophil attracting properties. They mediate the effect by binding to CXCR1 and CXCR2 expressed on neutrophils, monocytes, NK-cells and T-lymphocytes (9, 87). IL-8 has been reported to be involved in lymphocyte, eosinophil and basophile activation and migration to the inflammatory site, and suggested to play a role in asthma (52, 154). The transcription activation of the chemokines occurs by a variety of stimuli such as IL-1, TNF-α and bacterial endotoxin. Reactive oxygen species can also induce the transcription activity. The IL-8 gene contains, binding sites for several transcription factors, such as NFκB, nuclear factor IL-6 (NF-IL6), AP-1 AP-2 and AP-3 in its promoter region. NFκB together with either AP-1 or NF-IL6 is involved in the activation of the IL-8 gene (87). Additional pathways for neutrophil recruitment and activation have been described. LTB4 is a potent neutrophil chemoattractant from the arachidonic acid cascade (4).

Th_2 CYTOKINES

There is now evidence to suggest the existence of two different T cell responses based on the cytokine production. The Th_1 cytokines include IL-2, TNF-β, IFN-γ all involved in cell mediated responses and Th_2 cytokines, such as IL-4, IL-5, IL-10 and IL-13, and they are suggested to be important in allergic diseases and to mediate the antibody mediated responses. Diesel exhaust has been proposed to be involved in the increased incidence of respiratory allergic diseases. Many cell culture studies and studies combining allergen challenge with DEP have demonstrated that sensitisation to allergen may be enhanced by DEP exposure (46, 132, 133, 135). IL-13 is thought to be involved in a large number of inflammatory responses and share many properties with IL-4. IL-13 is often produced in larger amounts than IL-4 and exerts its biological effect through IL-13R and a complex of IL-4R-α-chain, and airway hyperreactivity may be increased following activation and mediated by IL-13R (36, 153). In mice, IL-13 stimulates B cells and enhances antibody production by these cells. Furthermore, IL-13 induces eotaxin expression by airway epithelial cell (79, 81). In addition IL-13 has a role in mucus secretion and may interact with epithelial growth factor receptor (EGFR) in the airway epithelium to cause mucus cell metaplasia and hyperplasia (121, 142). Diesel exhaust is suggested to cause inhibition of the LPS induced IFN-γ response (55). Among the cytokines involved in Th1 responses, IL- 18 has been shown to play a key role in inducing IFN-γ. Together with IL-12 it has been demonstrated that IL-18 has a synergistic effect on IFN-γ production (140). Mice challenged with lipopolysaccharide (LPS) showed a higher level of IL-18 expression while ovalbumin-challenged mice showed a lower level (30).

TRANSCRIPTION FACTORS

Regulation of gene expression is essential during different processes including cell responses to oxidative stress, inflammatory stimuli and cytokines. Transcription factors are specific DNA-binding proteins which have their binding sites in the promoter region of target genes (97). Signals and stimuli incoming from the cell surface become integrated into the nucleus through series of phosphorylation by kinases and activate transcription factor proteins in order to induce production of bioactive molecules such as cytokines, receptors, enzymes and other components (67, 72). The expression of cytokines and mediators in inflammatory responses is induced and regulated by a wide range of transcription factors such as NF-IL6, nuclear factor of activated T cell (NF-AT), NFκB and AP-1, which have their binding sites in the promoter regions for many cytokines.

Transcription factor NFκB consists of a heterodimer of the Rel family transcription factors which includes c-Rel, p50, p65,p52, and RelB (122). Some of these families have potent transactivational activities such as p65 and others such as p50 have potent DNA binding activities (122). Stimuli which activate NFκB include TNF-α, IL-1, LPS and oxidative stress. After activation, NFκB can regulate transcription of many inflammatory mediators, such as IL-6, IL-8, RANTES, TNF-α and IL-1 (15). The transcription factor NFκB in inactive form is located in the cytoplasm of cells in complex with Inhibitory κB (IκB) (14, 155). Following stimulation , phosphorylation and degradation of IκB, NFκB is released from the complex molecule and transported to the nucleus where it may bind to binding sites in the promoter regions of genes (155). The transcription factor AP-1 consist of subunits of Fos (c-fos, fosB, fra1, fra2) and Jun (c-jun, junB, junD) that build either heterodimers (Fos/Jun) or homodimers (Jun/Jun) in various combinations (130). This transcription factor together with NFκB are known as redox sensitive transcription factors and many studies have suggested an interaction between activating pathways of these transcription factors (51). Treatment of bronchial epithelial cell cultures with DEP cause cytokine production by these cells accompanied by activated NFκB and AP-1 (62, 106, 135, 137).

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs)

MAPKs are a group of protein kinases (enzymes) with important functions in transducing signals which mediate responses of cells by different stimuli and activate intracellular signaling cascades leading to a wide range of responses such as cytokine expression, cell proliferation and apoptosis. These enzymes transfer the γ-phosphate of ATP to serine (Ser) and threonine (Thr) residues in the target protein and are activated by dual phosphorylation on Threonine (Thr) and Tyrosine (Tyr) residues (35, 39, 107). The members of the MAPKs family include ERK, P38 and JNK which are activated in the last step of the intracellular phosphorylation cascades (39, 107). The MAPKs activation pathways are regulated in response to EGF, PDGF or other stimuli such as LPS through cell surface receptors including EGFR (10, 42, 86, 108). In addition seven-transmembrane spanning receptors that are linked to G-proteins are involved in these pathways, which lead to activation of the small G protein such as Ras and initiate activation of MAPKs (37, 38).

The P38 MAPKs have been shown to be activated by the exposure of cells to various stimuli including environmental stress, endotoxin and cytokines (60, 61, 108, 146). This

activation is regulated by its upstream activators, MKK3 and MKK6, and also by MKK4 an activator of JNK (70). There are other Ser/Thr kinases, TAK1 and ASK-1, that may act as MAPKKK for the p38 pathways (78). p38 MAPK is involved in the activation of many transcription factors, such as ATF-2, c-jun, and NFκB, (17, 32, 73, 104). P38 activity regulate expression and production of such cytokines as TNF-α, IL-1 IL-6 and IL-8 by (98, 159). The JNK MAPKs play an important role in the phosphorylation and activation of many transcription factors such as ATF-2, Elk and c-jun (138). The kinases which regulate activation of JNK are the upstream kinases MKK7 and MKK4 (39). The ability of particular matter in general and by DEP in particular, to modulate immune responses and production of ROS and RNS was the reason for investing molecular mechanisms behind these effects, and role of MAPKs in the signalling pathways

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

EGFR belongs to a family of cell surface receptors with protein tyrosine kinase (PTK) activity. The receptor tyrosine kinases (RTKs) are primary mediators of many of the signals during biological processes, such as proliferation, differentiation, motility and apoptosis. The EGFR consists of an extracellular ligand-binding domain and a membrane spanning domain followed by a cytoplasmic protein tyrosine kinase domain and Src homologi-1 (SH1) which mediate auto-phosphorylation of the carboxyl terminal (19, 145). The autophosphorylated tyrosine residues of the EGFR create binding sites for SH2 and phosphotyrosine-binding (PTB)-domain on proteins. These proteins which are phosphorylated and activated, are either enzymes, such as phospholipase C-γ (PLCγ) and phosphatidylinositol 3-kinase (PI3-K), or adapter molecules such as Shc (Src homology and collagen protein) and Grb2 (growth factor receptor-binding protein 2) which links receptor tyrosine to downstream signalling pathways (19, 59, 145). The EGF, TGF-α, amphiregulin, heparin-binding EGF-like growth factor and betacellulin are presently the identified ligands that bind to EGFR and lead to its activation. The tyrosine phosphorylation of EGFR and association with adaptor molecules Shc and Grb_2 lead to stimulation and activation of small G-proteins, such as Ras and activation of MAPKs (**Figure 2**).

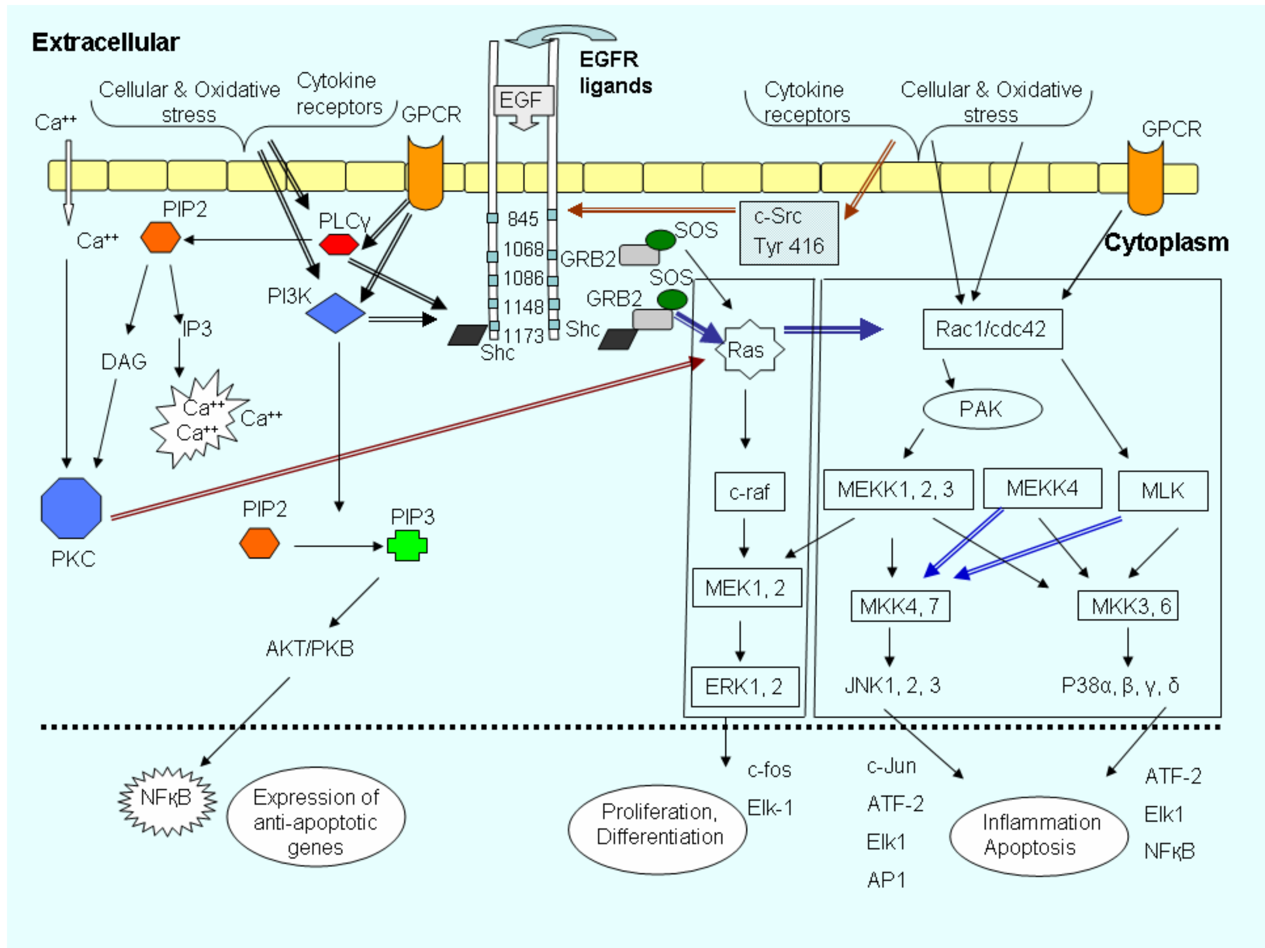

Figure 2: EGFR, protein tyrosine kinases and downstream signalling pathways. Extended and modified from Puddicombe SM. Clinical and Experimental Allergy, 2000;30:7-11.

AIMS

The overall aim of this thesis was:

To evaluate, the inflammatory effects of diesel exhaust in the airways of healthy human subjects, with focus on underlying regulatory mechanisms.

Specific aims were:

To investigate the effects of acute exposure to DE on cytokine expression in bronchial tissue in healthy subjects.

To determine neutrophil chemotactic pathways in the bronchial tissue following DE exposure

To evaluate whether exposure to DE would induce a shift towards a Th2 cytokine response profile in healthy non-atopic subjects.

To investigate the activation of redox-sensitive transcription factors and their associated upstream stress-related mitogen activated protein kinases (MAPKs) in relationship to DE exposure

To investigate the potential involvement of the epidermal growth factor receptor (EGFR) signaling pathway in the epithelial response to DE exposure.

To investigate airway inflammatory responses to diesel exhaust in healthy subjects, generated by a diesel engine during urban running conditions based on the standardized European Transient Cycle.

MATERIAL AND METHODS

In order to investigate the mechanisms involved in the inflammatory response to DE exposure, endobronchial biopsies were obtained from healthy non-atopic subjects exposed to air and DE in a blinded random order. Immunohistochemistry with appropriate antibodies were used in study I-IV on biopsies previously obtained. Biopsies, bronchial wash and bronchoalveolar lavage were sampled in study V from healthy subjects, in order to investigate the mechanisms attributed to the airway response to diesel exhaust.

SUBJECTS

Healthy, non-smoking volunteer (11 males and 4 females) mean age 24 years (range 21-28 years) were included in studies I-IV. Fifteen non-atopic, non-smoking healthy individuals (7 males and 8 females) mean age 26 (range 21-40 years) participated in study V.

They all had normal lung function (FEV1 and FVC) with negative skin prick tests to common airborne allergens and were free from respiratory tract infections for at least 6 weeks prior to or during the study period. The studies were performed according to the Declaration of Helsinki and approved by the local Ethics committee at Umeå University. All subjects gave their written informed consent.

STUDY DESIGN

In studies I-IV previously obtained bronchial biopsies from volunteers which had been exposed to DE and air were investigated. The subjects who had participated in the DE exposure studies acted their own control.

In study V new bronchial biopsies were obtained. In order to avoid any bias due to carry over effects from previous challenge, the exposures in this study were carried out at least three weeks apart and in a randomized blinded sequence. During exposure the subjects alternated mild exercise (V_E = 20L / min / m^2) on a bicycle ergonometer and rest at 15-minute intervals.

STUDY I

In this study the hypothesis that the leukocyte infiltration following exposure to DE was regulated by enhanced cytokine expression was based on earlier biopsy findings (112). The effect of a short time but high dose DE exposure (300 g/m^3 for 1h) on cytokine production, as reflected in bronchial wash cells and bronchial biopsies obtained by bronchoscopy performed six hours after exposures was investigated.

STUDY II

The relationship between DE and allergy has been investigated by in vitro and in vivo studies (45, 46, 48), and suggest that diesel exhaust particles may enhance the sensitization to allergen and development of allergic responses. This study was designed to examine the hypothesis that in healthy non-atopic subjects exposure to DE would induce a shift towards a Th_2 cytokine response in the bronchial mucosa, and/or suppress a Th_1 response.

STUDY III

The knowledge that oxidative effects of DEP and its content of metals, aromatic hydrocarbons, polyaromatic hydrocarbons (PAHs) and quinones might potentiate diesel exhaust effects were the background factors for this study. We hypothesized that the increased production and release of pro-inflammatory cytokines from the human airway after DE exposure could be attributed to the activation of redox-sensitive transcription factors (NFκB and AP-1) and their associated upstream stress-related MAP kinases (p38 and JNK). Thus, we evaluated the DE exposure effect on the activation of these transcription and MAPKs in the bronchial epithelium.

STUDY IV

The tyrosine kinases including receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR) mediate a wide range of cellular processes, including mitogenesis, apoptosis, migration, differentiation and proliferation. We hypothesized that the earlier demonstrated DE responses in studies I-III could be due to involvement of EGFR activation, and this was investigated in study IV.

STUDY V

The healthy subjects participated in this study were exposed to DE generated during city cycle running condition (ETC), and underwent bronchoscopies 6 hours after exposure. Bronchial biopsies, bronchial wash and bronchoalveolar lavage were sampled to investigate the effects of DE generated during city cycle condition.

DIESEL EXHAUST EXPOSURE

The diesel exhaust exposure set-up was originally developed and evaluated by Bertil Rudell during his work as a research fellow and was extensively described in his thesis (110) The diesel exhaust was generated by using an idling Volvo diesel engine (BM – Volvo T45, 4.5 l, 4 cylinders, 1991, 680r / min) and the used diesel fuel content was: cetane number 51; polycyclic aromatic hydrocarbons (PAHs) 0.5%, vol. sulphur 0.06% weight, carbon 86,4% weight, hydrogen 13.5% weight, nitrogen < 0.02%weight and oxygen < 0.1% weight. The 10% vol. boiling point was 200 °C, the 50% vol. boiling point was 282 °C and the 95% vol. boiling point was 355 °C. Over 90% of the exhaust was shunted away and the remaining

part was diluted with filtered air and fed into the exposure chamber. The exposure was standardized with the concentration of particulates with a mass median diameter of less than 10μm (PM_{10}) at 300μg/m^3. This was accompanied by levels of NO_2 of 1.6 ppm, NO of 4.5 ppm, CO of 7.5 ppm, total hydrocarbons of 4.3 ppm, formaldehyde of 0.26 mg/m^3 and 4.3x10^6 suspended particulates /cm^3. Diesel exhaust in city cycle running condition was generated using a Volvo diesel engine (Volvo TD40 GJE, 4.0 L, four cylinders, 1996), programmed to mimic the workload of an engine running in a European Transient Cycle (ETC) environment. The ETC test cycle has been introduced together with the European Stationary Cycle (ESC), for emission certification of heavy-duty diesel engines in Europe, starting in the year 2000 (2). The ETC comprise three different driving conditions, including urban, rural and motorway. In this study we used the urban driving part, representing city driving with a maximum speed of 50 km/h, frequent starts, stops, and idling (Figure 3). The diesel fuel used was Statoil class 1; cetane number 54; aromatics, 4% volume; polycyclic aromatic hydrocarbons, < 0.02 % volume; sulphur <1ppm. The initial boiling point was 195 °C and 95% volume boiling point was 280 °C. The low boiling point, contribute to almost complete combustion of fuel. More than 90% of the exhaust was shunted away and the remaining part was diluted with filtered air and fed to exposure chamber. The DE concentration of particlulates with a mass median diameter <10μm (PM_{10}) was 270 ± 46 SD. This was associated with concentrations of nitrogen dioxide (NO_2) of 0.35 ± 0.07 ppm, nitrogen oxide (NO); 5.9 ± 0.36 ppm, carbon monoxide (CO); 3.0 ppm, and total hydrocarbons (HC); 1.4 ± 0.12 ppm.

BRONCHOSCOPIES

Six hours after each exposure bronchoscopy was performed using a flexible video bronchoscope (Olympus BF type IT200, Tokyo, Japan), in alternating lungs on the two occasions. Prior to bronchoscopy topical anesthesia was applied by lidocaine, which was sprayed in the pharynx and instilled in the larynx and upper trachea. The airway sampling with airway lavages and endobronchial biopsies were carried out using a standardized protocol (112). The bronchoscopies in study V were performed 6 hours after diesel and filtered air exposures, including biopsy sampling, bronchial wash (BW, 2x20 ml) and bronchoalveolar lavage (BAL 3x60 ml) were carried out on the contra-lateral side, in a predetermined randomized way. The aspirates recovered from the first and second 20 ml instillations of the BW and the pooled BAL were collected into separate siliconised containers and immediately placed on ice. All lavage samples were filtered through nylon (pore diameter 100 μm) and centrifuged at 400 g for 15 minutes. Cell pellets were re-suspended in PBS at a cell concentration of 10^6 cells/ml. Differential cell counts were performed on cyto-centrifuge preparations stained with May-Grünwald Giemsa and 400 cells per slide were counted.

BIOPSY PROCESSING

Mucosal biopsies obtained at bronchoscopy were either used for a reverse transcriptase/polymerase chain reaction enzyme-linked immunosorbent assay (RT-PCR ELISA) or for immunohistochemical analysis. The biopsies used for RT-PCR ELISA were

immediately submerged in liquid nitrogen until further analysis. The biopsies used for immunohistochemical analysis were fixed overnight in chilled acetone (–20°C) containing the protease inhibitors iodoacetamide and phenylmethyl sulfonyl fluoride. After fixation, biopsies were processed into glycolmethacrylate (GMA) resin and stored at –20°C until cutting and immunostaining.

IMMUNOHISTOCHEMISTRY

Two sections from one biopsy with proper morphologic structures from each subject and exposure were cut at 2µm thickness and placed onto poly-L-Lysine treated slides. Endogenous peroxidases were inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide. Non-specific antibody binding was blocked with undiluted culture medium containing bovine serum albumin and fetal calf serum. For transcription factor- and phosphorylation, specific antibodies further blocking steps were performed by using rabbit or swine normal serum and incubation of 30 minutes, before applying primary antibodies and incubation at room temperature overnight. The primary antibodies were diluted in TRIS-buffered saline with 0.05% Triton-X-100 (TBST) with 1% bovine serum albumin (BSA), except for cytokines, cells and adhesion molecules stainings, for which dilutions have been performed in TRIS-buffered saline (TBS). In the rinsing steps, for staining with transcription factors, MAPKs and EGFR antibodies, TRIS-buffered saline with 0.1% Triton-X-100 (TBST) was used as washing solution, and TRIS-buffered saline (TBS) was used as rinsing buffer during staining with remaining antibodies. After overnight incubation with primary antibodies, the sections were rinsed with TBST or TBS for 3x5 minutes. The biotinylated rabbit anti-mouse (IgG $F(ab')_2$ Dako Glostrup, Denmark) were used as secondary antibodies on the slides immunostained with mAb, and biotinylated swine anti rabbit (Dako Glostrup, Denmark) were applied on slides on which the primary antibody source was rabbit, and incubated for 2 hours. After further rinsing with TBS 3x5 minutes, streptavidine-biotin horseradish-peroxidase complex (Dako) diluted in Tris-HCl was added and incubated for another 2 hours, followed by 3x5 minutes washes with TBS. In the sections for submucosal analysis, the sections were visualized with aminoethyl carbazole (AEC) in acetate buffer (pH 5.2), whereas sections for epithelial immunoreactivity measurement, diaminobenzidine (DAB) was used in the visualization step. The sections were then counter-stained with Mayer's Haematoxylin. IgG antibodies and diluents were used as negative controls.

QUANTIFICATION OF IMMUNOSTAINING

The immunoreactivity was quantified using a colour video camera (Sony DXC-950P 3-CCD three-chip power HAD) containing 380 000 effective picture elements (pixels) (Sony, Tokyo Japan). The camera was connected to a LEICA imaging workstation, with highly specific PC software (Leica Q500IW, Leica Cambridge UK). The image setting included a possibility to carefully adjust the individual colour components being displayed by the system in order to ensure a close match between what were being viewed directly and the image as displayed. Detection of an appropriate colour was quantified using binary definition of colour images as displayed on the screen. All biopsies with good morphologic areas and intact epithelium from both sections on a slide were used for quantification. The

appropriate areas were selected using image software which provides automatic area calculation. The immunoreactivity determined as positive staining was given as percentage of area selected with the image system. Nuclear translocation was determined and expressed as the number of positively stained nuclei / mm^2 of the selected epithelial area. The inflammatory cell were counted in epithelium and submucosal using light microscopy and expressed as cell /mm in the epithelium and cells/mm^2 in the submucosal respectively. The epithelium length and submucosal area was measured using the computer assisted image system. The endothelial adhesion molecules were quantified by expressing the proportion of stained adhesion molecules as a percentage of vessels stained with the endothelial marker EN4 in adjacent sections.

RT-PCR ELISA

To quantify relative changes in cytokine messenger RNA (mRNA) at the time of Study 1, the RT-PCR ELISA technique, described previously (113), was used. Total RNA was extracted from bronchial wash cells and biopsies using Trizol (Life Technologies, Paisly, Scotland) according the manufacture description. The equal amount of extracted total RNA from paired samples was used to produce complementary DNA (cDNA). The primer pair specific for the cytokines of interest and as a house keeping gene the constitutively expressed gene adenine phosphoribosyl transferase (APRT) were used to conduct PCR amplification of the cDNA. Taq DNA polymerase (Promega, UK) together with primer pairs and PCR digoxigenin (DIG) labeling mix (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate and DIG-11 deoxyuridine triphosphate) [Boehringer Mannheim, Mannheim, Germany] was used performing PCR amplification products. The PCR product was denatured with denaturation solution from the PCR ELISA kit (Boehringer Mannheim). The complementary DIG- labeled PCR product were hybridized to biotin labeled "capture probes" specific to each cytokine PCR product and immobilized on duplicate wells of streptavidin-coated microtiter plates. After washing to remove unbound PCR products, an anti-DIG antibody conjugated to horseradish peroxidase followed by reaction with the substrate 2,2´-Azino-di-(3-ethylbenzthiazoline sulfonate), was used to detect the bound PCR products. Cytokine expression was obtained by comparing the levels of cytokine transcripts to the level of APRT, and expressed as a percentage of APRT (level of cytokine products/level of APRT product x 100).

STATISTICAL EVALUATION

Wilcoxon´s non-parametric signed rank test for paired variables were used to compare data after air and diesel exhaust exposures. Correlation analyses were carried out using Spearman's-Rank-Order-Correlation.

MAIN RESULTS

STUDY I

In study I healthy subjects were exposed to DE (300 μg/m^3) and air during one hour on two separate occasions. Bronchoscopy with bronchial biopsies and bronchial wash was performed six hours after exposure. A significant upregulation of IL-8 mRNA in bronchial biopsies ($p=0.02$) and in the BW cells ($p=0.03$), without any significant effects on other cytokine mRNA was detected after DE exposure. Immunohistochemical analysis of protein production in the bronchial biopsies showed a significant increase in IL-8 ($p=0.04$) as well as in Gro-α ($p=0.01$). No changes were seen in GM-CSF or ENA-78 expression.

STUDY II

This study addressed Th_1 and Th_2 cytokine expressions in the bronchial mucosa after diesel and air exposures. Following DE exposure, immunohistochemical analysis of cytokine expression showed a significant increase in IL-13 in the epithelium ($p=0.009$). No difference was detected for the expressions of IL-10 or IL-18.

STUDY III

This study investigated the role of a number of transcription factors and kinases in the immune regulation after diesel and air exposures. Short term exposure to DE (300 μg/m^3) showed an increase in total (cytoplasmic plus nuclear) immunoreactivity of phosphorylated p38 MAP kinase in the bronchial epithelium, $p=0.03$. We also observed increased nuclear translocation of phosphorylated p38 and c-Jun NH_2-terminal kinase (JNK) MAP kinase in bronchial epithelium after DE ($p=0.01$ and $p=0.04$ respectively). The increased nuclear translocation of phosphorylated MAP kinases were accompanied by elevated nuclear phosphorylated tyrosine (p-Tyr) immunoreactivity, $p<0.05$. Together with changes in MAPKs expression a significant increase in nuclear translocation of the p65 subunit of NFκB ($p=0.02$) as well as the phosphorylated c-jun subunit of AP-1 ($p=0.02$) was seen after DE exposure. Comparison of these results with those previously obtained for epithelial IL-8, Gro-α and IL-13 immunoreactivity after air and diesel exhaust revealed that the expression of IL-8 was positively associated with nuclear phosphorylated p38 ($rs=0.74$, $p=0.002$) and c-jun ($rs=0.77$, $p=0.001$) and to a less degree nuclear p65 ($rs=0.61$, $p=0.02$) after DE, but not after filtered air.

STUDY IV

The role of EGFR in the bronchial epithelium after diesel and air exposure was addressed in this investigation of biopsies. Short term exposure to DE (300μg/m^3) induced a significant increase in the expression of EGFR ($p=0.004$), which was accompanied by an elevation of its autophosphorylation site Tyr 1173 ($p=0.02$). Other EGFR tyrosines, as well as Src and ERK pathway were not found to be affected by the exposures.

STUDY V

This study investigated the effects of diesel exhaust exposure from an engine running under a well defined urban running cycle (ETC). Analyses of bronchial mucosal biopsies sampled at 6 hours after exposures demonstrated significant increases in the endothelial expression of P-selectin and VCAM-1 after diesel compared to filtered air exposures ($p= 0.036$ and 0.030, respectively). No significant changes were seen in inflammatory cell numbers in the epithelium or submucosa. BAL analyses showed increased numbers of eosinophils ($p= 0.017$) without any changes in the number of the other cells in lavages.

DISCUSSION

DISCUSSION OF METHODS

Fifteen healthy, non-smoking volunteers were included in study I-IV and fifteen others were included in study V. For statistical evaluations each subject was his or her own control. The number of subjects included in these studies was almost twice that in our earliest experimental human studies (109) and were based on previous studies by this and other groups (16, 127, 128). Due to the limitations in recruiting subjects, in addition to practical purposes including staff resources, it was not possible to include substantially larger groups of subjects in these studies. Although the possibility that true effects can be missed by too few research subjects always is a possibility (type II error), it is not evident that the present studies would have benefited extensively from larger groups of subjects. The work on archived biopsy material eventually resulted in the biopsies being consumed.

The mucosal biopsies obtained at bronchoscopies were judged by their size and macroscopic structure to be of acceptable size and morphology. They were then processed in chilled acetone fixation and glycolmethacrylate (GMA) embedding. All GMA resin processed biopsies from each subject and brochoscopy were cut for microscopic evaluation. Biopsies containing proper epithelium and submucosal structure were accepted for the staining processes. This particular embedding in slow hardening resin gives the possibility to cut very thin sections. This has the advantage that the investigator is able to cut sequential sections and have the possibility to evaluate different inflammatory markers in sequential sections, even through the same cells. Two sections from a biopsy with properly morphologic structure from each subject and exposure, were cut at 2μm thickness, and placed onto poly-L-Lysine treated slides for immunostaing.

The antibodies used in the studies were commercially available and were mainly monoclonal antibodies. During staining processes fixed incubation time and room temperature were used. To obtain an optimum antibody dilution, a series of experimental dilutions have been tested. These tests were performed on tonsil tissues. The dilution with optimum staining intensity and minimal background staining were chosen for the biopsy stainings. An optimal balance between sensitivity and specificity was essential during the work. During the staining processes non-immunized immunoglobulin and antibody diluents were used as negative controls. The substrate, diaminobenzidine (DAB) which mainly was used in these studies, results in a brown end product with staining intensity that makes it easy to use in a computer aided image analysis. Detection and choice of an appropriate colour as positive staining was quantified using binary definition of colour images as displayed on the screen. This is an advantage in the quantification of immunostaining with a given antibody since the user is able to use the same binary definition of colour in the all tissue stained with a given antibody.

DISCUSSION OF MAIN RESULTS

Toxicological and inflammatory events caused by air pollution particles have been linked to both their size and chemical composition. These aspects have also been linked to adverse health effects of ambient particulate matter. DEP have been demonstrated to be toxicologically potent (90, 131), but ambient PM, and especially the metal rich residual oil fly ash (ROFA), have often shown an even higher potency. A variety of cell types and cell free systems have been utilized to investigate the toxicity of particles and the pathways that mediate their responses. The first surface to interact with DEP is the respiratory tract lining fluid and oxidative properties of DEP have been demonstrated. The alveolar macrophages and airway epithelial cells are the first cell types to encounter inhaled particles. Therefore, most investigators have focused on these cells (3, 20, 84, 96), and reported alterations of a variety of cytokines such as IL-1β, IL-8, GM-CSF, TGF-β and others by DEP stimulation. In vivo exposure studies in humans have reported neutrophilic responses and inflammatory mediator production following DE exposure (16, 92, 93, 127). Exposure to DE (PM_{10} 300 $\mu g/m^3$) induced a pronounced airway inflammatory response with recruitment of neutrophils, lymphocytes and mast cells into the bronchial mucosa. Together with inflammatory cell influx, up-regulation of adhesion molecules, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) was observed in the vascular endothelium (112). In this thesis, archived biopsies were used (study I-IV) to investigate the underlying biological mechanisms and inflammatory pathways in the DE induced cellular response reported previously (112), by evaluating chemokines, cytokines, transcription factors, MAPKs and upstream signalling pathways.

EFFECT OF DIESEL EXHAUST ON PRO-INFLAMMATORY CYTOKINES

DEP containing a variety of adsorbed organic components, such as aliphatic, aromatic and polyaromatic hydrocarbons, including pyrenes and quinones on their core, have been reported to induce airway inflammatory responses in subjects exposed to different concentration of the diesel exhaust (16, 92, 93, 112, 127). Furthermore, in vitro studies have reported increased production of cytokines such as IL-8 and GM-CSF, following exposure to DEPs or extracted organic components (12, 21, 74). DEPs are capable of causing oxidative stress, partly dependent on their content of quinones, and suggested to produce superoxide and hydroxyl radicals in mice. Pretreatment with antioxidants decreased the oxidative damage caused by DEP instillation (77, 111, 150). Additionally, metabolism of polyaromatic compounds through cytochrome P-450 may result in oxidative components. Semi-quinones may also undergo metabolism inducing oxidative capacity. Additionally, metabolism of polyaromatic compounds and expression of CYP1A1, a cytochrome P-450, is involved in this metabolism, thereby demonstrating the critical role of organic compounds in the DEP-induced proinflammatory responses (11, 22). Semiquinones may also undergo metabolism, generate ROS and give rise to oxidative damages (125). Furthermore, the capacity of DEP to generate oxidative stress in the respiratory tract induce activation of antioxidant defences, induction of Heme oxygenase-1, phase II enzyme expression in macrophages and activation of transcription factor Nrf2 (11,

82, 85). Signs of oxidative stress in the airways following DE challenge in human have been recently demonstrated (16, 90).

Induction of cytokines by DEP in vitro has been inhibited by radical scavengers and it has been suggested that adsorbed organic compounds are the main inducers of cytokine production (3, 20). Activation of transcription factors and TNF-α cytokine expression has been reported in macrophages exposed to ultrafine particles and has been suggested to be mediated by ROS and modulating intracellular calcium concentration (26).

In Study I, exposure to a high concentration of DE was associated with enhanced expression of IL-8 and Gro-α in the bronchial epithelium, but not ENA-78. It is well established that DE particles can elicit increased synthesis and release of pro-inflammatory cytokines from airway epithelial cells *in vitro* (3, 62), which is in accordance with our data. IL-8 and Gro-α are CXC chemokines with biological effects on various cell types, including activation and attraction of neutrophils. Increased neutrophil attractants after DE exposure were associated with a neutrophilic response, together with the upregulation of the vascular endothelial adhesion molecule ICAM-1 and the increase in the leukocyte expression of LFA-1 demonstrated in a previous study (112). Furthermore, IL-8 has been reported to play a role in lymphocyte, eosinophil and basophil activation and migration to inflammatory sites, such as asthma (52, 53, 154). LTB4 is an interesting chemoattractant produced by the arachidonic acid cascade. This has not been investigated after DE exposure, but represents one of several other components which potentially could have been addressed. The cytokines IL-4, IL-5, IL-6, TNF-α, GM-CSF and IFN-γ which were investigated in study I, showed no changes after DE exposure, except for a trend towards an increase in IL-5 gene transcription in the bronchial tissue. All this taken together, the available data demonstrate that DE exposure has the ability to stimulate expression of proinflammatory cytokines along with the upregulation of adhesion molecules and migration of inflammatory cells in human subject in-vivo.

EFFECTS OF DIESEL EXHAUST ON Th_2 CYTOKINES

A number of experimental studies, mainly in animals, have been performed to investigate possible immune modulating effects of DEP (115). In mice, immunization with allergen mixed with DEP caused enhanced IgE antibody production compared with allergen alone, indicating adjuvant activity of DEP (91). In humans, allergen provocation together with DEP have shown elevated Th_2 cytokines release (47, 48). Study II examined the hypothesis that exposure to DE would induce a shift towards a Th2 cytokine response in the bronchial mucosa and / or suppress Th1 responses in healthy non-atopic subjects. In asthmatics exposed to DE (PM_{10}, 300 μg/m^3) and filtered air, methacholine tests at 24 h demonstrated that diesel exhaust exposure caused a significant increase in the degree of bronchial hyperresponsiveness (94). A lower concentration of DE (PM_{10}, 108 μg/m^3 for 2 hours) in asthmatics induced a five fold elevation of the IL-10 expression in the bronchial epithelium (127). In non-atopic healthy subjects, exposure to DE (PM_{10}, 300μg/m^3) caused enhanced IL-13 expression in the epithelium (Study II). IL-18 is an IFN-γ inducer (140), and was selected to represent a Th_1 related response. IL-18 was unchanged after exposures as were IL-4 and IL-5. IL-10, which has been demonstrated to enhance Th_2 responses and to potentiate IgE production by B cells that have become committed to IgE production (69), was unchanged.

IL-13 is an important Th2 cytokine and is normally expressed in larger amounts than IL-4. These two cytokines share many properties (36, 153). IL-13 has been reported to have effects on chemokine expression in the lung and stimulate eotaxin production in the absence of TNF-α (81). In addition, DEP have been shown to enhance the eotaxin gene expression in human bronchial epithelial cells via a NFκB dependent pathway (135). IL-13 is involved in inducing proliferation of airway epithelial cells mediated by TGF- α (24). IL-13 plays a key role in the recruitment of leukocytes by inducing chemoattractant expression and is also of importance in mucin production and goblet cell metaplasia (76, 80, 121). The DE induced increase in IL-13 expression in non-atopic healthy subjects occurred in association with inflammatory cell infiltration, upregulation of the adhesion molecules ICAM-1 and VCAM-1 and the increased expression of chemokines IL-8 and Gro-α. DE enhanced IL-13 expression may potentially contribute to worsening of respiratory symptoms during pollutant episodes in subjects with asthma and COPD, due to enhanced Th_2 responses as well as mucus hypersecretion. Furthermore, the DE induced IL-13 expression may contribute to an enhanced risk of allergic sensitization in previously non-atopic individuals as well as enhanced sensitization and allergy development in allergic asthmatic subjects. It could consequently be one of the mechanisms underlying the clinical observation in subjects with respiratory diseases, who develop symptoms after an episode with increased pollution.

Following the studies of cytokine production after diesel exhaust in Study I and II, Studies III-IV investigated the signal transduction mechanisms behind the inflammatory events.

EFFECTS OF DIESEL EXHAUST ON TRANSCRIPTION FACTORS AND MAPKs

The diesel exhaust-induced cytokine release detected in studies I and II, requires activation of transcription factors and their upstream signalling pathways MAPKs, that modulate cytokine gene expression. The properties of PM in general and DEP in particular to elicit oxidative stress and activation of inflammatory pathways regulating these responses have been reported in several animal and *in vitro* studies (22, 23, 83, 84, 90). In addition, bronchial epithelial cells and macrophages challenged with DEP show increased expression of cytokines in association with NFκB and AP-1 that may be attenuated by antioxidant supplementation (62, 137, 157). The association of redox-sensitive transcription factor activation with their upstream MAPKs has been confirmed *in vitro* (62, 158). The involvement of the MAPKs, p38 and JNK, in the activation of transcription factors such as NFκB, AP-1 ATF2 and Elk and their downstream signalling for cytokine production, has been reported previously (17, 32, 98, 159). The capacity of DEP to generate free radicals by their metal (90), PAH (12) and quinone (125) content and the upregulation of redox-sensitive signalling pathways after DE exposure in study III are in accordance with the key role of NFκB and AP-1 and their upstream kinases in the regulation of DE effects (62, 136, 137). Upregulation of the redox-sensitive transcription factors NFκB and AP-1 and MAPKs signalling pathways, p38 and JNK, in the bronchial epithelium after DE exposure in healthy subjects, indicate that these pathways may be the molecular mechanisms involved in DEP-induced oxidative stress and the subsequent proinflammatory cytokine production and leukocyte recruitment.

EFFECTS OF DIESEL EXHAUST ON EGFR EXPRESSION AND ACTIVATION

In study IV, the signalling pathways, which activate and regulate the downstream signalling pathways found in study III, were investigated. Therefore, EGFR expression, activation and its downstream signalling were evaluated after inhalation of diesel exhaust. Receptor tyrosine kinases (RTKs) and EGFR are known as mediators of the incoming signals and have been shown to play key roles in cellular processes such as mitogenesis, proliferation, bronchial repair, remodelling, migration and inflammation, of importance in respiratory diseases such as asthma and COPD. There are several studies which have shown activation of EGFR by metals, organic components and oxidative stress, accompanied with activation of downstream MAPKs and transcription factors (29, 147-149). Recent studies have demonstrated the capacity of DEP to induce secretion of EGFR ligand, and its quinone compounds have been shown to activate Phospholipase A2 (PLA2). These signalling pathways could be blocked by PTK, EGFR inhibitors and antioxidants, which indicate the ability of DEP to activated by EGFR ligands and transactivate EGFR tyrosine residues, as a consequence of oxidative stress (18, 75). Cytokines may interact with EGFR, IL-13 has been shown play a key role in goblet cell metaplasia (142) and to activate neutrophils via EGFR interaction to increase goblet cell mucin production (121). Evaluation of EGFR in study IV, showed increased EGFR expression and phosphorylation of Tyr 1173. This autophosphorylation site is suggested to be involved in the association of EGFR with PLC-γ (34) and signal transduction coupled to PI3-K (6). Tyr 1173 also acts as a docking site for Shc (Src-homology and collagen protein), which in turn can bind to Grb2 (Growth factor receptor-binding protein 2) (101, 158), leading to MEK kinase-1 (MEKK1) activation and subsequent activation of JNK and p38 MAPKs (101, 105). These aspects together with the findings in study III indicate the ability of DEP to induce oxidative stress and cause activation / transactivation of EGFR. In addition, activation of EGFR, together with our finding of enhanced IL-13 expression in the bronchial epithelium following exposure to DE in humans, could be of particular importance for patients with asthma and COPD, who may experience exacerbation of their diseases after exposure to particulate matter air pollution (28, 94, 124).

The results from study I-V do, together with antioxidant data after diesel exhaust exposure (16), indicate that DEP may overcome the antioxidant defences locally in the airways, through their direct oxidative capacity. This oxidative stress to airways may be further augmented through the production of oxidative species after metabolisation of organics and ROS from activated macrophages and neutrophils. This may lead to EGFR transactivation as well as potential activation by EGFR ligands.

A variety of constituents of DEP have the potential to cause ROS generation and results in oxidative stress. These include metals and mainly organic compounds such as PAHs and quinones (64, 77). The organics constituents also have the ability to activate oxidative stress signalling pathways such as NFκB and p38 MAPK (22, 62). In addition, DEP may initially stimulate airway epithelial cells and macrophages to release pro-inflammatory cytokines that mediate inflammatory cell recruitment. The released cytokines may cause the release of EGFR ligands that bind and activate the EGFR through ligand dependent mechanisms. Activation or transactivation of EGFR results in tyrosine phosphorylation, such as Tyr 1173, and provide binding opportunities for adaptor proteins like Shc or enzymes such as

PLC and PI3-K. The adaptor protein Shc is able causing activation of small G protein, RAS and its downstream signalling pathways MEKK1, JNK, p38 and subsequent transcription factors. The cellular response to oxidative stress might increase the activity of the enzymes such as phospholipase A2, phospholipase C which regulate the activities of DAG and PI3-K, known to be activators of protein kinases. The enzymes PLC and PI3-K are involved in the activation of signalling pathways such as PKC, G protein coupled receptor (GPCR), small G protein and other signalling molecules, which could activate downstream signalling pathways in cooperation with EGFR. These mechanisms may be of importance particularly for sensitive populations with asthma and COPD that include EGFR-related mechanisms and airway remodelling in their pathophysiological characteristics.

EFFECTS OF DIESEL EXHAUST GENERATED AT EUROPEAN TRANSIENT CYCLE RUNNING CONDITION ON HUMAN AIRWAYS

Earlier diesel exposure studies have mainly used diesel exhaust generated during idling conditions, which gives higher organic contributions to the exhaust. Diesel exhaust with lower organic content and more carbon core particles is generated in traffic situations when engines work at higher speed. Study V was therefore performed to address the airway inflammatory responses to diesel exhaust, generated by a diesel engine during standardized ETC running conditions healthy human subjects. The present study demonstrated significant increases in the expression of the vascular endothelial adhesion molecules VCAM-1 and P-selectin which are important for recruitment and migration of inflammatory cells into the bronchial tissue. P-selectin is known to initiate the early phase of adhesion-rolling of inflammatory cells along the endothelial surface. The inflammatory cells can bind to ICAM-1 via LFA-1 and leads to migration of cells to the inflammatory site, but VCAM-1 mainly, bind to eosinophils, lymphocytes and monocytes via VLA-4. Absence of altered endothelial ICAM-1 expression and the finding of upregulated P-selectin, suggests an early phase of inflammatory cell recruitment into the airway mucosa. Whether a further development would occur during the forthcoming hours needs to be further addressed.

FINAL COMMENTS

Adverse health effect of PM and DE, as a major contributor of particulate air pollution, have been revealed by a number of epidemiological studies which have suggested that subjects with respiratory and cardio-vascular diseases comprise susceptible sub-populations. However, experimental studies are needed to elucidate and understand the underlying biological mechanisms behind these adverse effects. In this thesis, two separate diesel exposure set-ups were studied; diesel engine exhaust produced during idling as well as exhaust produced during city cycle running conditions. The aims of these studies were to investigate airway effects of DE exposure in healthy subjects and plausible biological mechanisms contributing to DE induced inflammatory outcomes in the human airways. Following exposure to DE generated by idling diesel engine, a neutrophilic inflammation accompanied with elevated adhesion molecules was reported in a previous study. This thesis shows that DE induced inflammation is mediated by enhanced chemokines and cytokines in the bronchial epithelium. The DE induced enhancement of cytokines may be regulated by augmentation of redox-sensitive transcription factor activity, in association with increased activation of their upstream MAPKs. An activation of redox-sensitive pathways and subsequent oxidative stress upon exposure to DE, may be mediated by metals, PAHs and quinones or ROS generated by respiratory cells and result in increased release of cytokines. The first study done with diesel under running cycle conditions demonstrated relatively similar effects as idling, but there were indications of a slower development of inflammation in the airways, that demand further attention. The finding of bronchoalveolar eosinophilia could be of certain importance for asthmatic subjects.

Combined, these findings may provide important links to the epidemiological findings of susceptibility of subjects with respiratory and cardio-vascular diseases, which already have ongoing oxidative stress responses. Importantly, this study demonstrates a key role for EGFR which can regulate migration, inflammation, repair, proliferation and remodelling following DE exposure. Complementary research also needed to study effects of diesel exhaust generated by various engines with and without particle filters and at various running conditions to better understand the toxicological features of diesel exhaust with different chemical and particulate properties.

CONCLUSIONS

The findings in this thesis indicate that diesel exhaust has potential to induce cascades of biological mechanisms in the airways of healthy subjects, which may be of particular importance for sensitive populations with respiratory and cardiovascular diseases.

It is concluded that:

Diesel exhaust generated during European transient cycle running condition as well as idling conditions, have the capacity to induce airway inflammatory events. Differences between the responses to the two types of exhaust may be due to the exhaust composition, dose and time kinetic aspects.

Diesel exhaust induced production of chemokines and cytokines with immunoregulatory abilities in the bronchial epithelium, resulting in a shift towards a Th_2 cytokine response. These finding highlight the regulatory role of the bronchial epithelium in the inflammatory response to diesel exhaust.

Diesel exhaust induced cytokine production was associated with increase in the redox-sensitive transcription factors NFκB, AP-1 as well as JNK and p38 MAPkinases in the bronchial epithelium.

Exposure to diesel exhaust was associated with increased EGFR expression and phosphorylation of the C-terminal Tyr 1173 residue which may reflect the ability of diesel exhaust to induce oxidative stress and cause activation or transactivation of EGFR.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to a number of people who's by giving their help, support and encouragement made writing this thesis possible:

Thomas Sandström, min handledare för Din entusiasm, uppmuntran och Ditt engagemang, för att jag fick möjligheten att komma in i forskningsvärlden och tack för trevliga konferenser världen över.

Anders Blomberg, min andra handledare, för Ditt stöd, engagemang och uppmuntran för Dina ovärderliga synpunkter, granskningar och språkliga hjälp. Tack för trevliga konferenser, planera och ordna olika utflyktsmål.

Ragnberth Helleday, för Din hjälp och Ditt stöd, tack för Din hjälp med redigering av min avhandling, för dina tillgängliga datorprogram som har hjälpt till att komma ut ur krissituationer och tack för Din uppmuntran.

Ellinor Ädelroth, för Ditt stöd och Din uppmuntran, tack också för Dina språkliga synpunkter och rättelse som gjorde min avhandling både lättare att förstå och bära. Tack för trevliga middagar på ATS resor, också tack för sportklubbkvällen och trevliga gruvarbetsveckor.

Charlotta Nordenhäll, för Din uppmuntran och att ledde mig in i forskningen och tack för trevlig snittnings- och färgningsvecka i Southampton.

Mina forskningskollegor och medarbetare, **Annelie Behndig**, för Ditt stöd, och räddaren i nöden med EndNote 9 i handen. **Maj-Cari Ledin**, tack för Din hjälp med exponeringar som är grunden för presenterade arbeten i avhandlingen. **Lena Åström**, tack för all hjälp och administreringsjobb som inte synts under åren. **Annika Johansson**, **Helena Bogseth** och **Frida Holmström** tack för allt stöd och hjälp med bronkoskopier och kringarbeten. **Jenny Bosson**, **Håkan Törnquist**, **Lena Skedebrant**, **Helén Bertilsson**, **Nikolai Stenfors**, **Lissi Thomasson**, **Ulf Hammarström**, **Bertil Rudell** och mina medarbetare på labbet **Ann-Britt Lundström**, **Maria Sehlstedt**, **Ester Roos-Engstrand**, tack för all hjälp, stöd och uppmuntran. **Henrik Fernerud**, tack för allt stöd, och tack för att Du tillsammans med **Ester Roos-Engstrand** och **Maria Sehlstedt,** ställer upp och ordnar disputationsfesten. Tack alla för trevliga flygresor, middagar, utflykter och poolkanter med en hel del av er. Tack **Kjell Holmgren**, **Stefan Frisk**, **Tage Norén**, **Joakim Grönlund och Hans Arvidsson** på Svenska Maskinprovning (SMP) för all hjälp och stöd i samband med exponeringarna.

Tack till alla på **Lung- och Allergikliniken, NUS** och **forskningsvåningen (UKBF)**, och alla som inte jag inte nämnt men ej heller glömt för all stöd, hjälp och uppmuntran som jag har fått.

My collaborators in the UK **Susan Wilson, Sundeep Salvi, Stephen Holgate** and **Tony Frew,** Southampton, **Ian Mudway** and **Frank Kelly,** London and in the USA **Samet J M,** Chapel Hill, for your help and support.

به پآد بؤد پدرو برادرم. با قدردانی از مادرم و
خواهرانم با خانوادشان. و با تشکر از خوپشاوندان و
دوستان که هر پک به طرپقی سهم در اپن کار دارند

Sist, men inte minst, min älskade familj, **Susan**, **Elham** och **Sam**, tack för all stöd och uppmuntran som ni har gett mig, för att ni har stått ut med mina arbetsrutiner, tack Elham för ditt stöd och att du har haft överseende med min frånvaro på dina födelsedagar, som jag har missat några av de under dessa år i samband med mina ATS resor, men 21 årsdagen är jag med.

This work was generously supported by grants from the Swedish Heart Lung Foundation, the Emission Research Programme (EMFO), the Swedish Road Administration, Umeå University, the Swedish National Air pollution Programme (SNAP), EU FP 5 programme grant QLRT-1999-01582.

REFERENCES

1. Committee of the Environmental and Occupational Health Assembly of the American Thoracic Society Health effect of outdoor air pollution. *Am J Resp Crit Care Med* 153(1): 3-50, 1996.

2. Directive 1999/96/EC of the European Parliament and of the Council of December 13 1999 Official Journal of the European Communities 16.2.2000.

3. **Abe S, Takizawa H, Sugawara I, and Kudoh S**. Diesel exhaust (DE)-induced cytokine expression in human bronchial epithelial cells: a study with a new cell exposure system to freshly generated DE in vitro. *Am J Respir Cell Mol Biol* 22: 296-303, 2000.

4. **Aoki Y, Qiu D, Zhao GH, and Kao PN**. Leukotriene B4 mediates histamine induction of NF-kappaB and IL-8 in human bronchial epithelial cells. *Am J Physiol* 274: L1030-1039, 1998.

5. **Atkinson RW, Anderson HR, Sunyer J, Ayres J, Baccini M, Vonk JM, Boumghar A, Forastiere F, Forsberg B, Touloumi G, Schwartz J, and Katsouyanni K.** Acute effects of particulate air pollution on respiratory admissions: results from APHEA 2 project. Air Pollution and Health: a European Approach. *Am J Respir Crit Care Med* 164: 1860-1866, 2001.

6. **Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS, and Rhee SG**. Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273: 4465-4469, 1998.

7. **Baeza-Squiban A, Bonvallot V, Boland S, and Marano F**. Airborne particles evoke an inflammatory response in human airway epithelium. Activation of transcription factors. *Cell Biol Toxicol* 15: 375-380, 1999.

8. **Baggiolini M**. Chemokines and leukocyte traffic. *Nature* 392: 565-568, 1998.

9. **Baggiolini M, Dewald B, and Moser B**. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Adv Immunol* 55: 97-179, 1994.

10. **Bauer GJ, Arbabi S, Garcia IA, deHingh I, Rosengart MR, and Maier RV**. Adherence regulates macrophage signal transduction and primes tumor necrosis factor production. *Shock* 14: 435-440, 2000.

11. **Baulig A, Garlatti M, Bonvallot V, Marchand A, Barouki R, Marano F, and Baeza-Squiban A**. Involvement of reactive oxygen species in the metabolic pathways triggered by diesel exhaust particles in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 285: L671-679, 2003.

12. **Baulig A, Sourdeval M, Meyer M, Marano F, and Baeza-Squiban A**. Biological effects of atmospheric particles on human bronchial epithelial cells. Comparison with diesel exhaust particles. *Toxicol In Vitro* 17: 567-573, 2003.

13. **Bayram H, Devalia JL, Sapsford RJ, Ohtoshi T, Miyabara Y, Sagai M, and Davies RJ**. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells in vitro. *Am J Respir Cell Mol Biol* 18: 441-448, 1998.

14. **Beg AA, and Baldwin AS, Jr.** The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. *Genes Dev* 7: 2064-2070, 1993.

15. **Beg AA, Finco TS, Nantermet PV, and Baldwin AS, Jr.** Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol Cell Biol* 13: 3301-3310, 1993.

16. **Behndig AF, Mudway IS, Brown JL, Stenfors N, Helleday R, Duggan ST, Wilson SJ, Boman C, Cassee FR, Frew AJ, Kelly FJ, Sandstrom T, and Blomberg A.** Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. *Eur Respir J* 27: 359-365, 2006.

17. **Beyaert R, Cuenda A, Vanden Berghe W, Plaisance S, Lee JC, Haegeman G, Cohen P, and Fiers W**. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. *Embo J* 15: 1914-1923, 1996.

18. **Blanchet S, Ramgolam K, Baulig A, Marano F, and Baeza-Squiban A**. Fine particulate matter induces amphiregulin secretion by bronchial epithelial cells. *Am J Respir Cell Mol Biol* 30: 421-427, 2004.

19. **Bogdan S, and Klambt C**. Epidermal growth factor receptor signaling. *Curr Biol* 11: R292-295, 2001.

20. **Boland S, Baeza-Squiban A, Fournier T, Houcine O, Gendron MC, Chevrier M, Jouvenot G, Coste A, Aubier M, and Marano F**. Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *Am J Physiol* 276: L604-613, 1999.

21. **Boland S, Bonvallot V, Fournier T, Baeza-Squiban A, Aubier M, and Marano F**. Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 278: L25-32, 2000.

22. **Bonvallot V, Baeza-Squiban A, Baulig A, Brulant S, Boland S, Muzeau F, Barouki R, and Marano F**. Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. *Am J Respir Cell Mol Biol* 25: 515-521, 2001.

23. **Bonvallot V, Baulig A, Boland S, Marano F, and Baeza A**. Diesel exhaust particles induce an inflammatory response in airway epithelial cells: involvement of reactive oxygen species. *Biofactors* 16: 15-17, 2002.

24. **Booth BW, Adler KB, Bonner JC, Tournier F, and Martin LD**. Interleukin-13 induces proliferation of human airway epithelial cells in vitro via a mechanism mediated by transforming growth factor-alpha. *Am J Respir Cell Mol Biol* 25: 739-743, 2001.

25. **Brain JD, and Valberg PA**. Deposition of aerosol in the respiratory tract. *Am Rev Respir Dis* 120: 1325-1373, 1979.

26. **Brown DM, Donaldson K, Borm PJ, Schins RP, Dehnhardt M, Gilmour P, Jimenez LA, and Stone V**. Calcium and ROS-mediated activation of transcription factors and TNF-alpha cytokine gene expression in macrophages exposed to ultrafine particles. *Am J Physiol Lung Cell Mol Physiol* 286: L344-353, 2004.

27. **Brunekreef B, Janssen NA, de Hartog J, Harssema H, Knape M, and van Vliet P**. Air pollution from truck traffic and lung function in children living near motorways. *Epidemiology* 8: 298-303, 1997.

28. Bucchier**i F, Puddicombe SM, Lordan JL, Richter A, Buchanan D, Wilson SJ, Ward J, Zummo G, Howarth PH, Djukanovic R, Holgate ST, and Davies DE.** Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am J Respir Cell Mol Biol* 27: 179-185, 2002.

29. **Burdick AD, Davis JW, 2nd, Liu KJ, Hudson LG, Shi H, Monske ML, and Burchiel SW**. Benzo(a)pyrene quinones increase cell proliferation, generate reactive oxygen species, and transactivate the epidermal growth factor receptor in breast epithelial cells. *Cancer Res* 63: 7825-7833, 2003.

30. **Cameron LA, Taha RA, Tsicopoulos A, Kurimoto M, Olivenstein R, Wallaert B, Minshall EM, and Hamid QA.** Airway epithelium expresses interleukin-18. *Eur Respir J* 14: 553-559, 1999.

31. **Carlos TM, and Harlan JM**. Leukocyte-endothelial adhesion molecules. *Blood* 84: 2068-2101, 1994.

32. **Carter AB, Knudtson KL, Monick MM, and Hunninghake GW**. The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem* 274: 30858-30863, 1999.

33. **Chalupa DC, Morrow PE, Oberdorster G, Utell MJ, and Frampton MW**. Ultrafine particle deposition in subjects with asthma. *Environ Health Perspect* 112: 879-882, 2004.

34. **Chattopadhyay A, Vecchi M, Ji Q, Mernaugh R, and Carpenter G**. The role of individual SH2 domains in mediating association of phospholipase C-gamma1 with the activated EGF receptor. *J Biol Chem* 274: 26091-26097, 1999.

35. **Cobb MH, and Goldsmith EJ**. How MAP kinases are regulated. *J Biol Chem* 270: 14843-14846, 1995.

36. **Cohn L, Homer RJ, MacLeod H, Mohrs M, Brombacher F, and Bottomly K**. Th2-induced airway mucus production is dependent on IL-4Ralpha, but not on eosinophils. *J Immunol* 162: 6178-6183, 1999.

37. **Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, and Gutkind JS**. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 81: 1137-1146, 1995.

38. **Coso OA, Teramoto H, Simonds WF, and Gutkind JS**. Signaling from G protein-coupled receptors to c-Jun kinase involves beta gamma subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. *J Biol Chem* 271: 3963-3966, 1996.

39. **Davis RJ**. Signal transduction by the JNK group of MAP kinases. *Cell* 103: 239-252, 2000.

40. **Dayer JM, Isler P, and Nicod LP**. Adhesion molecules and cytokine production. *Am Rev Respir Dis* 148: S70-74, 1993.

41. **Dean DC, Iademarco MF, Rosen GD, and Sheppard AM**. The integrin alpha 4 beta 1 and its counter receptor VCAM-1 in development and immune function. *Am Rev Respir Dis* 148: S43-46, 1993.

42. **Dean JL, Brook M, Clark AR, and Saklatvala J**. p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J Biol Chem* 274: 264-269, 1999.

43. **Devalia JL, Bayram H, Rusznak C, Calderon M, Sapsford RJ, Abdelaziz MA, Wang J, and Davies RJ**. Mechanisms of pollution-induced airway disease: in vitro studies in the upper and lower airways. *Allergy* 52: 45-51; discussion 57-48, 1997.

44. **Diaz-Sanchez D**. The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. *Allergy* 52: 52-56; discussion 57-58, 1997.

45. **Diaz-Sanchez D, Dotson AR, Takenaka H, and Saxon A**. Diesel exhaust particles induce local IgE production in vivo and alter the pattern of IgE messenger RNA isoforms. *J Clin Invest* 94: 1417-1425, 1994.

46. **Diaz-Sanchez D, Garcia MP, Wang M, Jyrala M, and Saxon A**. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol* 104: 1183-1188, 1999.

47. **Diaz-Sanchez D, Tsien A, Casillas A, Dotson AR, and Saxon A**. Enhanced nasal cytokine production in human beings after in vivo challenge with diesel exhaust particles. *J Allergy Clin Immunol* 98: 114-123, 1996.

48. **Diaz-Sanchez D, Tsien A, Fleming J, and Saxon A**. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J Immunol* 158: 2406-2413, 1997.

49. **Dockery DW, Pope CA, 3rd, Kanner RE, Martin Villegas G, and Schwartz J**. Daily changes in oxygen saturation and pulse rate associated with particulate air pollution and barometric pressure. *Res Rep Health Eff Inst* 1-19; discussion 21-18, 1999.

50. **Dockery DW, and Schwartz J**. Particulate air pollution and mortality: more than the Philadelphia story. *Epidemiology* 6: 629-632, 1995.

51. **Eder J**. Tumour necrosis factor alpha and interleukin 1 signalling: do MAPKK kinases connect it all? *Trends Pharmacol Sci* 18: 319-322, 1997.

52. **Erger RA, and Casale TB**. Interleukin-8 plays a significant role in IgE-mediated lung inflammation. *Eur Respir J* 11: 299-305, 1998.

53. **Erger RA, and Casale TB**. Tumor necrosis factor alpha is necessary for granulocyte-macrophage-colony-stimulating-factor-induced eosinophil transendothelial migration. *Int Arch Allergy Immunol* 115: 24-32, 1998.

54. **Fernandes MB, and Brooks P**. Characterization of carbonaceous combustion residues: II. Nonpolar organic compounds. *Chemosphere* 53: 447-458, 2003.

55. **Finkelman FD, Yang M, Orekhova T, Clyne E, Bernstein J, Whitekus M, Diaz-Sanchez D, and Morris SC**. Diesel exhaust particles suppress in vivo IFN-gamma production by inhibiting cytokine effects on NK and NKT cells. *J Immunol* 172: 3808-3813, 2004.

56. **Fujieda S, Diaz-Sanchez D, and Saxon A**. Combined nasal challenge with diesel exhaust particles and allergen induces In vivo IgE isotype switching. *Am J Respir Cell Mol Biol* 19: 507-512, 1998.

57. **Gauderman WJ, Avol E, Gilliland F, Vora H, Thomas D, Berhane K, McConnell R, Kuenzli N, Lurmann F, Rappaport E, Margolis H, Bates D, and Peters J.** The effect of air pollution on lung development from 10 to 18 years of age. *N Engl J Med* 351: 1057-1067, 2004.

58. **Gauderman WJ, Gilliland GF, Vora H, Avol E, Stram D, McConnell R, Thomas D, Lurmann F, Margolis HG, Rappaport EB, Berhane K, and Peters JM.** Association between air pollution and lung function growth in southern California children: results from a second cohort. *Am J Respir Crit Care Med* 166: 76-84, 2002.

59. **Hackel PO, Zwick E, Prenzel N, and Ullrich A**. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. *Curr Opin Cell Biol* 11: 184-189, 1999.

60. **Han J, Lee JD, Bibbs L, and Ulevitch RJ**. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265: 808-811, 1994.

61. **Han ZS, Enslen H, Hu X, Meng X, Wu IH, Barrett T, Davis RJ, and Ip YT**. A conserved p38 mitogen-activated protein kinase pathway regulates Drosophila immunity gene expression. *Mol Cell Biol* 18: 3527-3539, 1998.

62. **Hashimoto S, Gon Y, Takeshita I, Matsumoto K, Jibiki I, Takizawa H, Kudoh S, and Horie T**. Diesel exhaust particles activate p38 MAP kinase to produce interleukin 8 and RANTES by human bronchial epithelial cells and N-acetylcysteine attenuates p38 MAP kinase activation. *Am J Respir Crit Care Med* 161: 280-285, 2000.

63. **Hirota K, Murata M, Itoh T, Yodoi J, and Fukuda K**. Redox-sensitive transactivation of epidermal growth factor receptor by tumor necrosis factor confers the NF-kappa B activation. *J Biol Chem* 276: 25953-25958, 2001.

64. **Hiura TS, Li N, Kaplan R, Horwitz M, Seagrave JC, and Nel AE**. The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. *J Immunol* 165: 2703-2711, 2000.

65. **Hiyoshi K, Takano H, Inoue K, Ichinose T, Yanagisawa R, Tomura S, Cho AK, Froines JR, and Kumagai Y**. Effects of a single intratracheal administration of phenanthraquinone on murine lung. *J Appl Toxicol* 25: 47-51, 2005.

66. **Hiyoshi K, Takano H, Inoue KI, Ichinose T, Yanagisawa R, Tomura S, and Kumagai Y**. Effects of phenanthraquinone on allergic airway inflammation in mice. *Clin Exp Allergy* 35: 1243-1248, 2005.

67. **Hunter T, and Karin M**. The regulation of transcription by phosphorylation. *Cell* 70: 375-387, 1992.

68. **Janssen NA, Brunekreef B, van Vliet P, Aarts F, Meliefste K, Harssema H, and Fischer P**. The relationship between air pollution from heavy traffic and allergic sensitization, bronchial hyperresponsiveness, and respiratory symptoms in Dutch schoolchildren. *Environ Health Perspect* 111: 1512-1518, 2003.

69. **Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, and Bonnefoy JY**. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 160: 3555-3561, 1998.

70. **Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, and Han J**. Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J Biol Chem* 272: 30122-30128, 1997.

71. **Joost J. Oppenheim MF**. Introduction to the role of cytokines in innate host defense and adaptive immunity. *Cytokine References, academic press* 3-20, 2001.

72. **Karin M, and Smeal T**. Control of transcription factors by signal transduction pathways: the beginning of the end. *Trends Biochem Sci* 17: 418-422, 1992.

73. **Kawasaki H, Schiltz L, Chiu R, Itakura K, Taira K, Nakatani Y, and Yokoyama KK**. ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* 405: 195-200, 2000.

74. Kawasaki S, Takizawa H, Takami K, Desaki M, Okazaki H, Kasama T, Kobayashi K, Yamamoto K, Nakahara K, Tanaka M, Sagai M, and Ohtoshi T. Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 24: 419-426, 2001.

75. **Kikuno S, Taguchi K, Iwamoto N, Yamano S, Cho AK, Froines JR, and Kumagai Y**. 1,2-Naphthoquinone activates vanilloid receptor 1 through increased protein tyrosine phosphorylation, leading to contraction of guinea pig trachea. *Toxicol Appl Pharmacol* 210: 47-54, 2006.

76. **Kim S, Shim JJ, Burgel PR, Ueki IF, Dao-Pick T, Tam DC, and Nadel JA**. IL-13-induced Clara cell secretory protein expression in airway epithelium: role of EGFR signaling pathway. *Am J Physiol Lung Cell Mol Physiol* 283: L67-75, 2002.

77. **Kumagai Y, Arimoto T, Shinyashiki M, Shimojo N, Nakai Y, Yoshikawa T, and Sagai M**. Generation of reactive oxygen species during interaction of diesel exhaust particle components with NADPH-cytochrome P450 reductase and involvement of the bioactivation in the DNA damage. *Free Radic Biol Med* 22: 479-487, 1997.

78. **Kyriakis JM, and Avruch J**. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81: 807-869, 2001.

79. **Lai YH, and Mosmann TR**. Mouse IL-13 enhances antibody production in vivo and acts directly on B cells in vitro to increase survival and hence antibody production. *J Immunol* 162: 78-87, 1999.

80. **Laoukili J, Perret E, Willems T, Minty A, Parthoens E, Houcine O, Coste A, Jorissen M, Marano F, Caput D, and Tournier F**. IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. *J Clin Invest* 108: 1817-1824, 2001.

81. **Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, and Lo D**. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* 162: 2477-2487, 1999.

82. **Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di Stefano E, Slaughter N, Killeen E, Wang X, Huang A, Wang M, Miguel AH, Cho A, Sioutas C, and Nel AE.** Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. *J Immunol* 173: 3467-3481, 2004.

83. **Li N, Hao M, Phalen RF, Hinds WC, and Nel AE**. Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clin Immunol* 109: 250-265, 2003.

84. **Li N, Wang M, Oberley TD, Sempf JM, and Nel AE**. Comparison of the pro-oxidative and proinflammatory effects of organic diesel exhaust particle chemicals in bronchial epithelial cells and macrophages. *J Immunol* 169: 4531-4541, 2002.

85. **Li N, Venkatesan MI, Miguel A, Kaplan R, Gujuluva C, Alam J, and Nel A**. Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. *J Immunol* 165: 3393-3401, 2000.

86. **Manthey CL, Wang SW, Kinney SD, and Yao Z**. SB202190, a selective inhibitor of p38 mitogen-activated protein kinase, is a powerful regulator of LPS-induced mRNAs in monocytes. *J Leukoc Biol* 64: 409-417, 1998.

87. **Matsushima HIaK**. IL-8. *Cytokine References, academic press* 1062-1067.

88. **Miyabara Y, Ichinose T, Takano H, and Sagai M**. Diesel exhaust inhalation enhances airway hyperresponsiveness in mice. *Int Arch Allergy Immunol* 116: 124-131, 1998.

89. **Miyabara Y, Takano H, Ichinose T, Lim HB, and Sagai M**. Diesel exhaust enhances allergic airway inflammation and hyperresponsiveness in mice. *Am J Respir Crit Care Med* 157: 1138-1144, 1998.

90. **Mudway IS, Stenfors N, Duggan ST, Roxborough H, Zielinski H, Marklund SL, Blomberg A, Frew AJ, Sandstrom T, and Kelly FJ**. An in vitro and in vivo investigation of the effects of diesel exhaust on human airway lining fluid antioxidants. *Arch Biochem Biophys* 423: 200-212, 2004.

91. **Muranaka M, Suzuki S, Koizumi K, Takafuji S, Miyamoto T, Ikemori R, and Tokiwa H**. Adjuvant activity of diesel-exhaust particulates for the production of IgE antibody in mice. *J Allergy Clin Immunol* 77: 616-623, 1986.

92. **Nightingale JA, Maggs R, Cullinan P, Donnelly LE, Rogers DF, Kinnersley R, Chung KF, Barnes PJ, Ashmore M, and Newman-Taylor A**. Airway inflammation after controlled exposure to diesel exhaust particulates. *Am J Respir Crit Care Med* 162: 161-166, 2000.

93. **Nordenhall C, Pourazar J, Blomberg A, Levin JO, Sandstrom T, and Adelroth E**. Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. *Eur Respir J* 15: 1046-1051, 2000.

94. **Nordenhall C, Pourazar J, Ledin MC, Levin JO, Sandstrom T, and Adelroth E**. Diesel exhaust enhances airway responsiveness in asthmatic subjects. *Eur Respir J* 17: 909-915, 2001.

95. **Oberdorster G, and Utell MJ**. Ultrafine particles in the urban air: to the respiratory tract--and beyond? *Environ Health Perspect* 110: A440-441, 2002.

96. **Ohtoshi T, Takizawa H, Okazaki H, Kawasaki S, Takeuchi N, Ohta K, and Ito K**. Diesel exhaust particles stimulate human airway epithelial cells to produce cytokines relevant to airway inflammation in vitro. *J Allergy Clin Immunol* 101: 778-785, 1998.

97. **Pabo CO, and Sauer RT**. Transcription factors: structural families and principles of DNA recognition. *Annu Rev Biochem* 61: 1053-1095, 1992.

98. **Partrick DA, Moore EE, Offner PJ, Meldrum DR, Tamura DY, Johnson JL, and Silliman CC**. Maximal human neutrophil priming for superoxide production and elastase release requires p38 mitogen-activated protein kinase activation. *Arch Surg* 135: 219-225, 2000.

99. **Peters JM, Avol E, Gauderman WJ, Linn WS, Navidi W, London SJ, Margolis H, Rappaport E, Vora H, Gong H, Jr., and Thomas DC**. A study of twelve Southern California communities with differing levels and types of air pollution. II. Effects on pulmonary function. *Am J Respir Crit Care Med* 159: 768-775, 1999.

100. **Pilewski JM, and Albelda SM**. Adhesion molecules in the lung. An overview. *Am Rev Respir Dis* 148: S31-37, 1993.

101. **Pomerance M, Multon MC, Parker F, Venot C, Blondeau JP, Tocque B, and Schweighoffer F**. Grb2 interaction with MEK-kinase 1 is involved in regulation of Jun-kinase activities in response to epidermal growth factor. *J Biol Chem* 273: 24301-24304, 1998.

102. **Pope CA, 3rd, Burnett RT, Thurston GD, Thun MJ, Calle EE, Krewski D, and Godleski JJ**. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation* 109: 71-77, 2004.

103. **Pope CA, 3rd, and Dockery DW**. Acute health effects of PM10 pollution on symptomatic and asymptomatic children. *Am Rev Respir Dis* 145: 1123-1128, 1992.

104. **Ptashne M, and Gann A**. Transcriptional activation by recruitment. *Nature* 386: 569-577, 1997.

105. **Rao GN**. Hydrogen peroxide induces complex formation of SHC-Grb2-SOS with receptor tyrosine kinase and activates Ras and extracellular signal-regulated protein kinases group of mitogen-activated protein kinases. *Oncogene* 13: 713-719, 1996.

106. **Reibman J, Hsu Y, Chen LC, Kumar A, Su WC, Choy W, Talbot A, and Gordon T**. Size fractions of ambient particulate matter induce granulocyte macrophage colony-stimulating factor in human bronchial epithelial cells by mitogen-activated protein kinase pathways. *Am J Respir Cell Mol Biol* 27: 455-462, 2002.

107. **Robinson MJ, and Cobb MH**. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9: 180-186, 1997.

108. **Roos-Engstrand E, Wallin A, Bucht A, Pourazar J, Sandstrom T, and Blomberg A**. Increased expression of p38 MAPK in human bronchial epithelium after lipopolysaccharide exposure. *Eur Respir J* 25: 797-803, 2005.

109. **Rudell B, Blomberg A, Helleday R, Ledin MC, Lundback B, Stjernberg N, Horstedt P, and Sandstrom T**. Bronchoalveolar inflammation after exposure to diesel exhaust: comparison between unfiltered and particle trap filtered exhaust. *Occup Environ Med* 56: 527-534, 1999.

110. **Rudell B, Sandstrom T, Hammarstrom U, Ledin ML, Horstedt P, and Stjernberg N**. Evaluation of an exposure setup for studying effects of diesel exhaust in humans. *Int Arch Occup Environ Health* 66: 77-83, 1994.

111. **Sagai M, Saito H, Ichinose T, Kodama M, and Mori Y**. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radic Biol Med* 14: 37-47, 1993.

112. **Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, and Frew A**. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med* 159: 702-709, 1999.

113. Salvi S, Semper A, Blomberg A, Holloway J, Jaffar Z, Papi A, Teran L, Polosa R, Kelly F, Sandstrom T, Holgate S, and Frew A. Interleukin-5 production by human airway epithelial cells. *Am J Respir Cell Mol Biol* 20: 984-991, 1999.

114. **Samet JM, and Pope CA, 3rd**. Epidemiologic research needs for particulate air pollution. *J Toxicol Environ Health A* 66: 1873-1876, 2003.

115. **Sandström T BA, Helleday R, Rudell B**. Allergy and automobile pollutants: experiments in animals. *Rev Francaise d´allergologie* 40(1). : 47-51, 2000.

116. **Scheepers PT, and Bos RP**. Combustion of diesel fuel from a toxicological perspective. I. Origin of incomplete combustion products. *Int Arch Occup Environ Health* 64: 149-161, 1992.

117. **Scheepers PT, and Bos RP**. Combustion of diesel fuel from a toxicological perspective. II. Toxicity. *Int Arch Occup Environ Health* 64: 163-177, 1992.

118. **Schwartz J, and Dockery DW**. Increased mortality in Philadelphia associated with daily air pollution concentrations. *Am Rev Respir Dis* 145: 600-604, 1992.

119. **Schwartz J, Laden F, and Zanobetti A**. The concentration-response relation between PM(2.5) and daily deaths. *Environ Health Perspect* 110: 1025-1029, 2002.

120. **Schwartz J, Litonjua A, Suh H, Verrier M, Zanobetti A, Syring M, Nearing B, Verrier R, Stone P, MacCallum G, Speizer FE, and Gold DR.** Traffic related pollution and heart rate variability in a panel of elderly subjects. *Thorax* 60: 455-461, 2005.

121. **Shim JJ, Dabbagh K, Ueki IF, Dao-Pick T, Burgel PR, Takeyama K, Tam DC, and Nadel JA**. IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol* 280: L134-140, 2001.

122. **Siebenlist U, Franzoso G, and Brown K**. Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol* 10: 405-455, 1994.

123. **Snipes MB**. Long-term retention and clearance of particles inhaled by mammalian species. *Crit Rev Toxicol* 20: 175-211, 1989.

124. **Spix C, Anderson HR, Schwartz J, Vigotti MA, LeTertre A, Vonk JM, Touloumi G, Balducci F, Piekarski T, Bacharova L, Tobias A, Ponka A, and Katsouyanni K.** Short-term effects of air pollution on hospital admissions of respiratory diseases in Europe: a quantitative summary of APHEA study results. Air Pollution and Health: a European Approach. *Arch Environ Health* 53: 54-64, 1998.

125. **Squadrito GL, Cueto R, Dellinger B, and Pryor WA**. Quinoid redox cycling as a mechanism for sustained free radical generation by inhaled airborne particulate matter. *Free Radic Biol Med* 31: 1132-1138, 2001.

126. **Steerenberg PA, Zonnenberg JA, Dormans JA, Joon PN, Wouters IM, van Bree L, Scheepers PT, and Van Loveren H**. Diesel exhaust particles induced release of interleukin 6 and 8 by (primed) human bronchial epithelial cells (BEAS 2B) in vitro. *Exp Lung Res* 24: 85-100, 1998.

127. **Stenfors N, Nordenhall C, Salvi SS, Mudway I, Soderberg M, Blomberg A, Helleday R, Levin JO, Holgate ST, Kelly FJ, Frew AJ, and Sandstrom T.** Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. *Eur Respir J* 23: 82-86, 2004.

128. **Stenfors N, Pourazar J, Blomberg A, Krishna MT, Mudway I, Helleday R, Kelly FJ, Frew AJ, and Sandstrom T**. Effect of ozone on bronchial mucosal inflammation in asthmatic and healthy subjects. *Respir Med* 96: 352-358, 2002.

129. **Stohs SJ, and Bagchi D**. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* 18: 321-336, 1995.

130. **Suzuki T, Okuno H, Yoshida T, Endo T, Nishina H, and Iba H**. Difference in transcriptional regulatory function between c-Fos and Fra-2. *Nucleic Acids Res* 19: 5537-5542, 1991.

131. **Sydbom A, Blomberg A, Parnia S, Stenfors N, Sandstrom T, and Dahlen SE**. Health effects of diesel exhaust emissions. *Eur Respir J* 17: 733-746, 2001.

132. **Takano H, Ichinose T, Miyabara Y, Yoshikawa T, and Sagai M**. Diesel exhaust particles enhance airway responsiveness following allergen exposure in mice. *Immunopharmacol Immunotoxicol* 20: 329-336, 1998.

133. **Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, and Sagai M**. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. *Am J Respir Crit Care Med* 156: 36-42, 1997.

134. **Takenaka H, Zhang K, Diaz-Sanchez D, Tsien A, and Saxon A**. Enhanced human IgE production results from exposure to the aromatic hydrocarbons from diesel exhaust: direct effects on B-cell IgE production. *J Allergy Clin Immunol* 95: 103-115, 1995.

135. **Takizawa H, Abe S, Okazaki H, Kohyama T, Sugawara I, Saito Y, Ohtoshi T, Kawasaki S, Desaki M, Nakahara K, Yamamoto K, Matsushima K, Tanaka M, Sagai M, and Kudoh S.** Diesel exhaust particles upregulate eotaxin gene expression in human bronchial epithelial cells via nuclear factor-kappa B-dependent pathway. *Am J Physiol Lung Cell Mol Physiol* 284: L1055-1062, 2003.

136. **Takizawa H, Ohtoshi T, Kawasaki S, Abe S, Sugawara I, Nakahara K, Matsushima K, and Kudoh S**. Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: a review. *Respirology* 5: 197-203, 2000.

137. **Takizawa H, Ohtoshi T, Kawasaki S, Kohyama T, Desaki M, Kasama T, Kobayashi K, Nakahara K, Yamamoto K, Matsushima K, and Kudoh S.** Diesel exhaust particles induce NF-kappa B activation in human bronchial epithelial cells in vitro: importance in cytokine transcription. *J Immunol* 162: 4705-4711, 1999.

138. **Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, and Gutkind JS**. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. *J Biol Chem* 271: 27225-27228, 1996.

139. **Tobias HJ, Beving DE, Ziemann PJ, Sakurai H, Zuk M, McMurry PH, Zarling D, Waytulonis R, and Kittelson DB**. Chemical analysis of diesel engine nanoparticles using a nano-DMA/thermal desorption particle beam mass spectrometer. *Environ Sci Technol* 35: 2233-2243, 2001.

140. **Tominaga K, Yoshimoto T, Torigoe K, Kurimoto M, Matsui K, Hada T, Okamura H, and Nakanishi K**. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *Int Immunol* 12: 151-160, 2000.

141. **Tsien A, Diaz-Sanchez D, Ma J, and Saxon A**. The organic component of diesel exhaust particles and phenanthrene, a major polyaromatic hydrocarbon constituent,

enhances IgE production by IgE-secreting EBV-transformed human B cells in vitro. *Toxicol Appl Pharmacol* 142: 256-263, 1997.

142. **Tyner JW, Kim EY, Ide K, Pelletier MR, Roswit WT, Morton JD, Battaile JT, Patel AC, Patterson GA, Castro M, Spoor MS, You Y, Brody SL, and Holtzman MJ.** Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. *J Clin Invest* 116: 309-321, 2006.

143. **van Vliet P, Knape M, de Hartog J, Janssen N, Harssema H, and Brunekreef B**. Motor vehicle exhaust and chronic respiratory symptoms in children living near freeways. *Environ Res* 74: 122-132, 1997.

144. **Vedal S, Petkau J, White R, and Blair J**. Acute effects of ambient inhalable particles in asthmatic and nonasthmatic children. *Am J Respir Crit Care Med* 157: 1034-1043, 1998.

145. **Wells A**. EGF receptor. *Int J Biochem Cell Biol* 31: 637-643, 1999.

146. **Whitmarsh AJ, and Davis RJ**. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* 74: 589-607, 1996.

147. **Wu W, Graves LM, Jaspers I, Devlin RB, Reed W, and Samet JM**. Activation of the EGF receptor signaling pathway in human airway epithelial cells exposed to metals. *Am J Physiol* 277: L924-931, 1999.

148. **Wu W, Jaspers I, Zhang W, Graves LM, and Samet JM**. Role of Ras in metal-induced EGF receptor signaling and NF-kappaB activation in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 282: L1040-1048, 2002.

149. **Wu W, Samet JM, Ghio AJ, and Devlin RB**. Activation of the EGF receptor signaling pathway in airway epithelial cells exposed to Utah Valley PM. *Am J Physiol Lung Cell Mol Physiol* 281: L483-489, 2001.

150. **Xia T, Korge P, Weiss JN, Li N, Venkatesen MI, Sioutas C, and Nel A**. Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity. *Environ Health Perspect* 112: 1347-1358, 2004.

151. **Xiong C, and Friedlander SK**. Morphological properties of atmospheric aerosol aggregates. *Proc Natl Acad Sci U S A* 98: 11851-11856, 2001.

152. **Yang HM, Antonini JM, Barger MW, Butterworth L, Roberts BR, Ma JK, Castranova V, and Ma JY**. Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. *Environ Health Perspect* 109: 515-521, 2001.

153. **Yang M, Hogan SP, Henry PJ, Matthaei KI, McKenzie AN, Young IG, Rothenberg ME, and Foster PS**. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. *Am J Respir Cell Mol Biol* 25: 522-530, 2001.

154. **Yousefi S, Hemmann S, Weber M, Holzer C, Hartung K, Blaser K, and Simon HU**. IL-8 is expressed by human peripheral blood eosinophils. Evidence for increased secretion in asthma. *J Immunol* 154: 5481-5490, 1995.

155. **Zandi E, Rothwarf DM, Delhase M, Hayakawa M, and Karin M**. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* 91: 243-252, 1997.

156. **Zanobetti A, Schwartz J, and Dockery DW**. Airborne particles are a risk factor for hospital admissions for heart and lung disease. *Environ Health Perspect* 108: 1071-1077, 2000.

157. **Zhang Q, Kleeberger SR, and Reddy SP**. DEP-induced fra-1 expression correlates with a distinct activation of AP-1-dependent gene transcription in the lung. *Am J Physiol Lung Cell Mol Physiol* 286: L427-436, 2004.

158. **Zhou L, Tan A, Iasvovskaia S, Li J, Lin A, and Hershenson MB**. Ras and mitogen-activated protein kinase kinase kinase-1 coregulate activator protein-1- and nuclear factor-kappaB-mediated gene expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 28: 762-769, 2003.

159. **Zu YL, Qi J, Gilchrist A, Fernandez GA, Vazquez-Abad D, Kreutzer DL, Huang CK, and Sha'afi RI**. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. *J Immunol* 160: 1982-1989, 1998.

160. **Zweidinger RB**. Emission factors from diesel and gasoline powered vehicles: correlation with the Ames test. *Dev Toxicol Environ Sci* 10: 83-96, 1982.