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Ozone and Diesel Exhaust

Airway Signaling, Inflammation and Pollutant Interactions

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To Jason, Basil, Mom and Dad

Words ought to be a little wild for they are the assaults of thought on the unthinking.
John Maynard Keynes

We rarely think people have good sense unless they agree with us.
Francois de La Rochefoucauld

My goal in life is to be as good of a person my dog already thinks I am.
Anonymous

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ORIGINAL PAPERS

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I. **Bosson J, Stenfors N, Bucht A, Helleday R, Pourazar J, Holgate ST, Kelly FJ, Sandström T, Wilson S, Frew AJ, Blomberg A.**
Ozone-induced bronchial epithelial cytokine expression differs between healthy and asthmatic subjects
Clin Exp Allergy 2003;33:777-782

II. **Bosson J, Blomberg A, Pourazar J, Mudway I, Frew AJ, Kelly FJ, Sandström T.**
Early suppression of NFκB and IL-8 bronchial epithelium after ozone exposure in healthy human subjects
Submitted

III. **Bosson J, Pourazar J, Forsberg B, Ädelroth E, Sandström T, Blomberg A.**
Ozone enhances the airway inflammation initiated by diesel exhaust.
Respir Med 2006;Dec 28 *(E-pub ahead of print)*

IV. **Bosson J, Barath S, Pourazar J, Sandström T, Blomberg A, Ädelroth E.**
Diesel exhaust exposure enhances the ozone induced airway inflammation in healthy humans.
Submitted

ABSTRACT

It is well established that air pollution has detrimental effects on both human health as well as the environment. Exposure to ozone and particulate matter pollution, is associated with an increase in cardiopulmonary mortality and morbidity. Asthmatics, elderly and children have been indicated as especially sensitive groups. With a global increase in use of vehicles and industry, ambient air pollution represents a crucial health concern as well as a political, economical and environmental dilemma.

Both ozone (O_3) and diesel exhaust (DE) trigger oxidative stress and inflammation in the airways, causing symptoms such as wheezing, coughing and reduced lung function. The aim of this thesis was to further examine which pro-inflammatory signaling pathways that are initiated in the airways by ozone, as compared to diesel exhaust. Furthermore, to study the effects of these two ambient air pollutants in a sequential exposure, thus mimicking an urban profile. In order to investigate this in healthy as well as asthmatic subjects, walk-in exposure chambers were utilized and various airway compartments were studied by obtaining induced sputum, endobronchial biopsies, or airway lavage fluids.

In asthmatic subjects, exposure to 0.2 ppm of O_3 induced an increase in the cytokines IL-5, GM-CSF and ENA-78 in the bronchial epithelium six hours post-exposure. The healthy subjects, however, displayed no elevations of bronchial epithelial cytokine expression in response to the ozone exposure. The heightened levels of neutrophil chemoattractants and Th2 cytokines in the asthmatic airway epithelium may contribute to symptom exacerbations following air pollution exposure.

When examining an earlier time point post O_3 exposure (1½ hours), healthy subjects exhibited a suppression of IL-8 as well as of the transcription factors NFκB and c-jun in the bronchial epithelium, as opposed to after filtered air exposure. This inhibition of early signal transduction in the bronchial epithelium after O_3 differs from the response detected after exposure to DE.

Since both O_3 and DE are associated with generating airway neutrophilia as well as causing direct oxidative damage, it raises the query of whether daily exposure to these two air pollutants creates a synergistic or additive effect. Induced sputum attained from healthy subjects exposed in sequence to 0.2 ppm of O_3 five hours following DE at a PM concentration of 300 $\mu g/m^3$, demonstrated significantly increased neutrophils, and elevated MPO levels, as compared to the sequential DE and filtered air exposure.

O_3 and DE interactions were further investigated by analyses of bronchoalveolar lavage and bronchial wash. It was demonstrated that pre-exposure to DE, as compared to filtered air, enhances the O_3-induced airway inflammation, in terms of an increase in neutrophil and macrophage numbers in BW and higher EPX expression in BAL.

In conclusion, this thesis has aspired to expand the knowledge of O_3-induced inflammatory pathways in humans, observing a divergence to the previously described DE initiated responses. Moreover, a potentially adverse airway inflammation augmentation has been revealed after exposure to a relevant ambient combination of these air pollutants. This provides a foundation towards an understanding of the cumulative airway effects when exposed to a combination of ambient air pollutants and may have implications regarding future regulations of exposure limits.

Keywords: air pollution, ozone, diesel exhaust, airway inflammation, asthma, immunohistochemistry

SVENSK SAMMANFATTNING

De negativa miljö- och hälsoeffekter, som orsakas av trafikrelaterade luftföroreningar såsom dieselavgaser och ozon uppmärksammas alltmer. Sedan 1950-talet har epidemiologiska studier sammankopplat dessa luftföroreningar med ökad sjuklighet och dödlighet i såväl lung- som hjärt/kärlsjukdomar. I experimentella studier har både dieselavgaser och ozon visat sig leda till inflammation i luftvägarna. Barn samt individer med astma, KOL och hjärt/kärlsjukdomar är särskilt känsliga grupper och påverkas mer än friska individer.

Målsättningen med detta avhandlingsarbete har varit att undersöka vilka signaleringsvägar i luftvägsslemhinnan som ligger bakom den luftvägsinflammation som orsakas av ozon, men även att undersöka om det finns en koppling till de sedan tidigare kända bakomliggande mekanismerna till luftvägsinflammation orsakad av dieselavgaser. Ett ytterligare syfte var att studera huruvida exponering för dieselavgaser följd av en ozonexponering ger ett förstärkt inflammatoriskt svar i luftvägarna. Exponering för de båda luftföroreningarna gjordes i avsikt att efterlikna det mönster som finns i storstadstrafik - hög halt av dieselavgaser under rusningstrafik på morgonen följt av ökad ozonhalt på eftermiddagen.

Både dieselavgaser och ozon är oxidanter som kan övervinna antioxidantförsvaret i luftvägarna. Detta leder till produktion fria radikaler, och en obalans mellan oxidanter och antioxidantförsvar ger upphov till så kallad oxidativ stress. Få humana studier har tidigare utforskat de signalvägar som aktiveras vid luftvägsinflammation inducerad av dessa luftföroreningar, i synnerhet när det gäller ozon. Man har dock sett att dieselavgaser ger en ökning av oxidativ stress-känsliga transkriptionsfaktorer i luftvägsslemhinnan.

Alla exponeringar utfördes i särskilda exponeringskammare. Luftvägseffekterna av såväl dieselavgaser som ozon jämfördes med effekter av exponering för filtrerad luft.

Material för analys insamlades genom bronkoskopi med slemhinnebiopsier och sköljvätska (bronchial wash (BW) och bronkoalveolärt lavage (BAL) från luftrören, men även genom upphostat slem (inducerat sputum).

Samtliga exponeringar för dieselavgaser gjordes vid en partikelkoncentration på 300 $\mu g/m^3$ och pågick under 1 timme. Ozonkoncentrationen var 0,2 ppm och exponeringen 2 timmar. Båda koncentrationerna är relevanta för de som kan finnas i naturlig trafikmiljö, och har i tidigare studier visat sig ge inflammatoriska förändringar i luftvägarna.

Studie I avsåg att undersöka om graden av cytokinuttryck i luftvägsepitelet efter ozonexponering skiljde sig mellan friska individer och astmatiker. Sex timmar efter ozonexponering fann man hos astmatiker en ökning av neutrofilattraherande och

Th2- relaterade cytokiner. Ökningen av dessa proinflammatoriska signalmolekyler kan indikera att neutrofila och eosinofila celler kommer att rekryteras till luftvägarna. Detta skulle kliniskt kunna innebära att en astmatiker kan försämras i sin astma. Hos friska individer noterades däremot ingen ökning av signalämnen efter ozonexponering.

I Studie II var avsikten att utforska om det förelåg en tidig ökning av inflammationsreglerande signalvägar i luftvägsepitelet hos friska personer efter ozonexponering. En och en halv timme efter exponering för ozon noterades dock i stället ett minskat uttryck för signalvägarna NFκB, c-jun och IL-8, med en liknande tendens minskning av de flesta undersökta inflammatoriska markörerna. Denna hämning av signalvägar, som är associerade med oxidativ stress, visar på en tydlig skillnad i inflammatorisk signaltransduktion i luftvägarna mellan dieselavgaser och ozon.

Studie III avsåg att klargöra om det finns ett ökat luftvägsinflammatoriskt svar vid en kombinerad exponering för dieselavgaser och ozon, i strävan att efterlikna en realistisk trafikmiljö i storstad. Försökspersonerna genomgick två separata exponeringsserier bestående av exponering för dieselavgaser på morgonen och fem timmar senare exponering för antingen filtrerad luft eller ozon. Inducerat sputum (upphostat slem) uppsamlades 18 timmar efter det att den andra exponeringen var avslutad. Studien visade att exponering för dieselavgaser med efterföljande exponering för ozon gav en ökning av neutrofila celler och myeloperoxidas (MPO) och därmed tecken till neutrofilaktivering i luftvägarna. Fynden tyder på att ozon intensifierar den tidigare dieselavgasinducerade luftvägsinflammationen.

I Studie IV undersöktes vidare effekterna av dieselavgaser i kombination med ozonexponering. Här gjordes först en morgonexponering för dieselavgaser eller filtrerad luft som fem timmar senare följdes av ozonexponering. Bronkoskopi med luftvägssköljning utfördes 18 timmar efter avslutad ozonexponering. Resultaten visade att dieselexponering följt av ozonexponering gav en ökning av neutrofila celler i de större luftrören (BW). I de perifera luftvägarna (BAL) fanns ökade mängder av EPX, ett protein som indikerar aktivering av de eosinofila cellerna. Dessa fynd styrker hypotesen att exponering för en kombination av luftföroreningar ger en ökad luftvägsinflammation.

Denna avhandling har bidragit till att öka kunskapen om hur de mänskliga luftvägarna påverkas av exponering för oxidativa luftföroreningar. Studierna har undersökt signaleringsvägar vid ozonutlöst luftvägsinflammation och tidsaspekter härvidlag. Vidare har studierna indikerat att det finns betydande skillnader mellan de inflammatoriska signaleringsvägarna efter exponering för ozon jämfört med dieselavgaser. Dessutom visades att luftvägsinflammationen förstärktes av en kombination av exponering för dieselavgaser och ozon liknande den som finns i storstädernas trafikmiljö.

Fynden i denna avhandling utgör underlag till att vidare utforska och förstå de mekanismer som orsakar de negativa hälsoeffekterna av luftföroreningar.

LIST OF SELECTED ABBREVIATIONS

Δ	Delta, the change or difference between mathematical values
AP-1	Activator protein-1
BAL	Bronchoalveolar lavage
BW	Bronchial wash
CO, CO_2	Carbon monoxide, Carbon dioxide
COPD	Chronic obstructive pulmonary disease
DE	Diesel exhaust
DEPs	Diesel exhasut particles
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbant assay
ENA-78	Epithelial neutrophil activating peptide 78
EPA	Environmental Protection Agency
EPX	Eosinophil protein X
ERK	Extracellular signal regulated kinase
EU	European Union
FEF	Forced expiratory flow
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GAG	Glycosaminoglycan
GMA	Glycolmethacrylate
GM-CSF	Granulocyte/macrophage colony stimulating factor
Gro-α	Growth-related oncogene alpha
GSH	Reduced Glutathione
HNL	Human neutrophil lipocalin
H_2O_2	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule-1
IgE	Immunoglobulin E
IKKβ	IκB kinase β
IκB	Inhibitory kappa B
IL-	Interleukin
IQR	Interquartile range

JNK	Jun N-terminal kinase
LDH	Lactate dehydrogenase
LTB_4	Leukotriene B4
MAPK	Mitogen activating protein kinase
MIP-2	Macrophage inflammatory protein-2
MPO	Myeloperoxidase
MMP-9	Matrix metalloproteinase-9
1-NN	1- nitronaphthalene
NADPH-oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-IL-6	Nuclear factor IL-6
NFκB	Nuclear factor kappa B
NO, NO_x	Nitric oxide, Oxides of nitrogen
O_3	Ozone
·OH	Hydroxyl radical
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E2
PM	Particulate matter
PM_{10}	Particulate matter with an aerodynamic diameter of less than 10 μm
$PM_{2.5}$	Particulate matter with an aerodynamic diameter of less than 2.5 μm
PMNs	Polymorphonuclear neutrophilic cells (neutrophils)
ppb	Parts per billion
ppm	Parts per million
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
ROS	Reactive Oxygen Species
RTLF	Respiratory Tract Lining Fluid
TBS	TRIS-buffered saline;
TBST	TRIS-buffered saline with added Triton-X-100
Th2	T helper cell type 2
TNF-α	Tumor Necrosis Factor alpha
V_E	Volume of gas expired
VOC	Volatile Organic Compounds
WHO	World Health Organisation

INTRODUCTION

AMBIENT AIR POLLUTION

Recent years have seen a mounting concern about the environmental and health related impacts of air pollution. The air surrounding us is becoming an increasing threat and is the source of many harmful substances that may enter our bodies through the nose, mouth, skin, and the digestive tract. The World Health Organization (WHO) has estimated that 2 million people every year die prematurely as a consequence of urban air pollution [193].

Increased traffic, expanding cities, and industrialization have all contributed to a rise in emissions and growing levels of air pollution. Outdoor ambient air pollution has serious implications on respiratory health as well as the environment. Pollutants are linked to the destruction of ecosystems, formation of acid rain and instigators of global climate changes. Therefore making pollution not only an environmental and health problem, but also a hot political and economical issue

Two major contributors to traffic related air pollution are ozone (O_3) and diesel exhaust (DE). Many countries around the world have no regulations in effect for limiting these pollutants. However, even in countries where national standards exist, these are often exceeded. The American Lung Association has reported that almost 50% of Americans live in regions with health-threatening levels of ozone pollution and more than one-quarter live in areas where particle pollution reach harmful levels [7]. China, which has seen a doubling of vehicles within the last five years, is currently home to 16 of the planet's 20 most air-polluted cities [4]. In the EU in 2002, 12 of the 15 member states surpassed the limit values for the year. This indicates an increasing need not only for worldwide guidelines, but also the importance for achieving stricter adherence to air quality regulation already set in place.

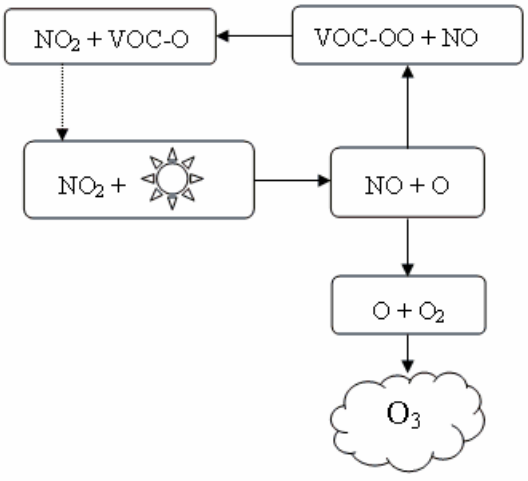
In general, air pollution is classified into two groups; direct release or subsequent air pollutants. The former refers to air pollutants that are directly emitted from a source, all of which are byproducts of combustion, such as carbon monoxide or sulfur dioxide. Whereas subsequent air pollutants are created in the atmosphere through chemical reactions involving direct release pollutants, where photochemical formation of ozone is a key example. Numerous combustion, atmospheric and meteorological conditions influence this complex mechanism of secondary aerosol formation [108, 198].

OZONE

Sources

Ozone (O_3), a tri-atomic odorless gas discovered in 1840, is an unstable allotrope of oxygen (O_2). It is ordinarily colorless, but turns pale blue at high concentrations. Ozone displays contrasting properties depending on where it is located in the atmosphere. The so-called 'ozone layer', otherwise known as stratospheric ozone, occurs 10-50 km above earth, where it is formed by an interaction between oxygen and ultraviolet (UV) rays. This highly concentrated, protective layer filters out approximately 90% of harmful UV wavelengths emitted from the sun. Paradoxically, tropospheric ozone, produced at ground level, is a powerful irritant and associated with numerous deleterious effects.

The infamous London smog in 1952 that is estimated to have killed 4000 people was in fact an antiquated definition of the term "smog". It was referring to a mixture of soot particles and sulfur dioxide trapped in fog, hence the name. However, in the second half of the 1950s the term came to be associated with photochemical smog, composed of a deleterious mixture of traffic related air pollutants in which tropospheric ozone is the major component. Photochemical smog also includes substances such as nitrogen oxides, volatile organic compounds (VOCs), carbon monoxide (CO), peroxyacyl nitrates (PAN), aldehydes (R'O), all highly reactive and oxidizing, as well as many being precursors to ozone. Emissions of volatile organic compounds (VOCs) and nitrogen oxides (NO_x) from such sources as motor vehicles and industrial factories create a complex cycle of photochemical reactions ultimately producing ozone.



Once it has been formed, ozone has a lifespan of 22 days, and elimination occurs by ground deposition as well as photolysis. This photodissociation in turn creates further harmful byproducts such as hydroxyl radicals and peroxacyl nitrates.

As the production of ozone is a sunlight driven reaction, it reaches its highest levels during sunny summer days as well as causing the peak concentration to occur in the afternoons. Photochemical smog tends to be regarded as a concern for most major urban centers but it can also affect sparsely populated areas since it easily travels with the wind.

Toxicology

A powerful respiratory oxidant and irritant, O_3 exists in gaseous form, allowing it to access further into lungs, thus impacting the entire respiratory tract. In the airway, ozone initially comes in contact with the respiratory tract lining fluid (RTLF). This fluid layer protects the airway epithelium and is made up of numerous constituents, such as antioxidants, various proteins and surfactant. Although ozone is a highly reactive gas, it does have a restricted aqueous solubility; therefore, unlike many other atmospheric gases, it is taken up by reactive absorption. Consequently, the amount of O_3 that will be consumed in the RTLF is directly correlated with how much oxidizable substrate is at hand [103, 139, 143]. If the oxidant exposure depletes these defenses, hence disturbing the redox environment stability, it will then go on to create secondary oxidation products via the oxidation of biomolecules such as proteins and lipids [57, 144]. These secondary oxidation products reach the epithelium, initiating an inflammatory cascade, which includes complex pathways of transcription factors, cytokines, and inflammatory cells.

The oxidative stress effects caused by ozone are thus brought about in two separate ways. Initially as an exogenous source directly reacting with the RTLF, and then indirectly via an endogenous source. This endogenous source is the formation by activated inflammatory cells of highly reactive molecules referred to as reactive oxygen species (ROS) (*Figure 1*).

This oxidant burden and ongoing airway inflammation can result in tissue damage as well as necrosis. These damaged cells are continually cleared and restored. However, it has been speculated that with continuous, chronic exposure to elevated levels of ozone, this process would create airway scarification and causing permanent respiratory consequences.

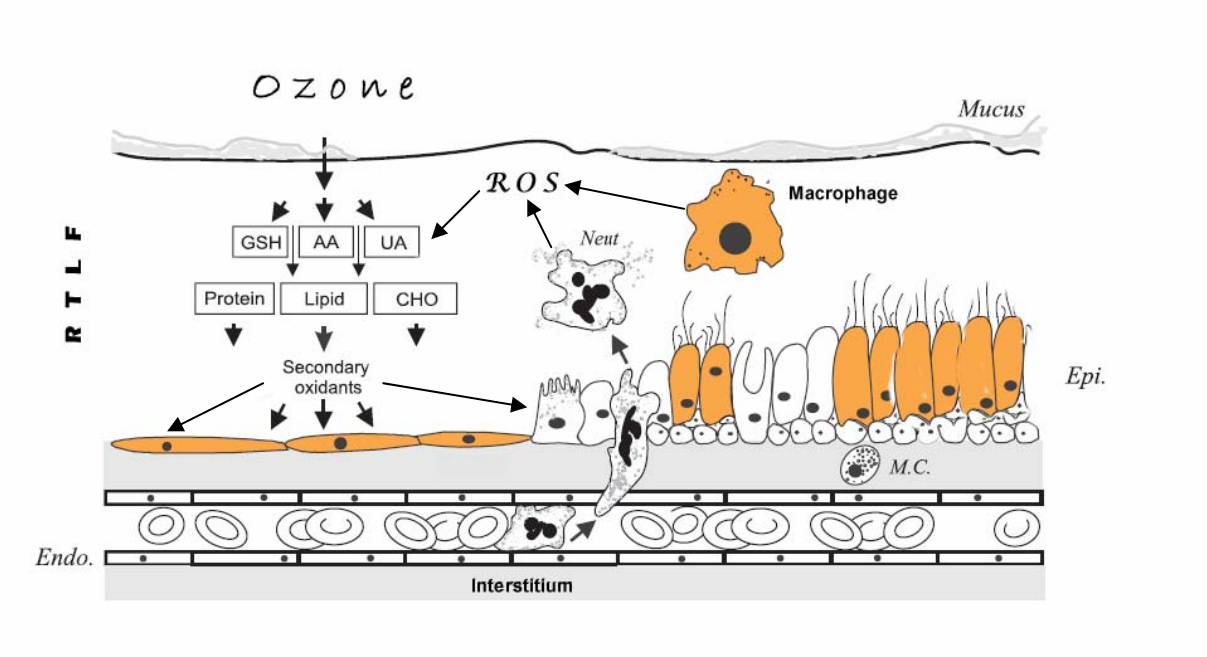


Figure 1: Schematic picture of the exogenous and endogenous oxidative stress in the airway tissues caused by ozone exposure (modified from Mudway IS, Kelly FJ. Ozone and the lung: a sensitive issue. Mol Aspects Med 2000).

Ozone and asthma

An estimated 300 million people worldwide suffer from asthma. There is a broad disparity in the prevalence of this disease when comparing individual countries, yet a clear rise in incidence has been seen on a global scale. Although the underlying reasons for this are not established, many look to the issue of air pollution as a key factor.

Childhood exposure to air pollution is of particular concern since children's lungs and immune system are not yet fully developed. The resulting damage could thus lead to persistent lifetime effects. A clear association exists between the reduction of lung function levels in children with exposure to high ozone concentrations [91, 135]. It has been observed in numerous epidemiological studies that increased ozone exposure when young heightens the risk of developing asthma, as a higher prevalence of (childhood) asthma is seen in more highly polluted areas [33, 115].

Ozone exposure has been linked with an increase in asthma-related hospitalizations and use of rescue medication [1, 61, 116, 169]. This is further supported by the observations noted during the 1996 Atlanta Olympic Games. As a result of traffic restrictions, peak ozone concentrations decreased from an average 81.3 ppb to 58.6 ppb, causing the number of asthma acute care visits and hospitalizations to decline by 44.1% [59].

Asthmatics have a chronic activity of inflammatory mediators in the bronchioles, in particular cytokines from Th2 cells. This results in an abnormal level in the airways of mast cells, eosinophils and lymphocytes. A rise in each of these cell types has

been associated with ozone exposure, giving further basis for the supposition of asthmatics being more at risk [56, 74, 85, 134].

Epidemiological studies

It has been a common praxis in air pollution epidemiological studies to focus on hospital admissions, since these are good indicators of increased morbidity as well as mortality. Total respiratory, asthma and cardiovascular admissions increase on the same day and/or on subsequent days after higher-level O_3 days, though especially with asthmatic patients, usually a one to two day lag time is observed [30, 169, 179, 190]. There is an indication that ozone exposure results in heightened susceptibility to allergens, virus and bacteria. This vulnerability may be a factor in the increased respiratory as well as cardiovascular mortality seen following periods of heightened ozone levels, particularly in already susceptible individuals such as those with asthma, COPD or pre-existing heart disease [62, 122].

As multiple aspects play a part in air pollution exposure, it is at times difficult to isolate the cause and effect association when investigating the effects of atmospheric pollutants on the respiratory system using epidemiological studies. Such confounding factors include second hand smoke, occupational exposure, and exposure to several pollutants.

Animal and in vitro studies

These studies are a valuable initial step in the investigation of the mechanistics behind tissue and cellular effects of ozone toxicity, as several logistical factors can be controlled for.

In both human cell lines and animals, ozone exposure has been found to produce epithelial injury and a subsequent acute inflammatory response in the upper and lower airway tissues. This reaction is characterized by an increase in inflammatory cells; in particular neutrophils, yet macrophages, mast cells, eosinophils and T lymphocytes also play a role [41, 92, 93, 105, 110, 184]. In conjunction, ozone exposure also elicits an enhancement of proinflammatory mediators, including TNF-α, MIP-2, IL-8, IL-6, IL-1β and ICAM-1 [37, 48, 105, 121, 180]. In vitro studies have revealed that airway epithelial cells react to ozone exposure earlier and with greater activity than macrophages. Epithelial inflammation has been found in vitro to be instigated by ozone levels as low as 0.1 ppm and as early as one hour post-exposure [44, 119].

Several studies have attempted to plot the intracellular signaling that mediates this inflammatory response, especially concerning oxidative stress. Jaspers *et al*, using a human alveolar type-II cell line exposed to 0.1 ppm of O_3, demonstrated that the DNA-binding of NFκB, NF-IL-6 and AP-1 increased, along with an enhancement of IL-8 mRNA and protein levels [84]. A following study also showed that ozone induced protein tyrosine kinase and protein kinase A [83]. It was hypothesized that this activation of protein kinases was instigated by the formation of lipid oxidation

products and reactive oxygen intermediates, thus leading to phosphorylation of transcription factors with ensuing IL-8 expression. Both lipid peroxidation and the creation of secondary free radicals have been reported after exposure to ozone [67, 88, 109, 145]. However, this comprehensive sequence of events has never been replicated in vivo.

In animals, exposure to ozone has also been linked to altered respiratory function and airway hyperresponsiveness [36, 132, 159]. These changes in pulmonary function are associated with cellular injury, influx of inflammatory cells and morphological damage. Moreover, these structural effects, including damaged mucociliary clearance cells, increase in airway permeability and impaired macrophage phagocytosis, leads to increased susceptibility to allergens, virus and bacteria [36, 105]. Ozone exposure has also been shown to alter the immunological response and demonstrates a dose-dependent increased vulnerability to infectious agents [64, 65].

Lung Function studies

Decreased lung function has been recorded in subjects exposed to O_3 concentrations above as well as below present ambient air quality standards and occupational exposure limits [54, 77]. Both FEV_1 and FVC decrements have been seen directly following ozone inhalation in asthmatics as well as in healthy. These impairments are reversible after acute exposures and return to pre-ozone values within approximately 24 hours [8, 55, 118]. Chronic ambient exposure, in particular during adolescence, result in permanently diminished levels of FEV_1, $FEF_{25–75}$ and FEF_{75} [91, 100, 173]. No discernable relationship has yet been found linking the intensity of the inflammatory response with the decrease in lung function [12, 22, 181].

Apart from susceptible groups, such as those with cardiorespiratory disease, the elderly and children, large variation in individual reactions to ozone exposure have been found to occur, involving both lung function and inflammatory consequences. Ozone responders are classified as those who experience a >15% decline from baseline in FEV_1 or a known >10% neutrophil influx following an ozone exposure (125 ppb) [56, 75, 117].

Human in vivo studies

Human exposure studies have investigated the airways from nasal passages to alveoli utilizing such methods as induced sputum, lavage fluids, and endobronchial biopsy samples. The results confirm the existence of a marked airway inflammatory reaction, associated with an increase in neutrophils, total protein, albumin, PGE_2, adhesion molecules (P-selectin, ICAM-1) and cytokines (IL-6, IL-8, GM-CSF, Gro-α) [8, 12, 21, 43, 56, 95, 97, 157, 168]. Studies in the 1990s to the present have tended to focus on relevant ambient concentrations of ozone, showing induced responses to O_3 levels as low as 0.08 ppm [43].

Evidence of early ozone reactions have been demonstrated in BAL and BW, where an immediate (≤ 1 hour) neutrophilia and increase of proinflammatory mediators such as IL-6, IL-8 and PGE_2 occurs [42, 56, 94, 157, 181]. Although, when observing ozone effects at a very early timepoint in endobronchial biopsies from healthy volunteers (1½ hours post exposure), no inflammatory cells were amplified, though a significant upregulation of P-selectin and ICAM-1 was found, indicating an initial step in neutrophil recruitment [22, 97]. The acute airway neutrophilia seen in BAL has been shown in healthy subjects to peak at approximately six hours post exposure; however significantly increased numbers have still been found in lavage after 18 to 24 hours [8, 43, 157]. In biopsies taken six hours after a 0.2 ppm O_3 challenge bronchial tissues displayed neutrophilia and continued upregulation of P-selectin and ICAM-1 [168]. This suggests that neutrophil response within the airway epithelium and submucosa may have a later culmination as compared to that seen in lavage. Further indication of this is that a continued upregulation has been demonstrated in biopsies at 18 hours, however, it should be noted that this was in response to a longer exposure time, thus creating a higher total O_3 exposure [8] (*Table 1*).

Ozone is consumed by substrates found in the RTLF, thus inducing oxidative stress on underlying tissues largely through a cascade of secondary ozonation products. The RTLF contains a variety of antioxidant defenses, including enzymatic, metal binding and low molecular weight antioxidants. The concentrations of these molecules differ between the upper and lower respiratory tract; fluid from the nasal cavity consists of large amounts of uric acid, whereas lining fluid in the lower airways mainly contains reduced glutathione (GSH) and ascorbate [90]. Therefore the consumption of RTLF antioxidants is a quantifiable marker of oxidative stress within the airways. Healthy subjects evaluated 1½ hours after a two hour exposure to 0.2 ppm of ozone displayed an increase of GSH in BAL and BW [22]. However, using an identical exposure pattern at six hours post exposure leukocytes obtained from BAL exhibited a significant ozone-induced loss of GSH [18]. Both glutathione and ascorbate concentrations in BAL and BW have been shown to be return to pre-exposure levels eighteen hours following an ozone challenge [49].

Experimental exposures in asthmatics have shown a tendency of these subjects to develop a more pronounced ozone-induced airway inflammatory response as compared to healthy individuals [14, 156]. However, previous studies have not detected a corresponding neutrophilia in the asthmatic subjects as in the healthy group at six and 18 hours post exposure [12, 168]. This finding suggests a possible discrepancy between the regulation of the O_3 induced inflammation in healthy and asthmatic subjects airways.

DIESEL EXHAUST

Sources and components

The diesel engine, patented in 1892, is today used to operate a variety of vehicles and equipment. Diesel exhaust is the product of the complete and incomplete combustion of diesel fuel, representing thousands of different chemical substances within its particle and gaseous components. Diesel exhaust particles contain a carbonaceous core which is coated by transition metals and organic chemicals, such as benzene, carbon dioxide (CO_2), formaldehyde, carbon monoxide (CO), polycyclic aromatic hydrocarbons (PAHs), sulphur dioxide, nitrogen dioxide, and nitrogen oxides (NO_x); nearly all of which are classified by the Environmental Protection Agency (EPA) as hazardous air pollutants substances.

Diesel engines are the major source of traffic related particulate matter less than 2.5 μm in diameter ($PM_{2.5}$) and ultrafine particles (<0.1 μm). Particulate matter is comprised of a suspension of diesel soot (elemental carbon) and aerosols such as ash particulates, metallic particles, sulfates, nitrates and silicates. When dispensed into the air, diesel PM can remain as individual particles or form chain aggregates, with approximately 90% in the imperceptible range of 100 nanometers.

Toxicology

Since the main particulate portion of diesel exhaust consists of ultrafine particles, they pose an eminent health risk when inhaled because of their deposition deep in the lungs, large surface area, and their possible progression into the blood stream [39, 130, 158, 161].

The large, uneven surface of ultrafine particles facilitates their combination with other toxins in the atmosphere, hence increasing the hazards of particle inhalation. PAHs and their oxidized derivatives as well as transition metals, also found in diesel exhaust, may be adsorbed onto the surface of the diesel particulate matter [53, 89, 162, 191]. An overload of particles in the airways causes impairment to pulmonary clearance, thus increasing the length of time that the airways are exposed to these harmful substances [142, 170, 171].

At high levels, diesel exhaust particles (DEPs) typically function as nonspecific airway irritants. However, at lower ambient concentrations, DEPs stimulate a release of cytokines, chemokines, immunoglobulins, and oxidants causing airway inflammation, serum leakage into the airways, as well as bronchial smooth muscle contraction in both the upper and lower airways [11, 41, 172]. Moreover, the TH_2 response phenotype, which is coupled with asthma and allergic disease, has been triggered as a result of DEP inhalation [35, 131]. Although much remains unanswered about whether the cytotoxic effects of DE are mainly driven by the particles or their surface associated chemical compounds.

Diesel exhaust is considered a significant occupational exposure hazard since a variety of professions entail a chronic exposure to the exhaust, for example bridge,

tunnel and loading dock workers, auto mechanics, toll booth collectors, truck and forklift drivers, bus drivers and people who work near areas where diesel powered vehicles are used, stored and maintained.

Epidemiological studies

Diesel exhaust research has generally concentrated on the measurement of diesel particulate matter present in the atmosphere of residential and occupational settings using such methods as gravimetric analysis. Traffic exposure has been most commonly used as a factor, since it has been difficult finding a specific and accurate biomarker or measurement of exposure in humans to focus on.

Exposures have been linked to acute short-term symptoms such as dizziness, nausea, headache, light-headedness, coughing, breathing difficulties, chest pain, and irritation of the eyes, nose and throat [9, 151]. Exposure to particulate matter has also been associated with hospitalizations and deaths due to numerous respiratory and cardiovascular diseases [46, 47]. Long-term recurrent diesel exhaust exposures can lead to chronic health problems such as cardiovascular or cardiopulmonary disease, and has also been linked to increased risk of developing lung cancer [29, 96].

Analysis regarding diesel exhaust exposure is further complicated by the fact that the exhaust components continually react with each other creating larger and more complex chemicals. As diesel exhaust is a complex mixture of particulate matter and gaseous air pollution, controlling for other interferences in the atmosphere is difficult, thus adding to the challenge to interpret its health effects with certainty.

Animal and in vitro studies

Conventionally, the results of animal studies have remained a crucial part in setting acceptable exposure limits for diesel exhaust. However, many of these studies use DE concentrations significantly higher than found in ambient air and questions still remain how well human risk can be characterized by animal findings.

Murine diesel exhaust health effects have been discovered throughout the body, affecting the reproductive system, lung, liver, skin and kidney. Within the airways much emphasis has been put into the evaluation of oxidative stress, inflammation, pro-allergic reactions and the association with asthma.

Oxidative stress has been proposed to be one of the underlying mechanisms of the detrimental effects on the airways inflicted by diesel exhaust. PM exposure is reported to lead to an excessive ROS production, arising from four possible mechanisms:

(1) Direct production induced by surface associated composites, primarily transition metals and organic molecules such as PAHs and quinones [15, 68, 99, 125, 152, 165, 183, 196].

(2) Direct production induced by the particle surface

(3) Modified function of NADPH-oxidase or mitochondria [107, 149]

(4) Activation of inflammatory cells capable of producing ROS [158]

Disproportionate ROS generation depletes the antioxidant defenses and contributes to the activation of MAPK and NFκB pathways, in turn elevating proinflammatory cytokines, chemokines and adhesion molecules. This premise is further strengthened by the evidence of intracellular triggering of redox sensitive pathways such as NFκB, p38 and JNK after DE exposure, coupled with the increased release of TNF-α, MIP-2, IL-6, IL-8, RANTES, GM-CSF, ICAM-1 and VCAM-1 [16, 17, 23, 113, 114, 146, 176, 177].

There is also evidence that DEPs are capable of directly elevating Th2 cytokine responses, and that asthmatic cells are more sensitive when it comes to releasing proinflammatory mediators. An adjuvant effect can be shown since the combination of exposure to a known allergen with a subsequent DEP challenge greatly increases the specific IgE concentration [45]. It is also been established that DEPs can adsorb aeroallergens to their surface, thus intensifying the retention in the airways. These aspects indicate a potential involvement of diesel exhaust in the pathogenesis of allergic disease. Moreover, the augmented inflammation induced by diesel exhaust exposure may alter the pulmonary susceptibility to viral or bacterial infections through modification of the lung host defense [69, 163].

Human exposure studies

Human in vivo studies have found DE toxicity to primarily affect the upper and lower airway epithelial cells, lung tissue, alveolar type II cells and the heart. Currently, there is no direct evidence of exposure to PM causing ROS production in humans. However, several recent findings demonstrate a convincing link between DE exposure and oxidative stress. CO, a marker for oxidative stress, has been found to be increased after DEP exposure in healthy [126]. In a recent study, short-term exposure to DE at a concentration of 300 $\mu g/m^3$ produced an upregulation of p38, together with an elevated nuclear translocation of p38, JNK, AP-1 and NFkB in healthy bronchial epithelium [140]. This indicates a possible oxidative stress related inflammatory pathway induced by DE exposure.

Controlled chamber studies have demonstrated a distinctive association between diesel exhaust inhalation and airway inflammation. At six hours post exposure, Salvi

et al showed that an exposure to a PM concentration of 300 μg/m^3 for one hour produced in healthy tissues and lavage significant increases in inflammatory cells (neutrophils, B lymphocytes, mast cells, CD3$^+$, CD4$^+$ and CD8$^+$ T lymphocytes) in conjunction with an upregulation of the vascular adhesion molecules (ICAM-1 and VCAM-1) and epithelial cytokines (IL-8, Gro-α) [153, 154]. Collaborating biopsy results were also illustrated at six hours by Stenfors *et al* using a lower PM concentration of 108 μg/m^3. Post DE exposure healthy subjects exhibited greater numbers of neutrophils, lymphocytes, as well as increased expression of IL-6, IL-8, P-selectin and VCAM-1 [167]. Furthermore, at 18 hours post exposure, using similar experimental conditions and a PM concentration of 100 μg/m^3, a significant increase in neutrophils and mast cells was observed in endobronchial mucosal biopsies. BW at this timepoint displayed a DE exposure induced rise in neutrophils, IL-6, IL-8 and MPO [19]. At the higher PM concentration of 300 μg/m^3, the DE mediated neutrophilia was still found to be present in lavage 24 hours after exposure [150].

Studies analyzing induced sputum after exposures to PM concentrations of 300 μg/m^3 also reveal interesting timeline and compartmental aspects of the DE stimulated airway inflammation. A marked increase in neutrophils and IL-6 was observed at six hours after exposure, however at 24 hours no DE related neutrophilia remained [129].

COMBINED EXPOSURE

Urban air pollution cycle

Reasonably, ambient exposure to several air pollutants, such as ozone and diesel exhaust, may cause these compounds to exhibit synergistic or additive properties.

Nitrogen oxides, formaldyde and hydrocarbons, all components of diesel exhaust, react with other atmospheric pollutants to subsequently form ozone precursors, resulting in an increase in O_3 levels.

Since diesel exhaust is a direct release pollutant, it tends to reach its highest concentrations at peak rush hour, such as mornings. However, as ozone is the product of photochemical reactions, it is predisposed to culminate in the afternoons.

Exposure limits for individual pollutants

Air quality measurements are now regularly tested and reported in most developed nations. Ozone and several components of diesel exhaust are classified by the U.S. Environmental Protection Agency (EPA) as a "criteria pollutant", meaning they are regarded as harmful to humans. This makes it essential to monitor and set rational standard exposure limits.

As of the revisions in 1997, the EPA set the standards for O_3 to 0.08 ppm over an 8- hour period. They set the annual standard of $PM_{2.5}$ at 15 $\mu g/m^3$ and the 24-hour $PM_{2.5}$ standard at 65 $\mu g/m^3$. The corresponding standards set for PM_{10} are 50 $\mu g/m^3$ and 150 $\mu g/m^3$ respectively [51]. Since these exposure limits have not been altered since 1997, the ozone standards are currently undergoing a review by the EPA and a report outlining revision recommendations is due out during this spring.

The WHO has contested that the present international averages are unacceptable in a global health perspective. According to the 2005 Air Quality Guidelines, the aspiration should be to limit O_3 8-hour standard to 100 $\mu g/m^3$ (approximately 0.05 ppm). The guidelines also propose an annual mean for $PM_{2.5}$ at 10 $\mu g/m^3$ and for PM_{10} at 20 $\mu g/m^3$; and a target 24-hour mean at 25 $\mu g/m^3$ and 50 $\mu g/m^3$ respectively. These new targets are set based on what studies have shown to be levels negatively affecting human health [192]. The EU is also presently debating a new energy strategy which will by 2020 reduce all greenhouse gas emissions by at least 20 percent below 1990 levels.

Sweden started measuring air quality in the lager urban areas in the early 1960s, yet regulations were not put into place until 1969. Today Sweden follows the EU standards for air quality, which are set at a 24-hour mean of 50 $\mu g/m^3$ for PM_{10} and a 110 $\mu g/m^3$ (approximately 0.06 ppm) 8-hour standard for ozone.

The air pollution standard limits for each pollutant are at present set independently of each other. As more data emerges showing the potentially damaging interactions these pollutants inflict upon one another, it might become relevant to instead set co-regulated limits.

Animal and in vitro studies

Only a few experimental studies have addressed the airway effects generated by the exposure to several pollutants.

Animal studies in this field have mainly focused on using simultaneous exposure models. Madden *et al* found that diesel exhaust particles exposed to 0.1 ppm of O_3 for 48 hours caused increased neutrophilia, lavage total protein, and LDH activity in rats, as opposed to unexposed DE particles. In contrast, exposing particles to higher concentrations of ozone (1.0 ppm) instead caused a decrease in particle-induced bioactivity [111]. Rats co-exposed to 0.8 ppm of O_3 and urban particles for four hours displayed an increase in airway macrophages and neutrophils, as well as more extensive epithelial cell damage, as compared to exposure to O_3 or particles alone [5]. A further study examined the combination effects of a long term O_3 exposure (0.8 ppm for >90 days) and 1-nitronaphthalene (1-NN), a component of DE, in the airways of rats. The long term O_3 exposure caused a chronic low level inflammation, probably leading to the heightened susceptibility to 1-NN. The subsequent 1-NN exposure resulted in an increase in primarily Th2 cytokines, such as IL-4, and GM-CSF [160]. These studies all show the potentiation of airway inflammatory changes when exposed to a combination of ambient air pollutants.

So far, only one study has been published involving pollutant interactions in human tissue in vitro. This study used A549 airway epithelial cells to examine if the known DE particle gene enhancement of IL-8, a neutrophil chemoattractant, would be affected by a subsequent O_3 exposure. The one-hour 0.5 ppm ozone exposure led to a significant rise in IL-8 gene expression as compared to DE particles alone, or filtered air. This enhancement was thought to be associated with previously described ozone-induction of the transcription factors NFkB and NF-IL6 [87].

INFLAMMATORY MECHANISMS

The immune system is a complex set of mechanisms designed to protect cells and tissues throughout the body from pathogens and external harmful stimuli. There are several methods of protection, including mechanical, chemical and biological barriers; as well as inflammation and biochemical cascades.

Within the lungs, one of the first barriers encountered by exogenous agents is the respiratory tract lining fluid (RTLF). This consists of two layers, the upper a mucus gel phase and the lower consisting of an aqueous sol phase. Both of these coverings contain antioxidants which manage oxidative stress, consequently delaying the oxidation of lipids, proteins and carbohydrates [34, 124, 185]. Since ozone and diesel exhaust are both potent oxidants, they react with the RTLF components generating secondary oxidation products, which impact the pulmonary epithelium [10, 15, 90].

The epithelium is not only a physical barrier, but also an important contributor to the innate immune defense. In response to endogenous or exogenous stimuli, pulmonary epithelial cells release an abundance of substances designed to influence airway defenses, among these antioxidants (*eg.* glutathione), proteins (*eg.* surfactant protein A), lipid mediators, and various growth factors [120, 175]. The epithelial cells also have the ability to release chemoattractants and cytokines, thus playing a pivotal role in recruiting and regulating inflammatory cells [175, 178].

Inflammatory cells

Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes (PMN), make up 50-60% of the circulating leukocytes and are therefore fundamental in the first line of defense upon injury or inflammatory stimuli. The recruitment of circulating PMNs is complex and highly regulated. Chemotactic factors, bioactive lipids and pro-inflammatory cytokines are released, signaling the location of impairment. This initiates the three step process of transendothelial migration. First, selectins mediate the loose adhesion of circulating neutrophils to the activated endothelial cells. Nearby cytokines and chemoattractants stimulate the neutrophils, causing

transmembrane integrins on the surface to bind to ICAM-1. Following this firm adhesion of the neutrophil to the vascular endothelium, chemoattractant gradients direct transmigration between the endothelial cells into the extracellular matrix. This process known as the leukocyte adhesion cascade can be applied to the recruitment of other leukocytes, such as monocytes and eosinophils. The release of specific and appropriate cytokines, adhesion factors and proinflammatory mediators offers selective control of the inflammatory action.

Neutrophil chemoattractant chemokines, such as IL-8 and Gro-α, play a vital role in the further migration of neutrophils through the tissues to the site of injury. Consecutively, activated PMNs release cytokines and proteases that continue to recruit both nonspecific and specific immune cells.

Macrophages

Macrophages are mononuclear phagocytes found throughout the body and are associated with various homeostatic, immunological, and inflammatory processes. Resident macrophages at different anatomical sites display unique characteristics and functions. Normally they exist within the tissue in a resting state, but following damaging or immunological stimuli they are activated, largely in response to cytokines such as IFN-γ and IL-1. Additional mobile macrophages can also be recruited as monocyte concentrations in the peripheral blood increase in response to the cytokine signaling. Circulating monocytes enter the tissue via the leukocyte adhesion cascade and undergo maturation into macrophages.

Activated macrophages perform several important immune functions aside from phagocytosis and antigen presentation to T cells. They can also initiate and prolong inflammation by releasing leukotrienes, cytokines, and other inflammatory mediators [27, 66]. Diesel exhaust particle ingestion by macrophages has been shown to cause the release of lysosomal enzymes and oxygen radicals, inflicting damage to nearby cells and enzymes [80]. It has also been suggested that air pollution exposure can suppress macrophage activity, either by direct damage or indirectly via altered microenvironment [71, 82, 150, 155].

Eosinophils

The eosinophil is characteristically associated with asthmatic and allergic airway inflammation. Eosinophil granules contain various mediators including eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil protein X (EPX), major basic protein (MBP), cysteinyl leukotrienes, platelet activating factor, and metalloproteinases [186-188]. The toxins from the granules are designed to kill parasites, yet in asthma the erroneous eosinophil accumulation and subsequent granule release causes damage to the airway tissues.

The Th2 cytokines IL-5 and GM-CSF are crucial as they essentially affect the entire life span of the eosinophil, from differentiation to activation and enhanced survival. Furthermore, IL-5 and GM-CSF display autocrine growth factor activity when

expressed by eosinophils. Other chemotactic factors, such as chemokines and leukotrienes, also play a role in the migration and degranulation of eosinophils in the tissue. Recent studies have shown that human eosinophils are capable of producing numerous cytokines, for instance IL-6, IL-8, TNF-α and IL-1α, giving them an active function in acute and chronic inflammatory responses [63, 195]. In addition, eosinophils potentiate the production of ROS in the presence of proinflammatory cytokines, displaying a more prolonged oxidant reaction as compared to neutrophils [70].

Lymphocytes

The three major types of this white blood cell are categorized into B cells, T cells and natural killer (NK) cells. T cells and NK cells are a part of the cell-mediated immune response, whereas B cells are associated with humoral immunity. The main purpose of NK cells is to release cytotoxic granules when a host cell signals that it is infected by presenting a foreign peptide on their cell surface.

When B cells encounter their initiating antigen accompanied by a collaborating helper T cell, they produce numerous plasma cells. Each B cell is encoded to create plasma cells which deliver a specific antibody, otherwise known as immunoglobulins. The T cells involved in the activation of B cells are known as regulatory T lymphocytes, mainly comprised of helper/inducer cells. These also stimulate other T cells, macrophages and NK cells primarily through the release of cytokines. In contrast, cytotoxic T cells attack invaded body cells, thus interacting directly with their target.

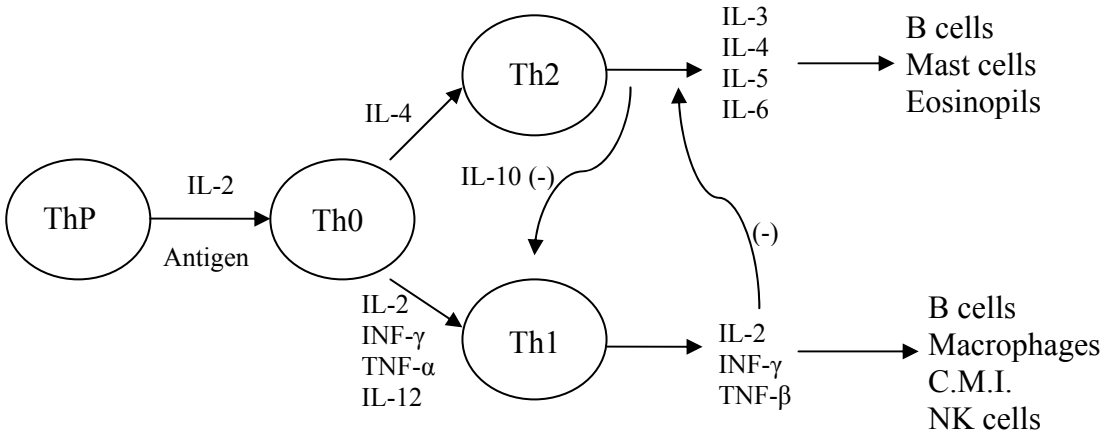
Cytokines

These chemical signals, mainly made up of proteins, glycoproteins or peptides, relay intercellular communication. They play a pivotal role in the immune system, conveying immunological, inflammatory and infectious information to a wide variety of cell types. Cytokines are formed and secreted in response to a stimulus by a multitude of diverse cell categories. Action is not limited to the production cell or neighboring cells, as they can affect cells throughout the body via circulation. Target cells are acted upon by each cytokine recognizing and binding to a specific membrane receptor, consequently triggering second messengers within the cell, frequently tyrosine kinases. This cascade may result in upregulation or suppression of cell surface receptors (either their own or for other molecules) or transcription factors, in turn leading to further cytokine regulation. Cytokines often evoke a cytokine cascade and can act synergistically or antagonistically on the same target. Typical cytokine characteristics are their redundancy and pleitrophism, causing them to be difficult to categorize.

However, some categories are still appropriate and relevant. Chemokines are small cytokines (8 to 16 kDa) with chemotactic properties. The inflammatory chemokines act mainly as chemoattractants for leukocytes, directing the migration

for neutrophils and monocytes. Their release is often stimulated by pro-inflammatory cytokines, such as IL-1, TNF and IL-6.

An additional classification is Th related cytokines. Naïve helper T cells (Th0) are activated by antigens presented on dendritic cells when allergens, infections or other foreign substances invade the body. Upon further stimulation, Th0 cells can differentiate into either Th1 or Th2 cells, based largely on the conditions in the microenvironment. Th1 cells are involved in the secretion and production of cytokines which increase Ig antibodies and stimulate a cell-mediated immune response. Th2 cytokines suppress macrophage activation and cell mediated immunity, instead tending to shift activation to a humeral immune response. Additionally, they are involved in promoting B cells to produce IgE antibodies. Excessive release of Th2 cytokines is associated with the development and worsening of asthma and allergy.



It has been established that ozone and DE exposures generate increases, as well as suppression of numerous cytokines predominantly secreted by macrophages and epithelial cells [21, 172, 176].

Transcription Factors

Transcription factors are proteins that bind either directly to DNA or to other already bound transcription factors; which in turn recruits RNA polymerase leading to the initiation of gene expression. Primary transcription factors, such as NFκB and c-Jun, are transcription factors which do not need to be synthesized upon cell stimulation, as they are continuously present in an inactive state. Therefore, since they can be rapidly employed, they are an important step in the reaction to harmful

stimuli as well as the acute immune response. Transcription factors are activated by an extensive range of cell surface receptors reacting to stimuli such as oxidative stress, cytokines, inflammation or infection.

The family of activating protein–1 (AP-1) transcription factors includes Jun, Fos and various additional subfamilies for instance, ATF2, ATF3/LRF1, B-ATF. Following cellular stimuli, these transcription factors are activated by phosphorylation of serine and threonine sites or regulated by their gene activation. The Jun protein can form a homodimer (Jun/Jun), or create a heterodimer with a Fos subunit (Jun/Fos), thus producing an especially stable formation with higher DNA binding affinity compared to the homodimer. These structures induce the production of numerous proinflammatory cytokines and mediators.

The Rel/NFκB family consists of the subunits p50, p52, p65, c-Rel, and RelB, which all exist as homo or heterodimers, most notably p50/p65 and p50/p50. In its inactive form in the cytoplasm, the inhibitor protein IκB is noncovalently bonded to NFκB, concealing the nuclear binding domain. The dissociation of IκB occurs in response to a broad array of exogenous as well as endogenous signals, for example cytokines, viruses and endotoxin. As these signals are transmitted within the cytoplasm, the IκB is phosphorylated by IKK, tagging it for degradation (*Figure 2*). There are three known catalytic subunits of IKK (α, β, γ), out of which IKKβ is induced by proinflammatory cytokines, in particular TNF-α. During oxidative stress, when there are high levels of ROS, IKKβ is inhibited leading to the repression of NFκB activity [148]. NFκB is known to be a redox sensitive factor, and is regulated on many levels in the cytoplasm as well as the nucleus by ROS production [86]. Ozone and diesel exhaust exhibit recognized oxidant forces on airway cells, both linked to increased ROS production [36, 106, 107, 123].

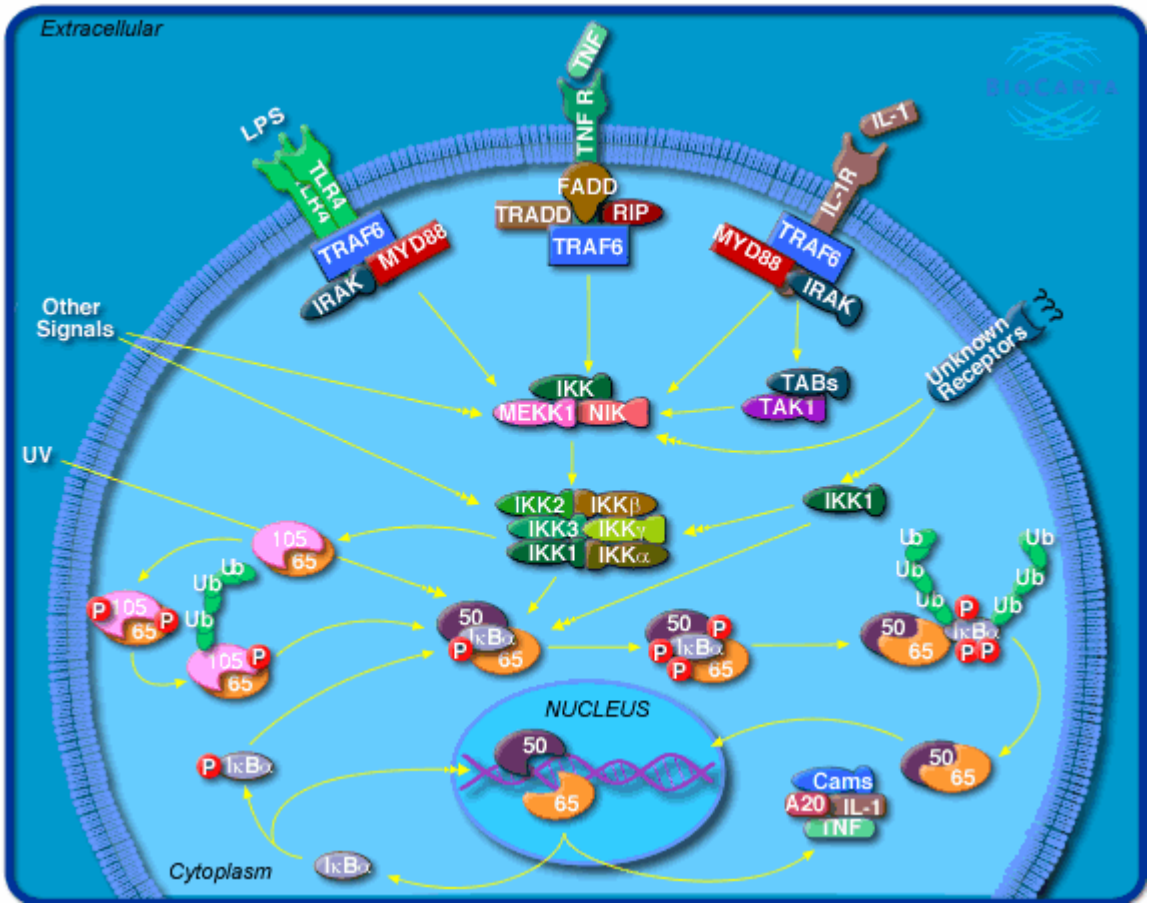


Figure 2: Activation and regulation of NFκB within the cytoplasm and nucleus. (Original picture Sujay Singh, PhD, Chun Wu, PhD, Usha Ponnappan, PhD. U of Arkansas, www.biocarta.com)

MAPK

The MAPK pathway is a complex signal transduction pathway that by means of various protein components links growth and stress signals conveyed to cell surface receptors with the intracellular response. These intracellular signaling cascades lead to immune responses such as activation of various transcription factors and cytokine production as well as controlling cell division and apoptosis.

Presently, there are five main established pathways classified as ERK1/2 (extracellular signal regulated kinase), ERK3/4, JNK (Jun-N-terminal kinase), p38 and BMK-1/ERK5 (Big MAP kinase) [60, 194]. The JNK and p38 pathways are predominantly generated by proinflammatory cytokines, endotoxins, and environmental & cellular stress, whereas the ERK1/2 pathway is known as the classic mitogenic cascade. BMK-1/ERK5 is triggered both by mitogenic activity as well as stress inducers. ERK3 is a nuclear protein kinase, however, little is known about its specific role or how it is regulated.

MAPKs are activated via dual phosphorylation, mediated within the cytoplasm by small G-proteins, and MAP kinase kinases (MEKs or MKKs) (*Figure 3*). The activated MAPKs can then either attach to cytoplasmic targets or translocate into the nucleus to stimulate transcription. ERK and p38 may pass directly into the

nucleus after activation. In contrast, JNK needs to phosphorylate the transcription factor c-Jun, which in turn gets translocated to the nucleus. The main targets for ERK phosphorylation and activation are pp90 ribosomal S6 kinase (Rsk), cytoplasmic phospholipase A2, and transcription factor Elk-1 [189]. The p38 pathway regulates several transcription factors including AP-1, ATF-2, Mac and MEF2 [79]. It also targets MAPKAPK2, which in turn triggers activation of heat-shock proteins [104, 128]. The JNK MAPK is involved in the promotion of transcriptional activity of AP-1, Elk-1 and ATF2 [40, 81]. The MAPKs activation and phosphorylation of these factors may intersect within the cell, however all of these pathways result in unique transcriptional activity for the specific external stress.

Soluble inflammatory mediators

MPO

Myeloperoxidase (MPO) is a protein extensively stored in the azurophilic granules of neutrophils. During the respiratory burst of the neutrophil degranulation upon activation, it generates cytotoxic substances such as superoxides, chloride anions (Cl^-), tyrosyl radicals and hypochlorous acid (HOCl). These products, although intended to eradicate bacteria and other pathogens, can have a detrimental effect on nearby cells and molecules, as well as initiating lipid peroxidation [199].

MMP-9

Matrix metalloproteinase-9 (MMP-9) is a proteolytic enzyme found in the gelatinase subgroup belonging to the MMP family of endopeptidases. Although several types of inflammatory cells can be the source of MMP-9, it is predominantly expressed and released by neutrophils and macrophages, thus playing a role in the acute inflammatory response. Its collagenolytic activity degrades extracellular matrix (ECM) components as well as non-ECM proteins within the airways. It is initially released as an inactive zymogenic proenzyme, which can be cleaved by numerous proteases. However, much remains unknown about the activation and regulation of this enzyme. It has been shown that an increase of T lymphocyte derived IL-17 induces a rise in concentration of active MMP-9 in murine airways [141].

sICAM

ICAM-1 is a membrane protein which upon cell activation is up regulated on the vascular endothelium by the stimulation of proinflammatory cytokines, such as IL-1 and TNF-α. This protein is fundamental for several cell interactions in the immune system following the onset of acute inflammation, such as mediating leukocyte adhesion to endothelial cells. Thus, soluble ICAM is a effective marker for examining the activation of leukocyte adhesion in inflammatory reactions and immune responses.

HNL

Human Neutrophil Lipocalin is a protein found in the neutrophil granules and regarded as a sensitive and specific marker demonstrating neutrophil granulocyte activation. Part of the large lipocalin family which exhibit diverse physiological roles, HNL is co expressed in granulocytes along with MMP-9. This protein could possibly function as part of the activation process of promatrix metalloproteinases, seeing as it shares a covalent bond to the proenzyme.

EPX

Eosinophil protein X is a glycosylated protein that functions as an index of eosinophil activation and degranulation. Numerous highly cationic proteins such as major basic protein, eosinophil peroxidase, eosinophil cationic protein (ECP), and eosinophil protein X (EPX) are incorporated within the eosinophilic granules. These positively charged particles make up about 90% of the granule proteins. EPX offers an excellent indication for eosinophil activation since it is released effectively and is stable during an extended time.

Albumin

The most prevalent serum-binding protein in the human body is albumin, typically found in the plasma where it helps to sustain colloid oncotic pressure as well as transporting various substances such as hormones and exogenous drugs. An increase of pulmonary albumin gives evidence of a dysfunction in the lung endothelial barrier, and is used as an indicator of pulmonary vascular permeability.

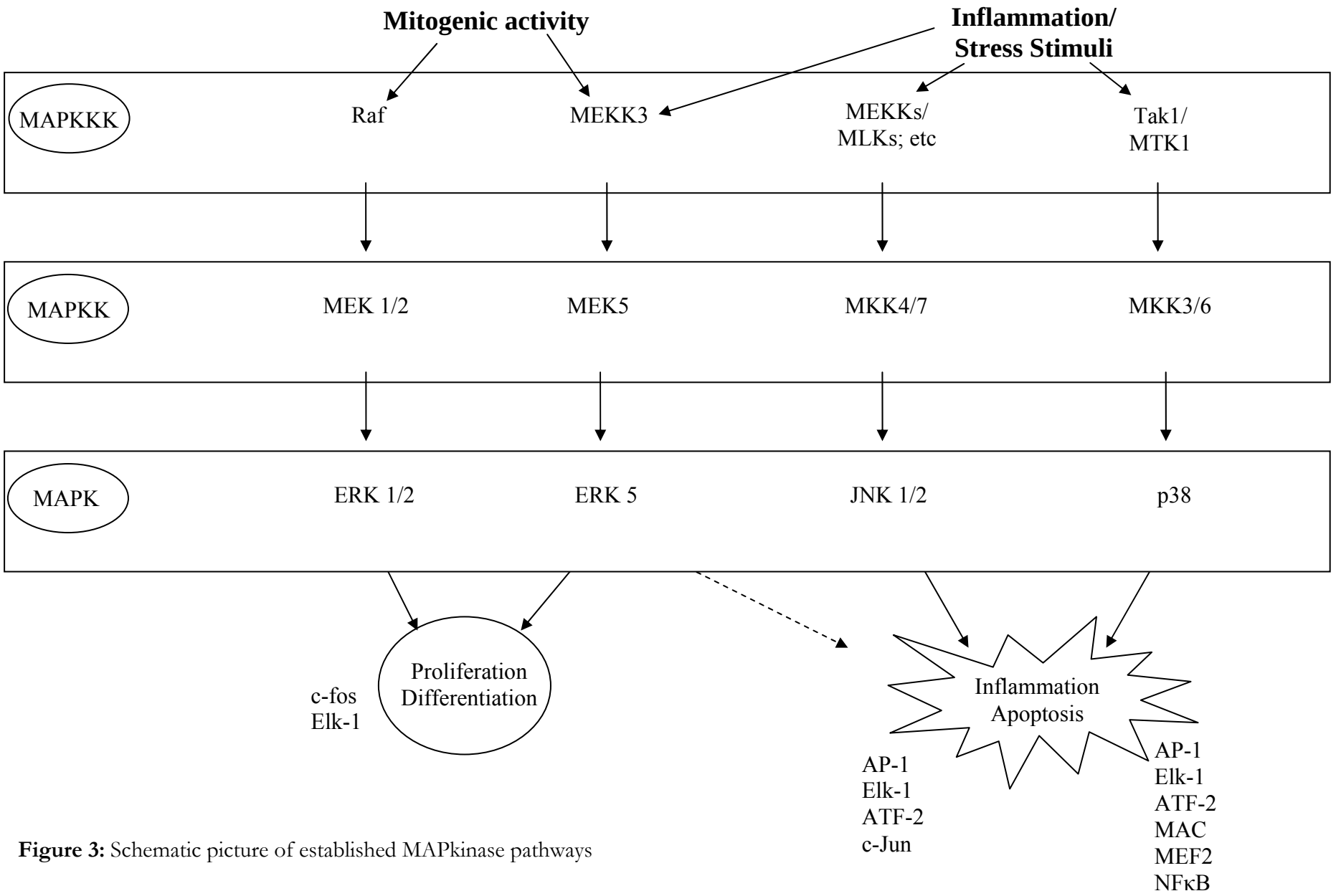


Figure 3: Schematic picture of established MAPkinase pathways

Time after exposure	Material	O_3 conc	Exposure time	Finding (O_3 effect)	Note
Immediately (≤1hour)	BAL	0.22 ppm	4 hours	↑ IL-6; IL-8	Peak; Frampton 97
		0.4 ppm	2 hours	↑ PMN, IL-6, PGE_2	Koren 91
		0.4 ppm	2 hours	↑PMN, LDH, protein, fibronectin, PGE_2, C3a, IL-6 ↓total cells, AM	Devlin 96
		0.22 ppm	4 hours	↑PMN ↑IL-6, IL-8 (peak)	Torres 97
		0.3 ppm	1 hour	↑PMN	Schelegle 91
	BW	0.3 ppm	1 hour	↑PMN	Schelegle 91
	NL	0.4 ppm	2 hours	↑ PMN, tryptase	Koren 90
75 minutes	Exhaled 99mTc-DTPA	0.4 ppm	2 hours	↑ epithelial permeability	Kehrl 87
1.5 hours	BAL	0.2 ppm	2 hours	↑GSH, α-tocopherol, ↓Total cells, macrophages, urate	Blomberg 99
	Biopsy	0.12 ppm	2 hours	↑ P-selectin	Krishna 97
		0.2 ppm	2 hours	↑ ICAM-1, P-selectin	Blomberg 99
3 Hours	BAL	0.4 ppm/0.6 ppm	2 hours	↑PMN, eicosanoids	Seltzer 86

Time after exposure	Material	O_3 conc	Exposure time	Finding (O_3 effect)	Note
6 Hours	BAL	0.3 ppm	1 hour	↑ PMN	Peak; Schelegle 91
		0.2 ppm	2 hours	↑PMN, epithelial cells, IL-8, Gro-α	Krishna 98
		0.2 ppm	2 hours	↑ PMN, MPO ↓ macrophages, lymphocytes	Mudway 99
		0.2 ppm	2 hours	↑ PMN, GSSG ↓Ascorbic acid	Mudway 01
		0.2 ppm	2 hours	↑ IL-6	Behndig 06
	BW	0.2 ppm	2 hours	↑ PMN	Stenfors 02
		0.2 ppm	2 hours	↑PMN	Behndig 06
		0.2 ppm	2 hours	↑ PMN, GSSG ↓ascorbic acid	Mudway 01
		0.2 ppm	2 hours	↑ PMN	Behndig 06
	Biopsy	0.2 ppm	2 hours	↑ PMN, Mast cells, P-selectin, ICAM-1	Stenfors 02

Time after exposure	Material	O_3 conc	Exposure time	Finding (O_3 effect)	Note
18 hours	BAL	0.22 ppm	4 hours	↑ PMN; lymphocytes; mast cells	Peak; Frampton 97
		0.4 ppm	2 hours	↑ PMN; protein, albumin, IgG, PGE_2, neutrophil elastase, LDH, fibronectin	Koren 89
		0.10 ppm	6.6 hours	↑PMN, protein, PGE_2, fibronectin, IL-6, LDH	Devlin 91
		0.08 ppm	6.6 hours	↑PMN, PGE_2, LDH, IL-6	Devlin 91
		0.2 ppm	4 hours	↑ PMN, total protein concentration	Balmes 97
		0.22 ppm	4 hours	↑PMN (peak) IL-6, IL-8, lymphocytes, mast cells, eosinophils	Torres 97
		0.4 ppm	2 hours	↑ PMN, albumin	Graham 90
		0.2 ppm	4 hours	↑ total cell count, LDH, PMN, albumin, protein, fibronectin, GM-CSF	Aris 93
	BW	0.2 ppm	4 hours	↑PMN	Balmes 97
		0.2 ppm	4 hours	↑ IL-8	Aris 93
		0.2 ppm	2 hours	↑PMN	Stenfors

Time after exposure	Material	O_3 conc	Exposure time	Finding (O_3 effect)	Note
18 hours	Biopsy	0.2 ppm	4 hours	↑ PMN	Aris 93
		0.2 ppm	4 hours	↑ ICAM-1	Balmes 97
	NL	0.2 ppm	2 hours	↑ PMN, albumin	Koren 90
24 hours	BAL	0.3 ppm	1 hour	↑ PMN	peak; Schelegle 91
	BW	0.3 ppm	1 hour	↑ PMN	Schelegle 91

Table 1 : Controlled ozone exposure chamber studies with healthy human subjects. The essential inflammatory results are outlined.
AM= alveolar macrophages

AIMS

The overall aim of this thesis was:

- To examine the ozone induced airway signaling and inflammatory pathways. Furthermore, to elucidate the effects of sequential exposure to diesel exhaust and ozone.

The specific aims were:

- To evaluate whether the bronchial epithelial cytokine expression would differ between healthy and allergic asthmatics after ozone exposure.

- To investigate the underlying rationale to the increased susceptibility and response to oxidative air pollutants of asthmatics.

- To observe whether an environmentally relevant ozone exposure would stimulate an early upregulation in the bronchial epithelial expression of redox sensitive transcription factors and kinases regulating neutrophil chemoattractants in healthy subjects.

- To explore whether ozone induced inflammatory signaling in the bronchial epithelium corresponds to the known pathways involved in diesel exhaust generated airway inflammation.

- To evaluate whether ozone exposure would cause an enhanced airway inflammatory response, in addition to the extensive airway inflammation established after diesel exhaust exposure.

- To further substantiate the airway inflammatory patterns when mimicking an urban profile of air pollution after sequential exposures to diesel exhaust and ozone.

SUBJECTS AND METHODS

SUBJECTS

Both asthmatic and healthy subjects included in the studies were non-smokers. They had normal physical examinations as well as ECG and lung function tests. All the volunteers were free from airway infection for at least six weeks prior to the first exposure and throughout the course of the study. In addition, non-steroid anti-inflammatory drugs and antioxidant supplements were not allowed 2 weeks before and during the entire study period. The studies were approved by the Umeå University Ethics Committee and subjects gave both verbal and written informed consent. All research performed was in compliance with the Declaration of Helsinki.

Study I

The study comprised healthy as well as asthmatic subjects. The healthy group consisted of fifteen non-atopic subjects, whereof six males and nine females with a mean age of 24 years, ranging from 19–31 years.

The asthmatic group comprised 15 subjects (nine males, six females; mean age 29 years, range 21–48 years) with intermittent to mild persistent disease diagnosed in accordance with the GINA guidelines [3]. The asthmatic subjects had normal lung function results with a mean FEV_1 of 90% of predicted (range 75–114%) and demonstrated bronchial hyper-responsiveness to methacholine (geometric mean PC_{20} 2.3 mg/mL). Furthermore, they had at least one positive skin prick test against a standard panel of common aeroallergens. With the exception of inhaled β-agonists on demand, they did not require any additional anti-asthma therapy.

Study II, III, IV

These studies all involved healthy non-atopic subjects inclusive of both sexes, numbering 15, 16 and 17 in the respective studies with an age range of 19-31 years. The criteria for these volunteers, in addition to those described above, included no history of asthma, allergy or other respiratory disease as well as negative skin prick tests.

STUDY DESIGN

All the exposures were administered in a randomized sequence with each exposure conducted at least three weeks apart; thus creating a single-blinded, crossover control, with the subject acting as their own control. The samples collected after exposures were coded enabling the investigators to be blinded as to exposure

sequences. Throughout the course of the exposure, subjects alternated at 15 minute intervals between rest and moderate exercise using a bicycle ergometer (V_E=20 L/min/m^2 body surface).

Study I

The study was performed in order to investigate differences between healthy and asthmatic bronchial epithelial responses to O_3 exposure. All volunteers were exposed to filtered air and 0.2 ppm of O_3 during two hours on two separate occasions. To assess the inflammatory effects on the airways with focus on epithelial cytokine expression, endobronchial mucosal biopsies were obtained via bronchoscopy 6 hours after each exposure. The prepared biopsy slides were stained for a panel of neutrophil chemoattractant and Th2-related cytokines (*Figure 4*)

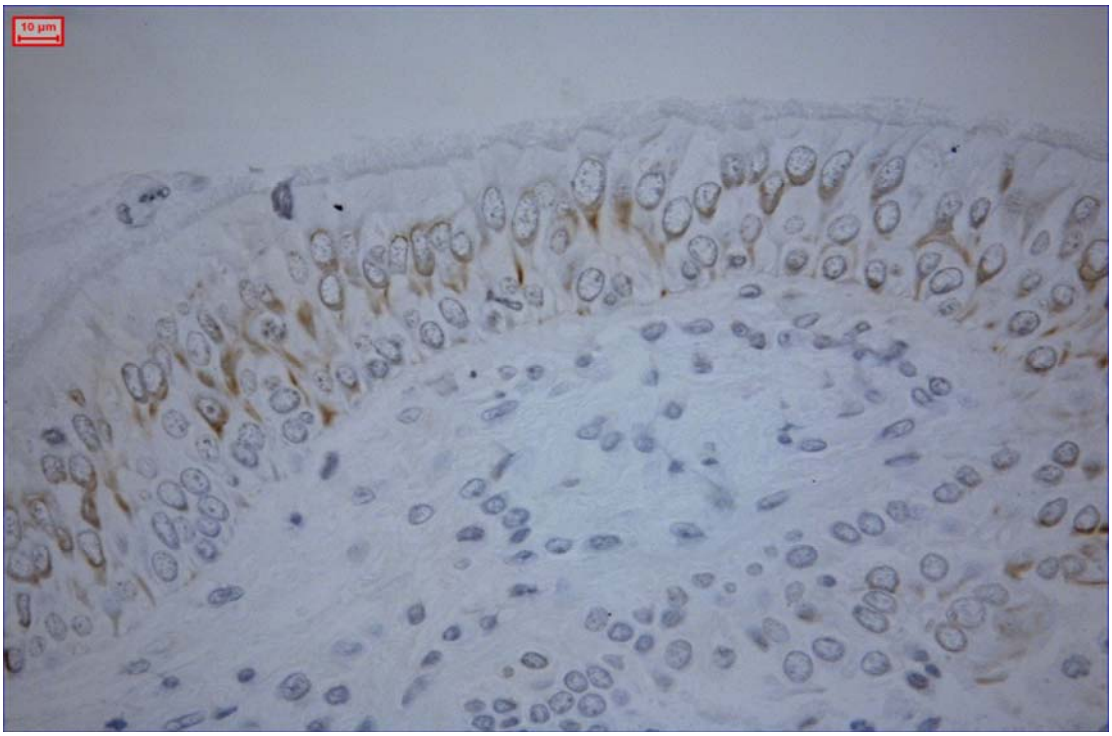


Figure 4: Immunohistochemical staining of cytokine IL-8 in the bronchial epithelium.

Study II

The design of this study allowed further examination of the time kinetics involved in the inflammatory pathways in the bronchial epithelium of healthy subjects exposed to ozone. Two independent exposures each lasting two hours were completed for each subject, once to filtered air and once to 0.2 ppm of O_3. Bronchoscopy to acquire bronchial biopsies was carried out one and a half hours after completed exposure. Biopsies were analysed for a panel of transcription factors and mitogen-activated protein kinases (*Figure 5*).

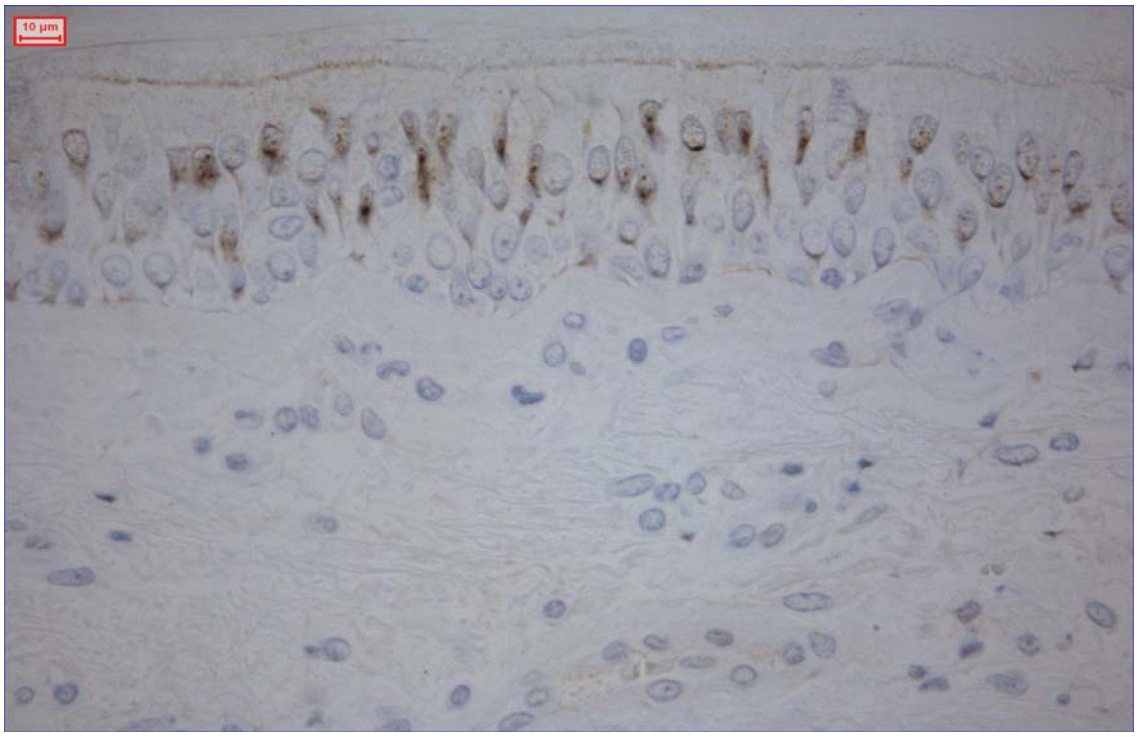


Figure 5: Immunohistochemical staining of MAPKinase JNK in the bronchial epithelium.

Study III

In order to observe the consequences of a sequential exposure pattern resembling an urban air pollution profile, subjects underwent two separate exposure series. These included a one-hour morning exposure to diesel exhaust (PM concentration 300 µg/m^3) followed five hours later by a two-hour exposure to filtered air or 0.2 of ppm O_3 (DE+air and DE+O_3 respectively). Induced sputum was collected 24 hours after the start of the initial exposure in each series. Sputum was evaluated for inflammatory cells and soluble inflammatory markers (*Figure 6*).

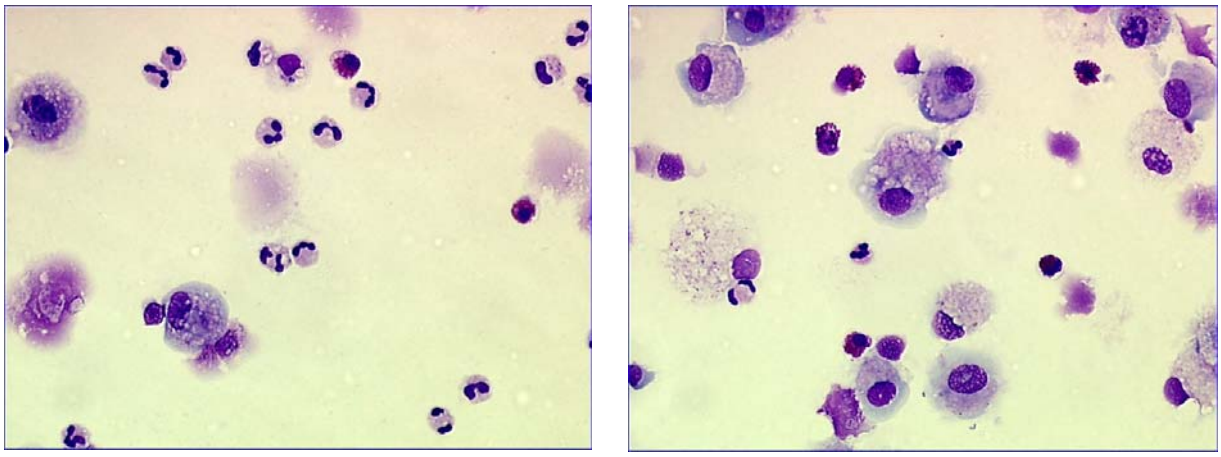


Figure 6: Inflammatory cells in induced sputum samples.

Study IV

To broaden the knowledge gathered in the previous study (Study III), subjects were again exposed in two separate exposure series. This time the one-hour morning exposure consisted of filtered air or diesel exhaust, and five hours later followed by a two hour exposure to 0.2 ppm ozone (air+O_3 and DE+O_3 respectively). Bronchial wash and bronchoalveolar lavage were obtained via bronchoscopy 24 hours after the start of each morning exposure. Expression of inflammatory cells and soluble mediators were assessed in the lavage samples.

EXPOSURE CHAMBERS

Both the diesel and ozone exposures were carried out in environmental "walk-in" chambers. The chambers are designed to create a steady flow of an established pollutant concentration. The environment in both DE and O_3 exposure chambers remained at a constant monitored temperature and humidity (20ºC and 50%, respectively). When entering the chambers subjects first enter a small vestibule and the external door is shut before access is granted to the inner door leading into the chamber, thus creating roughly an air lock system. Windows, in addition to an intercom system, provide subject and staff with a regular communication link.

Diesel Exhaust Chamber

The exposure chamber measures 2.5 x 2.5 x 2.5 m and the diesel exhaust was generated by an idling Volvo diesel engine (Volvo TD45, 4.5L, 4 cylinders, 1991, 68 rpm). Over 90% of the exhaust formed was shunted away and the remaining fraction was diluted with filtered air before being led into the exposure chamber, therefore generating a steady state air concentration of pollutant.

Throughout the exposure, air from the breathing zone of the subject was sampled and continuously monitored for concentrations of nitrogen monoxide (NO), nitrogen dioxide (NO_2), oxides of nitrogen (NO_x) and total gaseous hydrocarbons (measured as propane). The steady state concentrations of PM_{10} and gaseous components during the DE exposures were 285±17.1 μg/m^3 (PM_{10}), 1.65±0.17 ppm (NO), 0.51±0.05 ppm (NO_2), 2.16±0.21 ppm (NOx), 1.18 ± 0.02 ppm (hydrocarbons), expressed as means and SEM. The majority of PM mass quantified in the exposure chamber were fine particles under 1 μm, with a mass median particle diameter of 0.18 μm (*Figure 7*).

Ozone exposure chamber

The ozone exposure chamber has a volume of 14.1 m^3. O_3 was generated by a Fischer's O_3 generator 500 MM (Fischer Labor and Verfahrens-Technik, Bonn, Germany) and the concentration in the chamber was continuously monitored by a photometrical ozone analyzer (Dasibi model 1108, Dasibi Environmental Corp.,

California, USA). To maintain the continuous temperature and relative humidity, ambient air was continuously drawn through the exposure chamber at a ventilation rate of 30 m^3/h. During the exposures throughout the studies ozone concentrations remained stable at 0.199 ± 0.007ppm (mean ± SEM) (*Figure 7*).

Figure 7: Diesel exposure chamber (left) and ozone exposure chamber (right).

Filtered air exposures

In order to establish blinded exposures, the filtered air exposures were also performed in the same environmental exposure chambers. In studies I-III filtered air exposures were carried out in the ozone chamber, whereas in study IV it was located in the DE chamber. During these exposures, the concentrations of ozone and gaseous components of diesel exhaust were also continuously monitored to discount any uncertainties regarding particle or ozone contamination.

SAMPLING METHODS

Induced Sputum

Sputum induction was carried out in accordance with a method developed by Pin *et al* [137]. In order to prevent bronchoconstriction, all subjects received pre-treatment with an inhalation of 0.5 mg terbutaline. Hypertonic saline was placed in an ultrasonic nebuliser with an output of 1.5 ml/min (DeVILBISS Ultra-Neb 2000, DeVILBISS Health Care Inc., Somerset, PA, USA). Induced sputum was collected after three 7-minute inhalations of 3%, 4% and 5% saline solutions. After rinsing their mouths with water and blowing their noses, subjects expectorated sputum into a sterile container following each inhalation. FEV_1 was continually monitored prior to and after each inhalation period.

Bronchoscopy

Atropine (1 mg) was administered subcutaneously to all subjects as pre-medication 30 minutes prior to bronchoscopy. Lidocaine was subsequently given as topical anesthesia of the airways. An Olympus BF IT200, BF T10, BF T20 or BF IT160

fiberoptic or video bronchoscope (Olympus, Tokyo, Japan), was inserted into the airways via a mouthpiece with the subjects in the supine position.

Biopsies

Four to six endobronchial mucosal biopsies were obtained per session using fenestrated forceps (Olympus FB 21C). The biopsies were obtained either from the anterior aspect of the main carina and the subcarinae of the 2nd, 3rd and 4th generation airways on the right side or the posterior aspect of the main carina and the corresponding subcarinae on the left side. These sites were predetermined using a randomized approach, with the alternative site chosen for the second bronchoscopy, thus avoiding artefacts formed by earlier biopsy locations.

BW and BAL

Bronchial wash (BW) and bronchoalveolar lavage (BAL) were performed following biopsy procurement. Initially, a bronchial wash of 2 x 20 ml of sterile saline solution (pH 5.4 at 37°C) was instilled, followed by a bronchoalveolar lavage (BAL) of 3 x 60 ml of saline solution. The lavage fluids were obtained within the middle lobe when biopsies were taken from the left side or lingula lobe when biopsy samples were acquired from the right side. The recovered aspirate from the first and second 20 ml instillations of the BW and the combined BAL fluid were gathered into individual containers and immediately placed on ice.

PROCESSING AND ANALYSES

Induced Sputum

The sputum samples were processed utilizing the method based on Pizzichini *et al* [138]. The viscous portion of the sample was transferred to 10 ml siliconized tubes with Sputolysin and rocked for 15 minutes. This process dissolves the mucus and dissipates the cells. Phosphate buffered saline (PBS) was then added and the sample was rocked for an additional five minutes. The solution was filtered through a 48 μm Mesh nylon filter and centrifuged at 300 g at 4°C for 10 minutes forming a cell pellet. The supernatant was recentrifuged at 1000 g for 10 minutes so as to eliminate any additional debris. This batch was then stored at -70°C for future analyses.

The recovered cell pellet was resuspended in 1000 μl of PBS and using trypan blue, a hemocytometer determined total cell counts as well as cell viability. The cell dilution was adjusted to 0.5 x 10^6 cells/ml and 50 μl allocated per cup in a Shandon 3 cytocentifuge (Shandon Southern Instruments Inc., Sewikly, PA, USA). Cytospins were generated and stained with May–Grünwald Giemsa.

400 non-squamous cells were subsequently counted, with differential cell counts expressed as a total count as well as a percentage of the complete non-squamous

cell count. The percentage of squamous cell contamination was assessed in an additional general count of 400 cells. Sputum samples qualified for further analysis when containing < 20% squamous cells and the viability was > 50%. The total cell count was ultimately computed by dividing the number of cells with the volume of processed sputum (1 mg = 1 μl).

Quantification of the soluble inflammatory mediators myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9) and IL-6 where performed with commercial enzyme-linked inmmunosorbent assay kits using the sputum supernatant (ELISA; R&D Systems Inc., Abingdon, UK).

Biopsies

Processing of the endobronchial biopsies began by placing the tissue in chilled acetone containing the protease inhibitors phenyl-methyl-sulphonyl fluoride (2 mM) and iodoacetamide (29 mM) overnight. The biopsies were then processed into glycol methacrylate (GMA) resin in accordance with the method described by Britten *et al* [28]. These blocks were stored at -20ºC until immunohistochemistry could be implemented.

The first step was to trim sections from each block using a microtome (Leica, Cambridge, UK) and stain them with toluidine blue in order to ascertain epithelial quality within the biopsies. Using the biopsies with best morphological structure, two to four 2 μm sections were cut representing each subject and exposure and placed on poly-L-Lysine treated microscope slides. The slides stained for transcription factors and MAPKs were initially incubated with 0.3% Triton-X-100 (TBST), to facilitate nuclear permeability. In order to inhibit endogenous peroxidase activity, all slides were treated with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide, followed by three short washes. During the first day of the procedure, as well as the first set of washes on day two, the rinsing buffer for staining with transcription factors and MAPK was TRIS-buffered saline with 0.1% Triton-X-100 (TBST), the remaining antibodies instead used TRIS-buffered saline (TBS) as washing solution throughout the entire staining process. Non-specific antibody binding was blocked with undiluted culture medium containing bovine serum albumin and fetal calf serum. An added blocking step was necessary for transcription factor and MAPK stainings, requiring a 30 minute incubation with rabbit or swine normal serum before applying the primary antibodies to all slides and incubating at room temperature overnight. Primary antibodies for transcription factors and MAP kinases were diluted in Tris-buffered saline with 0.05% TBST with 1% bovine serum albumin (BSA), whereas for cytokine stainings dilutions were completed with TBS.

No later than 18 hours after antibodies were applied, the slides were rinsed three separate times with TBST or TBS, followed by a two hour incubation with a secondary antibody. Biotinylated swine anti rabbit (Dako Glostrup, Denmark) was applied to slides where the primary antibody source was rabbit, while biotinylated rabbit anti-mouse (IgG $F(ab')_2$ Dako Glostrup, Denmark) was used as a secondary

antibody on the mAb immunostained sections. Another set of three TBS rinses and then a further two-hour incubation using streptavidine-biotin horseradish-peroxidase complex (Dako) diluted in Tris-HCl, were followed by additional TBS washes.

In order to distinguish the epithelial immunoreactivity, 3,3-diaminobenzidine tetrahydrochloride (DAB) was applied, creating a brown hues corresponding with antibody reactions. The sections were then counter-stained with Mayer's Haematoxylin, generating a blue background staining so as to visualize morphology. Slides with sections of tonsil, treated in the same manner, apart from overnight incubation with TBS/TBST rather than primary antibody, were used as negative controls.

The epithelial expression of each factor was quantified using a computer-aided image analyser (Leica Microsystems Q500IW Image System, Leica, Cambridge, UK) (*Figure 9*). Cytokine expression was conveyed as the percentage of stained epithelial area, derived from the ratio of positive cytoplasmic and nuclear DAB immunostaining out of the total epithelial area.

Transcription factor and MAP kinase quantification were expressed in two different manners. The first, just as with cytokines, a percentage of total positively immunostained epithelium out of the entire intact epithelial area selected. Secondly, since the immunohistological method used allows for greater nuclear permeability, this enables a subsequent evaluation of positive staining of the nucleus or nuclear membrane, expressed as the number of positive nuclei/mm^2 epithelium.

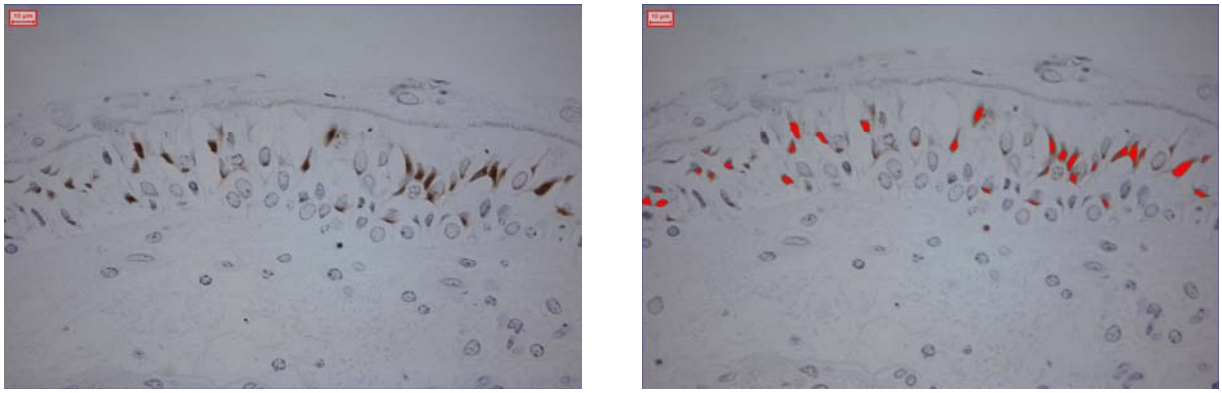


Figure 9: Immunohistochemical staining of transcription factor P-c-Jun (left) and the same staining as seen with Leica Image Analyzer (right).

BW and BAL

Inflammatory cells

Lavage fluids were filtered through a nylon filter (pore diameter 100 μm, Syntab Product AB, Malmö, Sweden) and centrifuged at 400 g for 15 minutes. The cell pellets were re-suspended in PBS to a concentration of 10^6 cells/ml, and total

number of cells counted using a Bürker chamber. Specimens were cytocentrifuged at 96 g for five minutes (Cytospin 3®, Shandon Southern Instruments Inc., Sewikly, PA, USA) and slides were prepared with 5 x 10^4 non-epithelial cells on each. 400 cells were counted on slides stained with May–Grünwald Giemsa, providing cell differential counts.

Cytokines and soluble mediators

The quantification of interleukin-6 (IL-6), interleukin-8 (IL-8), soluble intercellular adhesion molecule (sICAM), myeloperoxidase (MPO), and matrix metalloproteinase 9 (MMP-9) were determined using commercially available enzyme-linked immunosorbant assay (ELISA) kits. Albumin was measured using a commercially available kit from Boehringer Mannheim (Mannheim, Germany).

Analysis of human neutrophil lipocalin (HNL) and eosinophil protein X (EPX) were performed at the Department of Chemistry at Uppsala University, Sweden. HNL was assessed using a radio-immunoassay, while EPX was processed with ELISA [136, 197].

LUNG FUNCTION TESTING

Dynamic spirometry determining forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) was performed in the inclusionary stage of each study (Vitalograph-COMPACT; Vitalograph Ltd; Buckingham, UK). During all the studies, lung function measurements were consistently carried out pre and post air, ozone and diesel exhaust exposures respectively. All measurements were performed in triplicate, and the best value was recorded, in accordance with the recommendations of the American Thoracic Society [2].

SYMPTOMS

Throughout all exposures to filtered air, as well as pollutants, symptoms were assessed using a modified Borg's scale. Registration began immediately at the start of exposures and were performed at 30 minute intervals until termination [24]. The symptoms surveyed consisted of headache, dizziness, nausea, tiredness, chest pain, coughing, difficulty breathing, eye irritation, nose irritation, unpleasant smell, bad taste in mouth and throat irritation.

STATISTICS

All statistical calculations were processed SPSS® software version 13.0 for Windows® (SPSS Inc., Chicago, IL, USA). Significance was considered at $p \leq 0.05$. Values are expressed as medians with interquartile ranges (25^{th} to 75^{th} percentiles). Wilcoxon's non-parametric signed-rank sum test for paired observations was applied when evaluating coupled observations in the same individual, such as assessing O_3 and DE induced changes. In study I, group comparisons between asthmatics and healthy were calculated using the Mann-Whitney U test for analyzing independent samples. In study IV lung function comparisons were processed using T-Tests and symptomology was evaluated using General Linear Model, repeated measures ANOVA. The correlation analyses in study III were performed using the Spearman rank correlation test.

RESULTS

STUDY I

In this study healthy and mild asthmatic subjects were exposed to ozone (0.2 ppm) or filtered air for two hours during two randomized occasions. Bronchoscopy with biopsy acquirement was performed 6 hours after each exposure. Immunohistochemical methods were employed to analyze the cytokine expression in the bronchial epithelium.

Baseline results

When comparing the two groups after air exposure, asthmatics displayed a significantly greater baseline expression of IL-4 ($p=0.02$) and IL-5 ($p=0.02$). However, a significantly lower expression as compared to healthy subjects was observed for TNF-α ($p=0.02$), IL-10 ($p=0.001$), GRO- α ($p=0.006$), IL-8 ($p=0.003$) and ENA-78 ($p=0.006$). No significant baseline differences were detected for GM-CSF, IL-6 or fractalkine.

Cytokine responses

In the asthmatic group, O_3 exposure induced a significant increase in the expression of IL-5 ($p=0.03$), GM-CSF ($p=0.03$) and ENA-78 ($p=0.046$), with a trend suggestive of an IL-8 rise ($p=0.06$). No significant O_3-induced effects were seen in the healthy subjects, although IL-10 showed a tendency towards a decrease ($p=0.06$).

The O_3-induced changes within the two groups were then compared to one another and revealed that the increases in GRO-α ($p=0.011$), ENA-78 ($p=0.045$) and IL-8 ($p=0.004$) in the asthmatic group were significantly greater than the non-significant decreases seen in the healthy group. Similarly, the asthmatic group responded with a significantly greater response in IL-5 ($p=0.03$) and GM-CSF ($p=0.003$).

STUDY II

In this study, healthy subjects were exposed to ozone (0.2 ppm) or filtered air for two hours on two separate occasions. Endobronchial biopsies were procured via bronchoscopy 1½ hours after all exposures. The biopsy material was processed utilizing immunohistochemistry.

Cytokine responses

Ozone exposure brought about a significant suppression of the expression of IL-8 in the bronchial epithelium (p=0.018). No significant changes were noted in GRO-α, ENA-78, or TNF-α.

Transcription factor and MAPK responses

Exposure to O_3 resulted in a decrease both when examining total epithelial staining (p=0.003) as well as the number of positive nuclei for NFκB (p=0.02). Furthermore, a significant ozone-generated decline was seen in the total epithelial staining of p-c-Jun (p= 0.021), with a tendency towards a corresponding downward inclination in nuclear staining (p=0.07). No significant ozone induced responses were seen in C-fos, p-p38, p-JNK, or p-ERK.

STUDY III

In study III, healthy subjects underwent two separate exposure series, starting with a one hour exposure to diesel exhaust (300 μg/m^3) followed five hours later by a randomized O_3 (0.2 ppm) or filtered air exposure (DE+O_3 and DE+air respectively). Induced sputum collection occurred 24 hours after the initial exposure.

Differential cell counts

The DE+O_3 exposure was associated with a significant increase in the percentage of neutrophils (p=0.049), with an accompanying trend towards an increase in total neutrophil numbers (p=0.070). The neutrophilic increase was also reflected by a decrease in macrophage percentage (p=0.066). No changes in either cell counts or percentages were seen in lymphocytes, eosinophils or ciliated cells.

Soluble inflammatory markers

A significant increase in the concentration of MPO was seen after exposure to DE+O_3 (p=0.041), with a trend towards increase observed in MMP-9 concentrations (p=0.074). No changes were detected when evaluating IL-6.

Correlations

The differentials (Δ = DE+O_3 minus DE+air) between the two exposure series were analyzed and showed a significant association between the Δ of total PMNs with ΔMPO (r_S=0.759; p = 0.001) as well as ΔMMP-9 (r_S=0.535; p=0.033). Likewise, a strong correlation was observed between ΔMPO and ΔMMP-9 (r_S=0.803; p=0.000).

STUDY IV

In this study healthy subjects participated in two isolated exposure series, commencing with a systematically randomized one hour DE (300 μg/m^3) or filtered air exposure. Five hours later, on both occasions, the subjects underwent a two hour exposure to O_3 (0.2 ppm), creating a diesel exhaust+ozone and an air+ozone exposure series. Lung function testing was performed prior to and after each exposure. Bronchoscopy with BW and BAL was carried out 24 hours after the initial exposure.

BW responses

Following the diesel exhaust+ozone exposure there was a significant increase in the number of total cells ($p=0.011$), consisting of a clearly identifiable amplification in the number of neutrophils ($p=0.006$) and macrophages ($p=0.046$). An essential, yet non-significant, increase was seen in eosinophil numbers ($p=0.050$). No variation was seen in the lymphocyte count. Furthermore, no differences in the concentrations of IL-6, IL-8, sICAM, HNL, EPX, MMP-9, MPO or albumin were detected between the two series.

BAL responses

In BAL, the DE+O_3 exposure induced a heightened concentration of EPX ($p=0.04$). No other significant divergences were observed with regards to inflammatory cell counts, soluble proteins or cytokines.

Lung Function responses

Ozone exposure did not induce any significant changes to the subjects' lung function measurements regardless to if they were pre-exposed to DE or filtered air.

Study	Pollutant	Method	Subjects	Factors examined	Findings
I	Ozone	Endobronchial Biopsies	Healthy (H); Asthmatics (A)	**Cytokines:** IL-6, IL-5, IL-10, IL-8, Gro-α, ENA-78, TNF-α, GM-CSF, Fractalkine	**Baseline**: *A* ↑ IL-4 + 5 *H* ↑ TNF-α, IL-10, Gro-α, IL-8. ENA-78 **Air vs. O_3**: *A* ↑ IL-5, ENA-78, GM-CSF **Between groups**: IL-5, ENA-78, GM-CSF, GRO-α, IL-8
II	Ozone	Endobronchial Biopsies	Healthy	**Cytokines:** IL-8, GRO-α, ENA-78, TNF- α **Transcriptions factors + MAPKs:** NFκB, p-C-Jun, C-fos, p-p38, p-JNK, p-ERK	**%**: ↓ NFκB, p-C-jun **Nuclear**: ↓ NFκB **Cytokines**: ↓ IL-8
III	Ozone; Diesel	Induced Sputum	Healthy	**Cells:** MØ, PMNs, Lymphocytes, Eosinophils, Total cells **Soluble mediators:** MPO, MMP-9, IL-6	**After DE+O_3**: ↑ PMNs, MPO **Correlations**: Δ MPO-MMP-9- PMNs
IV	Ozone; Diesel	BW, BAL	Healthy	**Cells:** MØ, PMNs, Lymphocytes, Eosinophils, Total cells **Soluble mediators:** sICAM, IL-8, IL-6, MPO, MMP-9, HNL, EPX, Albumin **Lung function and symptoms**	**After DE+O_3:** *BW* ↑ Total cells, PMNs, MØ *BAL* ↑ EPX **Correlations:** *BAL* Total eosinophils – EPX

Table 2: Summary of results
Baseline = air differences between the groups; MØ = macrophages; %= percent positive staining of transcription factors within total epithelial area; Nuclear = number of positive nuclei/mm^2 in epithelium; Cytokine data all expressed as percent of positive staining in total epithelial area.

DISCUSSION

DISCUSSION OF METHODS

The exposures were meant to mimic a naturally occurring ambient exposure as closely as possible. Both exposure concentrations chosen, diesel exhaust at a PM concentration of 300 $\mu g/m^3$ and 0.2 ppm of ozone, represent realistic concentrations that can be encountered in an urban environment. In previous studies, these concentrations have also been observed to induce acute and measurable airway inflammatory effects.

The sequential exposure setup was chosen to resemble an urban cyclical profile of air pollution. Diesel exhaust, being a traffic-related pollutant, tends to peak at rush hours. Conversely, ozone production is mainly dependent on a photoreactive process causing it to reach a climax in the afternoon. Hence, a study design was selected with a diesel exhaust exposure in the morning and an ozone exposure taking place hours later in the afternoon.

Employing immunohistochemical techniques to endobronchial biopsies embedded in GMA allowed for the opportunity to study not only the cytokine, transcription factor and MAPK signaling pathways, but also gave an overview of the morphology. In studies I-II archived bronchial biopsies were examined and the sole focus was on inflammatory mediators within the epithelium. The airway epithelium has been suggested to play a central role in the defense and innate immune response of the lungs. It not only protects the airways via a barrier effect, but also serves as a target for inflammatory cell mediators and cytokines, as well as itself releasing several proinflammatory mediators and cytokines.

Induced sputum was selected as the investigative method in Study III since this gave us a proven, yet non-invasive, method to assess if any airway inflammatory effects would be present. Induced sputum has in previous studies been shown to be an effective means of evaluating inflammatory markers in the airways following separate air pollutant exposures consisting of DE or ozone [127, 129]. The cell counts obtained have been demonstrated to be highly repeatable. Sputum samples primarily represent the more proximal airways, though there are indices that it also provides information about effects in the lower airways as results have been comparable to those found in BAL [31, 112, 133].

Since enhanced inflammatory effects were shown in induced sputum after a diesel exhaust and subsequent ozone exposure, study IV took a further step in investigating the effects of combined sequential exposures. BW and BAL were obtained by means of flexible video bronchoscopy in order to evaluate airway inflammation. Whereas BW tends to represent more central airway sections, BAL principally corresponds to bronchoalveolar effects [147]. Consistently, DE induced airway neutrophilia tends to dominate in the bronchial segments, while neutrophilia

is found throughout the airway compartments after O_3 exposure [12, 73, 98, 124, 167]. Examining BW and BAL thus provides a useful method to distinguish distinctive effects found at various airway levels.

As it is always optimal to include as many subjects as possible, a balance has to be accomplished between what is useful from a statistical perspective versus what is ethically and practically feasible to carry out. Based on previous human exposure studies, it has been determined that a study population of fifteen subjects is a realistic and reasonable number of subjects when addressing air pollution induced airway inflammation [22, 168]. Each subject was exposed to filtered air and the chosen pollutant within the same exposure chamber, therefore acting as his or her own control.

Throughout the four studies non-parametric statistics tests were consistently chosen to test the hypotheses. The only exception to this was the use of t-test to evaluate lung function changes, as lung function is considered a normally distributed parameter. The use of non-parametric tests was selected since they offer two advantages, firstly they do not demand a normal distribution pattern and secondly they are less sensitive to outliers. When performing several statistical tests within a study, the concern of multiplicity arises. Multiple testing causes a potential increase of chance findings, since one in 20 tests may appear significant when the p-value is set to 0.05.

A solution would be to re-examine the significances using the Bonferroni post-hoc test. This correction maintains that if *n* independent hypotheses are tested on a set of data, then the p-value used should be *n* times smaller. Most studies with smaller subject numbers, such as fifteen, will encounter these statistical difficulties, and have a harder time holding up to the strictness of the Bonferroni correction than larger study groups.

However, if within the same study there are a considerable number of significances found within the results, it is more likely that these are true significances, such as in study I. Another factor to consider is whether the numbers and p-values are pointing to a trend. If the data show a clear tendency towards one direction, it can be presumed that the significances are less likely to be random and therefore more accurate. This consideration is applicable to the results found in all four studies, given that there were noticeable trends seen in each of the data sets, leading to an apparent premise. On the other hand, there are several p-values within the four studies that do hold up despite the rigorous Bonferroni correction, thus solidifying their validity.

DISCUSSION OF MAIN RESULTS

Study I-II

Inflammatory Responses Study I

Healthy subjects

Previous studies which have focused on the pulmonary effects of ozone exposure in healthy individuals, have found numerous markers in BW, BAL and biopsies indicating inflammation at various time points. These include the activation and expression of chemokines and adhesion molecules as well as inflammatory cells, primarily consisting of neutrophils [8, 13, 22, 43, 95, 157]. The inflammatory upregulation is seen as early as immediately after ozone exposure, and neutrophilia in lavage samples persists at 24 hours. Study I was carried out using archived biopsies from a previous study in healthy and asthmatic subjects [168]. Following ozone exposure, the upregulation of the expression of endothelial vascular adhesion molecules P-selectin and ICAM-1 expression was reported in healthy subjects. They also displayed an increase in neutrophil numbers in lavage as well as in the epithelium and submucosa.

Although ozone-stimulated neutrophil recruitment in the airways has been well established, little has been done to delineate the molecular mechanisms that precede this upregulation. Cytokines and chemokines play an essential role in the recruitment and regulation of neutrophils. In study I, no ozone-induced increase in cytokine expression was found in the bronchial epithelium of healthy individuals at six hours after exposure. At baseline, however, the expression of TNF-α, IL-10 and neutrophil chemoattractants were significantly increased in the healthy bronchial epithelium compared to the asthmatics. It is difficult to speculate what this might represent. Mild to moderate asthmatics, because of their persistent inflammation, may have a sustained underlying usage of the neutrophil chemoattractant factors, which in turn could be seen as a recession of these factors. Decreased levels of IL-10 have previously been found in sputum and BAL from asthmatic patients. IL-10 is a regulatory cytokine produced by T cells and macrophages, which relegates inflammatory responses. It has been theorized that the reduced production of this inhibitory cytokine is a factor in conditions with chronic underlying inflammation [25, 174].

Asthmatics

On account of a characteristically chronic airway inflammation and their heightened sensitivity to airway irritants, asthmatics have been indicated to be a particularly vulnerable group to the effects of ozone. It is presumed that ozone exposure heightens the already preexisting inflammatory condition, causing an exacerbation of symptoms. Enhanced ozone-induced airway inflammation in asthmatic airways as compared to healthy has been demonstrated. Notably, these

studies used longer exposures and thus employed a higher total ozone dose than the 0.2 ppm of ozone for 2 hours chosen in this study design [12, 14, 156]. In the study by Stenfors *et al* mentioned above, ozone exposure did not induce an airway neutrophilia in the asthmatic subjects, as was seen in the healthy group [168]. This raises the question if the ozone-induced airway inflammatory reaction in the asthmatic is delayed, and therefore finds itself in an earlier phase at 6 hours post exposure than in healthy airways.

As expected, the asthmatic group displayed a significantly higher expression already at baseline for IL-4 and IL-5, as compared to the healthy non-atopic group. Both these Th2 cytokines are strongly associated with the airway response seen in allergic asthma. IL-4 is crucial for IgE synthesis, and IL-5 plays a major part in eosinophil accumulation in asthmatic inflammation.

Ozone exposure induced an increase of IL-5 and GM-CSF, another cytokine which influences stimulation, activation and survival of eosinophils. This may mirror a later influx of eosinophils within the asthmatic airway. An ozone generated eosinophilia has been detected 18 hours after exposure in bronchial lavage from asthmatic subjects [134]. Significant associations have been found between the activation of eosinophils and the severity of asthma symptoms [50, 78]. This may provide an important cellular explanation to the epidemiological findings of increased asthma exacerbations following heightened ozone levels.

In addition, ozone exposure caused the upregulation of ENA-78 and IL-8 in the asthmatic epithelium. These chemokines have a central role in the activation and chemotaxis of neutrophils. Given that no corresponding neutrophil enhancement was seen in lavage or tissue at this time point, the amplified chemokines could denote that the asthmatics are still in the recruitment stage. This would suggest that the asthmatic airway does, in fact, respond slower to ozone-induced inflammation than in healthy subjects. This is in accordance with the lag effect in symptoms often noted in epidemiological studies, with emergency room visits increasing 1-2 days after a recorded ozone culmination period [30, 169, 179, 190]. Adaptations and alterations of endogenous mechanisms due to the already inflamed asthmatic airway could be involved with this delay. Another possible interpretation is that the chronic inflammation has exhausted the epithelial cells, thus causing a slower response to an exogenous irritant such as O_3.

Inflammatory responses Study II

There is limited knowledge about the time course involving the biochemical and cellular responses in human airways after ozone exposure. Ozone-induced elevations of neutrophils have been found in healthy subjects starting immediately after exposure (<1 hour) and persisting up to 24 hours [8, 12, 18, 42, 56, 94, 95, 98, 157, 181]. Although, it should be noted that most of these studies used higher ppm concentrations or longer exposure times, both resulting in a greater total ozone dose. We have previously observed an adhesion molecule upregulation at 1½ and 6 hours following a 0.2 ppm ozone exposure, with a subsequent neutrophilia at six

hours [22, 97, 168]. Given that no O_3 associated cytokine changes were seen in the healthy epithelium in Study I, further analysis was required to delineate the timeline aspect. We therefore analyzed archived healthy endobronchial mucosal biopsies sampled 1½ hours post-exposure, using an analogous exposure design to Study I. Unfortunately no asthmatic biopsies exist from this timepoint, although that would have been optimal.

Surprisingly, we found that O_3 significantly reduced the total epithelial expression of NFκB and c-Jun, as well as the nuclear expression of NFκB. Likewise, a significant decrease of IL-8 expression in the epithelium was discovered. The validity of these findings is strengthened by the consistency of the trends seen in the data. Overall, when looking at the medians, as well as spread of the interquartile ranges, there is a clear tendency towards suppression seen throughout the post-exposure data. This is also mirrored in the results for the healthy subjects in Study I. In general, these values also display a decline post-ozone, even though no significant O_3-induced changes were detected.

To the best of our knowledge, this is the first investigation of the early signal transduction and cytokine expression in the bronchial epithelium of human subjects after a controlled chamber ozone exposure. Therefore, to hypothesize about the possible underlying mechanisms of these unexpected results we must generally rely on conclusions formed in animal and in vitro studies.

Theories for NFkB suppression

Although several animal and in vitro studies have indicated that NFκB and AP-1 could be involved in the inflammatory response to ozone, there is no real experimental evidence for affirming that ozone causes changes to redox sensitive pathways in human airways. In addition, within the bronchial epithelium there has been no confirmation of increased expression of cytokines under the regulation of AP-1 or NFκB at either 1½ or six hours following a controlled ozone exposure [22, 26, 98].

As ozone interacts with components of the RTLF, it generates secondary oxidation products resulting in airway inflammation. Once activated, neutrophils can move into the RTLF, thus creating an endogenous oxidative stress by releasing ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). Ordinarily during inflammatory reactions the enzyme IκB kinase β (IKK β) phosphorylates IκB, which detaches from NFκB dimer. This allows for the activation, nuclear translocation and DNA binding of NFκB (*Figure 2*). It has been demonstrated that the presence of H_2O_2 leads to a reduction of IKK, thus causing a suppression of the kinase activity [148]. This negative regulation of NFκB is a reversible process, considered to be a protective mechanism during periods of intensified oxidation. Since neutrophils are found to be up regulated almost directly after an ozone challenge, it is possible that at 1½ hours they have become an endogenous source of H_2O_2 [42, 94, 157, 181].

This IKK decrement would entail that more IκB would stay bound to NFκB, therefore almost certainly masking the antibody binding site on the p65 epitope. This sustained bond to IκB also explains the decrease seen in the nucleus, since translocation for these complexes would be unachievable.

Another speculation to explain the ozone-induced decrease in NFκB could be based on in vitro studies that have shown an antioxidant induced inhibition at various stages of the TNF-α, NFκB and IL-8 pathway. Curcumin has been shown to increase GSH in epithelial cells and when GSH concentrations were high it effectively inhibited cytokine stimulated NFκB activation and subsequent IL-8 release [20]. This, in accordance with our previous finding of a significant increase in GSH in the RTLF at 1½ hours post ozone challenge, may be a key to explaining this initial suppression by an oxidant stimulus.

Theories for IL-8 suppression

Many in vitro studies that have focused on therapeutic or reactive oxidant inhibition of TNF-α or NFκB, have also reported a corresponding IL-8 repression [20, 182]. This is to be expected since the functional binding sites on the chemokine's promoter region are for NFκB and AP-1, thus causing these transcription factors to be vital for its gene expression.

Although, the decrease in IL-8 detected in this study could also be associated with its various characteristics. In the tissue IL-8 is found in different forms. Many of the free chemokines are monomers, yet many multimerize when higher concentrations arise. They can also be bound (as monomers) to their specific G-protein coupled receptor on the leukocyte or to cell surface and soluble glycosaminoglycans (as multimers) (*Figure 10*). Multimerization as well as forming chemokine-GAG complexes alter the biological function and the structural composition of the chemokine [52, 58, 76, 101, 164]. This could be an alternate explanation to the reduction in IL-8 seen in study II after ozone exposure, since much is still unknown about antibody specificity.

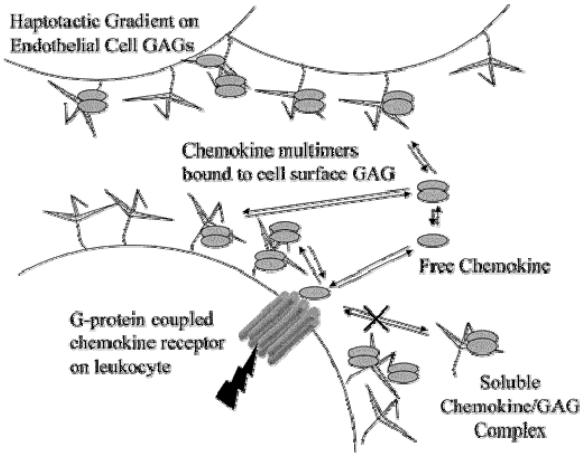


Figure 10: Schematic picture of IL-8 complexes and their affect on binding (Original picture Kuschert et al. Biochemistry, 1999; 38:12959-12968).

Theory for neutrophil upregulation at 6 hours

It appears that an environmentally relevant ozone exposure does not induce an upregulation of AP-1 or NFκB at the time point investigated in the present study. In spite of this, we do see neutrophil migration into the bronchial submucosa and epithelium. However, this could be stimulated via NFκB independent pathways.

As increasing doses of ozone come into contact with the RTLF, the antioxidant defenses decline. If the redox balance is overwhelmed in the RTLF, ozone can oxidize biomolecular components within the protective layer, causing the formation of bioactive oxidation products. Lipid ozonation products have displayed the capability to activate phospholipase A2 and 5-lipooxygenase, leading to the production of LTB_4, a potent neutrophil chemoattractant. Coffey *et al* demonstrated elevated levels of LTB_4 in BAL immediately, as well as 2 hours after an ozone challenge, which correlates well with the time point addressed in Study II [38].

ICAM-1 expression is an important factor in the neutrophil-mediated inflammatory response. We have in previous controlled chamber studies seen ozone-induced ICAM-1 upregulation in the vascular endothelium at 1,5 hours post exposure, yet not in the epithelium until six hours after challenge. In vitro studies have shown that, whereas TNF-α can cause an increase in ICAM-1 in both endothelial and epithelial cells, H_2O_2 mediated induction of ICAM-1 only occurs in endothelial cells [32, 102]. As no evidence has been found in humans at this timepoint of heightened TNF-α, H_2O_2 production could be a possible explanation for the early vascular endothelial expression of ICAM-1.

Pathways- DE vs. Ozone

When choosing what factors to examine in studies I and II, it seemed logical to see if DE and ozone exhibited any resemblance in the pathways they elicit in the airways, given their similar observed health outcomes. In addition, exposure to either of these oxidant pollutants has been shown to produce significant neutrophilia in the airway lavage and tissues. However, the inflammatory pathway triggered by DE has been more elucidated than that which ensues after an ozone exposure. It has been demonstrated in controlled chamber studies that DE generates an enhancement of neutrophil chemotaxis related chemokines (IL-8, Gro-α) in the bronchial epithelium. Moreover, it was demonstrated that DE initiates the p38 and JNK MAPK pathways as well as an increase in nuclear translocation of NFκB and AP-1 [140]. These findings may reflect that oxidative stress is involved in the underlying molecular mechanisms behind the DE-induced airway inflammation.

However, given that no such upregulation of cytokines, redox sensitive transcription factors or their upstream MAPKs were found in the epithelium, this does not appear to be the mechanism underlying the ozone airway neutrophilia. A current opinion is that ozone reactions are restricted to the RTLF, and that

secondary oxidation products are responsible for the ensuing inflammatory responses [139, 143, 144]. This would be in contrast with DE-mediated inflammation, which appears to involve direct cell contact with pro-oxidant elements.

Study III-IV

The evaluation of exposure to co-pollutants has been sparse and most of the knowledge is derived from a handful of animal and in vitro studies. Even though these tend to focus on a simultaneous exposure model, as opposed to a cyclical one, they form an interesting foundation for further study in humans as they indicate a potentiation effect. Using a simultaneous exposure pattern adds the element of pollutant interaction and subsequent modification of their properties. However, in the present studies we have chosen to asses an urban exposure situation and thus used a subsequent exposure model.

Inflammatory Results

In study III the two separate exposure series consisted of a DE challenge in the morning, followed by a filtered air or ozone exposure during the afternoon. Inflammatory factors were determined in induced sputum samples collected 18 hours after the second exposure. In study IV, the exposure sequence was DE or filtered air exposure in the morning, with a subsequent ozone exposure in the afternoon. Bronchoscopy was performed 18 hours after the second exposure (*Figure 11*).

In both studies subjects responded to a DE exposure and a subsequent ozone exposure with a significant increase in neutrophils, as compared with the sequence containing the filtered air exposure. Furthermore, in study III there was a significant association between neutrophils, MPO and MMP-9, a strong indication of neutrophil activation. Since the methods and study designs differ slightly between the two studies, there is a subtle, yet important difference in what the results denote. In study III the results indicate an ozone intensification of the DE-induced airway inflammation. Whereas in study IV, the ozone-generated inflammatory response is amplified by a pre-exposure to DE.

What comparisons can be drawn

Even though the intracellular mechanisms behind the DE and ozone induced inflammation appear to differ, airway neutrophilia is a well-established response to both these air pollutants. It would be unrealistic to complete a four-armed study in human volunteers in an attempt to include an air+air sequence and an air+O_3 (in study III) or a DE+air (in study IV). Therefore, it is feasible to instead rely on the results of previous controlled chamber studies performed under similar conditions to provide us with an idea of how these exposure series would appear.

As expected, the air+air exposure in all of our past experiments has established that this sequence of exposures does not produce any signs of significantly heightened

airway inflammation [19, 22, 26, 153, 168]. Primarily, environmentally applicable exposures to ozone begin recruiting neutrophilic to the airways early and airway neutrophilia is thought to peak around six to twelve hours. Though a study using similar exposure conditions as in study III, has demonstrated a sustained increase of neutrophils in healthy BW at 18 hours following an O_3 exposure [166]. Controlled chamber exposures to diesel exhaust in healthy subjects have, in similarity to ozone, observed neutrophilia in BW as late as 18-24 hours after exposure [19, 150].

EPX

Diesel exhaust and subsequent ozone exposure generated a modest, nonetheless significant, increase in EPX in BAL in study IV. This enhancement of EPX may signify an eosinophil activation, although a reflected rise in BAL eosinophils was not found. However, in BAL sampled after DE with subsequent ozone exposure there is a statistically significant correlation between EPX and total eosinophils. No eosinophilia has been reported in lavage samples from healthy subjects after DE or ozone under the exposure conditions applied here or at a comparable time point. This raises the question if the EPX increase is representative of a combination effect. If this indeed is an outcome due to a co-exposure of these pollutants in healthy subjects, it may well be even more pronounced in asthmatics. A heightened airway eosinophilia would in turn produce an augmentation of symptoms leading to an acute asthma exacerbation. If so, this would provide a critical link between an underlying mechanism and epidemiological data suggesting asthmatics to be particularly sensitive to air pollution.

Consequences

As NO emissions from traffic lessen, there is a reduced amount for O_3 interactions, thereby increasing the O_3 levels in cities. In addition, the continued effect of pollution on the global climate will likely lead to an increasingly warmer climate, thus generating higher urban ozone levels. Proposals of reducing particulate emissions may not be enough to reduce detrimental air pollution health effects. There is epidemiological evidence suggesting an adverse interaction between PM and O_3, as well as animal studies demonstrating O_3 increasing the susceptibility to exhaust particles [6, 72]. Although the interpretations of the results from study III and IV vary slightly, the outcomes from these studies both indicate an additive inflammatory effect when exposed to these two important ambient pollutants. Since air pollution levels are currently set individually for each pollutant, these findings could suggest that there needs to be consideration to the possibility that these pollutants should also be regulated in conjunction with each other. This signifies a valuable step forward in understanding the airway effects in humans exposed to an urban pollution profile.

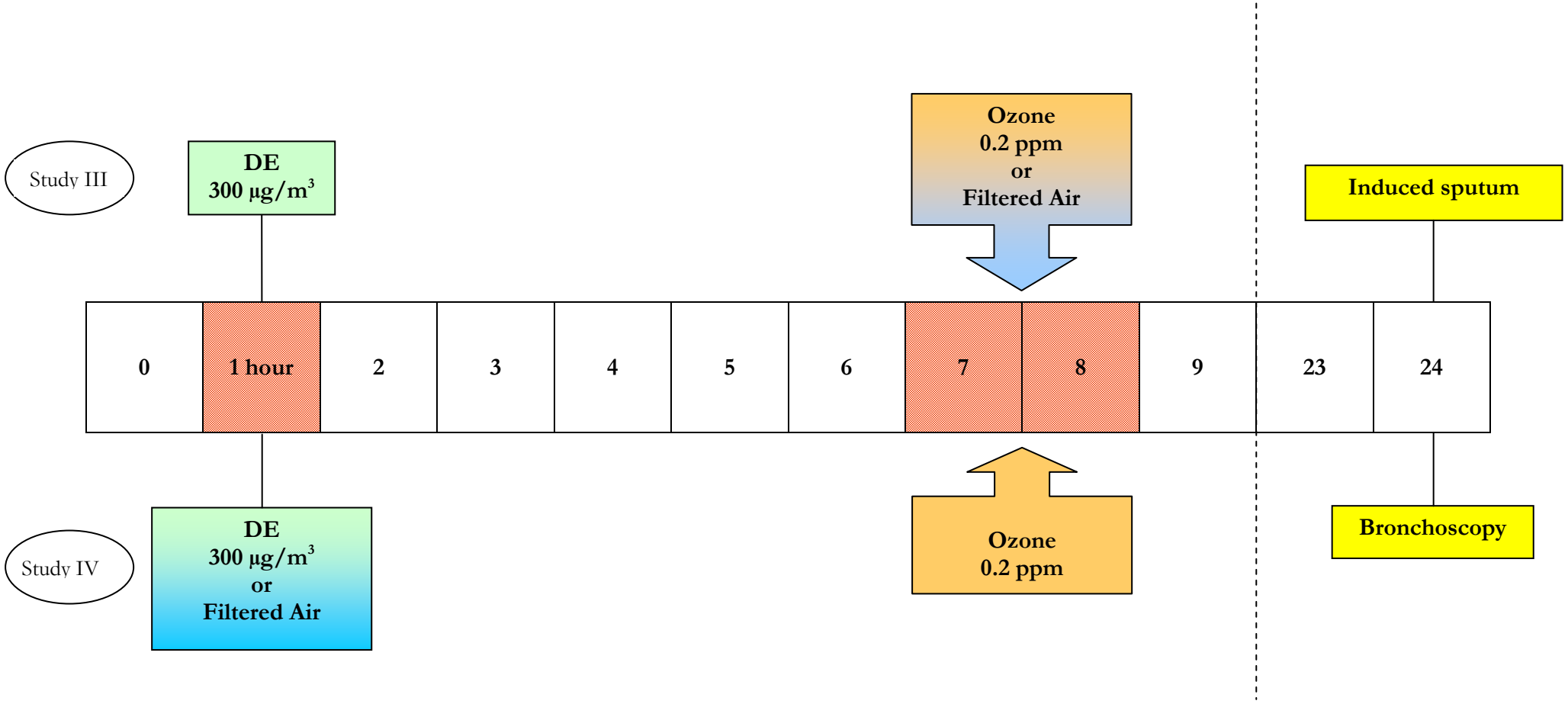


Figure 11: The top sequence of events is the setup for Study III, whereas the lower shows the exposure series for Study IV.

FINAL COMMENTS

Epidemiological evidence concerning the harmful health effects of air pollutants on the respiratory and cardiovascular systems has been accumulated over the last few decades. This is further supported by results from animal and in vitro studies demonstrating cellular and tissue damage after exposure to diesel exhaust as well as ozone. Human exposure studies have found clear associations between exposure to these oxidant air pollutants and airway neutrophilia. However, few human studies have been performed to elucidate the underlying biological mechanisms. In the first two studies of this thesis controlled chamber exposures were performed to evaluate the pro-inflammatory signaling pathways in the epithelium following ozone exposure.

In asthmatics a rise in the epithelial expression of neutrophil chemoattractants and eosinophil-related cytokines was present six hours after ozone exposure. However, this response was not found in biopsies from healthy subjects, where no ozone-induced cytokine upregulations were detected. This corresponds to a previous study showing ozone generated neutrophilia in biopsies from healthy subjects at this timepoint, yet no such similar reaction in the asthmatic tissue. As only the asthmatics displayed this pro-inflammatory cytokine enhancement at six hours, it was interpreted as a further indication of a time kinetic discrepancy or response pattern variability between ozone induced airway inflammation in healthy and asthmatic individuals.

Ozone and diesel exhaust have both been associated with generating oxidative stress and inflammation in the airways. Recently, diesel exhaust activation of redox sensitive pathways and enhancement of their associated cytokines have been demonstrated. Surprisingly, following an ozone exposure using a similar study design, endobronchial mucosal biopsies revealed an early suppression within the epithelium of transcription factors as well as IL-8, a chemokine regulated by their activity. This may support the presumption that ozone induced airway inflammation is mediated via secondary ozonation products, and therefore activates neutrophilia through an alternative signaling pathway. It could be speculated that this timepoint is too early to detect an activation by ozone of the investigated signaling pathways. As ozone did not seem to augment cytokine expression in healthy subjects at six hours, it appears likely that these molecular mechanisms may not be of importance in the epithelium, in contrast to the diesel exhaust mediated inflammation. In order to get a comprehensive picture of the timeline as well as the signaling pathways in the human airways after ozone exposure, additional time points and mediators must be investigated in both healthy and asthmatic subjects.

The last two studies in the thesis address the important issue of the cumulative inflammatory airway effects of exposure to an urban pollution profile. When exposed to a sequential diesel exhaust and ozone exposure model analysis of

inflammatory cells in induced sputum and BW showed an amplified neutrophilia. These novel findings reveal a clear additive effect on the proximal airway inflammation when exposed in sequence to environmentally relevant levels of these air pollutants. Future studies will further address these adjuvant effects within bronchial biopsies. It is of paramount importance to also investigate individuals with asthma, COPD and cardiovascular disease as they are known to be more susceptible to air pollution exposure.

As the detrimental effects of air pollution on our environment and health become more undeniable, new reforms concerning assessment and controls of emissions have started to emerge. Air pollution affects us all daily; therefore it is vital to continually increase our knowledge of the underlying mechanisms and co-pollutant impacts within the human body.

CONCLUSIONS

The findings in this thesis reveal:

- ∂ Asthmatic subjects displayed an augmentation within the bronchial epithelium of neutrophil chemoattractants and Th2 cytokines six hours following ozone exposure, in contrast to healthy subjects.

- ∂ The Th2 cytokine upregulation, along with the indication of an ensuing enhanced bronchial epithelial neutrophilia, may denote an explanation to the increased sensitivity of asthmatics to ambient levels of ozone.

- ∂ Exposure to ozone resulted in an early reduction in the expression of the redox sensitive transcription factors NFκB and c-jun as well as the neutrophil chemoattractant IL-8 in the bronchial epithelium.

- ∂ These findings suggest a divergence of the inflammatory signaling pathways in the bronchial epithelium after exposure to ozone and diesel exhaust.

- ∂ An ozone challenge following an initial diesel exhaust exposure generates an amplification of the established diesel exhaust induced neutrophilic airway inflammation, along with signs of neutrophil activation.

- ∂ Compared to air, diesel exhaust exposure enhanced the ozone induced airway inflammation, with increases in neutrophils, alveolar macrophages and eosinophil protein X in airway lavages.

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REFERENCES

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

2. Lung function testing: selection of reference values and interpretative strategies. American Thoracic Society. *Am Rev Respir Dis.* Nov 1991;144(5):1202-1218.

3. *National Institute of Health. Global Strategy for Asthma Management and Prevention.* Geneva: NHLBI/WHO 1995.

4. *World Development report*: World Bank; 2001.

5. **Adamson IY, Vincent R, Bjarnason SG**. Cell injury and interstitial inflammation in rat lung after inhalation of ozone and urban particulates. *Am J Respir Cell Mol Biol.* May 1999;20(5):1067-1072.

6. **Aga E, Samoli E, Touloumi G, Anderson HR, Cadum E, Forsberg B, Goodman P, Goren A, Kotesovec F, Kriz B, Macarol-Hiti M, Medina S, Paldy A, Schindler C, Sunyer J, Tittanen P, Wojtyniak B, Zmirou D, Schwartz J, Katsouyanni K**. Short-term effects of ambient particles on mortality in the elderly: results from 28 cities in the APHEA2 project. *Eur Respir J Suppl.* May 2003;40:28s-33s.

7. **ALA**. *State of the air*: American Lung Association; 2004.

8. **Aris RM, Christian D, Hearne PQ, Kerr K, Finkbeiner WE, Balmes JR**. Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. *Am Rev Respir Dis.* Nov 1993;148(5):1363-1372.

9. **Atkinson RW, Anderson HR, Sunyer J, Ayres J, Baccini M, Vonk JM, Boumghar A, Forastiere F, Forsberg B, Touloumi G, Schwartz J, 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.* Nov 15 2001;164(10 Pt 1):1860-1866.

10. **Babior BM**. Phagocytes and oxidative stress. *Am J Med.* Jul 2000;109(1):33-44.

11. **Baeza-Squiban A, Bonvallot V, Boland S, Marano F**. Airborne particles evoke an inflammatory response in human airway epithelium. Activation of transcription factors. *Cell Biol Toxicol.* 1999;15(6):375-380.

12. **Balmes JR, Aris RM, Chen LL, Scannell C, Tager IB, Finkbeiner W, Christian D, Kelly T, Hearne PQ, Ferrando R, Welch B**. Effects of ozone on normal and potentially sensitive human subjects. Part I: Airway inflammation and responsiveness to ozone in normal and asthmatic subjects. *Res Rep Health Eff Inst.* Jun 1997(78):1-37; discussion 81-99.

13. **Balmes JR, Chen LL, Scannell C, Tager I, Christian D, Hearne PQ, Kelly T, Aris RM**. Ozone-induced decrements in FEV1 and FVC do not correlate with measures of inflammation. *Am J Respir Crit Care Med.* Mar 1996;153(3):904-909.

14. **Basha MA, Gross KB, Gwizdala CJ, Haidar AH, Popovich J, Jr.** Bronchoalveolar lavage neutrophilia in asthmatic and healthy volunteers after controlled exposure to ozone and filtered purified air. *Chest.* Dec 1994;106(6):1757-1765.

15. **Baulig A, Garlatti M, Bonvallot V, Marchand A, Barouki R, Marano F, 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.* Sep 2003;285(3):L671-679.

16. **Bayram H, Devalia JL, Sapsford RJ, Ohtoshi T, Miyabara Y, Sagai M, 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.* Mar 1998;18(3):441-448.

17. **Becker S, Soukup JM, Gilmour MI, Devlin RB**. Stimulation of human and rat alveolar macrophages by urban air particulates: effects on oxidant radical generation and cytokine production. *Toxicol Appl Pharmacol.* Dec 1996;141(2):637-648.

18. **Behndig A**. *Adaptive antioxidant responses at the air lung interface in healthy humans following ozone exposure.*, Umeå University; 2006.

19. **Behndig AF, Mudway IS, Brown JL, Stenfors N, Helleday R, Duggan ST, Wilson SJ, Boman C, Cassee FR, Frew AJ, Kelly FJ, Sandstrom T, Blomberg A**. Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. *Eur Respir J.* Feb 2006;27(2):359-365.

20. **Biswas SK, McClure D, Jimenez LA, Megson IL, Rahman I**. Curcumin induces glutathione biosynthesis and inhibits NF-kappaB activation and interleukin-8 release in alveolar epithelial cells: mechanism of free radical scavenging activity. *Antioxid Redox Signal.* Jan-Feb 2005;7(1-2):32-41.

21. **Blomberg A**. Airway inflammatory and antioxidant responses to oxidative and particulate air pollutants - experimental exposure studies in humans. *Clin Exp Allergy.* Mar 2000;30(3):310-317.

22. **Blomberg A, Mudway IS, Nordenhall C, Hedenstrom H, Kelly FJ, Frew AJ, Holgate ST, Sandstrom T**. Ozone-induced lung function decrements do not correlate with early airway inflammatory or antioxidant responses. *Eur Respir J.* Jun 1999;13(6):1418-1428.

23. **Boland S, Baeza-Squiban A, Fournier T, Houcine O, Gendron MC, Chevrier M, Jouvenot G, Coste A, Aubier M, Marano F**. Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production. *Am J Physiol.* Apr 1999;276(4 Pt 1):L604-613.

24. **Borg G**. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc.* 1982;14(5):377-381.

25. **Borish L**. IL-10: evolving concepts. *J Allergy Clin Immunol.* Mar 1998;101(3):293-297.

26. **Bosson J, Stenfors N, Bucht A, Helleday R, Pourazar J, Holgate ST, Kelly FJ, Sandstrom T, Wilson S, Frew AJ, Blomberg A**. Ozone-induced bronchial epithelial cytokine expression differs between healthy and asthmatic subjects. *Clin Exp Allergy.* Jun 2003;33(6):777-782.

27. **Brain JD**. Mechanisms, measurement, and significance of lung macrophage function. *Environ Health Perspect.* Jul 1992;97:5-10.

28. **Britten KM, Howarth PH, Roche WR**. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. *Biotech Histochem.* Sep 1993;68(5):271-280.

29. **Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC, Jr., Tager I**. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation.* Jun 1 2004;109(21):2655-2671.

30. **Brunekreef B, Holgate ST**. Air pollution and health. *Lancet.* Oct 19 2002;360(9341):1233-1242.

31. **Brusasco V, Crimi E, Barisione G, Spanevello A, Rodarte JR, Pellegrino R**. Airway responsiveness to methacholine: effects of deep inhalations and airway inflammation. *J Appl Physiol.* Aug 1999;87(2):567-573.

32. **Bundy RE, Hoare GS, Kite A, Beach J, Yacoub M, Marczin N**. Redox regulation of p38 MAPK activation and expression of ICAM-1 and heme oxygenase-1 in human alveolar epithelial (A549) cells. *Antioxid Redox Signal.* Jan-Feb 2005;7(1-2):14-24.

33. **Byrd RS, Joad JP**. Urban asthma. *Curr Opin Pulm Med.* Jan 2006;12(1):68-74.

34. **Cantin AM, North SL, Hubbard RC, Crystal RG**. Normal alveolar epithelial lining fluid contains high levels of glutathione. *J Appl Physiol.* Jul 1987;63(1):152-157.

35. **Chang Y, Senechal S, de Nadai P, Chenivesse C, Gilet J, Vorng H, Legendre B, Tonnel AB, Wallaert B, Lassalle P, Tsicopoulos A**. Diesel exhaust exposure favors TH2 cell recruitment in nonatopic subjects by differentially regulating chemokine production. *J Allergy Clin Immunol.* Aug 2006;118(2):354-360.

36. **Chitano P, Hosselet JJ, Mapp CE, Fabbri LM**. Effect of oxidant air pollutants on the respiratory system: insights from experimental animal research. *Eur Respir J.* Aug 1995;8(8):1357-1371.

37. **Cho HY, Morgan D, Bauer AK, Kleeberger SR**. Signal Transduction Pathways of Tumor Necrosis Factor-Mediated Lung Injury Induced by Ozone in Mice. *Am J Respir Crit Care Med.* Jan 25 2007.

38. **Coffey MJ, Wheeler CS, Gross KB, Eschenbacher WL, Sporn PH, Peters-Golden M**. Increased 5-lipoxygenase metabolism in the lungs of human subjects exposed to ozone. *Toxicology.* Dec 18 1996;114(3):187-197.

39. **Daigle CC, Chalupa DC, Gibb FR, Morrow PE, Oberdorster G, Utell MJ, Frampton MW**. Ultrafine particle deposition in humans during rest and exercise. *Inhal Toxicol.* May 2003;15(6):539-552.

40. **Davis RJ**. Signal transduction by the JNK group of MAP kinases. *Cell.* Oct 13 2000;103(2):239-252.

41. **Devalia JL, Bayram H, Rusznak C, Calderon M, Sapsford RJ, Abdelaziz MA, Wang J, Davies RJ**. Mechanisms of pollution-induced airway disease: in vitro studies in the upper and lower airways. *Allergy.* 1997;52(38 Suppl):45-51; discussion 57-48.

42. **Devlin RB, McDonnell WF, Becker S, Madden MC, McGee MP, Perez R, Hatch G, House DE, Koren HS**. Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4 ppm ozone for 2 hr: a comparison of mediators found in bronchoalveolar lavage fluid 1 and 18 hr after exposure. *Toxicol Appl Pharmacol.* May 1996;138(1):176-185.

43. **Devlin RB, McDonnell WF, Mann R, Becker S, House DE, Schreinemachers D, Koren HS**. Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am J Respir Cell Mol Biol.* Jan 1991;4(1):72-81.

44. **Devlin RB, McKinnon KP, Noah T, Becker S, Koren HS**. Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. *Am J Physiol.* Jun 1994;266(6 Pt 1):L612-619.

45. **Diaz-Sanchez D, Garcia MP, Wang M, Jyrala M, Saxon A**. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J Allergy Clin Immunol.* Dec 1999;104(6):1183-1188.

46. **Dockery DW, Stone PH**. Cardiovascular risks from fine particulate air pollution. *N Engl J Med.* Feb 1 2007;356(5):511-513.

47. **Dominici F, Peng RD, Bell ML, Pham L, McDermott A, Zeger SL, Samet JM**. Fine particulate air pollution and hospital admission for cardiovascular and respiratory diseases. *Jama.* Mar 8 2006;295(10):1127-1134.

48. **Driscoll KE, Simpson L, Carter J, Hassenbein D, Leikauf GD**. Ozone inhalation stimulates expression of a neutrophil chemotactic protein, macrophage inflammatory protein 2. *Toxicol Appl Pharmacol.* Apr 1993;119(2):306-309.

49. **Dunster C, IS. M, A. B, Stenfors N, R. H, Sandstrom T, FJ. K**. Antioxidant kinetics in human respiratory tract lining fluid following exposure to ozone. *Am J Respir Crit Care Med.* 2003;167(A 127).

50. **Dusser D**. [Role of eosinophils in asthma]. *Rev Mal Respir.* Feb 2000;17(1 Pt 2):195-201.

51. **EPA**. National Ambient Air Quality Standards (NAAQS). http://www.epa.gov/air/criteria.html.

52. **Fernando H, Chin C, Rosgen J, Rajarathnam K**. Dimer dissociation is essential for interleukin-8 (IL-8) binding to CXCR1 receptor. *J Biol Chem.* Aug 27 2004;279(35):36175-36178.

53. **Finlayson-Pitts BJ, Pitts JN, Jr.** Tropospheric air pollution: ozone, airborne toxics, polycyclic aromatic hydrocarbons, and particles. *Science.* May 16 1997;276(5315):1045-1052.

54. **Folinsbee LJ, Horstman DH, Kehrl HR, Harder S, Abdul-Salaam S, Ives PJ**. Respiratory responses to repeated prolonged exposure to 0.12 ppm ozone. *Am J Respir Crit Care Med.* Jan 1994;149(1):98-105.

55. **Foster WM, Brown RH, Macri K, Mitchell CS**. Bronchial reactivity of healthy subjects: 18-20 h postexposure to ozone. *J Appl Physiol.* Nov 2000;89(5):1804-1810.

56. **Frampton MW, Morrow PE, Torres A, Voter KZ, Whitin JC, Cox C, Speers DM, Tsai Y, Utell MJ**. Effects of ozone on normal and potentially sensitive human subjects. Part II: Airway inflammation and responsiveness to ozone in nonsmokers and smokers. *Res Rep Health Eff Inst.* Jun 1997(78):39-72; discussion 81-99.

57. **Frampton MW, Pryor WA, Cueto R, Cox C, Morrow PE, Utell MJ**. Aldehydes (nonanal and hexanal) in rat and human bronchoalveolar lavage fluid after ozone exposure. *Res Rep Health Eff Inst.* Nov 1999(90):1-15; discussion 17-18.

58. **Frevert CW, Kinsella MG, Vathanaprida C, Goodman RB, Baskin DG, Proudfoot A, Wells TN, Wight TN, Martin TR**. Binding of interleukin-8 to heparan sulfate and chondroitin sulfate in lung tissue. *Am J Respir Cell Mol Biol.* Apr 2003;28(4):464-472.

59. **Friedman MS, Powell KE, Hutwagner L, Graham LM, Teague WG**. Impact of changes in transportation and commuting behaviors during the 1996 Summer Olympic Games in Atlanta on air quality and childhood asthma. *Jama.* Feb 21 2001;285(7):897-905.

60. **Garrington TP, Johnson GL**. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol.* Apr 1999;11(2):211-218.

61. **Gent JF, Triche EW, Holford TR, Belanger K, Bracken MB, Beckett WS, Leaderer BP**. Association of low-level ozone and fine particles with respiratory symptoms in children with asthma. *Jama.* Oct 8 2003;290(14):1859-1867.

62. **Gershwin LJ**. Effects of air pollutants on development of allergic immune responses in the respiratory tract. *Clin Dev Immunol.* Jun-Dec 2003;10(2-4):119-126.

63. **Giembycz MA, Lindsay MA**. Pharmacology of the eosinophil. *Pharmacol Rev.* Jun 1999;51(2):213-340.

64. **Gilmour MI, Park P, Doerfler D, Selgrade MK**. Factors that influence the suppression of pulmonary antibacterial defenses in mice exposed to ozone. *Exp Lung Res.* May-Jun 1993;19(3):299-314.

65. **Graham JA, Gardner DE, Blommer EJ, House DE, Menache MG, Miller FJ**. Influence of exposure patterns of nitrogen dioxide and modifications by ozone on susceptibility to bacterial infectious disease in mice. *J Toxicol Environ Health.* 1987;21(1-2):113-125.

66. **Granata F, Frattini A, Loffredo S, Del Prete A, Sozzani S, Marone G, Triggiani M**. Signaling events involved in cytokine and chemokine production induced by secretory phospholipase A2 in human lung macrophages. *Eur J Immunol.* Jul 2006;36(7):1938-1950.

67. **Hamilton RF, Jr., Li L, Eschenbacher WL, Szweda L, Holian A**. Potential involvement of 4-hydroxynonenal in the response of human lung cells to ozone. *Am J Physiol.* Jan 1998;274(1 Pt 1):L8-16.

68. **Han JY, Takeshita K, Utsumi H**. Noninvasive detection of hydroxyl radical generation in lung by diesel exhaust particles. *Free Radic Biol Med.* Mar 1 2001;30(5):516-525.

69. **Harrod KS, Jaramillo RJ, Rosenberger CL, Wang SZ, Berger JA, McDonald JD, Reed MD**. Increased susceptibility to RSV infection by exposure to inhaled diesel engine emissions. *Am J Respir Cell Mol Biol.* Apr 2003;28(4):451-463.

70. **Heiman AS, Allen-Gipson D**. Cytokines potentiate human eosinophil superoxide generation in the presence of N(omega)-nitro-L-arginine methyl ester. *Int J Immunopharmacol.* Feb 2000;22(2):171-181.

71. **Hiura TS, Kaszubowski MP, Li N, Nel AE**. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J Immunol.* Nov 15 1999;163(10):5582-5591.

72. **Hoek G, Brunekreef B, Verhoeff A, van Wijnen J, Fischer P**. Daily mortality and air pollution in The Netherlands. *J Air Waste Manag Assoc.* Aug 2000;50(8):1380-1389.

73. **Holgate ST, Sandstrom T, Frew AJ, Stenfors N, Nordenhall C, Salvi S, Blomberg A, Helleday R, Soderberg M**. Health effects of acute exposure to air pollution. Part I: Healthy and asthmatic subjects exposed to diesel exhaust. *Res Rep Health Eff Inst.* Dec 2003(112):1-30; discussion 51-67.

74. **Holz O, Mucke M, Paasch K, Bohme S, Timm P, Richter K, Magnussen H, Jorres RA**. Repeated ozone exposures enhance bronchial allergen responses in subjects with rhinitis or asthma. *Clin Exp Allergy.* May 2002;32(5):681-689.

75. **Holz O, Tal-Singer R, Kanniess F, Simpson KJ, Gibson A, Vessey RS, Janicki S, Magnussen H, Jorres RA, Richter K**. Validation of the human ozone challenge model

as a tool for assessing anti-inflammatory drugs in early development. *J Clin Pharmacol.* May 2005;45(5):498-503.

76. **Hoogewerf AJ, Kuschert GS, Proudfoot AE, Borlat F, Clark-Lewis I, Power CA, Wells TN**. Glycosaminoglycans mediate cell surface oligomerization of chemokines. *Biochemistry.* Nov 4 1997;36(44):13570-13578.

77. **Horstman DH, Ball BA, Brown J, Gerrity T, Folinsbee LJ**. Comparison of pulmonary responses of asthmatic and nonasthmatic subjects performing light exercise while exposed to a low level of ozone. *Toxicol Ind Health.* Jul-Aug 1995;11(4):369-385.

78. **Humbert M**. Pro-eosinophilic cytokines in asthma. *Clin Exp Allergy.* Feb 1996;26(2):123-127.

79. **Ichijo H**. From receptors to stress-activated MAP kinases. *Oncogene.* Nov 1 1999;18(45):6087-6093.

80. **Imrich A, Ning Y, Lawrence J, Coull B, Gitin E, Knutson M, Kobzik L**. Alveolar macrophage cytokine response to air pollution particles: oxidant mechanisms. *Toxicol Appl Pharmacol.* Feb 1 2007;218(3):256-264.

81. **Ip YT, Davis RJ**. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr Opin Cell Biol.* Apr 1998;10(2):205-219.

82. **Jakab GJ, Spannhake EW, Canning BJ, Kleeberger SR, Gilmour MI**. The effects of ozone on immune function. *Environ Health Perspect.* Mar 1995;103 Suppl 2:77-89.

83. **Jaspers I, Chen LC, Flescher E**. Induction of interleukin-8 by ozone is mediated by tyrosine kinase and protein kinase A, but not by protein kinase C. *J Cell Physiol.* Nov 1998;177(2):313-323.

84. **Jaspers I, Flescher E, Chen LC**. Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells. *Am J Physiol.* Mar 1997;272(3 Pt 1):L504-511.

85. **Jorres RA, Holz O, Zachgo W, Timm P, Koschyk S, Muller B, Grimminger F, Seeger W, Kelly FJ, Dunster C, Frischer T, Lubec G, Waschewski M, Niendorf A, Magnussen H**. The effect of repeated ozone exposures on inflammatory markers in bronchoalveolar lavage fluid and mucosal biopsies. *Am J Respir Crit Care Med.* Jun 2000;161(6):1855-1861.

86. **Kabe Y, Ando K, Hirao S, Yoshida M, Handa H**. Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal.* Mar-Apr 2005;7(3-4):395-403.

87. **Kafoury RM, Madden MC**. Diesel exhaust particles induce the over expression of tumor necrosis factor-alpha (TNF-alpha) gene in alveolar macrophages and failed to induce apoptosis through activation of nuclear factor-kappaB (NF-kappaB). *Int J Environ Res Public Health.* Apr 2005;2(1):107-113.

88. **Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M**. Induction of inflammatory mediators in human airway epithelial cells by lipid ozonation products. *Am J Respir Crit Care Med.* Dec 1999;160(6):1934-1942.

89. **Kanoh T, Fukuda M, Onozuka H, Kinouchi T, Ohnishi Y**. Urinary 1-hydroxypyrene as a marker of exposure to polycyclic aromatic hydrocarbons in environment. *Environ Res.* Aug 1993;62(2):230-241.

90. **Kelly FJ, Mudway IS**. Protein oxidation at the air-lung interface. *Amino Acids.* Dec 2003;25(3-4):375-396.

91. **Kinney PL, Thurston GD, Raizenne M**. The effects of ambient ozone on lung function in children: a reanalysis of six summer camp studies. *Environ Health Perspect.* Feb 1996;104(2):170-174.

92. **Kleeberger SR, Ohtsuka Y, Zhang LY, Longphre M**. Airway responses to chronic ozone exposure are partially mediated through mast cells. *J Appl Physiol.* Feb 2001;90(2):713-723.

93. **Koike E, Kobayashi T**. Ozone exposure enhances antigen-presenting activity of interstitial lung cells in rats. *Toxicology.* Mar 15 2004;196(3):217-227.

94. **Koren HS, Devlin RB, Becker S, Perez R, McDonnell WF**. Time-dependent changes of markers associated with inflammation in the lungs of humans exposed to ambient levels of ozone. *Toxicol Pathol.* 1991;19(4 Pt 1):406-411.

95. **Koren HS, Devlin RB, Graham DE, Mann R, McGee MP, Horstman DH, Kozumbo WJ, Becker S, House DE, McDonnell WF, et al.** Ozone-induced inflammation in the lower airways of human subjects. *Am Rev Respir Dis.* Feb 1989;139(2):407-415.

96. **Krewski D, Burnett R, Jerrett M, Pope CA, Rainham D, Calle E, Thurston G, Thun M**. Mortality and long-term exposure to ambient air pollution: ongoing analyses based on the American Cancer Society cohort. *J Toxicol Environ Health A.* Jul 9-23 2005;68(13-14):1093-1109.

97. **Krishna MT, Blomberg A, Biscione GL, Kelly F, Sandstrom T, Frew A, Holgate S**. Short-term ozone exposure upregulates P-selectin in normal human airways. *Am J Respir Crit Care Med.* May 1997;155(5):1798-1803.

98. **Krishna MT, Madden J, Teran LM, Biscione GL, Lau LC, Withers NJ, Sandstrom T, Mudway I, Kelly FJ, Walls A, Frew AJ, Holgate ST**. Effects of 0.2 ppm ozone on biomarkers of inflammation in bronchoalveolar lavage fluid and bronchial mucosa of healthy subjects. *Eur Respir J.* Jun 1998;11(6):1294-1300.

99. **Kumagai Y, Koide S, Taguchi K, Endo A, Nakai Y, Yoshikawa T, Shimojo N**. Oxidation of proximal protein sulfhydryls by phenanthraquinone, a component of diesel exhaust particles. *Chem Res Toxicol.* Apr 2002;15(4):483-489.

100. **Kunzli N, Lurmann F, Segal M, Ngo L, Balmes J, Tager IB**. Association between lifetime ambient ozone exposure and pulmonary function in college freshmen--results of a pilot study. *Environ Res.* Jan 1997;72(1):8-23.

101. **Kuschert GS, Coulin F, Power CA, Proudfoot AE, Hubbard RE, Hoogewerf AJ, Wells TN**. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry.* Sep 28 1999;38(39):12959-12968.

102. **Lakshminarayanan V, Beno DW, Costa RH, Roebuck KA**. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H2O2 and tumor necrosis factor-alpha in endothelial and epithelial cells. *J Biol Chem.* Dec 26 1997;272(52):32910-32918.

103. **Langford SD, Bidani A, Postlethwait EM**. Ozone-reactive absorption by pulmonary epithelial lining fluid constituents. *Toxicol Appl Pharmacol.* May 1995;132(1):122-130.

104. **Larsen JK, Yamboliev IA, Weber LA, Gerthoffer WT**. Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle. *Am J Physiol.* Nov 1997;273(5 Pt 1):L930-940.

105. **Leikauf GD, Simpson LG, Santrock J, Zhao Q, Abbinante-Nissen J, Zhou S, Driscoll KE**. Airway epithelial cell responses to ozone injury. *Environ Health Perspect.* Mar 1995;103 Suppl 2:91-95.

106. **Li N, Hao M, Phalen RF, Hinds WC, Nel AE**. Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clin Immunol.* Dec 2003;109(3):250-265.

107. **Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T, Froines J, Nel A**. Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. *Environ Health Perspect.* Apr 2003;111(4):455-460.

108. **Lim YB, Ziemann PJ**. Products and mechanism of secondary organic aerosol formation from reactions of n-alkanes with OH radicals in the presence of NOx. *Environ Sci Technol.* Dec 1 2005;39(23):9229-9236.

109. **Long NC, Suh J, Morrow JD, Schiestl RH, Murthy GG, Brain JD, Frei B**. Ozone causes lipid peroxidation but little antioxidant depletion in exercising and nonexercising hamsters. *J Appl Physiol.* Oct 2001;91(4):1694-1700.

110. **Longphre M, Zhang LY, Harkema JR, Kleeberger SR**. Mass cells contribute to O3-induced epithelial damage and proliferation in nasal and bronchial airways of mice. *J Appl Physiol.* Apr 1996;80(4):1322-1330.

111. **Madden MC, Richards JH, Dailey LA, Hatch GE, Ghio AJ**. Effect of ozone on diesel exhaust particle toxicity in rat lung. *Toxicol Appl Pharmacol.* Oct 15 2000;168(2):140-148.

112. **Maestrelli P, Saetta M, Di Stefano A, Calcagni PG, Turato G, Ruggieri MP, Roggeri A, Mapp CE, Fabbri LM**. Comparison of leukocyte counts in sputum, bronchial biopsies, and bronchoalveolar lavage. *Am J Respir Crit Care Med.* Dec 1995;152(6 Pt 1):1926-1931.

113. **Marano F, Boland S, Bonvallot V, Baulig A, Baeza-Squiban A**. Human airway epithelial cells in culture for studying the molecular mechanisms of the inflammatory response triggered by diesel exhaust particles. *Cell Biol Toxicol.* 2002;18(5):315-320.

114. **Mazzarella G, Ferraraccio F, Prati MV, Annunziata S, Bianco A, Mezzogiorno A, Liguori G, Angelillo IF, Cazzola M**. Effects of diesel exhaust particles on human lung epithelial cells: An in vitro study. *Respir Med.* Feb 3 2007.

115. **McConnell R, Berhane K, Gilliland F, London SJ, Islam T, Gauderman WJ, Avol E, Margolis HG, Peters JM**. Asthma in exercising children exposed to ozone: a cohort study. *Lancet.* Feb 2 2002;359(9304):386-391.

116. **McDonnell WF**. *U.S. Environmental Protection Agency's ozone epidemiology research program: a strategy for assessing the effects of ambient ozone exposure upon morbidity in exposed populations.*

117. **McDonnell WF, 3rd, Horstman DH, Abdul-Salaam S, House DE**. Reproducibility of individual responses to ozone exposure. *Am Rev Respir Dis.* Jan 1985;131(1):36-40.

118. **McDonnell WF, Kehrl HR, Abdul-Salaam S, Ives PJ, Folinsbee LJ, Devlin RB, O'Neil JJ, Horstman DH**. Respiratory response of humans exposed to low levels of ozone for 6.6 hours. *Arch Environ Health.* May-Jun 1991;46(3):145-150.

119. **McKinnon KP, Madden MC, Noah TL, Devlin RB**. In vitro ozone exposure increases release of arachidonic acid products from a human bronchial epithelial cell line. *Toxicol Appl Pharmacol.* Feb 1993;118(2):215-223.

120. **Message SD, Johnston SL**. Host defense function of the airway epithelium in health and disease: clinical background. *J Leukoc Biol.* Jan 2004;75(1):5-17.

121. **Mogel M, Kruger E, Krug HF, Seidel A**. A new coculture-system of bronchial epithelial and endothelial cells as a model for studying ozone effects on airway tissue. *Toxicol Lett.* Aug 1998;96-97:25-32.

122. **Molfino NA, Wright SC, Katz I, Tarlo S, Silverman F, McClean PA, Szalai JP, Raizenne M, Slutsky AS, Zamel N**. Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. *Lancet.* Jul 27 1991;338(8761):199-203.

123. **Mudway IS, Kelly FJ**. Ozone and the lung: a sensitive issue. *Mol Aspects Med.* Feb-Apr 2000;21(1-2):1-48.

124. **Mudway IS, Stenfors N, Blomberg A, Helleday R, Dunster C, Marklund SL, Frew AJ, Sandstrom T, Kelly FJ**. Differences in basal airway antioxidant concentrations are not predictive of individual responsiveness to ozone: a comparison of healthy and mild asthmatic subjects. *Free Radic Biol Med.* Oct 15 2001;31(8):962-974.

125. **Mudway IS, Stenfors N, Duggan ST, Roxborough H, Zielinski H, Marklund SL, Blomberg A, Frew AJ, Sandstrom T, Kelly FJ**. An in vitro and in vivo investigation of the effects of diesel exhaust on human airway lining fluid antioxidants. *Arch Biochem Biophys.* Mar 1 2004;423(1):200-212.

126. **Nightingale JA, Maggs R, Cullinan P, Donnelly LE, Rogers DF, Kinnersley R, Chung KF, Barnes PJ, Ashmore M, Newman-Taylor A**. Airway inflammation after controlled exposure to diesel exhaust particulates. *Am J Respir Crit Care Med.* Jul 2000;162(1):161-166.

127. **Nightingale JA, Rogers DF, Barnes PJ**. Effect of inhaled ozone on exhaled nitric oxide, pulmonary function, and induced sputum in normal and asthmatic subjects. *Thorax.* Dec 1999;54(12):1061-1069.

128. **Niwa M, Hotta K, Kanamori Y, Hatakeyama D, Hirade K, Katayama M, Hara A, Mori H, Ito H, Kato K, Matsuno H, Uematsu T, Kozawa O**. Involvement of p38 mitogen-activated protein kinase in heat shock protein 27 induction in human neutrophils. *Eur J Pharmacol.* Apr 18 2003;466(3):245-253.

129. **Nordenhall C, Pourazar J, Blomberg A, Levin JO, Sandstrom T, Adelroth E**. Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. *Eur Respir J.* Jun 2000;15(6):1046-1051.

130. **Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C**. Translocation of inhaled ultrafine particles to the brain. *Inhal Toxicol.* Jun 2004;16(6-7):437-445.

131. **Ohtani T, Nakagawa S, Kurosawa M, Mizuashi M, Ozawa M, Aiba S**. Cellular basis of the role of diesel exhaust particles in inducing Th2-dominant response. *J Immunol.* Feb 15 2005;174(4):2412-2419.

132. **Park JW, Taube C, Swasey C, Kodama T, Joetham A, Balhorn A, Takeda K, Miyahara N, Allen CB, Dakhama A, Kim SH, Dinarello CA, Gelfand EW**. Interleukin-1 receptor antagonist attenuates airway hyperresponsiveness following exposure to ozone. *Am J Respir Cell Mol Biol.* Jun 2004;30(6):830-836.

133. **Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE**. The use of induced sputum to investigate airway inflammation. *Thorax.* Jun 1997;52(6):498-501.

134. **Peden DB, Boehlecke B, Horstman D, Devlin R**. Prolonged acute exposure to 0.16 ppm ozone induces eosinophilic airway inflammation in asthmatic subjects with allergies. *J Allergy Clin Immunol*. Dec 1997;100(6 Pt 1):802-808.

135. **Peters JM, Avol E, Gauderman WJ, Linn WS, Navidi W, London SJ, Margolis H, Rappaport E, Vora H, Gong H, Jr., 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*. Mar 1999;159(3):768-775.

136. **Peterson CG, Venge P**. Purification and characterization of a new cationic protein--eosinophil protein-X (EPX)--from granules of human eosinophils. *Immunology*. Sep 1983;50(1):19-26.

137. **Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J**. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax*. Jan 1992;47(1):25-29.

138. **Pizzichini MM, Popov TA, Efthimiadis A, Hussack P, Evans S, Pizzichini E, Dolovich J, Hargreave FE**. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. *Am J Respir Crit Care Med*. Oct 1996;154(4 Pt 1):866-869.

139. **Postlethwait EM, Cueto R, Velsor LW, Pryor WA**. O3-induced formation of bioactive lipids: estimated surface concentrations and lining layer effects. *Am J Physiol*. Jun 1998;274(6 Pt 1):L1006-1016.

140. **Pourazar J, Mudway IS, Samet JM, Helleday R, Blomberg A, Wilson SJ, Frew AJ, Kelly FJ, Sandstrom T**. Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways. *Am J Physiol Lung Cell Mol Physiol*. Nov 2005;289(5):L724-730.

141. **Prause O, Bozinovski S, Anderson GP, Linden A**. Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. *Thorax*. Apr 2004;59(4):313-317.

142. **Pritchard JN**. Dust overloading causes impairment of pulmonary clearance: evidence from rats and humans. *Exp Pathol*. 1989;37(1-4):39-42.

143. **Pryor WA**. How far does ozone penetrate into the pulmonary air/tissue boundary before it reacts? *Free Radic Biol Med*. 1992;12(1):83-88.

144. **Pryor WA, Bermudez E, Cueto R, Squadrito GL**. Detection of aldehydes in bronchoalveolar lavage of rats exposed to ozone. *Fundam Appl Toxicol*. Nov 1996;34(1):148-156.

145. **Pryor WA, Das B, Church DF**. The ozonation of unsaturated fatty acids: aldehydes and hydrogen peroxide as products and possible mediators of ozone toxicity. *Chem Res Toxicol*. May-Jun 1991;4(3):341-348.

146. **Quay JL, Reed W, Samet J, Devlin RB**. Air pollution particles induce IL-6 gene expression in human airway epithelial cells via NF-kappaB activation. *Am J Respir Cell Mol Biol*. Jul 1998;19(1):98-106.

147. **Rennard SI, Ghafouri M, Thompson AB, Linder J, Vaughan W, Jones K, Ertl RF, Christensen K, Prince A, Stahl MG, et al.** Fractional processing of sequential bronchoalveolar lavage to separate bronchial and alveolar samples. *Am Rev Respir Dis*. Jan 1990;141(1):208-217.

148. **Reynaert NL, van der Vliet A, Guala AS, McGovern T, Hristova M, Pantano C, Heintz NH, Heim J, Ho YS, Matthews DE, Wouters EF, Janssen-Heininger YM**.

Dynamic redox control of NF-kappaB through glutaredoxin-regulated S-glutathionylation of inhibitory kappaB kinase beta. *Proc Natl Acad Sci U S A.* Aug 29 2006;103(35):13086-13091.

149. Romert L, Bernson V, Pettersson B. Effects of air pollutants on the oxidative metabolism and phagocytic capacity of pulmonary alveolar macrophages. *J Toxicol Environ Health.* Aug-Sep 1983;12(2-3):417-427.

150. Rudell B, Blomberg A, Helleday R, Ledin MC, Lundback B, Stjernberg N, Horstedt P, Sandstrom T. Bronchoalveolar inflammation after exposure to diesel exhaust: comparison between unfiltered and particle trap filtered exhaust. *Occup Environ Med.* Aug 1999;56(8):527-534.

151. Rudell B, Ledin MC, Hammarstrom U, Stjernberg N, Lundback B, Sandstrom T. Effects on symptoms and lung function in humans experimentally exposed to diesel exhaust. *Occup Environ Med.* Oct 1996;53(10):658-662.

152. Sagai M, Saito H, Ichinose T, Kodama M, Mori Y. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radic Biol Med.* Jan 1993;14(1):37-47.

153. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, 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.* Mar 1999;159(3):702-709.

154. Salvi SS, Nordenhall C, Blomberg A, Rudell B, Pourazar J, Kelly FJ, Wilson S, Sandstrom 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.* Feb 2000;161(2 Pt 1):550-557.

155. Saxena QB, Saxena RK, Siegel PD, Lewis DM. Identification of organic fractions of diesel exhaust particulate (DEP) which inhibit nitric oxide (NO) production from a murine macrophage cell line. *Toxicol Lett.* Aug 28 2003;143(3):317-322.

156. Scannell C, Chen L, Aris RM, Tager I, Christian D, Ferrando R, Welch B, Kelly T, Balmes JR. Greater ozone-induced inflammatory responses in subjects with asthma. *Am J Respir Crit Care Med.* Jul 1996;154(1):24-29.

157. Schelegle ES, Siefkin AD, McDonald RJ. Time course of ozone-induced neutrophilia in normal humans. *Am Rev Respir Dis.* Jun 1991;143(6):1353-1358.

158. Schins RP, Lightbody JH, Borm PJ, Shi T, Donaldson K, Stone V. Inflammatory effects of coarse and fine particulate matter in relation to chemical and biological constituents. *Toxicol Appl Pharmacol.* Feb 15 2004;195(1):1-11.

159. Schlesinger RB, Cohen M, Gordon T, Nadziejko C, Zelikoff JT, Sisco M, Regal JF, Menache MG. Ozone-induced modulation of airway hyperresponsiveness in guinea pigs. *Res Rep Health Eff Inst.* Jun 2002(109):1-40; discussion 41-51.

160. Schmelzer KR, Wheelock AM, Dettmer K, Morin D, Hammock BD. The role of inflammatory mediators in the synergistic toxicity of ozone and 1-nitronaphthalene in rat airways. *Environ Health Perspect.* Sep 2006;114(9):1354-1360.

161. Semmler M, Seitz J, Erbe F, Mayer P, Heyder J, Oberdorster G, Kreyling WG. Long-term clearance kinetics of inhaled ultrafine insoluble iridium particles from the rat lung, including transient translocation into secondary organs. *Inhal Toxicol.* Jun 2004;16(6-7):453-459.

162. **Sheu HL, Lee WJ, Lin SJ, Fang GC, Chang HC, You WC**. Particle-bound PAH content in ambient air. *Environ Pollut.* 1997;96(3):369-382.

163. **Siegel PD, Saxena RK, Saxena QB, Ma JK, Ma JY, Yin XJ, Castranova V, Al-Humadi N, Lewis DM**. Effect of diesel exhaust particulate (DEP) on immune responses: contributions of particulate versus organic soluble components. *J Toxicol Environ Health A.* Feb 13 2004;67(3):221-231.

164. **Solic N, Wilson J, Wilson SJ, Shute JK**. Endothelial activation and increased heparan sulfate expression in cystic fibrosis. *Am J Respir Crit Care Med.* Oct 1 2005;172(7):892-898.

165. **Squadrito GL, Cueto R, Dellinger B, Pryor WA**. Quinoid redox cycling as a mechanism for sustained free radical generation by inhaled airborne particulate matter. *Free Radic Biol Med.* Nov 1 2001;31(9):1132-1138.

166. **Stenfors N**. *Differential Airway Inflammatory Responses to Ozone and Diesel Exhaust in Healthy and Asthmatic Subjects* [Medical Dissertation]. Umeå: Dept. of Resp. Medicine and Allergy, Umeå University; 2002.

167. **Stenfors N, Nordenhall C, Salvi SS, Mudway I, Soderberg M, Blomberg A, Helleday R, Levin JO, Holgate ST, Kelly FJ, Frew AJ, Sandstrom T**. Different airway inflammatory responses in asthmatic and healthy humans exposed to diesel. *Eur Respir J.* Jan 2004;23(1):82-86.

168. **Stenfors N, Pourazar J, Blomberg A, Krishna MT, Mudway I, Helleday R, Kelly FJ, Frew AJ, Sandstrom T**. Effect of ozone on bronchial mucosal inflammation in asthmatic and healthy subjects. *Respir Med.* May 2002;96(5):352-358.

169. **Stieb DM, Burnett RT, Beveridge RC, Brook JR**. Association between ozone and asthma emergency department visits in Saint John, New Brunswick, Canada. *Environ Health Perspect.* Dec 1996;104(12):1354-1360.

170. **Strom KA, Garg BD, Johnson JT, D'Arcy JB, Smiler KL**. Inhaled particle retention in rats receiving low exposures of diesel exhaust. *J Toxicol Environ Health.* 1990;29(4):377-398.

171. **Strom KA, Johnson JT, Chan TL**. Retention and clearance of inhaled submicron carbon black particles. *J Toxicol Environ Health.* 1989;26(2):183-202.

172. **Sydbom A, Blomberg A, Parnia S, Stenfors N, Sandstrom T, Dahlen SE**. Health effects of diesel exhaust emissions. *Eur Respir J.* Apr 2001;17(4):733-746.

173. **Tager IB, Balmes J, Lurmann F, Ngo L, Alcorn S, Kunzli N**. Chronic exposure to ambient ozone and lung function in young adults. *Epidemiology.* Nov 2005;16(6):751-759.

174. **Takanashi S, Hasegawa Y, Kanehira Y, Yamamoto K, Fujimoto K, Satoh K, Okamura K**. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. *Eur Respir J.* Aug 1999;14(2):309-314.

175. **Takizawa H**. Airway epithelial cells as regulators of airway inflammation (Review). *Int J Mol Med.* Feb 1998;1(2):367-378.

176. **Takizawa H**. Diesel exhaust particles and their effect on induced cytokine expression in human bronchial epithelial cells. *Curr Opin Allergy Clin Immunol.* Oct 2004;4(5):355-359.

177. **Takizawa H, Ohtoshi T, Kawasaki S, Abe S, Sugawara I, Nakahara K, Matsushima K, Kudoh S**. Diesel exhaust particles activate human bronchial epithelial cells to express inflammatory mediators in the airways: a review. *Respirology.* Jun 2000;5(2):197-203.

178. **Thompson AB, Robbins RA, Romberger DJ, Sisson JH, Spurzem JR, Teschler H, Rennard SI**. Immunological functions of the pulmonary epithelium. *Eur Respir J*. Jan 1995;8(1):127-149.

179. **Thurston GD, Ito K, Kinney PL, Lippmann M**. A multi-year study of air pollution and respiratory hospital admissions in three New York State metropolitan areas: results for 1988 and 1989 summers. *J Expo Anal Environ Epidemiol*. Oct-Dec 1992;2(4):429-450.

180. **Todokoro M, Mochizuki H, Tokuyama K, Utsugi M, Dobashi K, Mori M, Morikawa A**. Effect of ozone exposure on intracellular glutathione redox state in cultured human airway epithelial cells. *Inflammation*. Apr 2004;28(2):105-114.

181. **Torres A, Utell MJ, Morow PE, Voter KZ, Whitin JC, Cox C, Looney RJ, Speers DM, Tsai Y, Frampton MW**. Airway inflammation in smokers and nonsmokers with varying responsiveness to ozone. *Am J Respir Crit Care Med*. Sep 1997;156(3 Pt 1):728-736.

182. **Valacchi G, Pagnin E, Phung A, Nardini M, Schock BC, Cross CE, van der Vliet A**. Inhibition of NFkappaB activation and IL-8 expression in human bronchial epithelial cells by acrolein. *Antioxid Redox Signal*. Jan-Feb 2005;7(1-2):25-31.

183. **Valavanidis A, Vlahoyianni T, Fiotakis K**. Comparative study of the formation of oxidative damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) adduct from the nucleoside 2'-deoxyguanosine by transition metals and suspensions of particulate matter in relation to metal content and redox reactivity. *Free Radic Res*. Oct 2005;39(10):1071-1081.

184. **van Bree L, Dormans JA, Koren HS, Devlin RB, Rombout PJ**. Attenuation and recovery of pulmonary injury in rats following short-term, repeated daily exposure to ozone. *Inhal Toxicol*. Aug 2002;14(8):883-900.

185. **van der Vliet A, O'Neill CA, Cross CE, Koostra JM, Volz WG, Halliwell B, Louie S**. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol*. Feb 1999;276(2 Pt 1):L289-296.

186. **Walsh GM**. Eosinophil granule proteins and their role in disease. *Curr Opin Hematol*. Jan 2001;8(1):28-33.

187. **Walsh GM**. Human eosinophils: their accumulation, activation and fate. *Br J Haematol*. Jun 1997;97(4):701-709.

188. **Walsh GM**. Mechanisms of human eosinophil survival and apoptosis. *Clin Exp Allergy*. May 1997;27(5):482-487.

189. **Waskiewicz AJ, Flynn A, Proud CG, Cooper JA**. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *Embo J*. Apr 15 1997;16(8):1909-1920.

190. **Weisel CP, Cody RP, Georgopoulos PG, Purushothaman V, Weiss SH, Bielory L, Gregory P, Stern AH**. Concepts in developing health-based indicators for ozone. *Int Arch Occup Environ Health*. Aug 2002;75(6):415-422.

191. **Westerholm R, Egeback KE**. Exhaust emissions from light- and heavy-duty vehicles: chemical composition, impact of exhaust after treatment, and fuel parameters. *Environ Health Perspect*. Oct 1994;102 Suppl 4:13-23.

192. **WHO**. WHO Air quality guidelines for particulate matter, ozone, nitrogen dioxide and sulfur dioxide - Global update 2005 - Summary of risk assessment

193. **WHO**. *World health report* Geneva: World health organization 2002.

194. **Widmann C, Gibson S, Jarpe MB, Johnson GL**. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev.* Jan 1999;79(1):143-180.

195. **Wong CK, Cheung PF, Ip WK, Lam CW**. Interleukin-25-induced chemokines and interleukin-6 release from eosinophils is mediated by p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor-kappaB. *Am J Respir Cell Mol Biol.* Aug 2005;33(2):186-194.

196. **Xia T, Korge P, Weiss JN, Li N, Venkatesen MI, Sioutas C, Nel A**. Quinones and aromatic chemical compounds in particulate matter induce mitochondrial dysfunction: implications for ultrafine particle toxicity. *Environ Health Perspect.* Oct 2004;112(14):1347-1358.

197. **Xu SY, Petersson CG, Carlson M, Venge P**. The development of an assay for human neutrophil lipocalin (HNL)--to be used as a specific marker of neutrophil activity in vivo and vitro. *J Immunol Methods.* May 16 1994;171(2):245-252.

198. **Zhang Q, Stanier CO, Canagaratna MR, Jayne JT, Worsnop DR, Pandis SN, Jimenez JL**. Insights into the chemistry of new particle formation and growth events in Pittsburgh based on aerosol mass spectrometry. *Environ Sci Technol.* Sep 15 2004;38(18):4797-4809.

199. **Zhang R, Brennan ML, Shen Z, MacPherson JC, Schmitt D, Molenda CE, Hazen SL**. Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. *J Biol Chem.* Nov 29 2002;277(48):46116-46122.