Respiratory and cardiovascular effects of exposure to oxidative air pollutants

Stefan Barath

Department of Public Health and Clinical Medicine
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to my beloved family

“and nothing else matters”

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Abstract

Background: The negative effects of air pollution on morbidity and mortality have been known since the mid 20th century. The two most well known examples are the Meuse Valley disaster in the 1930'ies and the London black fog in December 1952. Whilst there are numerous epidemiological studies, in which associations between morbidity and mortality and high levels of pollutants have been reported, the underlying mechanisms are not clear. Two of the main air pollutants are particulate matter (PM) mostly emanating from diesel exhaust (DE), and ozone, both of which are highly oxidative. Exposure to DE has resulted in adverse effects both in the respiratory tract and in the cardiovascular system. High ozone levels have also been shown to be associated with increased admissions to hospital for respiratory as well as cardiovascular conditions.

The main aim of this thesis was to investigate the respiratory and cardiovascular effects of a combination of exposures to ozone and DE. DE generated during the urban part of the standardized European Transient Cycle (ETC) was compared to DE generated by an idling engine. It was also evaluated whether an acute exposure to ozone would have any effects on the cardiovascular system as assessed by venous occlusion forearm plethysmography and heart rate variability (HRV). In addition, fraction of exhaled nitric oxide (FE_{NO}) was evaluated as a potential marker for acute exposure to ozone or DE.

Methods: Four double-blind randomized cross-over exposure studies were conducted to investigate the effects of ozone and DE on both the respiratory tract and the vascular function in healthy volunteers. All of the exposures were performed in purposely built "walk-in" chambers with strictly controlled exposures. In the first study, the volunteers were exposed to DE (300μg/m^3) generated by an idling engine or to air, for one hour in the morning and to ozone (200 ppb) for two hours in the afternoon. A bronchoscopy with bronchial wash (BW) and bronchoalveolar lavage (BAL) was performed 24 hours after the initial exposure. In study II and III, an assessment of vascular function using venous occlusion forearm plethysmography was performed after an exposure to DE (250 μg/m^3) generated under transient running conditions, compared to air exposure (study II) and ozone and air exposure (study III). HRV was assessed under a 24 hour period starting before each exposure (study III). In study IV, FE_{NO} measurements were conducted after DE and ozone exposures to investigate whether the previously established airway inflammation would be detectable by this non-invasive method.

Results: DE exposure enhanced the established ozone-induced airway inflammation in terms of a pronounced neutrophilia in BW. DE generated under transient running conditions, impaired vascular function in healthy volunteers, whereas exposure to ozone did not. HRV were not altered by exposure to ozone. Exposure to DE caused a significant increase in FENO at the 10 ($FENO_{10}$) and 50 ($FENO_{50}$) mL/s flow rates at 6 hours post-exposure, but ozone exposure did not affect FENO at any flow rate or time point.

Conclusion: We have tried to mimic real-life exposure to air pollutants. In the first study, an exposure to DE followed by an exposure to ozone in the afternoon resulted in an enhanced airway inflammation, suggesting an additive or synergistic effect, supporting the epidemiological findings of unfavorable effects of the combination of these two air pollutants.

DE generated by an engine running at the urban part of the standardized European Transient Cycle impaired two important and complementary aspects of vascular function, the regulation of vascular tone and endogenous fibrinolysis. This has previously been shown with DE generated at idling conditions. This suggests that the mechanisms behind the adverse effects can be found in the properties of the particles and not in the gaseous components.

In these studies, exposure to ozone did not impair vascular function in healthy subjects, or cause any alterations in HRV. This suggests that the epidemiological evidence for an increased risk of cardiovascular mortality following acute exposure to ozone might not be totally accurate. Previous controlled exposure studies with ozone have not shown an airway inflammation affecting the endothelium, at least not in the same time-frame as following DE exposure.

FENO could possibly be a useful tool for assessing airway inflammation caused by DE, whereas the powerful oxidant ozone did not affect FENO. This suggests that the airway inflammatory effects caused by these two pollutants are regulated via different mechanisms.

Selected abbreviations

AA: Ascorbic acid
Ach: Acetylcholine
BAL: Bronchoalveolar lavage
Bk: Bradykinin
Bpm: Beats per minute
BW: Bronchial wash
COPD: Chronic obstructive pulmonary disease
DE: Diesel exhaust
DEP: Diesel exhaust particles
EC: Elemental carbon
ETC: European transient cycle
EGFR: Epidermal growth factor receptor
FBF: Forearm blood flow
FENO: Fraction of exhaled nitric oxide
HRV: Heart rate variability
GSH: Reduced glutathione
NO: Nitric oxide
NOx: Oxides of nitrogen
NO_2: Nitrogen dioxide
MAPK: Mitogen-activated protein kinases
MI: Myocardial infarction
O_3: Ozone
OC: Organic carbon
OP: Oxidative potential
PAH: Polycyclic aromatic hydrocarbon
PPB: Parts per billion
PPM: Parts per million
PM: Particulate matter
SNP: Sodium nitroprusside
TC: Total carbon
tPA: Tissue plasminogen activator
Vp: Verapamil.

Sammanfattning på svenska

Bakgrund

Luftföroreningarnas negativa effekter på människors hälsa har varit kända sedan tidigt nittonhundratal. De två mest kända exemplen är katastrofen i Meusedalen på 30-talet och den svarta dimman i London i december 1952. Trots många epidemiologiska rapporter som beskrivit samband mellan ökad sjuklighet och död, och ökade halter av luftföroreningar så är de underliggande mekanismerna inte klarlagda. De två viktigaste luftföroreningarna är partiklar från framför allt diesel avgaser och ozon, båda med kraftig oxidativ kapacitet. Exponering för dieselavgaser ger negativa effekter både i luftvägarna och i hjärt/kärlsystemet. Höga halter av ozon är även relaterat till ökat antal akutbesök, både för luftvägs- och hjärt/kärlbesvär. Det övergripande syftet med denna avhandling är att studera effekterna av exponering för kombinationen av ozon och dieselavgaser på hjärt/kärlsystemet och luftvägarna. Effekterna av dieselavgaser genererade av en motor som programmeras att gå efter ett speciellt utformat program, sk. European Trancient Cycle (ETC), har jämförts med effekter av avgaser från en motor på tomgång. Vidare har effekter av ozonexponering på hjärt/kärl- systemet studerats med hjälp av venös ocklusionsunderarmspletysmografi, liksom effekter på hjärtats autonoma funktion, Heart Rate Variability (HRV). Fraktionerat utandat kväveoxid (FENO) har använts som metod att studera luftvägsinflammation orsakad av akut exponering för dieselavgaser eller ozon.

Metod

Fyra exponeringsstudier (dubbel-blinda cross-over studier) på friska försökspersoner har genomförts för att studera effekterna av ozon och dieselavgaser i både luftvägar och på kärlsystemet. Alla exponeringar har utförts i specialbyggda exponeringskamrar. I studie I exponerades försökspersonerna för dieselavgaser (300 µg/m^3), genererade av en motor på tomgång, eller för luft, under en timma på morgonen och för ozon (200 ppb) under två timmar på eftermiddagen. Nästa morgon genomfördes en bronkoskopi med bronkial wash (BW) och bronkoalveolärt lavage (BAL). I studie II och III genomfördes venös ocklusionsunderarmspletysmografi för att studera blodkärlsfunktionen efter exponering för dieselavgaser (250 µg/m^3) (ETC protokoll) jämfört med luftexponering (studie II) och ozon jämfört med luft (studie III). HRV studerades under 24 timmar från ozonexponeringens start och jämfördes med luftexponering (studie III).

Mätningar av FENO gjordes efter respektive dieselavgas- och ozonexponering .

Resultat

Exponering för dieselavgaser förstärker den redan etablerade ozoninducerade luftvägsinflammationen visat genom en ökning av neutrofila blodkroppar i BW. Dieselavgaser generade under ETC program försämrar kärlfunktionen hos unga friska försökspersoner medan ozon inte ger någon påverkan. HRV påverkas inte av exponering för ozon. Dieselavgaser ger en signifikant ökning av $FENO_{10}$ och $FENO_{50}$ 6 timmar efter exponering medan ozonexponering inte påverkade FENO vid något flöde eller tidpunkt.

Konklusion

Utomhusexponering för luftföroreningar har imiterats experimentellt. I den första studien noterades en förstärkning av luftvägsinflammation efter exponering av dieselavgaser när denna lades till ozonexponering. Detta talar för en additativ eller synergistisk effekt vilket stöder tidigare epidemiologiska studier, där ökande symtom, sjuklighet och död har beskrivits efter kombination av dessa två luftföroreningar.

Dieselavgaser genererade av en motor med ETC program, försämrade kärlfunktionen hos unga friska försökspersoners både mätt som kärltonus och endogen fibrinolys. Detta har tidigare visats med avgaser från en tomgångsmotor, vilket talar för att effekterna sannolikt medieras av partiklarna och deras ytkomponenter snarare än av gaskomponenten.

Ozonexponering påverkar inte HRV eller kärlfunktionen hos unga friska försökspersoner, vilket indikerar att de epidemiologiska rapporter som beskrivit samband mellan ökad dödlighet i hjärt/kärlsjukdom och ozon inte stämmer helt. Den luftvägsinflammation som beskrivits i tidigare studier påverkar inte blodkärlen, åtminstone inte under samma tidsperiod som efter dieselexponering.

FENO kan komma att bli användbart i studier för att upptäcka luftvägsinflammation efter exponering för diesel avgaser, men inte efter ozonexponering. Detta indikerar att luftvägsinflammationen som setts efter diesel- respektive ozon tycks regleras via olika mekanismer.

Original Papers

I. **Bosson J, Barath S, Pourazar J, Behndig AF, Sandström T, Blomberg A, Ädelroth E.**

Diesel exhaust exposure enhances the ozone-induced airway inflammation in healthy humans.

European Respiratory Journal 2008 Jun; 31(6):1234-40.

II. **Barath S, Mills NL, Lundbäck M, Törnqvist H, Lucking AJ, Langrish JP, Söderberg S, Boman C, Westerholm R, Löndahl J, Donaldson K, Mudway IS, Sandström T, Newby DE, Blomberg A.**

Impaired vascular function after exposure to diesel exhaust generated at urban transient running conditions.

Particle and Fibre Toxicology 2010 Jul 23; 7:19

III. **Barath S, Lundbäck M, Langrish JP, Bosson J, Blomberg A, Newby DE, Sandström T, Mills NL**

Ozone exposure does not impair vascular function or affect heart rate variability in healthy subjects.

Manuscript

IV. **Barath S, Mills NL, Ädelroth E, Olin A-C, Blomberg A**

Fraction of exhaled nitric oxide after experimental exposure to diesel exhaust and ozone in man.

Manuscript

Introduction

Background

Globally, air pollution is recognized as one of the main reasons for poor health and premature deaths both in children and adults. In the developing world, biomass burning for heating and cooking and exhaust from combustion engines are contributing to the polluted air. In the developed world, the air pollutants are mainly produced by traffic and factories. According to the WHO, more than three million people die every year because of polluted in- and outdoor air (1).

Since the mid 20th century, it has been known that air pollution cause serious health effects. The Meuse valley disaster in the 1930'ies and the London black fog in the 1950'ies, where the air was polluted with sulfuric acid and particulate matter (PM), are two of the most infamous incidents, in which people were taken ill or died from increased air pollution. Thick fog in the Meuse valley area was known even earlier, as an incident in 1911 killed most of the cattle in a smaller valley perpendicular to the Meuse valley. It has been estimated that over 4000 excess deaths occurred in London that particular week in December 1952 (2, 3). Today, the urbanization worldwide has led to very big, so called megacities, where air pollution is prevalent and visible to the inhabitants on a daily basis. In these cities, the background levels of fine particles often exceeds 200-500 $\mu g/m^3$ (24 h average), compared to USA and Europe, where the levels usually are between 5-30 $\mu g/m^3$ (4). This put in perspective, in a smoky bar, a passive smoker is exposed to 500-1500 $\mu g/m^3$ of fine particles (4). Many of these cities are geographically located in areas, where photochemical smog is prone to occur, for example Los Angeles, Mexico City and Beijing.

The described serious events in the 20th century were due to burning of charcoal for heating and cooking, as well as to an unfortunate combination of meteorological and geographical circumstances. Today the main pollutant

recognized is PM from combustion engines, both motor vehicles and industrial sources (4, 5). Increases in morbidity and mortality are strongly associated with high PM levels, especially in relation to the finer fractions of particles, below 10 μm (PM_{10}) (6). In larger cities, both in the developed and the developing world this is already and will continue to be an immense problem as for example in China. One of the biggest challenges for scientists and authorities today is to find ways to reduce the levels of ambient PM. Although there is strong epidemiological evidence linking exposure markers such as carbon content in macrophages to health effects, experimental studies are needed to understand the mechanisms for inducing health effects and to find threshold levels to set standards of limiting and preventing deleterious health effects (7).

Ozone, another important air pollutant present in areas preferably with sunshine and heavy traffic, can be present at extremely high levels and cause irritation of mucosal membranes and increased symptoms, especially in sensitive groups such as asthmatics. Numerous epidemiological studies have shown decreased lung function and airway symptoms in children exposed to high ozone levels both in healthy and asthmatic children. High ozone levels have also been shown to be associated with increased hospital admissions for both respiratory and cardiovascular conditions. However, so far there are no published experimental exposure studies with ozone employing cardiovascular endpoints.

Diesel exhaust

Particulate Matter

According to both the United Nations and the WHO, the single most important air pollutant is PM, mainly derived from combustion engines. The particle size is important, but recently there have been increased interest in the complex chemistry of the particle surfaces such as the various polycyclic aromatic hydrocarbons (PAH) with their different oxidative capacity. The particles consists of a core of elemental carbon covered with various

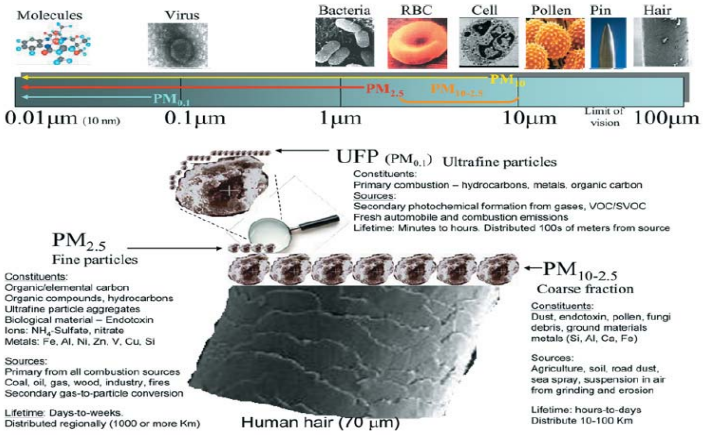
compounds including, transition metals from engine wear. Transition metals such as copper, iron and lead are considered highly oxidative and have the capacity to cause injury deep in the lungs. (8).

Exhaust from incomplete combustion of diesel fuel consists of particulate matter, carbon monoxide, carbon dioxide, sulfur dioxide, nitric oxide and nitrogen dioxide in a changing mix depending mainly on the engine workload. Both particle size and chemical compositions are of toxicological importance (9).

Particles are described based on their aerodynamic diameter and noted as coarse, fine and ultrafine. The coarse fraction consists of particles in the range 2.5 to 10 μm, the fine fraction <2.5 μm and the ultrafine <0.1 μm. The coarse fraction is derived from road, tyre and tare, while the fine and ultrafine fractions mainly consist of combustion-derived particles.

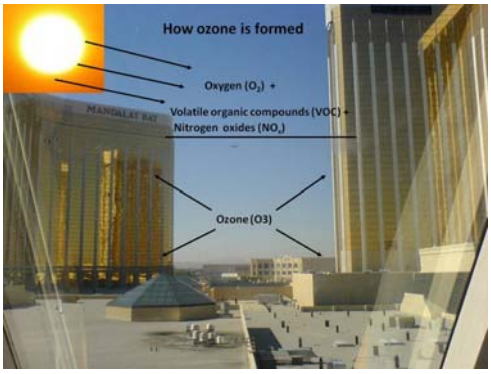
The fine fraction includes conglomerates of secondary emission particles, whereas the ultrafine fraction consists of primary emission. Time and distance from the emission source play a significant role in how reactive the particles are, regardless of size. The particles in the ultrafine fraction, less than 0.1μm, are commonly called nanoparticles and have recently been recognized as being particularly reactive, as they have the capacity to be deposited in the alveolar spaces (10). Nanoparticles are also discussed in terms of possible translocation from the alveolar space into the blood vessels and may therefore very rapidly be able to induce a systemic inflammatory process as well as causing acute adverse effects on the endothelium (11, 12).

Figure 1



(Adapted from *Brook R, Cardiovascular effects of Air pollution, Clinical Science (2008) 115 175-187)*

Ozone



Ozone an allotrope of oxygen (O_2) was initially described in the 1840ies. The odorless, usually colorless gas, often referred to as stratospheric ozone is mainly known for the protection of the earth from harmful UV-light in the stratosphere 15 km above the earth. The ozone produced at ground level is called tropospheric ozone and is a very powerful oxidant. The production is driven by the sun and, thus the highest level occurs in summertime with peaks in the afternoon. In combination with traffic related and industry emission of gases such as nitrogen oxides, carbon monoxide, peroxyacyl nitrates, aldehydes and volatile organic compounds, ozone forms a complex pollutant referred to as photochemical smog. Examples of this smog is the haze often seen over Los Angeles in summertime or the “fog” in Beijing during the last Olympic summer games.

Epidemiological Studies

PM

There are a large number of studies dealing with *long-term exposure* to air pollution and the association to increased cardiopulmonary morbidity and mortality (13, 14). Epidemiological studies have described the relationship between long-term exposure to $PM_{2.5}$ and increases in cardiopulmonary mortality (fig 1) (15). In the updated statement on air pollution and cardiovascular disease by the American Heart Association (AHA), the evidence summary on cohort studies states that for every 10 μg/m^3 elevation in average long-term $PM_{2.5}$ exposure, there is a 10 % increase in all-cause mortality (16).

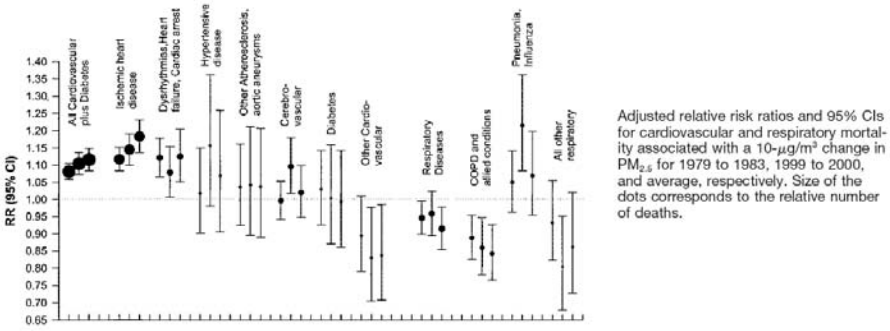
Long-term exposure to air pollution has also been shown to be a risk factor for developing chronic obstructive pulmonary disease (COPD) but decreases in lung function has also been shown to be associated with living within 100 meters of roads with heavy traffic (17). A recent paper from Wallace *et al.* concluded a relationship between living close to a highway and neutrophilic bronchitis, an increased risk of asthma diagnosis, asthma exacerbations and lower lung function (18).

Short-term exposures to air pollutants have been associated with an increased risk of stroke, arrhythmias, heart failure and ischemic heart disease. Pope *et al.* have described an increase in both respiratory and cardiovascular mortality for every increase in 10 μg/m^3 PM_{10} concentration (fig 2) (19). In a pivotal study by Peters *et al.*, an increased risk of having a myocardial infarction two hours after exposure to traffic was shown when subjects were seeking medical help in emergency rooms after having spent time in busy traffic (20). Acute exacerbations of COPD and chronic bronchitis have been associated with short-term exposure to increased levels of PM (21, 22). In a cohort study from Mexico City Barazza-Villarreal *et al.* followed 158 asthmatic and 50 non-asthmatic children over 22 weeks and

concluded that air pollution decreased lung function and caused an acute airway inflammation in both groups after short term exposure to $PM_{2.5}$ (23).

In another study from Mexico City, effects on lung growth were associated with increasing levels of PM_{10}, ozone and NO_2, with deficits in FEV_1 and FVC growth over a 3-year follow-up in a cohort of 170 8-year old children. From another cohort study in Southern California, Gauderman *et al*. found that children living within 500 meters from a motorway had substantial deficits in 8-year growth of FEV_1 compared to children living at least 1500 meters form a motorway (24, 25).

Figure 2



Adjusted relative risk ratios and 95% CIs for cardiovascular and respiratory mortality associated with a 10-$\mu g/m^3$ change in $PM_{2.5}$ for 1979 to 1983, 1999 to 2000, and average, respectively. Size of the dots corresponds to the relative number of deaths.

(Adapted from, Pope CA III *et al.* (2004) Cardiovascularmortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. Circulation 109: 71–77)

Ozone

To investigate effects of ozone in an epidemiological setting is very difficult; in fact, it is more or less impossible due to too many confounding factors. Increased number of hospital admissions for respiratory and cardiovascular conditions have been noted on days with high levels of ambient ozone or during the immediate next few days. There are indications that the susceptibility to airway infections is heightened after exposure to high levels

of ozone or smog. Children with asthma are reporting worsening of symptoms during the days following high levels of ozone.

In a recent study from Portugal, Almeida *et al*. reported a positive association between short-term exposure to ozone and cardiovascular mortality, showing an increase by 0.89 % in total mortality for every increase in 8-hour average by 5 ppm (26).

Experimental Studies

PM

PM and studies in healthy subjects

Our research group has performed numerous exposure studies with DE and ozone in healthy subjects. The PM mass concentration has varied from 100 μg/m^3 to 350 μg/m^3 in different studies. The same setup with an idling Volvo diesel engine has been used. The studies have included investigations of the respiratory tract with bronchial wash (BW), bronchoalveolar lavage (BAL) and endobronchial mucosal biopsies but also of cardio-vascular function. In the airways, analyses of mucosal biopsies have revealed an inflammatory response with recruitment of the neutrophils and up-regulated expression of various proinflammatory cytokines from 6 up to 24 hours after exposure (27).

The work by Pourazar *et al*. has described the importance of the epidermal growth factor receptor, (EGFR) and the signaling cascade of mitogen-activated protein kinases (MAPKs) as probable trigger mechanism in regulating the DE-induced airway inflammation (28, 29). Vascular dysfunction with attenuation of vasodilatation after infusion of vasoactive drugs and decreased tissue plasminogen activator (tPA) secretion has been shown after DE exposure with 300 μg/m^3 from an idling engine, indicating that short term exposure to DE impairs two important aspects of vascular

function, vasomotor function and endogenous fibrinolysis, and provides a possible link between air pollution and the pathogenesis of acute myocardial infarction (MI) (30).

Short-term exposure to DE also enhanced thrombus formation within 2 h after exposure, associated with increased platelet activation, which provides an even stronger link between DE exposure and AMI (31).

In a recent study by Mills *et al*. heart rate variability (HRV) was assessed after experimental exposure to DE in both healthy subjects and patients with stable coronary heart disease. Neither patients nor healthy subjects showed any serious arrhythmias or alterations in HRV (32).

PM and studies in subjects with respiratory disease

In controlled exposure studies in subjects with asthma, a different inflammatory response has been shown compared to that seen in healthy subject. An increase in the epithelial expression of the cytokine IL-10 was found along with an increased bronchial responsiveness 24 hours after exposure to DE, whereas the preexisting asthmatic inflammation was unaffected (33). In subjects with COPD there is, to the best of our knowledge, no published experimental exposure data as of yet. We have performed a study in subjects with moderate COPD using induced sputum to investigate airway inflammation and spirometry to assess lung function. Here we could not find any signs of an aggravated airway inflammation induced by the DE exposure or any deterioration of the lung function (data not published) (34).

PM and studies in subjects with cardiovascular disease

Exposure to DE generated by an idling engine impairs endogenous fibrinolysis in individuals with a stable coronary heart disease measured as decreased tPA secretion. When these patients exercised mildly on a bicycle ergometer where the workload were standardized to maintain estimated minute ventilation of 15 L/min/m^2 body surface a ST-segment depression was noted very rapidly after the onset of the exercise. All of the patients had gone through previous PCI and were taking full relevant medication according to recent guidelines but still a significant effect on the heart was seen (35). This suggests that even mild exercise during exposure to curb side levels of DE can cause "micro-ischemia" in the heart muscle. The same population has been assessed regarding alterations in heart rhythm and HRV and no such alterations were found (32).

Ozone

Ozone and studies in healthy subjects

There are many experimental exposure studies with healthy subjects exposed to ozone. In bronchoscopy studies, BAL and BW have shown neutrophilia as well as an increase of inflammatory mediators such as IL-6, IL-8 and PGE_2. Endobronchial mucosal biopsies have shown an up-regulation of adhesion molecules such as P-selectin and ICAM-1 as evidence for an early recruitment of neutrophils into the airways. Lung function studies have shown reversible FEV_1 and FVC decrements after exposure to ozone concentrations both above and below air quality standards (120 ppb, 1 hour average, http://www.epa.gov/glo/standards.html). The lung function has been normalized within 24 hours (36-41).

Ozone and studies in subjects with respiratory disease

Studies in asthmatics have failed to show a more aggravated airway inflammation of ozone compared to healthy subjects. No exaggerated neutrophil recruitment or exacerbation of pre-existing allergic inflammation was seen 6 hours after a 2-hour exposure to 200 ppb of ozone (42). This study suggested that there might be a difference in time-kinetics between healthy and asthmatic subjects in their response to ozone.

Ozone and studies in subjects with cardiovascular disease

There are to our knowledge no published experimental studies of exposure to ozone in individuals with cardiovascular disease.

Studies of the combination of ozone and DE

In a study of healthy subjects in which airway responses were investigated by induced sputum, a significant increase in neutrophilia was shown when subjects were exposed to ozone preceded by an exposure to DE, but not when the ozone exposure was preceded by an air exposure. This suggests that ozone enhances the airway inflammatory response induced by DE (42).

In a vascular assessment of healthy subject using high resolution ultrasonography of the brachial artery, alterations in the diameter of the artery were measured after exposure to concentrated ambient fine particles (CAP) at a concentration of 150 μg/m^3 plus ozone 120 ppb and compared to effects by exposure to filtered air. An acute arterial vasoconstriction by the combined CAP + ozone exposure was registered (43). This indicates that an exposure to curbside concentrations of the combination of these two pollutants can cause alterations in vascular tone which in turn may be one of the mechanisms explaining the observations from epidemiological studies linking air pollution to acute cardiac events. So far no studies have addressed effects on HRV in relation to ozone exposure.

Aims

Overall aim;

The aim of this thesis was to investigate the effects on respiratory and cardiovascular functions in healthy subjects of two of the most powerful oxidative air pollutants, DE and ozone.

Specific aims;

to investigate whether a real-life sequential exposure to DE and ozone would enhance an already established airway inflammation caused by DE

to study whether DE generated by an diesel engine running at transient load consistent with urban driving would induce similar cardiovascular effects as DE generated at idling

to elucidate whether short-term exposure to ozone would cause endothelial dysfunction in or affect heart rate variability in healthy subjects

to evaluate whether FE_{NO} measurements at multiple flow rates could be employed as a biomarker for air pollution-induced airway inflammation in healthy subjects

Subjects and methods

Subjects

All subjects were healthy, nonsmoking individuals with a normal physical examination, normal spirometry and negative skin prick tests against a standard panel of ten common aeroallergens. In the first study, 5 women and 9 men were included; whereas study 2, 3 and 4 included men only. None reported any symptoms from the respiratory tract at least six weeks prior to the first visit.

All studies were approved by the local Ethics Review Board and all volunteers gave their written informed consent and the studies were performed in accordance to the Declaration of Helsinki. The ambition was to include both men and women. However, this was unfortunately not to be fully realized.

Study Design

All studies were carried out in a double-blind crossover fashion with two visits at least two weeks apart, to reduce any possible carry over effects. Each subject served as his or her own control. During all exposures, the subjects alternated between rest and mild exercise on a bicycle ergometer in 15 minute intervals. The workload was individualized for each subject to give an estimated minute ventilation of 20 L/min/m^2 body surface.

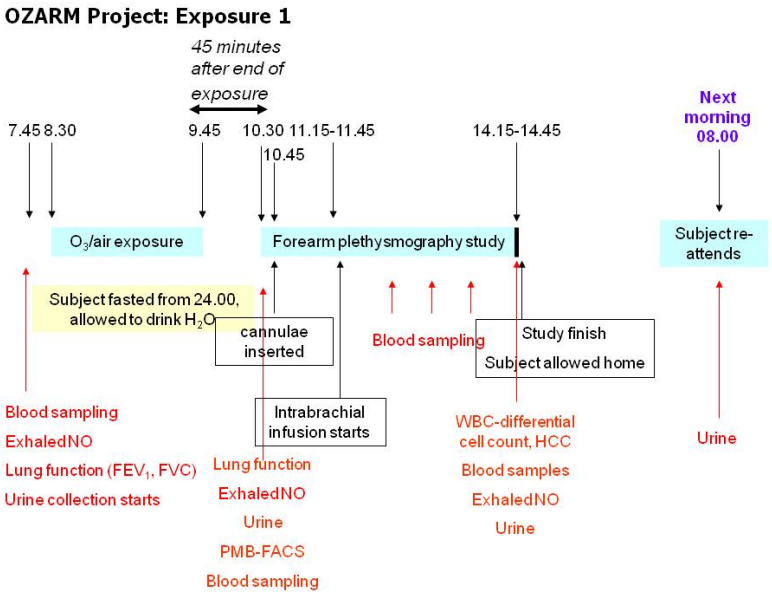
Study 1

To this study fourteen healthy individuals were recruited. Five women and nine men, with a mean age of 25 years, all without significant illness and free from airway infections at least 6 weeks prior to the study were included. The

subjects were not allowed to use any non-steroid anti-inflammatory drugs or vitamins within 2 weeks prior to the first visit and throughout the study. Each subject was randomized to either DE or air exposure in the morning for one hour, followed by a 2 hour ozone exposure 5 hours later.

Videobronchoshopy with BW and BAL were performed 24 hours after the initial exposure. Two to three weeks later the second exposure visit with subsequent bronchoshopy was performed.

Study 2 and 3



Study flow chart study 3

In total eighteen healthy, men were recruited, with a mean age of 27 years. Women were not included due to the potential for cyclical hormones to affect the vascular responses. All individuals were randomized to either air or DE exposure for 1 hour. Vascular assessment using venous forearm plethysmography was performed 6-hours after exposure according to a protocol used in previous studies. A mean of 42 days, range 22-62 days, after

the first exposure, a second exposure to DE or air was performed in the same manner.

All subjects were fitted with Holter electrocardiographic monitors (Reynolds Medical Lifecard, Delmar Reynolds, United Kingdom) before exposures with ECG monitoring continuing for 24 hours.

All individuals were asked to refrain from alcohol containing beverages for 24 hours and caffeine drinks for at least 4 hours prior to the exposure. This was done to secure that uncontrolled effects of vasoconstriction or vasodilatation caused by alcohol or caffeine was avoided.
Strenuous exercise during the day of exposure was not allowed and the subjects were asked to stay indoors to avoid any uncontrolled DE exposure.

The vascular studies were carried out in a temperature-controlled room maintaining a temperature of 22°C to 24°C to ensure as little effect as possible on temperature-related blood flow alterations.

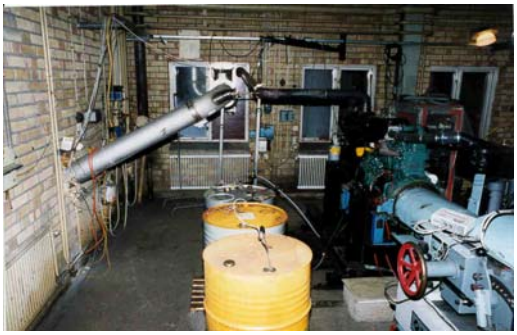
Study 4

In this study thirty-six men were exposed to ozone and ten to DE. The unfortunate unequal distribution of subjects between the two exposures was due to technical problems, beyond our control at the time. Standardized duplicate Fraction of exhaled nitric oxide (FENO) was measured at the expiratory flow rates of $FENO_{10}$, $FENO_{50}$, $FENO_{100}$ and $FENO_{270}$ mL/s before, at 6 and 24 hours after the end of exposure by using a chemiluminescence analyzer (NIOX-system; Aerocrine AB; Stockholm; Sweden). All exposures took place in the morning and all individuals were asked to fast from midnight. A standardized breakfast containing food without nitrates to minimize the source for NO production was served after the exposure.

Exposure

Diesel exhaust

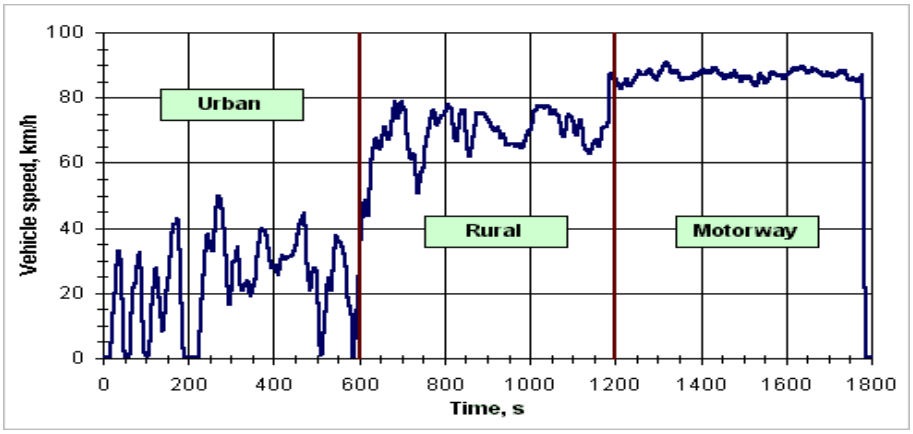
All exposures were performed in a specially designed walk-in chamber. The DE in study 1 was generated from an idling Volvo diesel engine (Volvo TD45, 4.5L, 4 cylinders, 1991, 68 rpm) and mass concentration were maintained at 300 µg/m^3 PM_{10}, monitored online with a Tapered Element Oscillating Microbalance instrument (TEOM).



Ninety percent of the exhaust was shunted away and the remaining 10 % was mixed with filtered air and fed into the chamber. The air in the exposure chamber was continuously monitored for nitrogen oxides (NO, NO2), carbon monoxide (CO), particulates (number/cm^3), and total hydrocarbons. In study 2 and 4 the DE was generated from another Volvo engine (Volvo TD40, 4.0L, 4 cylinders, 1991) connected to a dynamometer and running under the control of a computer program to mimic real life situation. The program with acceleration and breaking is called the European Transient Cycle (ETC), where the urban part of the program was used (fig 3). The monitoring and the technique to feed the chamber with the exhaust were the same as in study 1. In study 2 and 4 the mass concentration was

maintained at 250 µg/m^3 PM_{10}, the lower mass concentration is due to the running cycle and the different combination of gases.

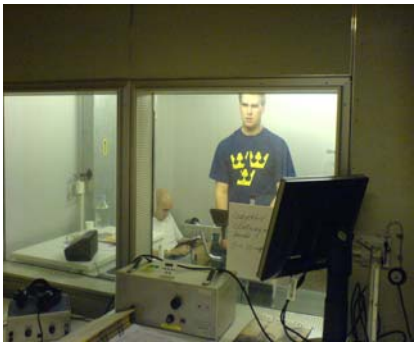
Figure 3



ETC (Directive 1999/96/EC of December 13, 1999)

Ozone

The ozone was generated by a Fischer's ozone generator 500 MM (Fischer Labor and Verfahrens-Technik, Bonn, Germany) and the chamber concentration was monitored and maintained at 200 ppb. All ozone exposure took place in a walk-in chamber designed for this particular purpose.

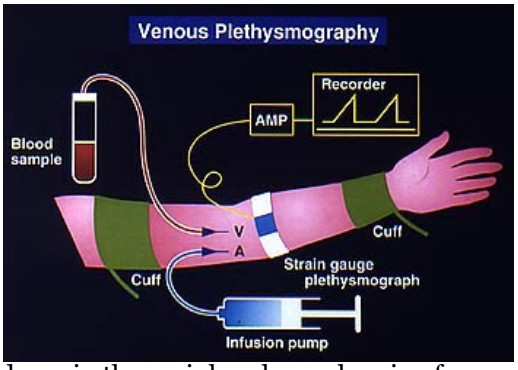


Bronchoscopy

A standard flexible video bronchoscope (Olympus BF 1T 160, Tokyo, Japan) was introduced orally with the subjects in the supine position. All subjects had been pre-medicated with 1 mg of atropine subcutaneously. For topical anesthesia lidocain was used. The subjects received supplementary oxygen (flow 2L/min) through a nasal cannula throughout the session.

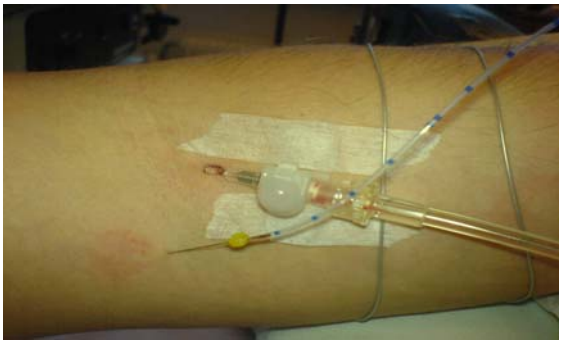
BW was performed by an initial instillation of 2x20 mL sterile saline solution followed by a BAL with 3x60 mL saline solution. The BAL was performed either the in lingual or the right middle lobe, with sides randomly selected and switched to the opposite side at the second visit. The recovered aspirate from the first and second 20 mL instillations of BW and the collective BAL fluid were accumulated and immediately placed on ice. The samples were used for total and differential cell counts as well as examination of soluble mediators. The BAL fluid was filtered to remove mucus (pore diameter 100 mm; Syntab Product Ab, Malmö, Sweden) and centrifuged at 400xg for 15 min in order to separate cellular components from the supernatant, which was then divided into aliquots and stored at -80°C until analysis.

Forearm venous occlusion plethysmography



When assessing endothelial dysfunction the coronary arteries are of most interest. However, coronary arteries are difficult to investigate invasively but there is a close relationship between the responses to the vasoactive drugs in the peripheral vessels using forearm occlusion plethysmography. This method can be used as a surrogate and has become the golden standard

method as it is well described and has shown excellent reproducibility (44-45). The technique makes it possible to cannulate the brachial artery by using a very fine needle, and locally infuse vasoactive drugs with minimal systemic effects. The simplicity of the method makes it robust. Initially, the venous blood is stopped by an upper arm cuff inflated to a pressure of 30-40 mmHg in a cycle of alternating 8 seconds inflated and 4 seconds deflated during blood flow measurement. Arterial blood flow is remained unaltered and because of this, blood enters the forearm but cannot return immediately. During the procedure the circumference of the forearm increases proportionally to the blood flow. By using a special strain gauge made of mercury-in-silicon connected to a computer, the blood flow can be calculated. Because of the differences of the capillary network in the hand and it's susceptibility to temperature changes, the blood flow is stopped during measurement. This is achieved by a second inflatable cuff which is placed around the wrist and inflated to 200 mmHg, producing a microcirculation in the forearm only. Both arms are used in the experiment, but only one arm is used for infusion and the other arm serves as a control for systemic effects of the infused drugs. The subjects are in the supine position with the arms resting on pillows above the level of the heart to ensure venous blood return. Since blood flow is very dependant on temperature and the levels of excitement of the subject, it is very important that the room in which the study is performed has a controlled temperature and is peaceful and quiet. For this reason the subjects are not allowed to sleep, read or watch movies during the study.



Fraction of exhaled nitric oxide (FENO)

After an at least 4-hour fast, all individuals were given a standardized breakfast, free of nitrates. FENO was then measured before, at 6 hours and 24 hours after exposure to DE, ozone or air according to the ATS/ERS guidelines (46). The measurement was performed by using a chemiluminescence analyzer (NIOX-system; Aerocrine AB; Stockholm; Sweden) and exhaled nitric oxide was measured during a slow single exhalation against an oral pressure of 5 cm of H_2O. Four different flow rates were used $FENO_{10}$, $FENO_{50}$, $FENO_{100}$ and $FENO_{270}$ mL/s (± 10%). Each exhalation lasted for 10 seconds and FENO was measured during the 6^{th} to the 10^{th} second of the exhalation phase. Duplicate measurements were secured, all within a 10% variation, and the mean concentration of nitric oxide (NO) in parts per billion (ppb) was registered.

Heart rate variability (HRV)

All subjects were fitted with Holter electrocardiographic monitors (Reynolds Medical Lifecard, Delmar Reynolds, United Kingdom) before exposures and ECG monitoring continued for 24 hours.

To assess the acute effects of exposure on heart rate variability, the subjects were asked to rest supine in a quiet, temperature controlled room maintained at 22-24°C for 20 min immediately prior to, and 2 and 6 hours following the start of each exposure. All volunteers also underwent blood pressure measurements and a vascular assessment.

Electrocardiographic recordings were analysed using the Reynolds Medical Pathfinder Digital 700 Series Analysis System (Delmar Reynolds, United Kingdom). An experienced single operator, blinded to both subject characteristics and exposure, verified any abnormal rhythms and performed manual editing of aberrant beats and electrical interference prior to generating RR data tables. If more than 95% of the RR data was valid, the recording was analysed. RRdata were analysed using the HRV Tools software package (DelMar Reynolds, United Kingdom).

Standard time-domain measures were calculated including mean NN-interval (time interval between consecutive sinus beats), standard deviation of NN-interval values (SDNN; an index that expresses overall variability), percent successive NN-interval differences >50 ms (PNN50), root-mean-square of successive NN-interval differences (RMSSD) and the triangular index (an estimate of overall heart rate variability). SDNN, PNN50 and RMSSD are measures of high-frequency variation mediated primarily by the vagus nerve. Frequency domain analysis determined the low frequency (LF; 0.1 Hz) and high frequency (HF; 0.25 Hz) components of the power spectrum in absolute values of power (ms^2). LF and HF were also expressed in normalized units (LFn and HFn), to account for variation in the total power and very low frequency components, as well as the HF/LF ratio.

Main results

Study 1

Significant increases in neutrophil and macrophage numbers were found in BW after DE followed by ozone exposure in comparison to when exposure to air was preceding ozone exposure. Total cell count macrophages (7.1 (4.0–9.5) 8.2 (4.2–10.9) x 10^4 cells x L^{-1} respectively; p= 0.046. DE before ozone also raised eosinophil protein X levels in BAL compared to air preceding ozone. Median (IQR) concentration 0.25 (0.00–0.49) and 0.41 (0.00–0.85) cells x L^{-1}, respectively; p=0.04.

Study 2

Diesel exhaust exposure attenuated the vasodilatation to bradykinin ($p<0.05$), sodium nitroprusside ($p<0.05$), acetylcholine ($p<0.001$) and to verapamil ($p<0.001$) compared to air exposure. The net release of tPA during bradykinin infusion was also impaired following DE exposure ($P < 0.05$). No effects on heart rate, blood pressure or resting blood flow were seen. The results indicate that short-term exposure to DE generated by an urban running mode caused endothelial dysfunction in the same way as after an exposure to exhaust from an idling engine.

Study 3

Exposure to 300 ppb of ozone for 75 minutes did not alter heart rate, blood pressure or resting forearm blood flow at either 2 or 6 h after exposure. Ozone exposure did not affect vasodilation to bradykinin (p=0.75), sodium nitroprusside (p=0.90), acetylcholine (p=0.92) or to verapamil (p=0.13) 2 hours after exposure. However, 6 hours after exposure, the vasodilation to acetylcholine ($p<0.02$) and sodium nitroprusside ($p<0.005$) was augmented

whilst the vascular response to bradykinin and verapamil were unaffected. The subjects did not experience any symptoms or serious arrhythmias during either exposure or during the 24 hour study period. Exposure to ozone did not affect time or frequency domain measures of heart rate variability over the 24-hour period. There were no differences in either time or frequency domain measures of heart rate variability at 2 or 6 hours between exposures.

Study 4

Exposure to diesel exhaust for one hour increased FENO at 6 hours compared to filtered air at expiratory flow rates of 10 mL/s (mean±SEM 60.8 ± 6.0 ppb *versus* 50.2 ± 5.9 ppb; P=0.01) and at 50 mL/s (18.6 ± 1.6 ppb *versus* 15.9 ± 1.5 ppb; P=0.011), but concentrations had normalised at 24 hours. FENO concentrations were not affected by diesel exhaust exposure or filtered air at $FENO_{100}$ or $FENO_{270}$, the two higher flow rates.

Exposure to ozone did not affect FENO at any flow rate or time point in either cohort of healthy volunteers.

Statistics

Study 1

Cell count and soluble protein data of filtered air followed by ozone versus DE followed by ozone were analyzed with Wilcoxon Signed-Rank Test, a non-parametric test for paired observations. Correlations were assessed using the Spearman correlation test. A p-value less than 0.05 were considered statistical significant. Values are presented as medians with interquartile ranges (IQRs).

Study 2 and 3

Plethysmographic data were analyzed as described previously. The net release of tPA antigen was defined as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused FBF) and the concentration difference between the infused and non-infused arms as described previously (44). Data were analyzed by 2-way ANOVA with repeated measures or 2-tailed Student's t-testswhere appropriate, using GraphPad Prism (GraphPad Software, Version 4 for Macintosh) and SPSS (SPSS inc. Chicago, IL, USA, version 15).

Study 4

Data are presented as mean ± SEM. A repeated measures analysis of variance (General Linear model) with two within-subject factors (time and exposure) was used, with pre-exposure FENO data used as reference using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). In order to avoid type-I errors due to two comparisons, the level of significance was adjusted by dividing the set significance level by two (Bonferroni correction) and therefore statistical significance was taken at $P<0.025$.

Discussion

Air pollution is of major global concern. In the years to come, it is evident that air pollution will be an even larger problem when it comes to the "new" economies and their potential to contribute to bad air quality. Efforts have been made to find ways to reduce PM in ambient air. The introduction of retrofit exhaust traps (47) and legislations against private driving in city traffic are possibilities discussed. The London Summer Olympics in 2012 will be a challenge for research on how to reduce PM in a large city, where smog development is prone.

It is essential to elucidate the underlying mechanisms behind the reported excess in mortality and morbidity associated with increasing air pollution. As a scientific community, we need to provide the legislators with safe air pollution threshold levels and evaluate risks of exposure to different air pollutants and their possible interactions in ambient air. Epidemiology only provides data on associations between air pollutants and health effects, whereas experimental research is crucial to elucidate the underlying biological mechanisms. The present thesis is based on research dealing with associations provided by epidemiology.

The first study is based on the assumption that ozone and diesel exhaust interact in causing airway inflammation. Since the two air pollutants usually coexist and as ozone levels commonly peak in the afternoon, a previous study protocol was designed to mimic ambient conditions. Subjects were exposed to DE in morning (when they go to school or work) and ozone or air in the afternoon (on returning home). In that study Bosson *et al.* showed that ozone exposure magnified the DE-induced airway inflammation assessed by induced sputum (42). In the present study, we exposed the subject to DE or air in the morning followed by ozone exposure in the afternoon, in order to elucidate whether pre-exposure to DE would enhance the ozone-induced airway inflammatory responses. Bronchoscopy was performed the next

morning and showed an increase in BW-neutrophils when exposure to DE was carried out pre-ozone exposure, indicating an additional effect. These two studies cannot be fully compared, since the assessments were different (induced sputum vs. bronchoscopy) but both studies show an increase in inflammatory markers when subjects were exposed to an additional pollutant. These results indicate the need for further studies on combined air pollution exposures. The study presented in this thesis was designed as a sequential exposure but it would have been interesting to perform an experimental exposure study with both ozone and DE simultaneously, as in real life. There are logistical and toxicological issues concerning a simultaneous exposure study. The ozone would rapidly oxidize the surface components in a chamber environment and probably change their oxidative potential. Another problem would be to determine what exposure levels to employ. Logistically, such a study design would be very complicated as it would require four study arms in a double-blind and cross-over fashion; air, air + DE, air + ozone, DE + ozone, which would be both expensive and time-consuming.

The second study deals with the fact that, so far, all DE exposure studies employing cardiovascular endpoints have been performed with the diesel engine operating at idling conditions. In real-life however, exposure to DE curbside mainly arises from moving trucks and cars and not primarily from idling engines. The PM chemical and physiological properties differ between the exhaust from these two running conditions. The PM from an idling engine exhaust is richer in organic carbon, whereas the PM from transient running engines mainly consists of elemental carbon, i.e. soot. These different characteristics might have different effects on humans. Sehlstedt *et al*. showed an increase in eosinophils in BAL after exposure to DE generated during the ETC compared to air, something which has not been reported after exposure to DE during idling (49). This study, although comparisons are made to historical findings, suggests a different airway inflammatory response between the two running conditions. We therefore designed a study similar to the previous by Mills *et al*. but employed exposure to DE

generated during transient running, i.e. the urban part of the ETC (30) to further investigate this issue.

The study showed that the vascular effects were similar between the two engine running modes, however, not identical. In the previous studies, vasodilatation during infusion of BK, ACH and SNP was attenuated after DE exposure, whereas vasodilatation was not affected during VP-infusion. As several studies employing DE exposure at idling engine conditions show no attenuation of the vasodilatation during VP-infusion, this suggests, although close to statistical significance in one study (p-value just above 0.05), that this is a true lack of VP response to exposure to DE generated during idling (32, 35, 47, 48, 50). The present study (study II) showed a similar vascular response after DE exposure during transient running, but with attenuated vasodilatation during VP-infusion as well. In a recent study by Lucking *et al.* (47), the same protocol was employed and resulted in an identical attenuation of vasodilatation during VP-infusion, indicating that this is a true effect and a possible difference in response to DE generated by the two different engine running modes.

Verapamil exerts its effects by blocking the calcium channels in smooth muscles in the blood vessels (51). The VP effects seen after exposure to DE generated during transient running could be associated with exhaust properties and different chemical composition of the particles themselves, for example, a higher content of soot and absorbed organic compounds. Although the mass concentration between the two running modes was similar, the particles were larger and fewer in number and had a lower oxidative potential during the transient running mode. The PAH content was also lower compared to the idling situation and the gaseous components differed with higher NO_x levels during the transient running mode. Whilst there are some differences in the effects on the vasculature between idling and transient running conditions, the main effects, impairment of vascular function and endogenous fibrinolysis, were similar. This finding is clinically

relevant since it provides a possible link between increases in air pollution and increased cardiovascular morbidity.

In a recent study by Mills *et al.*, in which healthy subjects were exposed to diesel exhaust, pure carbon nano-particulate, filtered DE and filtered air, vascular effects were shown only after DE exposure, suggesting, that the adverse vascular effects are mediated by combustion-derived DE particles (50). In that study, the DE was generated by a generator engine powered by diesel fuel and operated at idling. As in previous studies employing the idling situation, there was no attenuation of vasodilatation during infusion of VP (50).

It has been hypothesized that the adverse effects of DE exposure on the vascular endothelium is caused by the oxidative stress exerted by the PM itself or by its surface components (5). The oxidative stress produces superoxide radicals which form peroxynitrite with endothelial NO. This reaction leads to consumption of NO and thus, reduces NO-bioavailability (30, 52).

Thus, as the effects on the vasculature are similar in five studies (30, 47, 48, 50) three studies employing an idling engine and two running at a transient mode, it can be concluded that the adverse vascular effects are comparable regardless of engine or running mode. This suggests that the particle fraction of the exhaust is the culprit.

In the third study, it was found that a brief exposure to ozone did not impair vascular function or affect heart rate variability. Rather, the vasomotor response to two of the infused agonists was enhanced 6-8 hours after ozone exposure. These findings are in contrast to previous DE studies and suggest that the increase in cardiovascular events and mortality demonstrated after exposure to ozone and motor engine exhaust in traffic is mediated through different pathways and mechanisms.

Previously, experimental human studies have shown that exposure to ozone increases the oxidative burden in the airways and trigger a pronounced neutrophil-dominated inflammatory response (37, 53-55). Already at 1.5 hours after ozone exposure, the expression of P-selectin was up-regulated in the bronchial endothelium. This was followed by the up-regulation of endothelial ICAM-1 expression and inflammatory cell recruitment into the bronchial mucosa and bronchoalveolar spaces at 6 hours, with the inflammatory response peaking around 12-24 hours following exposure (37, 55). The ozone-induced airway responses coincide with the time course of the development of airway inflammation following exposure to DE, and include the up-regulation of vascular endothelial adhesion molecule expression and a neutrophil-dominated inflammation, accompanied by CD4+ and CD8+ cell influx (27).

The systemic inflammation seems to be more pronounced after ozone than after diesel exhaust. In a study by Bosson *et al.* healthy subjects were exposed to 200 ppb of ozone for 2 hours, and results showed a significant increase in peripheral blood neutrophils 6 hours after exposure compared to air (unpublished data) (56). This is in contrast to a study in which DE exposure did not cause systemic neutrophilia but increased serum levels of TNF-alfa and IL-6 24 hours after exposure (48).

We have previously reported that diesel engine exhaust generated during idling or transient running conditions caused endothelial dysfunction, increased arterial stiffness, impaired endogenous fibrinolysis, increased platelet activation, and enhanced *ex-vivo* thrombus formation in human subjects 6 hours after exposure, with some vascular effects maintained up to 24 hours (30, 48, 57). We have also demonstrated that patients with coronary heart disease experienced ST-segment depression when exercising during exposure to diesel exhaust as compared with air, despite stable disease conditions and relevant preventive medication (35). These effects are consistent with the reported immediate and delayed association between traffic exposure and the onset of acute myocardial infarction (MI) (20).

The development of MI after ozone exposure has not been described in detail as after traffic exposure. There are studies in which ozone has been linked with MI and mortality during the day of exposure. Because of similarities in the airway oxidative and inflammatory responses between ozone and diesel exhaust, it has been proposed that vasomotor and thrombogenic functional changes would potentially also occur in parallel (26, 58). Associations between ozone and mortality are difficult to disentangle from the effects of PM, and ozone and particles do indeed interact to enhance airway inflammation as previously reported (42).

Whilst effects on airway inflammation induced by ozone and diesel exhaust exposure show several similarities, there are some important aspects to address. The DE effect is thought to be mediated via epidermal growth factors receptor (EGFR), tyrosine 1173 transphosphorylation, and redox-sensitive elements causing NFκB activation and the subsequent up-regulation of IL-8, GRO-alpha, ICAM-1 and NOS II in the bronchial mucosa (29, 59). Ozone appears to react directly with components of the respiratory tract lining fluid (RTLF) and leads to the production of secondary free-radical-derived ozonation products that cause cellular damage (60). Exposure to ozone does not seem to involve NFκB-activation in the bronchial epithelium in humans (61).

In contrast to the study hypothesis, an ozone-induced increased vasomotor response to acetylcholine and sodium nitroprusside was demonstrated. This suggests a maintained or even improved NO bioavailability after ozone exposure. In some studies, oxidative stress has been shown to increase NO bioavailability through NOS III activation (62-63).

In the fourth study, FENO was, for the first time, introduced at multiple flow rates in experimental exposure studies employing DE and ozone. The rationale for this, is that the various flow rates are thought to reflect different parts of the bronchial tree, with the lower flow rates ($FENO_{10}$ and $FENO_{50}$)

corresponding to the central or bronchial airways and the higher flow rate ($FENO_{270}$) to the distal airways (64).

Healthy subjects exposed to DE for one hour showed an increase in FENO at 6 hours after exposure. Only FENO concentrations obtained at lower flow rates ($FENO_{10}$ and $FENO_{50}$) were affected, suggesting that the central airways, but not the peripheral airways, are principally involved. This observation is in accordance with previous studies, in which diesel exhaust induced increases in inflammatory cells and cytokines in mucosal biopsies and BW obtained from the central airways, but not in BAL (65-66).

In contrast, exposure to ozone did not affect FENO at any flow rate or any time point. To increase the power of the study to detect a small ozone-induced effect on FENO, the study was repeated and the number of subjects was increased from 18 to 36, but still no consistent effect of ozone on FENO in either cohort or in the combined data set could be detected. These findings are consistent with two previous studies in healthy subjects, suggesting that exposure to ozone does not affect FENO (67-68).

Associations reported between exposure to ozone and FENO in field studies are in contrast with our findings (69). In real life, people can be exposed to ozone repeatedly during many days but also to a mixture of many air pollutants. As it is impossible to distinguish the effects of a single air pollutant in field studies, it is not surprising that augmented effects on airway inflammation are seen when exposures to different air pollutants are combined (42). When it comes to FENO, a single ozone exposure does not seem to cause a noticeable acute effect as seen after DE.

Exhaled NO production is thought to be under the regulation of three endothelial NOS isoforms. NOS I and II are predominantly expressed in healthy subjects, while NOS II, the corticoidsteroid-sensitive inducible NOS, is up-regulated in patients with asthma. Recently, there is evidence that exhaled NO is associated with a genetic variant of NOS III in patients with

asthma, suggesting that both NOS II and NOS III are important in determining the exhaled NO in this patient group (70). Endothelial nitric oxide synthetase (NOS III) is regulated under the influence of the oxidative stress-sensitive transcription factor NFκB (71).

Both diesel exhaust and ozone are considered oxidant air pollutants and exert their effects on the airways through oxidative stress (5, 72-73). The ozone molecule is highly reactive and does not reach the airway epithelium but reacts with components in the respiratory tract lining fluid and causes a cascade of secondary free radical-derived ozonation products (60), whereas the DEPs are deposited on the airway epithelium and induce a local inflammatory response and may also translocate to affect the local vascular endothelium (5).

NFκB activation and up-regulation of NOS III occur in endothelial cells exposed to reactive oxygen species (74). NFκB, along with AP-1 and MAPkinases, are activated by diesel exhaust exposure (10), and in turn this might lead to up-regulation of NOS III. Exposure to ozone has not been found to activate NFκB in human airway epithelium, suggesting a different induction of airway inflammatory responses between ozone and DE (14). These observations suggest that ozone exposure may not influence FE_{NO}, as it does not by itself activate NFκB and up-regulate NOS III.

Mehta *et al* studied the levels of exhaled NO following infusion of the NO precursor L-Arginine and found increased levels indicating that exhaled NO may reflect endogenous production of NO (75). Interestingly, the baseline concentrations and changes in exhaled NO were similar in that study to the increase in FE_{NO} following exposure to diesel exhaust in the present study.

Previously, we have hypothesized that the DE-induced oxidative stress and the subsequent adverse cardiovascular health effects are mediated through reduced NO bioavailability (30). Oxidative stress caused by exposure to DE and subsequent consumption of vascular NO may evoke homeostatic

mechanisms to normalize vascular function through the up-regulation of NOS III, which in turn may increase FENO.

All the studies in this thesis contend with experimental exposure to oxidative air pollutants. Sequential exposure of DE and ozone has been applied. To mimic real-life urban driving a novel mode to generate DE for exposure has been used. Both the cardiovascular system and the airways have been investigated. In the airways, ozone-induced inflammation seems to be more pronounced after sequential exposures, but ozone alone does not increase the inflammatory marker FENO or cause endothelial dysfunction, as shown after exposure to DE. This suggests different underlying mechanisms and pathways and necessitates further research.

The time-points and exposure levels applied in this thesis have been chosen based on previous investigations. All air pollutant exposures are equivalent to high ambient levels, but realistic real-life levels, as they often can be measured curbside or in tunnels etc. during rush hour traffic. All studies were performed in healthy subjects. The first study included both men and women, since it was a bronchoscopy and not a vascular assessment study. In the second and third studies only men took part as it is well known that the cyclic changes of hormones in women clearly affect vascular function, especially fibrinolysis. Another important detail is the difficulty to cannulate the brachial artery in women. As in other clinical research the fact that women often are excluded limits the possibility to draw general conclusions. An additional limiting factor is that only healthy subjects were included in these studies and thus, it cannot be excluded that other effects might be relevant in patients with asthma, COPD and cardiovascular diseases.

Final comments

The research group to which I belong has conducted human exposure studies since the late 1980'ies. The studies have included exposure to sulphur dioxide, nitrogen dioxide, ozone and diesel exhaust generated by a truck engine, both during idling and transient running conditions. The exposures have been performed in well validated walk-in exposure chambers and because of the vast experience that our staff has acquired over the years, the technical standard of the studies has been kept high.

In this thesis, exposures to the highly oxidative air pollutants ozone and diesel exhaust have performed and both respiratory and cardiovascular effects have been assessed. The respiratory effects were investigated by means of bronchoscopy with bronchial wash and bronchoalveolar lavage, methods which have been shown to be valid and highly reproducible.

The use of venous occlusion forearm plethysmography to assess vascular function has been established through a very productive collaboration with the Department of Cardiovascular Research in Edinburgh. This method is thoroughly validated and robust as an invasive technique. The effects of vasodilation and excretion of tPA in the forearm circulation, i.e. brachial artery, is closely associated with the effects in the coronary circulation which makes this method very appropriate to assess cardiovascular endpoints after exposure to air pollution. A standard method for evaluating heart rate variability has also been acquired.

The study results bring novel insights into this research field. Whilst the airway inflammatory response after exposure to DE or ozone is similar, there are significant differences in the response in the vasculature and in FE_{NO} suggesting different mechanisms and pathways for inducing the airway inflammation. It is important to reduce combustion-derived PM in ambient air and regardless how the particulate is generated, it still has a big impact on human health. An acute single exposure to ozone does not alter the heart

rate variability or rhythm, which is one of the hypotheses for the association between mortality and increases in ambient ozone levels. This could be crucial information for legislators when it comes to recommendations on air quality.

For the first time, airway inflammation by means of fraction of exhaled nitric oxide, FENO, measured at multiple flow-rates, was used in an experimental ozone and DE exposure study. The significant results in FENO changes after DE exposure in healthy humans are too small to have a significant clinical impact. This raises the question of the mechanisms behind the association between increased FENO and increased levels of air pollution. This study emphasizes the importance of PM. Since there is a strong association between PM and increasing levels of ozone and NO_2, it cannot be excluded that the measured air pollution-related effects on FENO are due to $PM_{2.5-10}$ or the combination of PM-ozone and not to ozone alone. For research purposes and speculations on underlying mechanisms FENO still may play a role.

Studies on FENO are mainly performed in asthmatic subject showing an augmentation of FENO in relation to increases in symptoms. Human exposure studies employing different patient groups are needed to clarify the mechanisms behind the reported adverse health effects. Vascular studies following exposure to DE, ozone or the combination are needed, but logistically problematic in patients. It is known that the ischemic burden increases in patient with coronary heart disease when they are exercised during exposure for DE. The cardiovascular impact of ozone in patients with coronary disease is unknown, but given the lack of response in young healthy subjects, perhaps such a study has a low priority at present.

Patients with COPD comprise a very heterogenetic group with different clinical phenotypes. Therefore such studies would be difficult to interpret, but it is a well known fact that COPD patients have an increased cardiovascular morbidity and mortality compared to smokers without COPD (76).

This thesis has determined that it is essential to reduce PM in ambient air. Studies with intervention have already been conducted. Rudell *et al.* showed an attenuation of symptoms using a cabin air-inlet filter in cars (77) and the results have been confirmed in a very recent study (unpublished data). Furthermore, Lucking *et al.* have shown that a retrofit particle trap reduces the endothelial dysfunction and the increased prothrombotic effect seen after exposure to DE (57).

This suggests that models for PM reduction are effective and that the hypothesis that PM and its chemical and physiological properties are driving the adverse health effects, is plausible and that ozone might act as a booster of these effects.

Conclusions

It is hereby concluded that:

- ✓ Exposure to ozone preceded by exposure to diesel exhaust at levels that can be encountered curb-side, enhances the already established DE-induced neutrophil-dominated airway inflammation in healthy subjects

- ✓ Exposure to diesel exhaust generated by an engine running at a transient mode causes a similar vascular response as exposure to diesel exhaust generated during idling

- ✓ An acute single exposure to ozone does not cause endothelial dysfunction or alterations in heart rate variability in healthy subjects

- ✓ Fraction of exhaled nitric oxide, F_{ENO}, measured at multiple flow-rates, may be used as a marker of acute diesel exhaust-induced, but not ozone-induced, airway inflammation

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