THE UNIVERSITY *of* EDINBURGH

THE ACUTE CARDIOVASCULAR EFFECTS OF EXPOSURE TO AIR POLLUTION: COMPONENTS, VASCULAR MECHANISMS AND PROTECTING THE PUBLIC

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For Emily, Nicholas and William

ABSTRACT

Exposure to air pollution, particularly fine and ultrafine particulate matter derived from combustion sources, has been consistently associated with increased cardiovascular morbidity and mortality. Recent controlled exposure studies demonstrate that short-term exposure to diesel exhaust, which can contribute up to 40% of urban particulate air pollution, results in impaired vascular endothelial and fibrinolytic function in healthy volunteers, and increased exercise-induced myocardial ischaemia in patients with coronary heart disease. These observations may, in part, explain the observed increase in cardiovascular events following exposure to air pollution.

Despite these observations there remain uncertainties regarding the key constituents of the air pollution mixture that mediate these adverse effects, and the underlying physiological and biological pathways involved. In these studies, using two controlled exposure facilities, I explored the vascular effects of the most prevalent gaseous component of the air pollution mixture – nitrogen dioxide – and the mechanisms responsible for impaired vasomotor function following exposure to diesel exhaust. Furthermore, I investigated the effect of acute exposure to "real-world" urban air pollution in both healthy volunteers and patients with coronary heart disease, and the effect of reducing that exposure using a simple facemask.

In total, 10 healthy volunteers were exposed to nitrogen dioxide, and 29 healthy volunteers exposed to dilute diesel exhaust in a series of double-blind randomised crossover studies. Exposure to nitrogen dioxide had no effect on either vasomotor function or endogenous fibrinolysis, providing indirect evidence that the adverse vascular effects are predominantly driven by particulate components. Following exposure to diesel exhaust there was no up regulation of endothelin-1 production, although there was increased vasoconstriction to intra-arterial infusion of endothelin-1. Following endothelin A receptor antagonism, there was attenuated vasodilatation following exposure to diesel exhaust as compared to air, an effect abrogated by endothelin B receptor antagonism. My findings suggest that the endothelin system does not play a central role in the adverse vascular effects of air pollution, but given the tonic interaction between the endothelin and nitric oxide systems, these observations could be explained by reduced nitric oxide bioavailability. Following diesel exhaust inhalation, plasma nitrite concentrations (as a marker for nitric oxide generation) are markedly increased without changes in haemodynamics or basal blood flow consistent with increased nitric oxide consumption. In the presence of a nitric oxide clamp, and without endogenous nitric oxide release, the vascular responses to vasodilators are similar. This perturbation of nitric oxide consumption and release appears to underlie the observed vascular endothelial effects.

Fifteen healthy volunteers and 98 patients with coronary artery disease were recruited in Beijing, China. Subjects walked along a predefined city centre route for 2 hours in the presence and absence of a highly efficient facemask to reduce personal particulate air pollution exposure in an open label randomised crossover study. When wearing a facemask, there was an attenuation of exercise-induced increases in blood pressure, an improvement in heart rate variability, reduced myocardial ischaemia and subjects reported fewer symptoms.

My findings have identified the biological mechanisms underlying the adverse vascular effects of exposure to diesel exhaust, and have helped to clarify the components responsible for these effects. Moreover, I have identified important benefits of reducing personal exposure to particulate matter using a simple facemask that have the potential to reduce cardiovascular events in patients living in urban or industrialised areas. Ongoing research in this area will provide further insight into the underlying vascular mechanisms, and the potential benefits of reducing particulate air pollution exposure, and may result in important targeted interventions to reduce the impact of air pollution on cardiovascular health.

CONTENTS

APPENDICES

I declare that this thesis has been entirely composed by myself, Dr Jeremy Langrish, and that it has not been submitted for any other degree or professional qualification. The work presented in this thesis was performed as part of an international research collaboration between the University of Edinburgh, Umeå University in Sweden, the National Institute of Public Health and the Environment in the Netherlands and the Fuwai Hospital and Cardiovascular Institute in Beijing. I helped to formulate the research questions and design all studies, analysed all data and drafted all manuscripts presented in this thesis. Healthy volunteers and patients were recruited in Beijing by Li Xi, a fellow PhD student based at Peking University and in Sweden by the research nursing staff Annika Johansson and Frida Holmström. Data were collected primarily by myself including ambient air pollution monitoring and aerosol sampling in Beijing. Toxicological analysis of the particles was performed by TNO in The Netherlands.

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ABBREVIATIONS

ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
ANOVA	Analysis of variance
BK	Bradykinin
CHD	Coronary heart disease
CIMT	Carotid intima-media thickness
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ET-1	Endothelin-1
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
HEPA	High efficacy particulate air filter
HRV	Heart rate variability
L-NMMA	L-N^G-monomethylarginine
PAH	Polyaromatic hydrocarbons
$PM_{2.5}$	Particulate matter (mean aerodynamic diameter of ≤2.5 μm)
PM_{10}	Particulate matter (mean aerodynamic diameter of ≤10 μm)
RMSSD	Root mean square differences of successive RR intervals
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_x	Oxides of nitrogen
SDMA	Symmetrical dimethylarginine
SDNN	Standard deviation of successive RR intervals
SO_2	Sulphur dioxide
SNP	Sodium nitroprusside
UNEP	United Nations Environment Programme

WHO	World Health Organisation

CHAPTER 1

Introduction

Extracts from this chapter have been published in two editorials:

1. Langrish JP, Mills NL and Newby DE. Air pollution: the new cardiovascular risk factor. *Intern Med J* 2008;38:875-878.
2. Langrish JP, Mills NL and Newby DE. Heat and haze: a forecast for myocardial infarction? *Heart* 2009;95:1721-1722.

"…how much the Respiration is perturb'd, and concern'd, when the Lungs are prepossessed with these grosse and dense vapours, brought along in the Aer; which on the other side being pure and fitly qualified, and so conducted to them, is there commixed with the circulating blood, insinuating it self into the left ventricle of the heart by the Arteria Venosa, to rarifie and subtilize that precious vehicle of the Spirits and vital flame: The Vena Arteriosa disposing themselves into many branches through the Pulmonique lobes, for its Convoy the Aer (as we say'd) being first brought into them out of the Bronchia (together with the returning blood) to the very Heart it self; so as we are not at all to wonder, at the suddain and prodigious Effects of a poysonous or less wholesome Aer, when it comes to invade such noble Parts, Vessells, Spirits, and Humours, as it visits and attaques, through those subtile and curious passages…

…this acrimonious Soot produces another sad effect, by rendring the people obnoxious to Inflammations, and comes (in time) to exulcerate the Lungs, which is a mischief so incurable that it carries away multitudes by Languishing and deep Consumptions, as the Bills of Mortality do Weekly inform us…

…The Consequences then of all this is, that (as was said) almost one half of them who perish in London, dye of Phthisical and Pulmonic distempers; That the Inhabitants are never free from Coughs and importunate Rheumatisms, spitting of Impostumated and corrupt matter: for remedy whereof, there is none so infallible, as that, in time, the Patient change his Aer, and remove into the Country: Such as repair to Paris (where it is excellent) and other like Places, perfectly recovering of their health; which is a demonstration sufficient to confirm what we have asserted…"

John Evelyn Esq., 1661

1.1 OVERVIEW

Air pollution is a major public health concern and is robustly and consistently associated with an increase in cardiovascular morbidity and mortality. The World Health Organisation estimate that exposure to outdoor urban air pollution results in around 800,000 premature deaths each year, and indoor air pollution (mainly as a result of biomass combustion) to a further 2.4 million annual deaths [United Nations Environment Programme 2006]. In all, this represents about 5% of the total worldwide deaths each year.

It is evident that the link with increased cardiovascular morbidity and mortality is strongest for inhaled fine and ultrafine particulate matter. The pathophysiological mechanisms underlying these observed adverse effects remain unclear, but there is evidence of enhanced atherosclerosis in both animal models and in man. Acute exposures to particulate air pollution have been associated with increases in blood pressure and central arterial stiffness, alterations in the autonomic control of the heart, platelet activation and increased thrombogenicity. In addition, vascular endothelial responses – vasomotor function and endogenous fibrinolysis – are shown to be impaired by exposure to particulate air pollution.

Whilst many questions remain as to the exact pathophysiological mechanisms underlying these observed responses, in combination these effects go some way to explaining the epidemiological data.

1.2 AIR POLLUTION AND CARDIORESPIRATORY RISK

In August 2008, the XXIXth Olympic Games held in Beijing China brought the hazards of air pollution exposure into the public eye. International headlines highlighted the poor air quality and the potential health effects that might have an adverse impact on the performance of athletes, as well as on those with chronic cardiorespiratory diseases. At least one high profile marathon runner declared that he would not run in Beijing because of the poor air quality, and on arrival to Beijing, the United States of America cycling team wore close-fitting face masks designed to reduce personal exposure to airborne particles. So was this international concern justified?

Air pollution represents a major public health concern, and has been recognised as being detrimental to health for many years. Indeed, John Evelyn Esq. presented Charles II with a treatise entitled "Fumifugium: or the inconvenience of the aer and smoake of London Dissipated" in which he proposed that air pollution would shorten the lives of Londoners in 1661 (extract reproduced on title page) [Evelyn 1661].

Between 5th and 8th December 1952 prevalent atmospheric conditions along with major industrial emissions resulted in Greater London being enveloped by dense smog. It was later recognised that this "pollution episode" was accompanied by a sharp rise in mortality, and that around 4000 more people died during those four days than would have been expected under normal conditions. Overall it is estimated that an additional 12,000 deaths resulted from this "Great Smog" [Greater London Authority 2002; Dooley 2002].

This episode focussed attention on air quality standards, and resulted both in scientific interest in the adverse effects of air pollution and the introduction in the UK of the Clean Air Act 1956 [Greater London Authority 2002]. Since the introduction of this act, which regulated the burning of fossil fuels, controlled the heights of chimneys and introduced "smokeless zones" air quality standards have improved dramatically. However, pollution remains a major public health concern and currently the World Health Organisation estimates that each year it results in around 3 million premature deaths worldwide [United Nations Envorinment Programme 2006], representing around 5% of the 55 million annual deaths worldwide. Here in the UK the government has estimated that around 8,000 excess deaths occur each year in the UK as a result of air pollution exposure [COMEAPS 2000].

1.3 CHRONIC AIR POLLUTION EXPOSURE

1.3.1 Epidemiology

A number of large, well-conducted epidemiological studies have now shown clear associations between chronic air pollution exposure and mortality. Whilst estimates vary, increasing chronic exposure to particulate air pollution by 10 $\mu g/m^3$ (equivalent to the difference between background particulate air pollution concentrations in London and Edinburgh) increases all cause mortality by between 2 and 4% [Anderson, *et al.* 1996; Pope, *et al.* 2002]. In their study of mortality across six cities in the USA Dockery and colleagues demonstrated that, after adjustment for smoking and other risk factors, living in the most polluted city as compared to the least polluted was associated with a mortality-rate ratio of 1.26 (95% confidence interval 1.08 to 1.47) [Dockery, *et al.* 1993].

Perhaps surprisingly, given that after inhalation the air pollution is delivered directly to the respiratory tract, much of this excess mortality is attributed to cardiovascular causes. Typical estimates suggest that for every 10 $\mu g/m^3$ increase in background particulate air pollution, cardiovascular mortality increases by between 8 and 18% [Pope, *et al.* 2004], although in one recent study of 65,893 women without clinically identified cardiovascular disease enrolled in the Women's Health Initiative (WHI) study it was shown that the same increase in background air pollution concentrations resulted in a 76%

increase in cardiovascular mortality and a 24% increased risk of acute cardiovascular events [Miller, *et al.* 2007].

Conversely by compiling data on life expectancy, demographics, and socioeconomic status along with matching data on particulate air pollution concentrations from the 1970s to the early 2000s, Pope and colleagues have demonstrated that for each 10 $\mu g/m^3$ reduction in background fine particulate air pollution concentrations, life expectancy in the USA increased by approximately 7 months [Pope, *et al.* 2009].

So how does the risk attributable to air pollution compare to traditionally quoted cardiovascular risk factors? The well-defined risk factors of hypertension, hypercholesterolaemia, diabetes mellitus, family history and smoking have been studied in depth in large epidemiological studies. In comparison to these variables, chronic exposure to fine particulate air pollution gives a dose-dependent and similar risk to hypertension or hypercholesterolaemia (Figure 1.1).

Evidence from these epidemiological studies, and the observation that there appears to be no absolute threshold for this increased risk prompted reanalysis of environmental health standards and implementation of strict guidelines across Europe and the USA [Pope, *et al.* 2002]. Indeed following implementation of these standards in the USA, the Environmental Protection

Agency were challenged by industry groups in the courts, but an independent review of the evidence by the Health Effects Institute [Krewski, *et al.* 2005; Krewski, *et al.* 2005] reaffirmed the associations and the case was lost [Kaiser 1997; Ware 2000]. In fact, recent evidence suggests that the association between exposure and mortality is not linear, and is relatively steep at low levels of exposure and flattens out towards higher levels [Pope, *et al.* 2009] – making these controls in Europe and the USA (where air quality standards are generally relatively good compared to China and the Indian subcontinent [Table 1.1]) even more relevant.

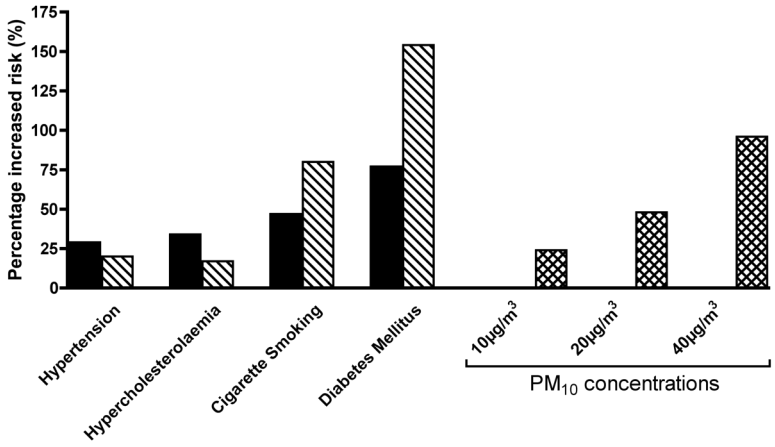

Figure 1.1. Percentage increase in population attributable risk with traditional cardiovascular risk factors for an acute cardiovascular event from the Framingham (solid bars) and QRESEARCH (striped bars) cohorts [Hippisley-Cox, *et al.* 2008]. For comparison, risk attributed to each 10µg/m^3 increase in $PM_{2.5}$ concentrations (hashed bars) is shown [Miller, *et al.* 2007]. Diabetes Mellitus refers to type II diabetes. Hypertension means incidence of hypertension in Framingham study and per 20 mmHg increase in systolic blood pressure in QRESEARCH. All other parameters refer only to their incidence.

Rank	Country	City	Average PM_{10} Concentration, µg/m^3 (2004)
1	Egypt	Cairo	169
2	India	Delhi	150
3	India	Kolkata	128
4	China	Tianjin	125
5	China	Chongqing	123
6	India	Kanpur	109
7	India	Lucknow	109
8	Indonesia	Jakarta	104
9	China	Shenyang	101
10	China	Zhengzhou	97
11	China	Jinan	94
12	China	Lanzhou	91
13	China	Beijing	89
14	China	Tiyuan	88
15	China	Chengdu	86
16	India	Ahmadabad	83
17	China	Anshan	82
18	China	Wuhan	79
19	Thailand	Bangkok	79
20	China	Nanchang	78
50	*Greece*	*Athens*	*43*
74	*Italy*	*Rome*	*29*
85	*Germany*	*Berlin*	*22*
91	*UK*	*London*	*21*
107	*France*	*Paris*	*11*
110	*Sweden*	*Stockholm*	*11*

Table 1.1. Average air quality in the world's cities showing the top 20 most polluted cities, with European cities included for comparison. Data published by the World Bank [World Bank 2007].

1.3.2 Components of the air pollution mixture

Air pollution is a complex mixture of gases, particulate matter and volatile agents, which may be derived from a variety of sources. The exact components responsible for the harmful effects are not yet known, but the epidemiological link is strongest for fine particulate matter [Laden, *et al.* 2006; Samet, *et al.* 2000]. Indeed reanalysis of data from the US six cities study after chemical analysis and source apportionment of the air pollution in each location has shown that fine particulate air pollution resulting from combustion (mobile and coal) sources is associated with increased mortality whereas crustal particles (soil and road dust) is not [Laden, *et al.* 2000].

Similar positive associations between exposure and cardiovascular mortality have been demonstrated for long-term exposure to nitrogen dioxide, sulphur dioxide and ozone [Anderson, *et al.* 1996; Elliott, *et al.* 2007; Hoek, *et al.* 2001; Rosenlund, *et al.* 2008]. Nitrogen dioxide and sulphur dioxide are major constituents of combustion-derived air pollution, and the associations of gaseous exposure with adverse outcomes has usually been attributed to the close association of fine particulate matter with gaseous concentrations – suggesting that concentrations of these gaseous pollutants are merely markers for fine particulate air pollution [Sarnat, *et al.* 2001]. However, isolated real life exposures to nitrogen dioxide have been linked to respiratory illness and susceptibility to airway infection [Guidotti 1978; Love, *et al.* 1982; Mostardi, *et al.* 1981], and controlled exposure to nitrogen dioxide

induces airway inflammation and modifies antioxidants in the respiratory tract lining fluid [Kelly, *et al.* 1996], and it may therefore be premature to state that these gaseous co-pollutants are inert.

Concordant with the hypothesis that it is combustion-derived fine particulate matter that is responsible for the increased cardiorespiratory mortality, a reduction in fine particulate air pollution is associated with reduced total mortality [Laden, *et al.* 2006]. This is elegantly demonstrated by Clancy and colleagues who studied the effect of a public health intervention in Dublin, Ireland designed to reduce the levels of combustion-derived particulate air pollution [Clancy, *et al.* 2002]. At the time of the study, domestic households were predominantly using bituminous coal that was cheap and readily available. However, concerns over air quality resulted in the government banning the marketing, sale and distribution of bituminous coal within the city of Dublin, and a subsequent 70% reduction in daily average black smoke concentrations, along with a 15% reduction in respiratory and a 10% reduction in cardiovascular deaths.

1.3.3 Underlying mechanisms

Epidemiological studies can, by their very nature, describe only association and interpreting this evidence requires a plausible underlying mechanism. One explanation is that chronic exposure to particulate air pollution may increase atherosclerotic burden. Chronic exposure to particulate air pollution is associated with the degree of coronary atherosclerosis and coronary artery calcium scores using electron-beam computed tomography [Hoffmann, *et al.* 2007]. Consistent with this, particulate air pollution exposure has been linked to increased carotid intima-media thickness (CIMT; measured using high-resolution B-mode ultrasound scanning), with the CIMT increasing by nearly 6% for each 10 $\mu g/m^3$ increase in background fine particulate matter ($PM_{2.5}$) exposure [Kunzli, *et al.* 2005]. Furthermore, the same investigators recently reported that progression of CIMT is closely associated with particulate air pollution exposure [Kunzli, *et al.* 2010].

Animal studies have shown that apolipoprotein-E deficient (Apo $E^{-/-}$) mice exposed daily to concentrated ambient particles collected near a freeway (in Los Angeles and Tuxedo, New York) exhibited larger early atherosclerotic lesions than mice exposed to just filtered air [Araujo, *et al.* 2008; Sun, *et al.* 2005]. Watanabe heritable hyperlipidaemic rabbits exposed to urban dust (Ottowa particles, EHC-93) by intrapharyngeal instillation twice weekly for four weeks resulted in the development of larger and more advanced atherosclerotic plaques [Suwa, *et al.* 2002].

1.4 ACUTE EXPOSURE TO AIR POLLUTION

1.4.1 Epidemiology

In a case-crossover study of 12,865 patients in Utah USA, Pope and colleagues showed that for each 10 $\mu g/m^3$ increase in background $PM_{2.5}$ concentrations the risk of an acute ischaemic coronary event increased by 4.5% [Pope, *et al.* 2006]. Krishnan Bhaskaran and colleagues have recently subjected this association to careful systematic review. The authors performed a robust systematic analysis of the current evidence linking short-term exposure to air pollution and the incidence of acute myocardial infarction and identified 26 studies that demonstrate an effect of exposure to particulate matter, ozone, carbon monoxide, nitrogen oxides or sulphur dioxide. The authors included only those studies that specifically addressed the incidence of myocardial infarction as an outcome and carefully controlled for potential confounders. They conclude that there is "fairly persuasive evidence of [a] short-term effect on myocardial infarction risk", with the risk of increasing by 5-17% for each 10 $\mu g/m^3$ increase in $PM_{2.5}$ (particulate matter with a mean diameter of ≤ 2.5 μm) exposure [Bhaskaran, *et al.* 2009]. Similarly, short-term exposure to fine particulate air pollution has been linked to increased hospital readmissions among survivors of myocardial infarction [von Klot, *et al.* 2005] and in patients with congestive cardiac failure [Mann, *et al.* 2002; Morris 2001; Pope, *et al.* 2008].

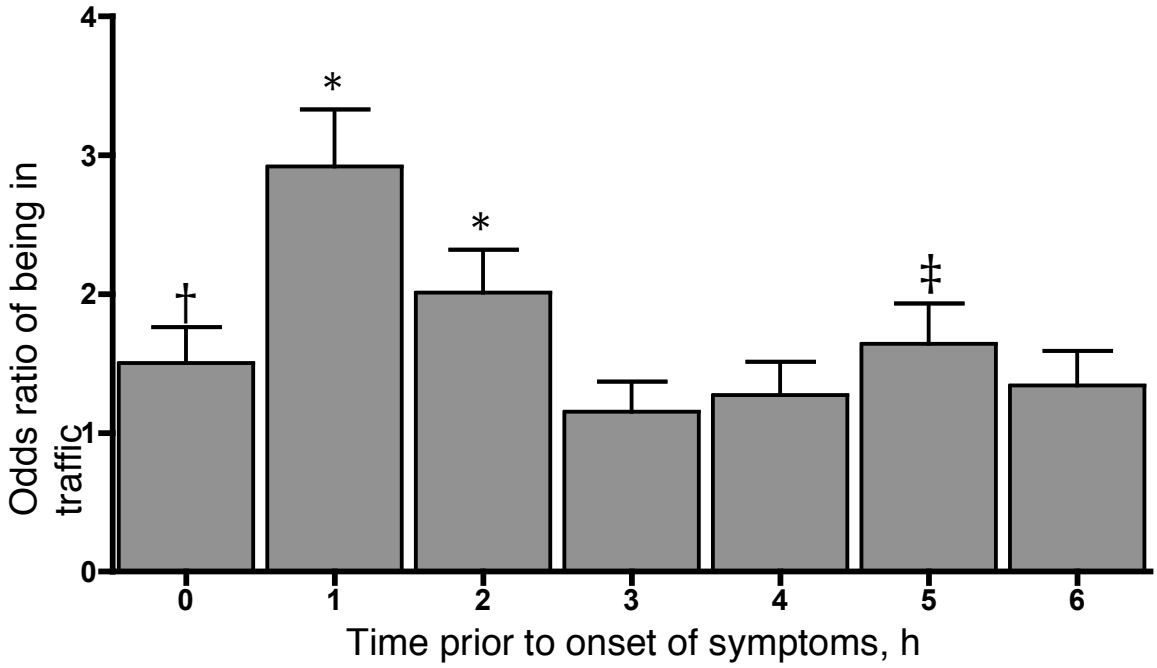

Figure 1.2. Odds ratio of being in traffic in the hours leading up to the onset of symptoms in 691 survivors of myocardial infarction. Data shown as mean ± standard deviation. Data adapted from Peters, *et al.* 2004. †P=0.02, *P<0.0001 and ‡P=0.004.

In a pivotal study by Peters and colleagues, 691 patients admitted with myocardial infarction in Augsburg, Germany completed a standardized interview-based diary following their admission to hospital, where they were asked to record their activities in the hours leading up to admission [Peters, *et al.* 2004]. The authors found that patients were nearly three times more likely to have been in traffic in the 2 hours prior to the onset of symptoms (Figure 1.2). A similar pattern was seen during an equivalent study by the same authors in Boston, USA [Peters, *et al.* 2001]. The authors suggest that acute exposure to traffic-derived air pollution may trigger the onset of acute myocardial infarction. Recent evidence from the UK using the MINAP (Myocardial Infarction National Audit Project) database has strengthened

this hypothesis, and the authors demonstrate a dose-dependent increase in risk of myocardial infarction 1 to 6 hours after exposure to particulate air pollution [Bhaskaran, *et al.* 2011]. A recent meta-regression analysis of factors associated with the triggering of acute myocardial infarction suggests that on a population-wide level, exposure to traffic-derived air pollution emerges as the leading risk factor given the ubiquitous nature of exposure, with an effect similar in magnitude to well accepted risk factors such as physical exertion [Nawrot, *et al.* 2011].

1.4.2 Mechanisms

Whilst increased coronary atherosclerotic burden may go some way towards explaining the increase in cardiovascular risk, there remain many unanswered questions, with regards to how and why atherosclerotic plaque formation is increased and why plaques should become more unstable resulting in an increase in cardiovascular events and mortality. There is now a large body of work that has begun to explore these mechanisms in depth.

1.4.2.1 Human exposure systems

Ideally studies looking at the mechanisms underlying the acute cardiovascular responses to air pollutants would be performed using controlled exposures to urban ambient air pollution at atmospheric concentrations. The major advantage of such an approach is the realistic nature of the exposure, and the generalisability of any results to similar

exposure scenarios. However, these studies may be limited by the day-to-day variability in ambient pollutant concentration and chemical composition, depending on traffic density, activity of nearby industry, weather conditions and a multitude of other factors. Because of this inherent variability, and the fact that ambient air exposures are comprised of complex mixtures of air pollutants, an alternative approach is needed in order to study the biological and pathophysiological effects of individual inhaled air pollutants.

Facilities have been developed to enable the carefully controlled exposure of human subjects to commonly encountered air pollutants, resulting in a stable and predictable exposure [Langrish, *et al.* 2010]. Such exposures have been carried out in both large room-sized facilities, as in Umeå, Sweden [Salvi, *et al.* 1999], Rochester, NY [Morrow, *et al.* 1988; Utell, *et al.* 1984] and Seattle, Washington [Gould, *et al.* 2008], and in a modified body-box as in the mobile exposure facility described by Mills et al. [Mills, *et al.* 2008].

It has been demonstrated that the link between cardiovascular morbidity and mortality and air pollution exposure is strongest for exposure to combustion-derived fine and ultrafine particulate matter from coal and mobile sources [Laden, *et al.* 2000]. In the urban environment, up to 40% of airborne particulate matter is derived from diesel exhaust [Zheng, *et al.* 2007], a fraction that is likely to increase with the increasing restrictions on industry

and the current drive for "green" taxation on road vehicles linked to carbon dioxide production.

Diesel engines produce a cloud of carbon-centred nano-particulate that includes unburnt fuel, lube oil, polycyclic aromatic hydrocarbons, metals and sulphates [Scheepers, *et al.* 1992; Sydbom, *et al.* 2001]. Although diesel engines produce less carbon monoxide and carbon dioxide than gasoline engines, they produce more nitrogen oxides and aldehydes, and the particle emissions are up to 100 times greater – of which >80% (by particle number) are within the ultra-fine fraction (<100 nm in diameter), proposed to be the most closely associated with adverse health effects [Donaldson, *et al.* 2001].

Exposure to diluted diesel exhaust has been used as a stable and predictable model of pure combustion-derived air pollution in the study of the health effects of particulate air pollution in controlled human exposure studies [Gould, *et al.* 2008; Mills, *et al.* 2005]. Briefly, fresh diesel exhaust is produced by a commercial diesel engine under either idling conditions, or during transient running cycles such as the European City Cycle. More than 90% of the exhaust is vented externally, with a small fraction collected and delivered to a conditioning chamber where it is mixed with clean HEPA-filtered air. The conditioning chamber allows complete mixing and the maintenance of a stable temperature and humidity within the aerosol. This is then drawn through an airtight exposure chamber in which the subject is situated. The

air within the chamber is sampled from the breathing zone of the subject and monitored as described previously. The concentration of airborne particulate is normally maintained at 200-300 μg/m^3 and can be adjusted by altering the amount of exhaust drawn into the conditioning chamber (Figure 1.3). Control exposures to filtered air are generated in the same facility, without adding any diesel exhaust to the chamber.

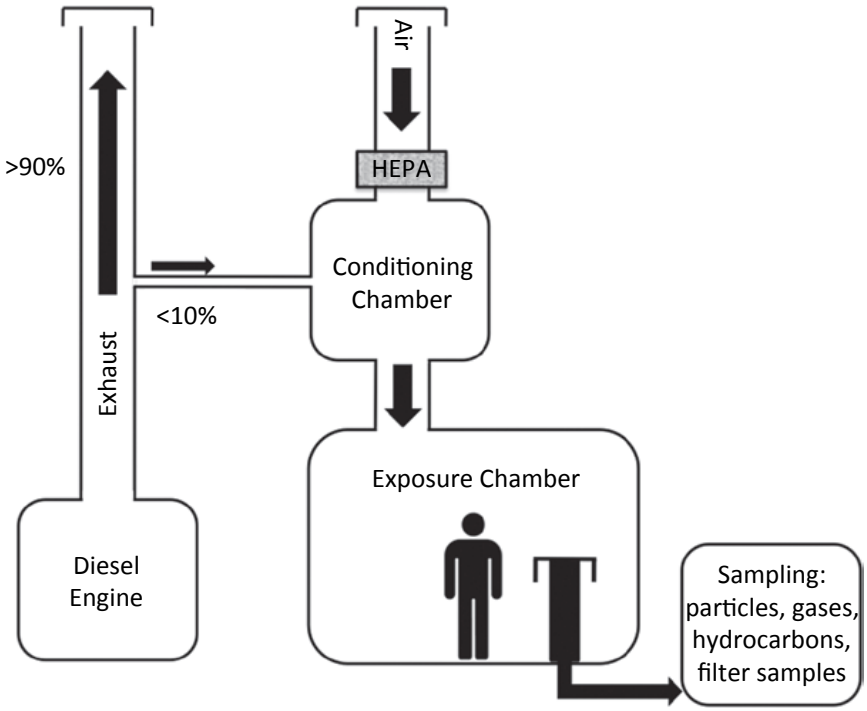

Figure 1.3. Schematic diagram of a diesel exhaust exposure facility.

Using a predictable model of exposure, such as diesel exhaust, offers many advantages. In order to ensure consistency of approach, exposures can be performed identically on different study days, thus making interpretation of the results much easier. The inherent variability of chemical composition and particulate load seen with concentrated ambient particles is not an issue. The

technology required to produce a diesel exposure does not rely on complex impactors that are sensitive to environmental and weather conditions, meaning that studies can be carried out on any particular day – a definite advantage in scheduling clinical experiments involving human subjects. For all these advantages, diesel exhaust exposures will always be open to criticism. Diesel exhaust is used as a model of ambient combustion-derived air pollution. Whilst it is true that diesel exhaust can make up a large proportion of an individual's exposure to air pollution in urban areas, exposure to pure diesel exhaust (or indeed pure combustion-derived particulate) is artificial. Therefore, extrapolating the results from experimental diesel exhaust exposure chamber studies to the ambient exposure situation is fraught with uncertainty. However, to gain important mechanistic information on air pollution-induced health effects, it should be emphasised that experimental exposure chamber studies should be used to complement "real-world" exposures.

1.4.2.2 Pulmonary and systemic inflammatory response

The mechanisms underlying the adverse effects of inhaled particulate matter are still unclear, and particularly the pathway of effect following inhalation of particles into the lungs. It was hypothesised that inhalation of airborne particulate matter causes alveolar inflammation and subsequent release of inflammatory cytokines that then result in exacerbation of chronic lung

diseases and affect blood coagulability and thus increase acute cardiovascular events [Seaton, *et al.* 1995].

The ability of inhaled particulate matter to cause local inflammation is now well established. In animal models, intratracheal instillation of urban ambient particulate matter (PM_{10}) in rats results in an influx of neutrophils, a depletion of antioxidants (GSH, glutathione) and an increase in tumour necrosis factor α (TNF-α) release within the alveolae [Li, *et al.* 1996]. Similar findings have been shown after inhalation of concentrated ambient particulate matter [Elder, *et al.* 2004].

In clinical studies of healthy volunteers exposed to concentrated ambient particulate matter in a whole body exposure chamber similar to described above for 2 hours, there is an increased neutrophil count in bronchoalveolar lavage fluid along with increased plasma fibrinogen in blood 18 hours after exposure [Ghio, *et al.* 2000]. Similarly, 6 hours after exposure to dilute diesel exhaust there is an increase in both neutrophil and lymphocyte cell count in bronchoalveolar fluid, and evidence of early inflammatory change on bronchial biopsies [Salvi, *et al.* 1999].

In vitro studies on alveolar macrophages incubated with particulate matter demonstrate dose-dependent release of the proinflamatory cytokines TNF-α, interleukin (IL)-1β, IL-6 and granulocyte macrophage colony-stimulating

factor (GM-CSF) [van Eeden, *et al.* 2001]. Co-culture experiments of alveolar macrophages with human bronchial epithelial cells have shown amplification of this inflammatory response, and supernatants from these co-cultures instilled into the lungs increase circulating polymorphonuclear leucocytes through the bone marrow in rabbits [Fujii, *et al.* 2002], suggesting that this local inflammatory stimulus can generate a systemic response. Ultrafine carbon particles instilled into rat lung cause an increase in both plasma fibrinogen and circulating neutrophils in both normal [Elder, *et al.* 2004] and spontaneously hypertensive rats [Cassee, *et al.* 2005].

Concordant with these findings, clinical panel studies have demonstrated an inflammatory response following exposure to ambient air pollution. Blood samples taken from 30 healthy male volunteers during a "pollution episode" (the forest fires in South Asia in 1997) and again once the haze had cleared showed an increase in the proinflammatory cytokines IL-1β, IL-6 and GM-CSF in peripheral blood during the high exposure to particulate air pollution seen during the fires. In subjects investigated during an air pollution episode in Augsburg Germany, plasma viscosity [Peters, *et al.* 1997] and C-reactive protein (CRP) [Peters, *et al.* 2001] were significantly increased suggesting an acute phase response. Increasing exposure to nitrogen dioxide and black smoke is also associated with an increase in plasma fibrinogen, again reflective of a non-specific systemic inflammatory response. [Pekkanen, *et al.* 2000].

Controlled exposure studies in man have been less conclusive. In healthy volunteers exposed to dilute diesel exhaust (300 $\mu g/m^3$) for 1 hour with intermittent exercise, plasma concentrations of TNF-α and IL-6 are increased at 24 hours [Törnqvist, *et al.* 2007], but not at 6 hours [Mills, *et al.* 2005], although these changes are not associated with an increase in peripheral leucocyte counts.

It is proposed that the inflammatory response to inhaled particulate matter is driven by oxidative stress. Diesel exhaust particles are able to generate large amounts of oxygen-centred free radicals in the absence of tissue, as measured by electron paramagnetic resonance [Miller, *et al.* 2009]. In vitro studies have shown that diesel exhaust particles are able to oxidise LDL cholesterol particles [Ikeda, *et al.* 1995], thus increasing the inflammatory and atherogenic properties of the particles. Diesel exhaust particles cause an upregulation of the antioxidant enzyme haem-oxygenase-1 (HO-1) and upregulation of the MAPK/NF-κB (mitogen-activated protein kinase/nuclear-factor kappa light chain enhancer of activated B cells) pathways when incubated with human epithelial cell lines, effects that can be inhibited with co-administration of antioxidants [Bonvallot, *et al.* 2001; Hirano, *et al.* 2003; Li, *et al.* 2000; Marano, *et al.* 2002]. Moreover, instillation of diesel exhaust particles into mouse lungs causes a dose-dependent increase in systemic 8-hydroxy-2'-deoxyguanosine (8-OHdG; an oxidative DNA adduct and a marker of

systemic oxidative stress) concentrations [Ichinose, *et al.* 1997], and effect that can be offset in vitro using superoxide dismutase or desferrioxamine [Arimoto, *et al.* 1999]. Indeed, systemic 8-OHdG concentrations are associated with background particulate matter exposure in 76 healthy students studied in Taipei Taiwan [Chuang, *et al.* 2007].

It is possible that local inflammation with a subsequent systemic inflammatory response may contribute to the adverse vascular responses to inhaled air pollutants (see sections 1.4.2.4 and 1.4.2.5) and hence to the increased cardiovascular morbidity and mortality described. However, this effect is of slow onset and cannot be the whole story as effects on the vascular system can be robustly demonstrated immediately after any exposure – and indeed during the exposure itself. Therefore there must be an additional explanation.

1.4.2.3 Particle translocation

One such hypothesis is the direct translocation of particles into the bloodstream after inhalation, resulting in a direct effect on the cardiovascular system from within blood vessels. Airborne particulate matter in the fine ($PM_{2.5}$) and ultrafine fractions (UFP, particles with a mean diameter of 0.1 μm or less) can penetrate deeply into the lungs, largely bypassing filtration in the nasal tract and by bronchial cilia. Interestingly, it is these fine and UFP fractions that appear to be most closely associated with the demonstrated

adverse effects [Donaldson, *et al.* 2001]. Once deep in the lungs, any inhaled particles (which are approximately the same size as an LDL-cholesterol particle) are separated from the bloodstream by only a monolayer of alveolar cells. It is proposed that these particles may then translocate through the epithelium into the blood stream and from there mediate their effects.

Previous attempts to identify particle translocation have proved inconclusive. Human studies have used an aerosol of technetium-99m (99mTc)–labeled carbon nanoparticles, Technegas (Vita Medical Ltd., Sydney, Australia), which is used routinely in clinical practice for radionuclide lung ventilation imaging to try and address this issue. Nemmar and colleagues demonstrated that following inhalation of Technegas, thin-layer chromatography of whole blood identified the presence of 99mTc-labeled carbon nanoparticles in the bloodstream as early as 1 min after Technegas inhalation [Nemmar, *et al.* 2002]. However, Technegas generation has many technical challenges. It is generated in a pure argon atmosphere to avoid the formation of the soluble, unbound sodium pertechnate. It was subsequently suggested that the very rapid detection of a signal in the bloodstream was suggestive of a significant amount of soluble pertechnate in the inhaled aerosol, and further attempts at repeating the study (with very careful attention to the exclusion of oxygen from the generation atmosphere) did not show systemic translocation and demonstrated instead that the labeled carbon particles remained within the lung at 6 hours post inhalation

[Mills, *et al.* 2006]. Möller and colleagues recently reported a similar study in smokers and patients with chronic obstructive lung disease in which inhaled Technegas remained within the lung parenchyma even up to 48 hours after the exposure, without significant evidence of systemic translocation [Moller, *et al.* 2008]. However, in both of the latter studies, a small amount of technetium was detectable in the systemic circulation, consistent with the leaching of a small amount of soluble sodium pertechnate from the labeled particles despite the careful attention to the test atmosphere. It is difficult to be absolutely sure that a small fraction of these particles did not in fact translocate, and animal studies have clearly demonstrated evidence of systemic translocation for a range of nanoparticles following inhalation and intra-tracheal installation [Kreyling, *et al.* 2002; Nemmar, *et al.* 2004; Nemmar, *et al.* 2001; Oberdorster, *et al.* 2002] and this hypothesis remains to be refuted.

1.4.2.4 Vascular vasomotor function

Vascular endothelial dysfunction, which is generally characterised by the reduced bioavailability of endothelium-derived nitric oxide, is an independent risk factor for cardiovascular morbidity and mortality [Schachinger, *et al.* 2000; Suwaidi, *et al.* 2000]. Indeed, endothelial dysfunction – manifesting as an attenuated vasomotor response – is an early marker for atherosclerosis and may be present long before structural changes within blood vessels are apparent on clinical investigation [Davignon, *et al.* 2004; Ross 1999]. The vascular vasomotor response is complex, and is the result of

the interaction of tonic vasoconstrictors and vasodilators working in balance to maintain normal vascular tone (Figure 1.4).

A 2-hour exposure to concentrated ambient particles (approximately 150 $\mu g/m^3$) and ozone (120 ppb) [Brook, *et al.* 2002] or dilute diesel exhaust [Peretz, *et al.* 2008] causes acute arterial vasoconstriction, measured immediately after completing the exposure. Similarly, a transient increase in central arterial stiffness as measured using applanation tonometry has been demonstrated following a 1-hour exposure to dilute diesel exhaust (approximately 350 $\mu g/m^3$) [Lundback, *et al.* 2009]. A 1-hour exposure to dilute diesel exhaust (300 $\mu g/m^3$) results in an attenuated vascular vasomotor response to infused vasodilators, measured using venous occlusion plethysmography, 2-hours after the exposure [Törnqvist, *et al.* 2007]. This effect is still present 6-hours following the exposure [Mills, *et al.* 2005], although is largely resolved by 24-hours [Törnqvist, *et al.* 2007].

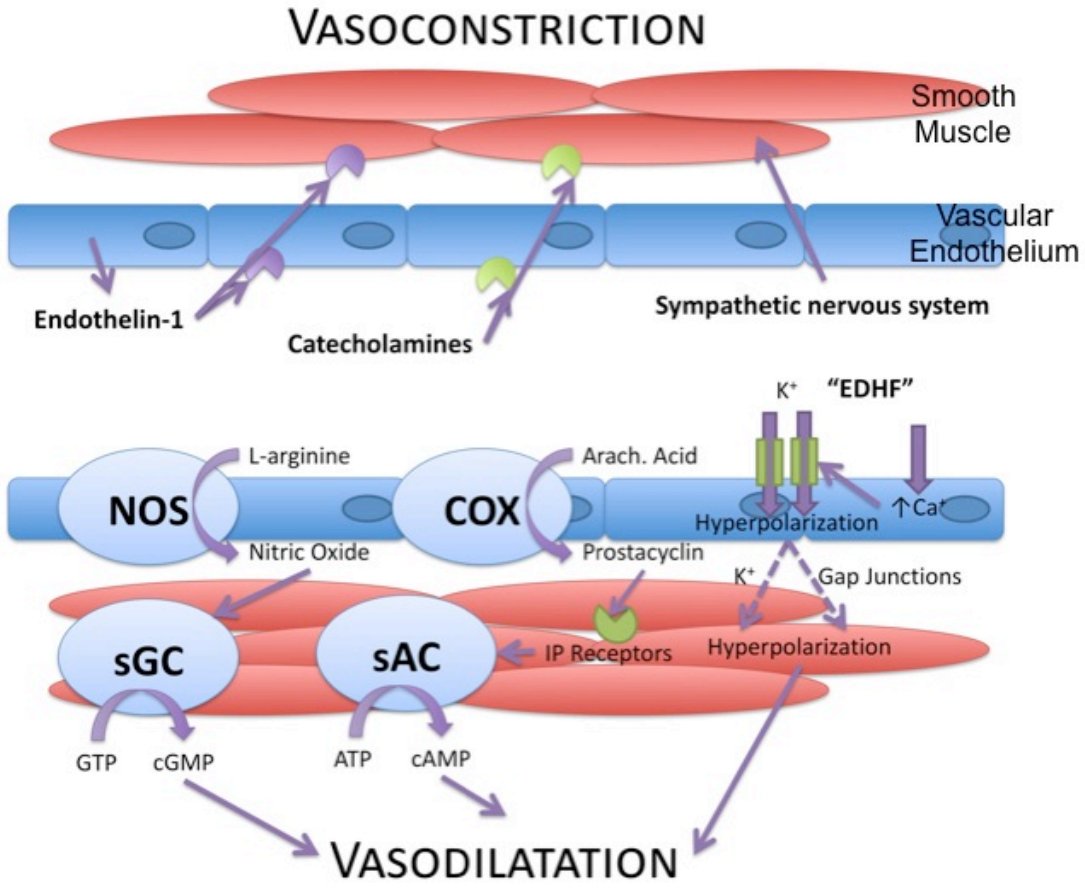

Figure 1.4. Schematic drawing showing the major vasoconstrictor and vasodilator influences on basal vascular tone and the vascular vasomotor response. Blue cells represent the vascular endothelium, the red cells the underlying smooth muscle cells. EDHF: endothelium-derived hyperpolarisation factor; Arach. Acid: arachadonic acid; K^+: potassium ions; Ca^{2+}: calcium ions; GTP: guanosine triphosphate; cGMP: cyclic guanosine monophosphate; ATP: adenosine triphosphate; cAMT: cyclic adenosine monophosphate; NOS: nitric oxide synthase; COX: cycloxygenase; sGC: soluble guanylate cyclase; sAC: soluble adenylyl cyclase

The mechanisms underlying these adverse vasomotor responses remain unclear. Preclinical studies have demonstrated a reduced release of nitric oxide from vascular endothelium following treatment with diesel exhaust

particles [Miller, *et al.* 2009; Nurkiewicz, *et al.* 2009], an effect proposed to be driven by the generation of oxygen-centred free radicals [Miller, *et al.* 2009] and the uncoupling of nitric oxide synthase [Knuckles, *et al.* 2008]. However, it has also been proposed that these responses may be driven by endothelin-1 (ET-1).

The endothelin system plays a major role in cardiovascular and renal physiology [Goddard, *et al.* 2004], and although its actions are complex, ET-1 is a powerful endogenous vasoconstrictor which contributes to the maintenance of basal vascular tone and blood pressure in man [Haynes, *et al.* 1994; Haynes, *et al.* 1996]. Recent work has suggested that plasma ET-1 concentrations are increased by exposure to air pollution. Rats raised with daily exposure to diesel exhaust particles and urban particulate matter have increased blood pressure, plasma ET-1 concentrations [Vincent, *et al.* 2001], and endothelin-A receptor expression in cardiac tissue [Ito, *et al.* 2008]. In children from Mexico City, plasma ET-1 concentrations correlated with the degree of air pollution exposure [Calderón-Garcidueñas, *et al.* 2007]. Peretz *et al* recently demonstrated elevated plasma ET-1 concentrations in a heterogeneous population of healthy volunteers and patients with the metabolic syndrome, 3 hours after a controlled 2-hour resting exposure to diesel exhaust [Peretz, *et al.* 2008].

1.4.2.5 Thrombogenicity

Short-term exposure to particulate air pollution has been linked to the triggering of acute myocardial infarction, which is usually a result of atheromatous plaque rupture and subsequent thrombus formation leading to vessel occlusion. It has been demonstrated in a pre-clinical arterial injury model that instillation of diesel exhaust particles into the lungs of hamsters results in enhanced arterial thrombus formation and significantly increased platelet activation [Nemmar, *et al.* 2003]. Similarly, using an ex-vivo model of arterial thrombus formation – the Badimon chamber [Badimon, *et al.* 1987] – a 1-hour exposure to dilute diesel exhaust (approximately 350 $\mu g/m^3$) causes increased platelet activation and increased thrombus formation in man [Lucking, *et al.* 2008]. As well as causing enhanced arterial thrombus generation, inhalation of diesel exhaust reduces the fibrinolytic activity of the vascular endothelium, with reduced release of tissue-plasminogen activator (t-PA) [Mills, *et al.* 2007].

Interestingly, there is now some evidence that venous clotting may also be enhanced, with small reductions in prothrombin times with increasing background particulate air pollution exposure [Baccarelli, *et al.* 2007] and an increased risk of developing deep vein thrombosis when subjects lived closer to a major road [Baccarelli, *et al.* 2009].

1.4.2.6 Blood pressure changes

Population studies have confirmed a link between increases in blood pressure and particulate air pollution exposure. In 2,607 adults studied in Augsburg Germany during an air pollution episode in January 1985, systolic blood pressure was seen to rise by nearly 2 mmHg for every 90 $\mu g/m^3$ increase in airborne particulate matter, an effect further exaggerated in those with high resting heart rates or increased plasma viscosity [Ibald-Mulli, *et al.* 2001]. Similarly, among 5,112 adults without cardiovascular disease studied in the Multi-Ethnic Study of Athersclerosis (MESA) study, linear increases in both pulse-pressure and systolic blood pressure were found with increases in $PM_{2.5}$ concentrations [Auchincloss, *et al.* 2008].

Controlled exposure studies in man have also demonstrated changes in blood pressure. Twenty-three healthy non-smoking adults were exposed to concentrated ambient particles (approximately 150 $\mu g/m^3$) and ozone (120 ppb) for 2-hours whilst resting in an exposure chamber. During the exposure diastolic blood pressure (and mean arterial blood pressure) increased proportionately to the organic carbon content of the concentrated particles (measured as a marker for combustion-derived particulate matter) [Urch, *et al.* 2005]. More recently, the same investigators describe similar acute changes in diastolic blood pressure during controlled exposures in 81

subjects during exposure to concentrated ambient particles with and without ozone in Toronto and Ann Arbor [Brook, *et al.* 2009].

1.4.2.7 Myocardial ischaemia

Particulate air pollution exposure is associated with exercise-induced myocardial ischaemia in patients with coronary artery disease. In one study, repeated clinic recorded exercise stress tests performed by patients with stable treated coronary artery disease were paired with mean regional ambient particle concentrations as a crude assessment of pollution exposure [Pekkanen, *et al.* 2002]. The authors demonstrated a three-fold increase in the risk of developing more than 1 mm ST segment depression during exercise with increasing particulate air pollution exposure. More recently, 48 patients were investigated up to 4 times during the first 12 months following a percutaneous intervention for myocardial infarction, acute coronary syndrome or stable angina with ambulatory electrocardiography. The risk of developing clinically-relevant ST segment depression (>1 mm) during the monitoring period was increased around 1.5-fold for each interquartile increase in the black carbon level (used as a measure of combustion-derived particulate air pollution) over the preceding 24-hours [Chuang, *et al.* 2008].

In a controlled exposure study, 20 patients with asymptomatic coronary heart disease who had suffered a prior myocardial infarction were exposed to dilute diesel exhaust (300 $\mu g/m^3$) or filtered air. During the exposure they

alternated between periods of rest or moderate exercise. Myocardial ischaemia was quantified by ST-segment analysis using continuous 12-lead electrocardiography. This study demonstrated a three-fold increase in ST segment depression and increased ischaemic burden during the exposure to diesel exhaust [Mills, *et al.* 2007].

1.4.2.8 Autonomic regulation of the heart

Short-term increases in ambient particulate air pollution exacerbate chronic cardiovascular conditions, and lead to increased hospital admissions with cardiac arrhythmias [Hoek, *et al.* 2001] as well as myocardial infarction and heart failure. In patients with implantable cardiac defibrillators, there is a relationship between ambient particulate matter and the incidence of ventricular tachycardia and fibrillation [Berger, *et al.* 2006; Dockery, *et al.* 2005; Peters, *et al.* 2000].

Autonomic control of the cardiac cycle has been linked to the incidence of arrhythmias and to cardiovascular mortality [Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996]. The simplest, and therefore most widely used measurement of autonomic control of the heart, is heart rate variability (HRV) – a measure of the variation in the interval between consecutive heart beats, and a measure of the contrasting effects of the sympathetic and parasympathetic nervous systems. Reduced HRV suggests either an increase

in sympathetic drive or a decrease in vagal parasympathetic tone and has been linked to cardiovascular morbidity and mortality in both healthy individuals [Tsuji, *et al.* 1996] and patients following acute myocardial infarction [Kleiger, *et al.* 1987].

More than 30 panel studies have now been reported showing an effect of exposure to particulate air pollution on measures of HRV, although the reported effects are far from consistent, with marked variation in the nature, magnitude, direction and duration of these associations (a summary of some of these is illustrated in Table 1.2). Liao and colleagues were the first to report an association between particulate air pollution and reduced HRV in a small panel of elderly residents of Baltimore Maryland [Liao, *et al.* 1999], where they showed evidence of an effect on both high-frequency (related to parasympathetic activity) and low-frequency (related to sympathetic activity) components of HRV. In the largest study to date, the same authors subsequently reported a weak association between particulate air pollution and reduced HRV in 6,784 healthy adults – with a one standard deviation increase in ambient fine particulate matter being associated with a 0.32 beats per minute reduction in heart rate and a 1.03 ms decrease in SDNN (standard deviation of successive RR intervals) [Liao, *et al.* 2004].

Few controlled exposure studies have been performed to investigate the effect of exposure to particulate air pollution on HRV. Zareba and colleagues

investigated the effect of a 2-hour exposure to carbon ultrafine particles on electrocardiographic parameters including HRV. They reported a small decrease in RMSSD (root mean square of the difference in RR intervals) and suggested a trend towards an increase in parasympathetic tone (based on analysis of T-wave morphology) in their healthy young subjects [Zareba, *et al.* 2008]. Similarly, in healthy volunteers and patients with coronary heart disease exposed to pure carbon particles (50 $\mu g/m^3$), Routledge and colleagues demonstrated a fall in heart rate and an increase in high-frequency components of HRV suggesting increased parasympathetic vagal stimulation of the heart [Routledge, *et al.* 2006]. In 31 healthy volunteers investigated in Toronto Canada, a 2-hour exposure to concentrated ambient particles resulted in a reduction in SDNN, RMSSD, high-frequency power and low-frequency power measures of HRV during the exposure itself [Brook, *et al.* 2009]. Concordantly, Gong and colleagues also reported modest changes in HRV consistent with parasympathetic stimulation following a 2-hour exposure to concentrated ambient particles in both healthy volunteers and patients with asthma in Los Angeles California [Gong, *et al.* 2003].

However when using dilute diesel exhaust as a model exposure, Peretz and colleagues recently demonstrated that a 3-hour exposure to dilute diesel exhaust was not associated with changes in HRV [Peretz, *et al.* 2008], and this is consistent with the findings of our group [Mills, *et al.* 2011]. Therefore the

effects of air pollution on HRV remain controversial, inconsistent and undefined.

Table 1.2. The acute effect of exposure to particulate air pollution on heart rate variability.

Author	Population	Number	Pollutant	HR	SDNN	RMSSD	HF	LF	HF/LF
[Liao, *et al.* 1999]	Elderly	26	$PM_{2.5}$	-	↓	-	-	-	-
[Pope, *et al.* 1999]	Healthy	7	PM	↑	↓	↑	-	-	-
[Gold, *et al.* 2000]	Elderly	21	PM	↓	↓	↓	-	-	-
[Magari, *et al.* 2001]	Occupational	40	$PM_{2.5}$	↑	↓	-	-	-	-
[Brauer, *et al.* 2001]	COPD	16	PM_{10}	↔	↔	↔	-	-	-
			$PM_{2.5}$	↔	↔	↔	-	-	-
[Holguin, *et al.* 2003]	Elderly	34	$PM_{2.5}$	-	-	-	↓	↓	↑
[Pope, *et al.* 2004]	Elderly	88	$PM_{2.5}$	↔	↓	↓	-	-	-
[Liao, *et al.* 2004]	Community	6,784	PM_{10}	↑	↓	-	↓	↔	-
[Chan, *et al.* 2004]	Healthy	19	$PN_{0.02\text{-}1}$	-	↓	↓	↓	↓	-
[de Paula Santos, *et al.* 2005]	Occupational	48	PM_{10}	↔	↔	↔	-	-	-
[Park, *et al.* 2005]	Elderly	497	$PM_{2.5}$	-	-	-	↓	↔	↑
[Schwartz, *et al.* 2005]	Elderly	28	$PM_{2.5}$	-	↔	↓	-	-	↔
[Sullivan, *et al.* 2005]	Elderly	34	$PM_{2.5}$	-	-	↔	↔	-	-
[Vallejo, *et al.* 2006]	Young	40	$PM_{2.5}$	-	↔	-	-	-	-
[Timonen, *et al.* 2006]	CHD	84	PN	-	↔	↔	↔	-	↓
			$PM_{2.5}$	-	↔	↔	↔	-	↔
[Lipsett, *et al.* 2006]	CHD	19	$PM_{2.5}$	-	↔	↔	↔	↔	-
			$PM_{2.5\text{-}10}$	-	↓	↑	↔	↔	-
[Luttmann-Gibson, *et al.* 2006]	Healthy	32	$PM_{2.5}$	↑	↓	↓	↓	↓	-
[Park, *et al.* 2006]	Elderly	518	$PM_{2.5}$	-	-	-	↓	-	-
[Chuang, *et al.* 2007]	Young	76	PM_{10}, $PM_{2.5}$	-	↔	↔	↔	↔	-
[Chahine, *et al.* 2007]	Elderly	539	$PM_{2.5}$	-	↓	-	↓	↔	-
[Min, *et al.* 2008]	Community	1,349	PM_{10}	-	↓	-	↔	↓	-
[Cavallari, *et al.* 2008]	Occupational	36	$PM_{2.5}$	-	↓	-	-	-	-
[Cardenas, *et al.* 2008]	Young	52	$PM_{2.5}$	-	-	-	↓	↓	-
[Baccarelli, *et al.* 2008]	Elderly	549	$PM_{2.5}$	-	↔	-	↔	↔	-
[Cavallari, *et al.* 2008]	Occupational	26	$PM_{2.5\ metal}$	-	-	↓	-	-	-
[Chang, *et al.* 2007]	Elderly	15	$PM_{1\text{-}2.5}$	↑	↔	↔	-	↓	↑
			$PM_{2.5\text{-}10}$	↔	↓	↓	-	-	↑

SDNN = SD of NN-interval values; RMMSD = root mean square of successive NN interval differences; HF = high frequency; LF – low frequency; CHD = coronary heart disease; COPD = chronic obstructive pulmonary disease; PM = particulate matter; PN = particle number

1.4.2.9 Summary of acute effects of particulate air pollution

It is now well accepted that short-term exposure to increases in ambient particulate air pollution, and particularly that associated with combustion, is associated with an increase in acute cardiovascular events. Following a number of controlled exposure experiments, plausible mechanisms are now proposed that might underlie these observations (summarised in Figure 1.5).

During the exposure itself, there is evidence of both an acute rise in blood pressure, with concordant arterial vasoconstriction and increased central arterial stiffness – most plausibly the result of alterations in autonomic control of the heart and the vascular tree. Within 2-hours of the exposure there is an impairment of vascular endothelial function, with impaired vasomotor responses. Concordant with this there is an increase in thrombogenicity with platelet activation along with a reduction in activity of the endogenous fibrinolytic system. The events leading to these later changes are unclear – they may be due to a direct oxidative effect of particles on the vascular system and platelets if they are indeed able to translocate into the systemic circulation, or maybe via early inflammatory influences mediated via alveolar macrophages. Later there is evidence of established local inflammation within the lung and a coincident systemic inflammatory response.

Taken together, these responses can help to explain the increase in cardiovascular morbidity and mortality consistently observed following exposure to ambient air pollution, and the triggering of myocardial infarction (Figure 1.6), although questions still remain as to the exact mechanisms mediating the adverse vascular responses demonstrated.

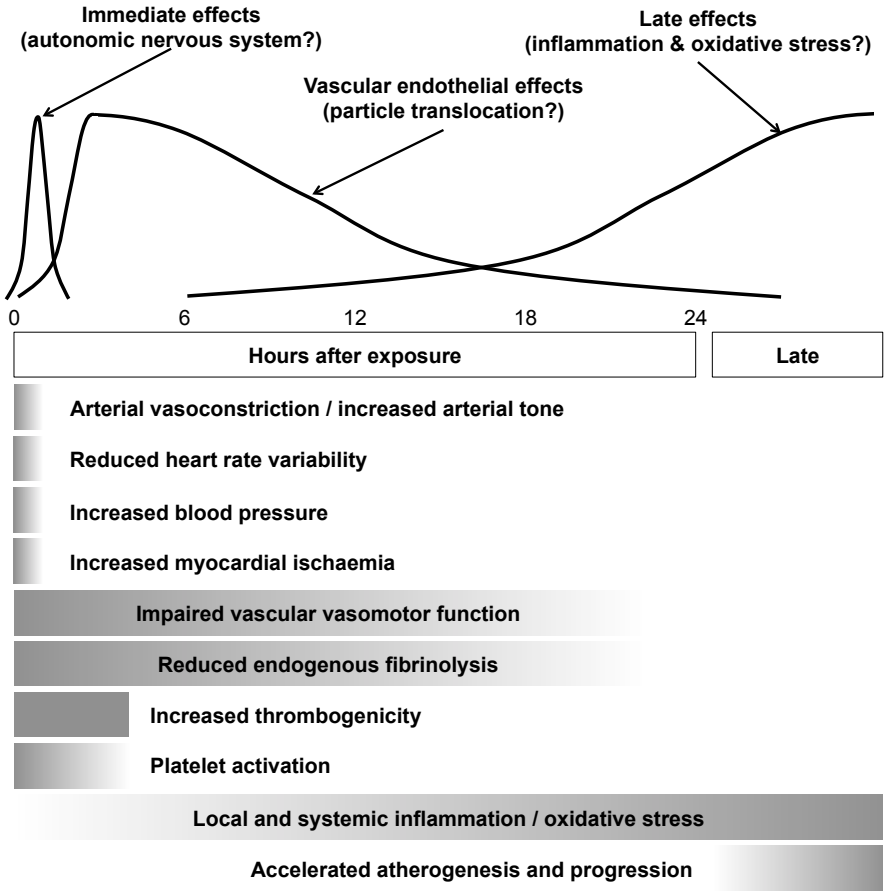

Figure 1.5. The acute cardiovascular responses to inhalation of fine particulate air pollution over the first 24-hours.

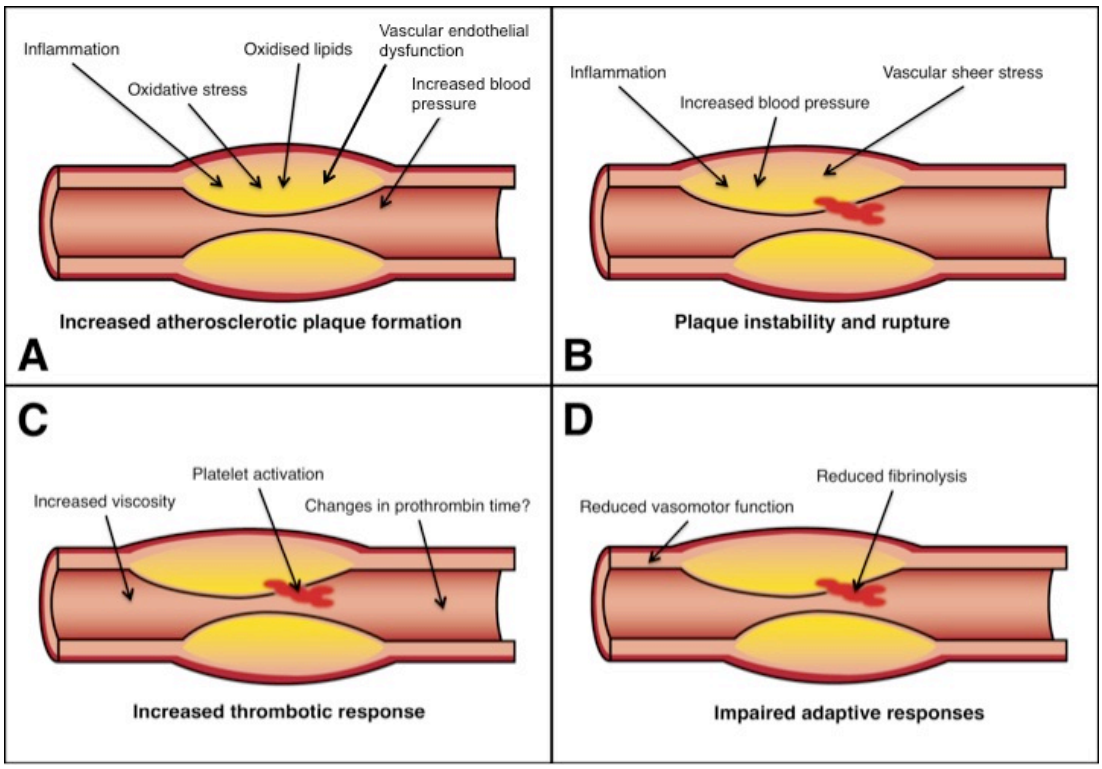

Figure 1.6. Schematic drawing showing how the demonstrated responses to particulate air pollution exposure may result in increased cardiovascular morbidity and mortality. (A) shows the effects of chronic exposure to air pollutants resulting in increased atherosclerotic burden. The factors that may predispose to subsequent plaque instability and rupture are shown in (B). (C) and (D) summarise the abnormal thrombotic and adaptive responses to plaque rupture following exposure to airborne particulate matter that may explain the observed increase in acute cardiovascular events.

1.5 AIMS AND HYPOTHESES

Air pollution is now well accepted as a risk factor for increased cardiovascular mortality and morbidity, and through a series of observational studies and controlled exposure studies and understanding of some of the mechanisms underlying these effects is emerging.

In a series of clinical studies in both healthy volunteers and patients with cardiovascular disease, I aim to further the understanding of the mechanisms that lead to vascular endothelial dysfunction following exposure to particulate matter. In addition, I intend investigate the importance of one of the major gaseous co-pollutants found in both ambient settings and within the controlled exposure facilities: nitrogen dioxide. Finally, I intend to examine the "real-world" effects of acute exposure to particulate air pollution, and begin to address the important of how we can protect susceptible populations from the adverse effects of air pollution.

The following hypotheses will be tested:

1. The adverse effects of exposure to particulate air pollution and diesel exhaust are a result of the presence of nitrogen dioxide. An exposure to pure nitrogen dioxide (4 ppm) will attenuate vascular endothelium-dependent vasodilatation (Chapter 3);
2. Diesel exhaust inhalation causes an increase in plasma endothelin(ET)-1 and big ET-1 concentrations, increases ET-1 induced

vasoconstriction and the contribution of ET-1 to the maintenance of basal vascular tone (Chapter 4);

3. Following inhalation of diesel exhaust, vasoconstriction to nitric oxide synthase inhibition will be enhanced and plasma nitrite concentrations suppressed. In the presence of a nitric oxide clamp, the responses to infused vasodilators will be equivalent following exposure to diesel exhaust or filtered air (Chapter 5);
4. Wearing a simple facemask to reduce personal exposure to particulate air pollution reduces blood pressure and increases heart rate variability in healthy volunteers exposed to high ambient air pollution (Chapter 6);
5. Wearing a simple facemask to reduce personal exposure to particulate air pollution reduced blood pressure, improves heart rate variability, reduces myocardial ischaemia and symptoms of angina in a susceptible population with coronary heart disease exposed to high ambient air pollution (Chapter 7).

CHAPTER 2

Methodology

2.1 SUBJECT RECRUITMENT

2.1.1 Ethical considerations

All studies were reviewed an approved by the local research ethics committees at Umeå University, Sweden or the Chinese Academy of Medical Sciences in Beijing as appropriate. All subjects were provided with written information and gave their written informed consent to participate in the studies in accordance with the Declaration of Helsinki. Further details of the ethical review process can be found at http://www.ClinicalTrials.gov/ (NCT00774514, Chapter 3; NCT00745693, Chapter 4; NCT00845767, Chapter 5; NCT00809432, Chapter 6; NCT00809653, Chapter 7).

2.1.2 Healthy volunteers

Healthy volunteers (aged between 18 and 40 years) were recruited by either local advertisement or from volunteer databases held at the Department for Respiratory Medicine and Allergy, University Hospital, Umeå or the Fuwai Cardiovascular Hospital, Beijing China. All subjects took no regular medication (except for the oral contraceptive pill), had normal lung function and were non-smokers. Those with an intercurrent illness were excluded and all subjects were free of the symptoms of respiratory tract infection for more than 6 weeks before participation.

Subjects participating in the vascular studies attended the Department of Respiratory Medicine and Allergy, University Hospital, Umeå for a

screening visit prior to their enrolment. At this visit standard lung function tests, comprising FEV_1 (forced expiratory volume in 1 second), FVC (forced vital capacity) and slow vital capacity, were performed. Subjects had a baseline full blood count performed and a 12-lead electrocardiogram. Subjects then performed a standardised cardiopulmonary exercise stress test on a bicycle ergometer to determine the exercise intensity required to maintain an average ventilation rate of 20 L/min/m^2. The determined workload was then used during the subjects' exposures to produce an equivalent exposure in all. Subjects were instructed to abstain from alcoholic beverages for the 24-hours prior to attendance for all their visits, and from caffeinated drinks for at least 8-hours. All subjects were then fasted for at least 4-hours prior to performing the vascular studies.

2.1.3 Patients with coronary heart disease

Patients were recruited from the outpatient departments of 5 local hospitals in the city of Beijing, China – coordinated by the Fuwai Cardiovascular Hospital - or from the HPS2-THRIVE (more information available at http://clinicaltrials.gov/ct2/show/NCT00461630) patient database held at the Fuwai Hospital. All patients were non-smokers and had a history of coronary heart disease, having had a previous myocardial infarction, percutaneous coronary intervention or coronary bypass surgery. Subjects were maintained on their normal medical therapy throughout the studies. The exclusion criteria for the study were a history of arrhythmia (including

atrial fibrillation), severe three-vessel coronary artery disease or left main stem stenosis that had not been revascularised, a resting conduction abnormality on the electrocardiogram, digoxin therapy, uncontrolled hypertension, renal or hepatic failure or an acute coronary syndrome within the preceding 3 months.

2.2 DIESEL EXHAUST EXPOSURES

Exposures to dilute diesel exhaust were performed in Umeå, Sweden in a specially built and well-characterised human exposure facility [Rudell, *et al.* 1996] that is situated within the Svensk Maskinprovning AB facility (http://www.smp.nu). This comprises of a ~15.6 m^3 (2.5 x 2.5 x 2.5 m) room chamber into which the test atmosphere is introduced (Figure 2.1). Diesel exhaust was generated from an idling diesel engine (Volvo TD40 GJE, 4.5 L, four cylinders, 680 rpm) situated in the room adjacent to the exposure chamber. More than 90% of the exhaust was shunted away, and the remaining part diluted with filtered air in a conditioning chamber of ~1 m^3 to allow complete mixing before being introduced into the exposure chamber.

The aerosol within the chamber was sampled continuously during the exposures from the breathing zone of the participants (Figure 2.1), and monitored for concentrations of nitric oxide (NO), nitrogen dioxide (NO_2) and total oxides of nitrogen (NO_x) using a chemiluminescent technique (ECO Physics CLD700, AL Med, Switzerland) and total hydrocarbons (measured as propane) using a flame-ionization detection method (Hydrocarbon Analyszer model 3-300, JUM Engineering Co., Oakland, CA, USA). Particle mass concentration was measured in real-time with a tapered-element oscillating microbalance (TEOM 1400, Rupprecht & Patashnick, East Greenbush, NY, USA) at 50 °C. In addition to this, the particle mass concentration was determined gravimetrically using glass fibre filter sampling. Particle number concentration was determined in real-time using a

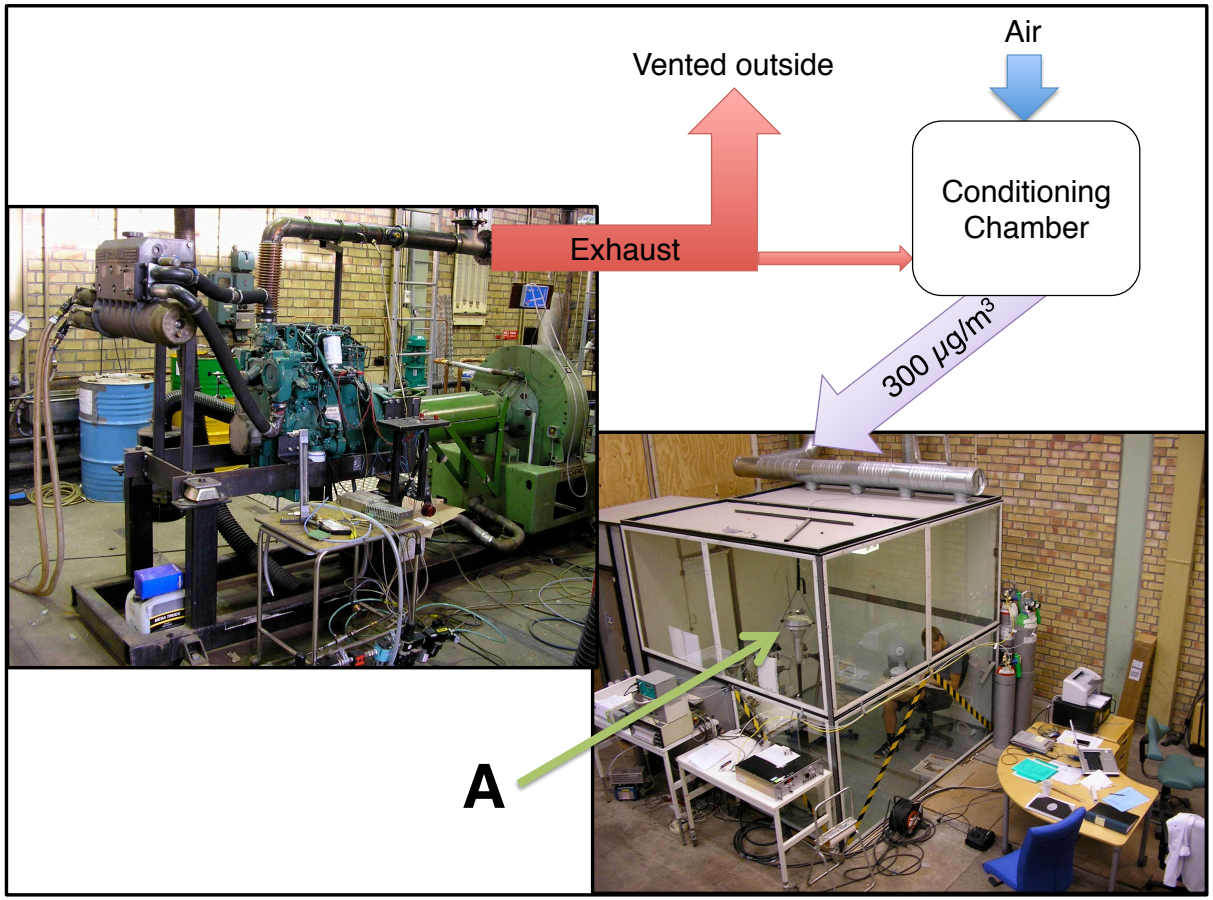

Figure 2.1. The human exposure facility in Umeå showing diesel engine used and exposure chamber, along with schematic description. The exercise bicycle can be seen in the middle of the chamber. (A) shows the inlet from which the aerosol is sampled during the exposure.

condensation particle counter (CPC Model 3010, TSI Instruments Inc., St Paul, MN, USA). During the exposures, the particle mass concentration was maintained at approximately 300 $\mu g/m^3$ which equates to a particle number concentration of approximately 1.2×10^6 particles/cm^3 [Mills*, et al.* 2005]. The chamber was maintained at a constant temperature of 22 °C and at 50% relative humidity.

Filtered air control exposures were performed in the same exposure chamber, and to ensure adequate blinding the engine was running as during the diesel exhaust exposure. However, the valve allowing the side-flow from the exhaust pipe to enter the conditioning chamber remained closed throughout the exposure.

2.3 NITROGEN DIOXIDE EXPOSURES

Nitrogen dioxide exposures were performed in a second specially built exposure chamber [Kelly, *et al.* 1996], situated at the Medical Division of the National Institute for Working Life in Umeå. Ambient air was drawn through this large room-sized exposure chamber at a constant flow rate of 30 m^3/h after HEPA filtration, which was maintained at a temperature of 20 °C and a relative humidity of 50%. Bottled nitrogen dioxide (AGA Special Gas, Lidingö, Sweden) was passed through a mass flow controller (Model 5850TR, Brooks Instrument BV., Veenendaal, Netherlands) and a flow meter (Rota, Hannover, Germany) to control the gas flow before being introduced into the ventilating duct just before the air reached the exposure chamber. Air within the chamber was monitored within the breathing zone of the subjects and analysed constantly for nitrogen dioxide concentration using an oxides of nitrogen analyser (CSI-1600, Columbia Scientific Industries, Austin, Tx, USA). The concentration of NO_2 in the chamber was kept constant at 4 ppm. Control exposures were produced in the same facility, without the introduction of additional nitrogen dioxide to the incident air flow.

2.4 VASCULAR STUDIES

Vascular endothelial dysfunction, which is generally characterised by the reduced bioavailability of endothelium-derived nitric oxide, has been shown to be an independent risk factor for cardiovascular morbidity and mortality [Schachinger, *et al.* 2000; Suwaidi, *et al.* 2000]. Endothelial cell dysfunction results in adaptive changes within the vasculature that lead to increased leucocyte adhesion, an enhanced inflammatory response, and increased permeability – changes that eventually lead to the development of atherosclerotic lesions [Ross 1999]. Indeed, it has been shown that endothelial dysfunction – manifesting as an attenuated vasomotor response – is an early marker for atherosclerosis and may be present long before structural changes within blood vessels are apparent on clinical investigation [Davignon, *et al.* 2004; Ross 1999]. Whilst it would be ideal to study vascular vasomotor responses within the coronary arteries, this is clearly not without risk. However, it has been demonstrated that the responses of peripheral resistance vessels are well correlated with coronary responses and can be used an accurate and validated surrogate [Anderson, *et al.* 1995].

The technique of venous occlusion plethysmography to measure forearm blood flow was first described by Hewlett and van Zwaluwenburg in 1909 and has been used widely since then. The use of venous occlusion plethysmography along with unilateral intra-brachial infusion of vasoactive mediators has allowed the in-depth study of vascular homeostatic

mechanisms and vascular pharmacology, and is now regarded as the "gold-standard" method of determining vascular vasomotor function [Wilkinson, *et al.* 2001].

2.4.1 Venous occlusion plethysmography

Venous occlusion plethysmography is a technique of measuring blood flow by determining the change in volume of the forearm with time, and can be applied to both arms simultaneously. An occluding cuff, inflated above systolic blood pressure (usually 200 mmHg), is placed around the wrist to exclude the hand – in which there are a large number of arterio-venous connections and where the blood flow is highly temperature-dependent resulting in a different physiology and pharmacology to the rest of the forearm [Wilkinson, *et al.* 2001]. A second cuff is placed around the upper arm and is inflated and deflated cyclically (40 mmHg) to occlude venous outflow from the arm. When this cuff is inflated, blood flows into the arm but does not escape, and a linear increase in forearm volume is seen. This is detected by the use of a mercury-in-silastic strain gauge placed around the widest part of the forearm – effectively measuring stretch. The change in volume (calculated by assuming the forearm is a truncated cone in shape, with the widest part at the strain gauge) can then be expressed as blood flow per 100 mL tissue per minute (Figure 2.2). In resting conditions, it is known that around 70% of the blood flow goes to skeletal muscle, the remaining 30% to the skin [Cooper, *et al.* 1955]. As skin blood flow is dependent on

temperature, it is important that ambient temperature is constant, and for all these studies the room was maintained at 22-24 °C.

Here, vascular studies were carried out two hours after termination of the exposure (to diesel exhaust or nitrogen dioxide). Previous studies have demonstrated pronounced and reproducible effects of exposure on vascular endothelial function by this 2-hour time point, that are still present at 6-hours but largely resolved by 24-hours [Mills, *et al*. 2005].

Subjects lay supine on a bed with both arms supported in a quiet, warm room. Forearm blood flow was measured in both arms using venous-occlusion plethysmography during unilateral intra-brachial infusion of vasoactive mediators.

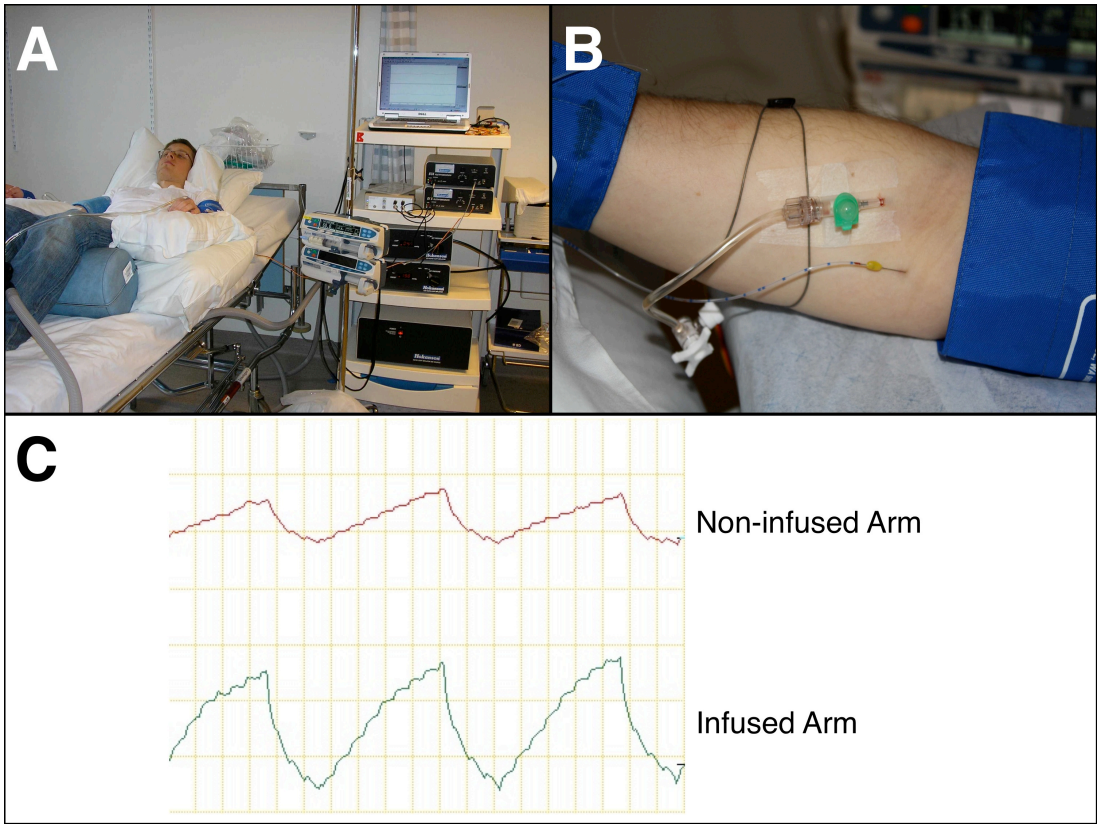

Figure 2.2. (A) Shows the subject resting supine on the bed, attached to the plethysmography equipment. (B) The occluding wrist cuff and upper arm cuff in situ. The mercury-in-silastic strain gauge can be seen around the forearm. In this subject, a venous cannula is placed in a large ante-cubital vein for venous blood sampling and the brachial artery is cannulated with a 27G needle. (C) Representative trace obtained showing the linear change in volume with time in the infused and non-infused arms (here with substance P at 8 pmol/min).

2.4.2 Brachial artery cannulation

The brachial artery of the non-dominant hand was cannulated at the start of the vascular studies using a sterile 27-standard wire gauge steel needle (Coopers Needle Works Ltd, Birmingham, UK) under aseptic techniques. This needle was attached to a sterile epidural catheter and secured with sticky dental wax. This technique has been shown to be safe and simple and allows infusion of vasoactive drugs into the forearm with minimal trauma to the vessel itself.

2.4.3 Venous blood sampling

At the start of the study, subjects had 17 or 18 gauge intravenous cannulae inserted into a large antecubital vein in each arm. This enabled venous blood sampling in both the infused and non-infused arms during the study without further venepuncture.

2.4.4 Intra-arterial vasoactive mediators

After a baseline infusion of 0.9% sodium chloride (to allow a true baseline forearm blood flow measurement to be obtained) subjects received intra-arterial infusions of endothelin-1 (5 pmol/min), endothelin-receptor antagonists (BQ-123, 10 nmol/min; BQ-788, 1 nmol/min), L-N^G-monomethylarginine (L-NMMA, 2-8 μmol/min), sodium nitroprusside (SNP, 90 ng/min – 8 μg/min), acetylcholine (ACh, 5-20 μg/min) or bradykinin (100-1000 pmol/min) as per the individual study protocol. These

doses are much lower than would be required to result in a systemic effect, as they need to act only within the forearm – a major advantage of this model. Subjects' blood pressure was monitored throughout the studies to assess for systemic spill over of drug using a validated, semi-automated, oscillometric sphygmomanometer (Omron Healthcare Ltd., Japan) placed around the upper part of the non-infused arm. Forearm blood flow was determined at defined timepoints during the infusion of all drugs. A 20 minute 0.9% saline infusion separated multiple drug used during a single protocol to allow blood flow to return to baseline. The infusion rate was kept constant at all times at 1 ml/min.

2.5 FACEMASK SELECTION

Masks designed for use by cyclists, pedestrians and occupational settings were tested for penetrance of fresh diesel exhaust particulate. Diesel engine exhaust was generated from the idling (1500 rpm) engine (F3M2011, Deutz Ag, Köln, Germany) of a 35KVA generator (Bredenoord, Apeldoorn, Netherlands). The exhaust was diluted with filtered air to obtain a mass concentration of 75±12 $\mu g/m^3$ (as measured by gravimetric analysis) and a particle number concentration of 500,000 particles/cm^3 (condensation particle counter [CPC] model 3022, TSI Instruments, High Wycombe, UK). Sections of each mask filter were mounted in a filter holder. After 5 min of baseline measurements, filters were introduced between the exhaust and the CPC that sampled at a flow rate of 1.5 L/min. Particle number was recorded for 5 min and penetrance defined as the percentage of particles passing through the filter compared to baseline.

Mask penetrance was highly dependent on mask type (Figure 2.3). The 3M Dust Respirator (Model 8812, 3M, St Pauls, USA) was selected for the subsequent intervention studies as it provided good filtration performance, was extremely efficient, comfortable to wear and inexpensive.

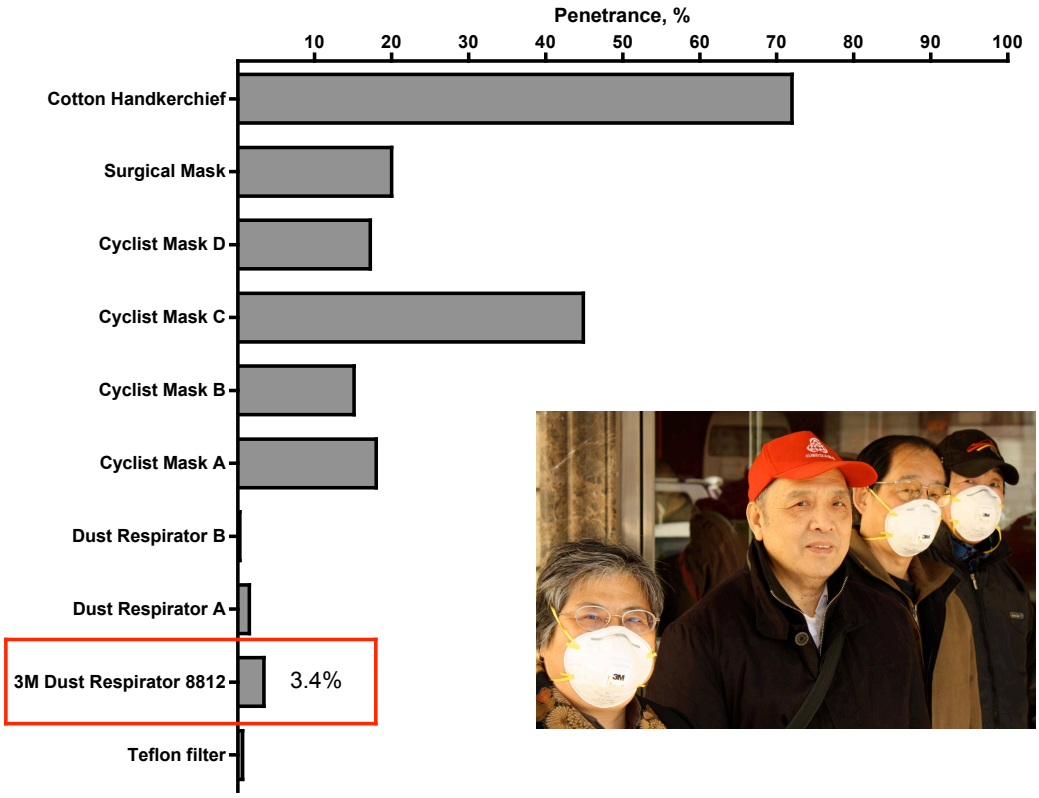

Figure 2.3. Penetrance of commercially available filters: 3M Dust Respirator 8812, Dust Respirators A and B, Cyclist Masks A to D. The Teflon filter is an industry standard filter for aerosol studies included as a control. Cotton handkerchiefs and surgical masks are often seen worn in public areas in parts of Asia. Inset: subjects wearing the mask at the beginning of a study day.

2.6 AMBIENT POLLUTION MONITORING

One of the problems with air pollution research in ambient settings, and the reason why model exposure systems such as the diesel exhaust system described previously are widely used in toxicological and mechanistic studies, is that there are myriad factors that affect local pollution levels including traffic density, activity of nearby industry and weather conditions. Indeed, particulate air pollution measured at a background monitoring station away from the roadside may give a significantly false impression of an individual's real exposure to air pollution when walking along a busy roadside [Buzzard, *et al.* 2009; Watt, *et al.* 1995].

In light of this we collected ambient air pollution data both from the local background monitoring stations, but we also measured personal exposure to air pollutants using portable air pollution monitoring equipment contained within a backpack, sampling from near the breathing zone of the participants (Figure 2.4). During the patient study we also collected particles in size fractions to allow gravimetric quantification and chemical analysis.

Figure 2.4. Pollution monitoring equipment contained within a small backpack, sampling from the tubes and sensors on the outside of the bag.

2.6.1 Personal pollution exposure assessment

Using a similar approach to that described before by McCreanor and colleagues who measured personal exposure to air pollutants using equipment pushed in a handcart [McCreanor, *et al.* 2007], personal exposure to air pollutants was monitored using a collection of portable monitoring equipment mounted in a backpack. Particle mass concentration (particle diameter <2.5 μm; $PM_{2.5}$) was measured in using a light-scattering nephelometric method with a DataRAM monitor (pDR-1500, Thermo Scientific, Franklin, USA). Particle number was measured using a handheld condensation particle counter (CPC 3007, TSI Instruments Ltd, High Wycombe, UK). Ambient temperature and relative humidity were recorded using a sensor on the outside of the backpack (Omegaette® HH-314, Omega Engineering Ltd, Connecticut, USA). Gaseous pollutants were measured using a multigas analyser (X-am 7000, Dräger Safety, Pittsburgh, USA) measuring carbon monoxide (CO), nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) using electrochemical sensors with a sensitivity of 1 part per million.

2.6.2 Activity assessment

Physical activity was assessed using a portable global positioning system (GPS) monitor secured to the outside of the bag (eTrex Summit HC, Garmin, USA). This recorded the route taken by volunteers, their total distance travelled, and average speed. This information was used, along with baseline

anthropometric measurements, to calculate the energy expended during the walk in kilocalories and metabolic equivalents (METS).

2.6.3 Background pollution exposure assessment

In addition to the personal exposure measurements, during the patient study background air pollution exposure was recorded from permanent city centre monitoring stations in the same district as the patients' walk (Xicheng for the Fuwai Hospital, ChaoYang for the ChaoYang Hospital); freely available online at http://www.bjepb.gov.cn/air2008/olympic.aspx. A MOUDI cascade impactor was set up in the front of the Fuwai Hospital (Figure 2.5), at the start point of the 2-hour walk, and attached to a high-volume sampling pump running at 30 L/min. Particles were collected onto Teflon filters (Pall Corp., Ann Arbor, MI, USA) in three size fractions: coarse (with mean aerodynamic diameter 2.5-10 μm), fine (0.18-2.5 μm) and ultrafine (<0.18 μm). Samples were collected over 24 hours and stored in airtight containers prior to analysis. The mass collected on the filters was determined gravimetrically for each size fraction.

Figure 2.5. (A) Shows representative filter samples from MOUDI impactor in the three size fractions: ultrafine, fine and coarse from left to right. (B) Shows MOUDI impactor in situ at the Fuwai Hospital entrance at the start of the walk, indicated by the yellow circle in panel C. (D) shows the location of the impactor looking out onto the roadside, adjacent to a major traffic interchange.

2.6.4 Particle chemical and toxicological analysis

Collected particulate samples were analysed for total carbon content, as well as elemental and organic carbon fractions, using the Sunset method (NIOSH 5040; http://www.cdc.gov/niosh/docs/2003-154/pdfs/5040.pdf). Metals and cations were determined using inductively-coupled plasma mass spectrometry (ICP-MS) following pre-treatment with nitric acid. Nitrate and sulphate anions were determined following extraction with water using LC-ICP-MS (liquid chromatography paired with ICP-MS). Organic matter was extracted from filters by ultrasonification with toluene and analysed using gas chromatography-mass spectrometry (GC-MS).

Oxidative potential of particles was assessed using electron paramagnetic resonance (EPR) [Miller, *et al.* 2009]. EPR was used to establish oxygen-centred free radical generation from the collected particulate matter in the absence of tissue. Filters for all size fractions were pooled and sonicated in phosphate buffered saline to give a final concentration of particles of 1 mg/mL. Solutions were incubated with the spin-trap 1-hydroxyl-2,2,6,6,-tetramethyl-4-oxo-piperidine (Tempone-H; 1 mM), loaded into a capillary tube and assessed at 37 ^{o}C in an X-band EPR spectrometer (Magnettech MS-200, Berlin, Germany) as described previously [Miller, *et al.* 2009]. Pyrogallol (100 μM) was used as a positive control, and samples were compared to the NIST (National Institute of Standards and Technology, Gaithersburg, MD,

USA) standard reference materials: urban dust (SRM-1649a; 1 mg/mL) and diesel exhaust particles (SRM-2975; 10 μg/mL).

2.7 PHYSIOLOGICAL PARAMETERS

2.7.1 Ambulatory blood pressure

Subjects were fitted with an ambulatory blood pressure monitor (Model 90217, Spacelabs Healthcare, Chesham, UK) at the beginning of the study days (Figure 2.6). During the controlled exposure studies, this recorded blood pressure every 30 minutes during the day (07:00 to 22:00) and every hour overnight (22:00 to 07:00). When taking part in the ambient studies, blood pressure was determined every 15 minutes during the 2-hour walk, then every 30 minutes for the rest of the day and every hour overnight as above. Data were stored and analysed using Spacelabs Healthcare proprietary software.

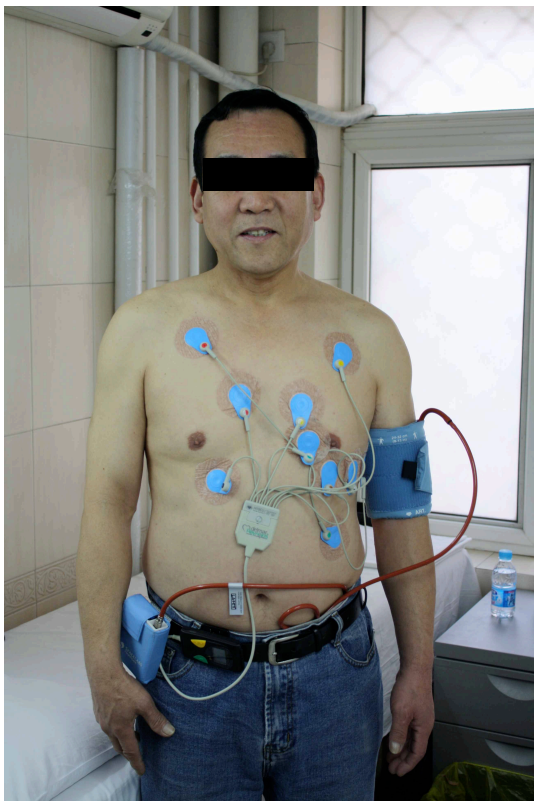

Figure 2.6. Subject wearing 12-lead electrocardiography Holter monitor and ambulatory blood pressure monitor at the start of a study day.

2.7.2 Electrocardiography

Subjects were fitted with a 12-lead continuous electrocardiographic Holter monitor (Lifecard 12, Spacelabs Healthcare, Chesham, UK) at the beginning of the study (Figure 2.6), which they were asked to wear for 24 hours. Holter electrocardiographic traces were analysed using DelMar Reynolds proprietary software packages. The quality of the trace was visually assessed before automated arrhythmia analysis was performed using the Pathfinder package. Identified arrhythmias were individually inspected and verified or deleted as appropriate. Heart rate variability was assessed using the HRV Tools software package in the time and frequency domains, with identified arrhythmias excluded from this analysis.

During the patient study, ST segment analysis was performed over the whole 24-hour period and during the 2-hour walk. Three representative leads were chosen, leads II, V2 and V5, and analysed separately for each subject. The baseline ST segment amplitude was defined as the average over the entire 24-hour period, and all subsequent measurements were compared to this. ST segment deviation was determined at the J-point +80 ms. The maximal ST segment depression during the two time periods was recorded and ischaemic burden determined (calculated as the product of the change in ST segment amplitude and the duration of the recording) for each lead individually and as a composite [Mills, *et al.* 2007]. Clinically relevant ST segment events were recorded and defined as horizontal or downsloping ST

segment deviation of at least 1 mm (100 μV), lasting at least 60 s and separated from the next event by at least 60 s [Cohn, *et al.* 2003; Quyyumi, *et al.* 1993].

2.8 BIOCHEMICAL ANALYSES

2.8.1 Endothelins

Blood samples were collected into ethylene diamine tetra-acetic acid and kept on ice until centrifuged at 3000 rpm for 30 min. Plasma samples were immediately frozen and stored at -80 °C. Plasma ET-1 and big-ET-1 concentrations were measured according to an acetic acid extraction technique using a commercial radioimmunoassay with rabbit anti-human ET-1 or big-ET-1 (Peninsular Laboratories Europe Ltd, St Helens, UK) as described previously [Adam, *et al.* 2001].

2.8.2 Nitrites

Blood samples were obtained for the measurement of plasma nitrite from both infused and non-infused arms during the vascular assessment via indwelling 17-gauge venous cannulae inserted into a large antecubital vein on each arm. Samples were obtained before and after each incremental dose of L-NMMA, acetylcholine and SNP and were collected into lithium heparin, immediately transferred to eppendorf tubes prewashed with Milli-Q de-ionised water and immediately centrifuged at 5000 *g* for 1 minute. Plasma was then transferred into a dark coloured eppendorf tube containing 100 μL of a solution containing 1 mM diethylenetriaminepentaacetic acid and 62.5 mM N-ethylmaleimide before being snap-frozen on dry ice and stored at -80°C prior to further analysis.

Plasma nitrite samples were defrosted and deproteinised using acetonitrile and chloroform as described previously [Romitelli, *et al.* 2007]. Aliquots of the aqueous fraction were injected into a reaction vial containing glacial acetic acid and ascorbate [Nagababu, *et al.* 2007]. Nitric oxide generated in the reaction chamber was driven off by a continual stream of nitrogen and detected using a chemiluminescent analyzer (Seivers, Melbourne, Australia) with a detection limit of 10-20 nM. Analyses were performed in quadruplicate.

Blood samples for arginine, homoarginine (nitric oxide synthase substrates), ADMA and SDMA (endogenous nitric oxide synthase antagonists) were collected into ethylene diamine tetra-acetic acid and kept on ice until centrifuged at 3000 rpm for 30 min. Plasma samples were immediately frozen and stored at -80°C. Plasma concentrations were determined by high-performance liquid chromatography as described previously [Blackwell, *et al.* 2009].

2.8.3 Fibrinolytic system

Blood samples were obtained during the forearm vascular study from indwelling 17-gauge venous cannulae inserted into a large antecubital vein on both arms before and after each dose of bradykinin. Bradykinin is an endothelium-dependent vasodilator that also stimulates the release of stored

tissue-plasminogen activator (t-PA) from the vascular endothelium [Brown, *et al.* 2000; Brown, *et al.* 1999]. Samples were collected into acidified buffered citrate (Stabilyte, Biopool International) for analysis of t-PA and into citrate (BD Vacutainer) for plasminogen-activator inhibitor type 1 (PAI-1) assays to assess the activity of the endogenous fibrinolytic pathway.

t-PA and PAI-1 antigen concentrations were determined by commercially available ELISA assays (t-PA combi Actibind, Technoclone, Vienna, Austria; Elitest PAI-1, Hyphen BioMed, Neuville-sue-Oise, France). As much of the t-PA present in the circulation is bound to PAI-1, thus rendering both inactive and leading to hepatic clearance [Chandler, *et al.* 1997], both t-PA and PAI-1 activity were assessed using enzymatic assays (t-PA combi Actibind, Technoclone, Vienna, Austria; Zymutest PAI-1, Hyphen BioMed, Neuville-sur-Oise) as described previously [Mills, *et al.* 2005].

2.9 DATA ANALYSIS AND STATISTICS

For the vascular studies, nursing staff at the clinical research facility at University Hospital, Umeå, Sweden, randomised exposure type and the associated vascular study protocol. The investigators were blinded to the exposure received. Plethysmography data were analysed as described previously [Newby, *et al.* 1999]. Estimated net release of t-PA activity and antigen was calculated as described previously [Newby, *et al.* 1997] as below, where FBF if forearm blood flow, Hct is haematocrit, $t\text{-}PA_{inf}$ and $t\text{-}PA_{non}$ the concentrations of t-PA (antigen or activity) in the infused and non infused arms respectively:

$$\text{Estimated net t-PA release} = FBF \times (1 - Hct) \times ([t\text{-}PA]_{inf} - [tPA]_{non})$$

Statistical analyses were performed using paired Student's *t*-tests and two-way analysis-of-variance (ANOVA) with repeated measures including time and exposure as variables where appropriate.

For the ambient studies, subjects were randomised to wearing a mask on their first or second visit on recruitment by the use of a random number generator. Data were compared using paired Student's *t*-tests or Wilcoxon matched pairs signed rank test as appropriate. Symptom data were collected using a visual analogue scale, from 0 to 10. Occurrence of arrhythmias, reported symptoms and ST segment event frequency were compared using

the Chi-squared analysis and subsequent average event data compared using Mann-Whitney U tests.

All data were analysed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA) on a Macintosh personal computer. Statistical significance was taken as a two-sided P value <0.05.

CHAPTER 3

Exposure to nitrogen dioxide is not associated with vascular dysfunction in man

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3.1 SUMMARY

Exposure to air pollution is associated with increased cardiorespiratory morbidity and mortality. It is unclear whether these effects are mediated through combustion-derived particulate matter or gaseous components, such as nitrogen dioxide. The objective of this study was to investigate the effect of nitrogen dioxide exposure on vascular vasomotor and fibrinolytic function.

Ten healthy male volunteers were exposed to nitrogen dioxide at 4 ppm or filtered air for 1 hour during intermittent exercise in a randomised double-blind crossover study. Bilateral forearm blood flow and fibrinolytic markers were measured before and during unilateral intrabrachial infusion of bradykinin (100-1000 pmol/min), acetylcholine (5-20 μg/min), sodium nitroprusside (2-8 μg/min) and verapamil (10-100 μg/min) 4 hours after the exposure. Lung function was determined before and after the exposure, and exhaled nitric oxide at baseline, 1 and 4 hours after the exposure.

There were no differences in resting forearm blood flow after either exposure. There was a dose-dependent increase in forearm blood flow with all vasodilators but this was similar after either exposure for all vasodilators ($P>0.05$ for all). Bradykinin caused a dose-dependent increase in plasma tissue-plasminogen activator, but again there was no difference between the

exposures. There were no changes in lung function or exhaled nitric oxide following either exposure.

We conclude that inhalation of nitrogen dioxide does not impair vascular vasomotor or fibrinolytic function and therefore does not appear to be a major arbiter of the adverse cardiovascular effects of air pollution.

3.2 Introduction

Exposure to air pollution is a major public health problem, and is associated with an increased risk of cardiorespiratory morbidity and mortality. Epidemiological studies have consistently shown a strong association of cardiorespiratory illness and fine particulate matter [Dockery, *et al.* 1993; Miller, *et al.* 2007; Pope, *et al.* 2002], although similar positive associations have been shown for long-term exposure to nitrogen dioxide [Anderson, *et al.* 1996; Hoek, *et al.* 2001; Rosenlund, *et al.* 2008]. Nitrogen dioxide and other oxides of nitrogen are major constituents of combustion-derived air pollution, such as diesel exhaust, and the associations of nitrogen dioxide with adverse outcomes have usually been attributed to the close association of fine particulate with nitrogen dioxide concentrations [Sarnat, *et al.* 2001]. However, isolated real life exposures to nitrogen dioxide have been linked to increases in respiratory illness and susceptibility to airway infection [Guidotti 1978; Love, *et al.* 1982; Mostardi, *et al.* 1981]. Controlled exposure to nitrogen dioxide induces airway inflammation and modifies antioxidants in the respiratory tract lining fluid [Kelly, *et al.* 1996].

The cardiovascular effects of inhaled diesel exhaust are well documented. We have demonstrated that controlled exposures to diesel exhaust, as a model of combustion-derived fine particulate air pollution, causes acute vascular endothelial effects [Mills, *et al.* 2005; Törnqvist, *et al.* 2007]. In these models, the actual exposure is a complicated mixture of carbon centred

particulate matter, volatile organic compounds and gaseous pollutants [Scheepers and Bos 1992]. The predominant gaseous components are nitrogen dioxide and other oxides of nitrogen, with NO_x concentrations reaching around 4 ppm in previous controlled exposures to diesel exhaust [Behndig, *et al.* 2006; Mills, *et al.* 2005; Salvi, *et al.* 1999]. These models have consistently demonstrated an impairment of vascular endothelial function and endogenous fibrinolysis following exposure to diesel exhaust, that appears to be mediated through increased oxidative stress and reduced nitric oxide bioavailability [Miller, *et al.* 2009; Mills, *et al.* 2005]. Although particulate matter appears to be a major arbiter of these adverse vascular effects, the question remains as to the independent effect of a pure exposure to nitrogen dioxide. Nitrogen dioxide is itself a powerful oxidising species, and this is proposed to underlie the airway inflammatory and antioxidant responses, as well as the vascular dysfunction previously described [Blomberg, *et al.* 1997; Patel and Block 1986].

The aim of this study was to explore the effect of a pure exposure to 4 ppm of nitrogen dioxide on vascular endothelial and fibrinolytic function, and to test the hypothesis that the previously demonstrated adverse effects are driven by nitrogen dioxide.

3.3 Methods

3.3.1 Subjects

Ten healthy male volunteers were recruited into the trial. Women were not included to avoid the potential confounding influence of cyclical changes in estrogen on vascular endothelial function [Ganz, 2002]. One subject developed a respiratory tract infection during the study and was excluded and replaced. All subjects were non-smokers, had no intercurrent illness, took no regular medication, and all had normal lung function. All subjects had been free of symptoms of upper airway infection for at least 6 weeks prior to the study. All subjects gave their written informed consent, and the trial was approved by the local research ethics committee in accordance with the Declaration of Helsinki.

3.3.2 Exposure protocol

The exposures to nitrogen dioxide or filtered air took place at the Medical Division of the National Institute for Working Life in Umeå as described previously [Kelly, *et al.* 1996]. Briefly, ambient air was drawn continuously through a large exposure chamber at a constant rate of 30 m^3/h, which was maintained at a temperature of 20 ˚C and relative humidity of 50%. Nitrogen dioxide (AGA Special Gas, Lidingö, Sweden) was passed through a mass flow controller (Model 5850TR, Brooks Instrument B.V., Veenendaal, Netherlands) and a flow meter (Rota, Hannover, Germany) to control the gas flow, and was introduced to the ventilating duct just before reaching the

chamber. Air was sampled in the breathing zone of the subjects and analysed continuously for nitrogen dioxide, nitric oxide and total NO_x using a CSI 1600 oxides of nitrogen analyser (Columbia Scientific Industries, Austin, Tx, USA). The concentration of nitrogen dioxide within the chamber was maintained at 4 ppm, a concentration selected to match that of NO_x concentrations seen in controlled exposures to diesel exhaust as described previously [Behndig, *et al.* 2006; Mills, *et al.* 2005; Salvi, *et al.* 1999]. Filtered air exposures were performed by HEPA filtration of air prior to introduction into the exposure chamber without introduction of additional nitrogen dioxide.

3.3.3 Study design

Subjects attended the clinical research facility at Umeå University Hospital on two occasions, each at least one week apart. In a randomised double-blind cross over trial, subjects were exposure to either filtered air or nitrogen dioxide at 4 ppm for 1 hour with intermittent exercise. Subjects performed exercise on a bicycle ergometer whilst in the chamber at a pre-designated workload to achieve a mean ventilation rate of 20 L/min/m^2, based on a pre-study screening exercise ventilation stress test. Subjects then returned to the clinical research facility for forearm vascular plethysmography studies with active drug infusions commencing 4 hours after the exposure.

3.3.4 Outcome measures

3.3.4.1 Vascular assessments

Subjects underwent forearm venous occlusion plethysmography as described previously [Wilkinson and Webb 2001]. The brachial artery of the non-dominant arm was cannulated with a 27-gauge steel needle under aseptic technique and local anaesthesia. After a 30-minute baseline saline infusion, forearm blood flow was recorded during infusion of the endothelial-dependent (acetylcholine, 5, 10 and 20 μg/min; bradykinin, 100, 300 and 1000 pmol/min) and –independent (sodium nitroprusside, 2, 4 and 8 μg/min; verapamil, 10, 30 and 100 μg/min) vasodilators. The vasodilators were infused for 6 minutes at each dose, with the blood flow determined for last 3 minutes of the infusion. Vasodilators were administered in a random order, except for verapamil that was always administered last given its prolonged vascular action, and were separated by a washout period of 20 minutes during which saline was infused.

3.3.4.2 Biochemical analyses

Peripheral venous blood samples were obtained at baseline and at 4 and 6 hours after exposure for total and differential cell counts. Analysis was performed on an autoanalyser by the local haematology reference laboratory (Department of Clinical Chemistry, University Hospital, Umeå). Blood samples were obtained during the forearm vascular study from indwelling 17-gauge venous cannulae inserted into a large antecubital vein on both arms before and after each dose of bradykinin. Bradykinin is an endothelial-

dependant vasodilator that also stimulates the release of stored tissue-plasminogen activator (t-PA) from the vascular endothelium [Brown, *et al.* 2000; Brown, *et al.* 1999]. Samples were collected into acidified buffered citrate (Stabilyte, Biopool International) for analysis of t-PA and into citrate (BD Vacutainer) for plasminogen-activator inhibitor type 1 (PAI-1) assays to assess the activity of the endogenous fibrinolytic pathway.

t-PA and PAI-1 antigen concentrations were determined by commercially available ELISA assays (t-PA combi Actibind, Technoclone, Vienna, Austria; Elitest PAI-1, Hyphen BioMed, Neuville-sue-Oise, France). As much of the t-PA present in the circulation is bound to PAI-1, thus rendering both inactive and leading to hepatic clearance [Chandler, *et al.* 1997], both t-PA and PAI-1 activity were assessed using enzymatic assays (t-PA combi Actibind, Technoclone, Vienna, Austria; Zymutest PAI-1, Hyphen BioMed, Neuville-sur-Oise) as described previously [Mills, *et al.* 2005].

3.3.4.3 Exhaled nitric oxide and lung function

The standardised fractional exhaled nitric oxide (FE_{NO}) was measured in duplicate at expiratory flow rates of 10, 50, 100 and 250 mL/s at baseline, 1 hours and 4 hours after both exposures using a chemiluminescence analyser (NiOX, Aerocrine AB, Stockholm, Sweden) [ATS/ERS 2005]. Lung function was measured using simple spirometry (Vitalograph, Bucks, UK) at baseline

and after the exposure, recording forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), and slow vital capacity (VC).

3.3.5 Statistical analysis

We calculate, based on previous studies [Mills, *et al.* 2005], that a sample size of 10 gives an 80% power of detecting a difference in forearm blood flow responses of 20% at a 2-sided statistical significance level of 5%. All investigators were blinded to the exposure received. Plethysmography data were analysed as described previously [Newby, *et al.* 1999]. Data were analysed using 2-way analysis-of-variance (ANOVA) with repeated measures. The variables included in the ANOVA were dose and exposure for the forearm blood flow data, and time and exposure for the other outcome measures. All data are expressed as mean ± standard deviation unless otherwise stated. Statistical significance was taken as a two-sided P value of 0.05. Data were analysed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA) on a Macintosh personal computer.

3.4 Results

Ten subjects, median age 24 years, completed the study (Table 3.1). There were no changes in any indices of lung function seen following the nitrogen dioxide exposure (Table 3.2). Exhaled nitric oxide (FE_{NO}), measured as a surrogate of airway inflammation, was unchanged at all time points following either nitrogen dioxide or filtered air exposure (Table 3.2).

Table 3.1. Baseline characteristics of the 10 subjects completing the study.

Age, years (median, range)	24 (22-28)
Male sex, %	100%
Height, cm	181±5
Weight, kg	79±10
BMI, kgm^{-2}	24±2
FEV_1, L	4.95±0.33
FVC, L	5.99±0.52
VC, L	5.91±0.47
Systolic blood pressure, mmHg	136±11
Diastolic blood pressure, mmHg	67±8
Heart rate, bpm	60±12
Haemoglobin, g/L	146±7
White cell count, $x10^9/L$	4.9±1.5
Platelet count, $x10^9/L$	224±53
PAI-1 antigen, ng/mL	6.87±4.04
PAI-1 activity, U/mL	0.27±0.22
t-PA antigen, ng/mL	2.82±1.85
t-PA activity, U/mL	1.25±0.54

Data expressed as mean ± standard deviation unless otherwise stated. Slow vital capacity, VC; forced expiratory volume in 1 second, FEV_1; forced vital capacity, FVC. Plasminogen activator type 1 (PAI-1) and tissue plasminogen activator (t-PA) concentrations measured during forearm study on control air day before infusion of bradykinin.

Table 3.2. Basic spirometry and exhaled nitric oxide (FE_{NO}) at flow rates of 10, 50, 100 and 250 mL/s after 1 hour of nitrogen dioxide (4 ppm) or filtered air exposure with intermittent exercise.

	Time	NO_2	Air	P Values	
				Time	Exposure
VC, L	Pre exposure	5.82±0.41	5.91±0.47	0.959	0.153
	Post exposure	5.88±0.44	5.87±0.48		
FEV_1, L	Pre exposure	4.95±0.24	4.95±0.33	0.979	0.920
	Post exposure	4.95±0.33	4.94±0.33		
FVC, L	Pre exposure	5.93±0.47	5.99±0.52	0.882	0.570
	Post exposure	5.93±0.49	5.92±0.54		
FE_{NO} 250mL/s, ppm	Pre exposure	6.28±2.77	6.49±2.36	0.796	0.424
	1 hours	5.80±2.31	6.14±2.10		
	4 hours	6.27±1.69	6.83±2.53		
FE_{NO} 100mL/s, ppm	Pre exposure	10.23±5.62	10.53±3.22	0.867	0.819
	1 hours	10.33±5.45	10.52±5.32		
	4 hours	11.28±4.44	11.44±5.43		
FE_{NO} 50mL/s, ppm	Pre exposure	16.55±9.67	15.71±9.02	0.863	0.700
	1 hours	16.94±9.71	16.34±8.32		
	4 hours	18.25±9.01	17.81±9.48		
FE_{NO} 10mL/s, ppm	Pre exposure	51.15±32.65	51.87±32.59	0.829	0.992
	1 hours	55.74±32.00	54.28±33.16		
	4 hours	58.57±27.16	59.48±35.41		

Data shown as mean ± standard deviation. P values shown from 2-way ANOVA with repeated measures showing effect of time and exposure. Slow vital capacity, VC; forced expiratory volume in 1 second, FEV_1; forced vital capacity, FVC.

The forearm vascular studies revealed a dose-dependent increase in blood flow in the infused arm compared to the non-infused arm in all subjects after each infused vasodilator. There was no difference in the vascular responses to any of the vasodilators (endothelium-dependent or –independent) following air or nitrogen dioxide exposure (Figure 3.1). Bradykinin stimulated a dose-dependent increase in release of plasma tissue-plasminogen activator (t-PA) although the response was similar following each exposure (Table 3.3). Plasma plasminogen-activator inhibitor 1 (PAI-1) concentrations were unchanged (P>0.05 for both infused and non-infused arms, data not shown) following infusion of bradykinin with no differences following either exposure (P>0.05 for all, data not shown).

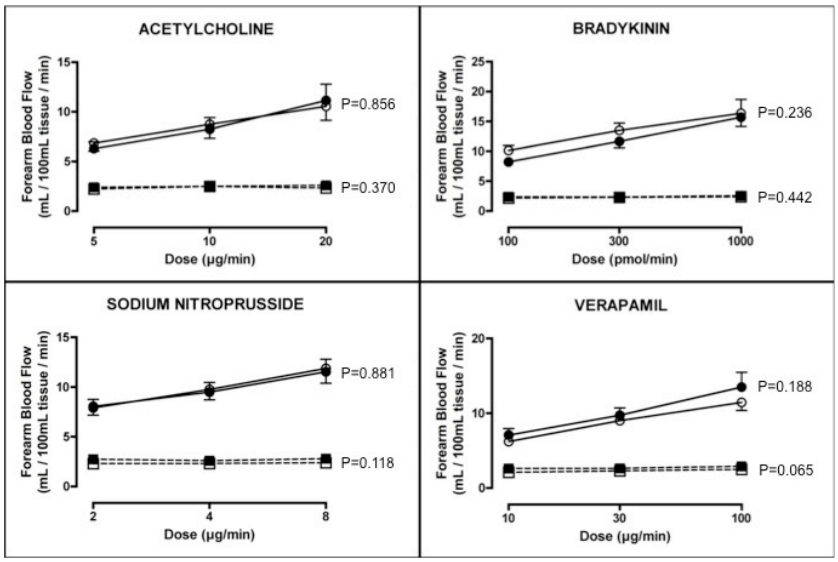

Figure 3.1. Forearm venous occlusion plethysmography with intra-arterial infusion of vasodilators performed 4 hours after exposure. Data plotted as mean and T-bars show standard error of the mean. Solid lines show infused arm, dotted lines show non-infused arm after exposure to air (○) or nitrogen dioxide (●) at 4 ppm. P values shown from 2-way ANOVA with repeated measures showing effect of exposure for both infused and non-infused arms.

Table 3.3. Plasma tissue-plasminogen activator (t-PA) antigen concentrations and activity and estimated net t-PA release following infusion of bradykinin after 1 hour of nitrogen dioxide (4 ppm) or filtered air exposure with intermittent exercise. Data shown as mean ± standard deviation. P values shown from 2-way ANOVA with repeated measures showing effect of time and exposure (Exp.).

Bradykinin (pmol/min)		Air				NO_2				P Value	
		Baseline	100	300	1000	Baseline	100	300	1000	Time	Exp.
t-PA antigen ng/mL	Infused arm	2.82±1.85	2.46±1.26	4.22±2.78	8.52±4.74	3.01±1.98	2.67±3.32	3.32±2.42	5.98±3.10	**0.001**	0.098
	Non-infused arm	2.50±1.54	1.81±1.01	2.08±1.16	2.24±1.23	2.82±1.88	1.99±1.44	2.17±1.35	2.36±1.51	0.672	0.256
	Estimated net t-PA release (ng/100mL tissue/min)	0.43±0.67	3.78±1.87	16.54±13.90	54.27±30.52	0.33±0.64	2.93±4.59	8.89±12.29	36.71±25.87	**<0.001**	0.083
t-PA activity U/mL	Infused arm	1.25±0.54	1.81±0.55	2.60±0.72	3.48±1.57	1.25±0.43	1.82±0.77	2.11±1.11	2.95±1.01	**<0.001**	0.213
	Non-infused arm	0.98±0.49	1.08±0.32	1.20±0.36	1.54±0.49	1.00±0.34	1.04±0.44	1.13±0.42	1.53±0.54	**0.001**	0.819
	Estimated net t-PA activity release (U/100mL tissue/min)	0.43±0.42	4.82±2.66	12.24±6.59	17.29±10.14	0.39±0.26	3.97±3.23	7.60±5.73	15.09±10.20	**<0.001**	0.216

There was no difference in white cell count, differential cell count, or platelet count through the study following either exposure (Table 3.4). We did observe a small fall in systemic haemoglobin concentrations through the study period consistent with repeated venesection, although there was no difference between the study exposures (Table 3.4).

Table 3.4. Differential cell count and hemoglobin concentrations before and after a 1 hour nitrogen dioxide (4 ppm) or filtered air exposure with intermittent exercise.

	Time	NO_2	Air	P Values	
				Time	Exposure
Haemoglobin (g/L)	Pre exposure	148±8	146±7	***0.012****	0.284
	4 hours	142±8	143±7		
	6 hours	139±7	137±7		
White cell count ($x10^9$/L)	Pre exposure	4.8±0.9	4.9±1.5	0.243	0.434
	4 hours	5.6±1.5	5.7±1.6		
	6 hours	6.2±2.1	5.7±1.5		
Platelet count ($x10^9$/L)	Pre exposure	209±44	224±53	0.836	0.237
	4 hours	214±55	226±50		
	6 hours	209±46	209±45		
Neutrophils ($x10^9$/L)	Pre exposure	2.37±0.75	2.34±1.14	0.158	0.296
	4 hours	3.14±1.10	3.15±1.21		
	6 hours	3.57±1.78	3.00±1.22		
Lymphocytes ($x10^9$/L)	Pre exposure	1.89±0.34	1.91±0.47	0.623	0.676
	4 hours	1.91±0.55	1.92±0.44		
	6 hours	2.12±0.54	2.04±0.48		
Monocytes ($x10^9$/L)	Pre exposure	0.39±0.11	0.40±0.10	0.669	0.965
	4 hours	0.41±0.15	0.43±0.13		
	6 hours	0.45±0.16	0.43±0.12		

Data shown as mean ± standard deviation. P values shown from 2-way ANOVA with repeated measures showing effect of time and exposure.

3.5 Discussion

We have demonstrated for the first time that direct exposure to nitrogen dioxide in isolation is not associated with vascular vasomotor or fibrinolytic dysfunction. This suggests there are components other than nitrogen dioxide that are responsible for the previously documented adverse cardiovascular effects of air pollution.

3.5.1 Vascular Effects

In this study we have employed a robust and well-validated technique to assess vascular endothelial function following a short-term exposure to nitrogen dioxide. Exposure to nitrogen dioxide, a major component of combustion-derived air pollution, has been linked to cardiovascular morbidity and mortality in epidemiological studies. Why then did we not observe adverse vascular effects? Many workers have attributed the epidemiological link with nitrogen dioxide to a bystander association or epi-phenomenon rather than a casual relationship. Nitrogen dioxide is a co-pollutant that correlates tightly with fine and ultrafine particulate matter [Sarnat, *et al.* 2001]. Our current and previous findings are in agreement with this hypothesis. Using controlled exposures to dilute diesel exhaust, we have previously demonstrated marked adverse effects on vascular endothelial function [Mills, *et al.* 2005] that are present 2 and 6 hours after the exposure but have largely resolved by 24 hours [Törnqvist, *et al.* 2007]. In the present study, performed 4 hours after the exposure and well within the time

window of the previously demonstrated adverse vascular effects, we aimed to separate the effects of the major gaseous co-pollutant, nitrogen dioxide, by using a pure gaseous exposure.

It is important to note that the nitrogen dioxide concentration employed in this study has been previously demonstrated to cause airway inflammatory responses [Blomberg, *et al.* 1997; Sandstrom, *et al.* 1991]. In homes with gas stoves and in certain industries, nitrogen dioxide concentrations may peak at 1-2 ppm [Samet, *et al.* 1987]. Alongside busy roads, nitrogen dioxide concentrations may reach 0.6 ppm [WHO, 1999]. Whilst the concentration of nitrogen dioxide chosen for this study was matched to the overall concentration of oxides of nitrogen from previous diesel exhaust exposure studies [Mills, *et al.* 2005; Salvi, *et al.* 1999], in these studies nitrogen dioxide concentrations reached an average of 1.6 ppm. Therefore, even at levels above those encountered in dilute diesel exposures, when pronounced adverse vascular effects were demonstrated, we have clearly demonstrated that short-term exposure to pure nitrogen dioxide is not associated with vascular endothelial dysfunction in healthy young male subjects, although we cannot rule out a small effect in women or elderly people with co-morbidities who may be more sensitive to environmental pollutants.

Nitrogen dioxide is a powerful oxidising species, and we have demonstrated that its inhalation is associated with mild airway inflammation and changes

in airway antioxidant responses [Blomberg, *et al.* 1997; Kelly, *et al.* 1996]. Moreover, after exposure to nitrogen dioxide in *in vitro* studies, porcine pulmonary artery and aortic endothelial cells have been shown to suffer a significant oxidant injury, with lipid peroxidation and impaired cell membrane function [Patel and Block 1986]. We have suggested that the vascular effects of air pollution exposure are mediated by oxidative stress [Miller, *et al.* 2008], so why has inhalation of nitrogen dioxide failed to have an effect on vascular function? Nitrogen dioxide is relatively insoluble, and does not easily diffuse across to the bloodstream from the lungs. In fact, its penetration of the alveolar space is further inhibited by the normal respiratory tract lining fluid [Postlethwait, *et al.* 1991]. Therefore it is likely that inhaled nitrogen dioxide is confined mainly to the lungs where it exerts a localised and specific pulmonary inflammatory stimulus [Blomberg, *et al.* 1997], without any significant vascular or systemic oxidative insult.

In light of these findings, we suggest that the vascular endothelial effects we have previously demonstrated following exposure to diesel exhaust are driven by exposure to fine and ultrafine particulate matter, rather than any gaseous component.

3.5.2 Thrombotic effects

Thrombosis plays a central role in coronary heart disease. Formation of thrombus on disrupted atherosclerotic plaques may cause acute vessel

occlusion, and acute coronary syndromes. Exposure to air pollution is proposed to be procoagulant. Exposure to ambient nitrogen dioxide and carbon monoxide has been associated with increased plasma fibrinogen [Pekkanen, *et al.* 2000] and a reduced prothrombin time [Baccarelli, *et al.* 2007], and ambient sulphur dioxide with plasma viscosity [Peters, *et al.* 1997], but again the question of whether exposure to nitrogen dioxide and carbon monoxide is a surrogate for exposure to combustion-derived particulate matter arises. We have previously demonstrated that short term exposure to dilute diesel exhaust results in increased platelet activation and enhanced thrombus formation [Lucking, *et al.* 2008], and at the same time impairs vascular release of tissue-plasminogen activator (t-PA), an endogenous fibrinolytic enzyme [Mills, *et al.* 2005] responsible for local dissolution of formed blood clot.

In this study we have not shown impaired release of tissue-plasminogen activator, or any increase in its endogenous inhibitor plasminogen-activator inhibitor type 1 (PAI-1), following exposure to nitrogen dioxide. Therefore we propose that the previously demonstrated procoagulant and antifibrinolytic effects of air pollution exposure are primarily driven by exposure to fine and ultrafine particulate matter rather than the gaseous co-pollutants.

3.5.3 Pulmonary Effects

Our study demonstrated no change in exhaled nitric oxide (FE_{NO}) or simple spirometry following exposure to nitrogen dioxide. Spirometry provides a basic measure of lung function, assessing airway restriction and obstruction and forms a critical part of the diagnosis of chronic lung conditions such as asthma and chronic obstructive pulmonary disease. Small effects on lung function have been demonstrated in patients with asthma following exposure to inhaled ambient air pollution [McCreanor, *et al.* 2007]. In light of this, we chose to assess changes in airway reactivity using simple spirometry in this group of healthy volunteers. Our study findings of no change in spirometry indices are in concordance with similar studies performed after exposure to diesel exhaust in healthy volunteers [Nightingale, *et al.* 2000].

Exhaled nitric oxide is proposed as a marker of airway inflammation, especially in asthmatic patients [ATS/ERS, 2005], and is closely correlated with eosinophilic inflammation [Lim and Mottram 2008]. As a marker it is helpful in obtaining a diagnosis of asthma [Dupont, *et al.* 2003] and may be used to track response to treatment [Yates, *et al.* 1995]. Whilst FE_{NO} has been used in other airway conditions, such as chronic obstructive pulmonary disease, interstitial lung diseases and allergic rhinitis [ATS/ERS, 2005], its usefulness as a marker of airway inflammation in healthy volunteers is unclear. Although exposure to the strong oxidative air pollutant ozone does not affect exhaled nitric oxide concentrations at an ozone dose known to

induce a pronounced neutrophilic airway inflammation [Olin, *et al.* 2001], we have recently demonstrated a significant increase in FE_{NO} following a 1-hour exposure to dilute diesel exhaust in young healthy volunteers [Barath, *et al.* 2007] and for this reason we chose FE_{NO} as a marker of airway inflammation in this study.

In healthy volunteers we have previously demonstrated a mild airway inflammatory response, with increases in interleukin-8 (IL-8) concentrations in bronchial washings 90 minutes after a 4-hour exposure to nitrogen dioxide (2 ppm). At 6 hours after the exposure we demonstrated increased IL-8 concentrations and neutrophil counts within bronchial washings but no signs of inflammatory cell recruitment into the endobronchial mucosa [Blomberg, *et al.* 1997]. We therefore believe that our measurements of FE_{NO} were performed within the established timeframe of an early airway inflammatory response.

Allowing for the limitations of FE_{NO} as a sensitive marker of airway inflammation as a result of air pollution exposure, we suggest that our study provides no strong evidence for an early marked airway inflammatory response following short-term nitrogen dioxide exposure. However, we also acknowledge that measurements of FE_{NO} can be highly variable [Olin, *et al.* 2001], and therefore that our small study may be underpowered to detect a clinically significant change. We therefore suggest that further investigation

into the ability of short-term high-dose nitrogen dioxide to cause an inflammatory response is warranted.

3.5.4 Conclusions

Using a robust controlled study design we have demonstrated for the first time that exposure to nitrogen dioxide in isolation is not associated with any vascular vasomotor or fibrinolytic dysfunction. The adverse cardiovascular effects of combustion-derived air pollution appear to be mediated via components other than nitrogen dioxide, and it is plausible that these vascular effects are rather driven by the fine and ultrafine particle fractions. It remains possible that nitrogen dioxide is a synergistic factor, and is required in addition to the presence of particles to result in the previously demonstrated vascular effects.

CHAPTER 4

Contribution of endothelin-1 to the vascular effects of diesel exhaust inhalation

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4.1 SUMMARY

Diesel exhaust inhalation impairs vascular function and, although the underlying mechanism remains unclear, endothelin-1 and nitric oxide are potential mediators. The aim of this study was to identify whether diesel exhaust inhalation affects the vascular actions of endothelin-1 in man.

In a randomised double-blind cross-over study, 13 healthy male volunteers were exposed to either filtered air or dilute diesel exhaust (331±13 $\mu g/m^3$). Plasma concentrations of endothelin-1 and big-endothelin-1 were determined at baseline, and throughout the 24-hour study period. Bilateral forearm blood flow was measured 2 hours after the exposure during infusion of either endothelin-1 (5 pmol/min) or the endothelin A receptor antagonist, BQ-123 (10 nmol/min) alone and in combination with the endothelin B receptor antagonist, BQ-788 (1 nmol/min).

Diesel exhaust exposure had no effect on plasma endothelin-1 and big-endothelin-1 concentrations ($P>0.05$ for both), or 24-hour mean blood pressure or heart rate ($P>0.05$ for all). Endothelin-1 infusion increased plasma endothelin-1 concentrations by 58% ($P<0.01$) but caused vasoconstriction only following diesel exhaust exposure (-17% *versus* 2% after air; $P<0.001$). In contrast, diesel exhaust exposure reduced vasodilatation to isolated BQ-123 infusion (20% *versus* 59% after air; $P<0.001$)

but had no effect on vasodilatation to combined BQ-123 and BQ-788 administration ($P>0.05$).

Diesel exhaust inhalation increases vascular sensitivity to ET-1 and reduces vasodilatation to ET_A receptor antagonism despite unchanged plasma ET-1 concentrations. Given the tonic interaction between the endothelin and nitric oxide systems, we conclude that diesel exhaust inhalation alters vascular reactivity to endothelin-1 probably through its effects on nitric oxide bioavailability.

4.2 INTRODUCTION

Exposure to combustion-derived fine particulate air pollution is a recognised risk factor for cardiorespiratory mortality and morbidity [Dockery, *et al.* 1993; Miller, *et al.* 2007]. There is a strong relationship between acute exposure to traffic-derived particulate matter and the incidence of acute myocardial infarction [Peters, *et al.* 2004] and hospital readmission in survivors of myocardial infarction [von Klot, *et al.* 2005]. The WHO estimate that annually around 3 million deaths worldwide can be attributable to air pollution [UNEP, 2006].

Recent controlled exposure studies have demonstrated that inhalation of concentrated ambient particles and ozone causes acute arterial vasoconstriction 2 hours after the exposure [Brook, *et al.* 2002]. Inhalation of diesel exhaust, a major component of fine particulate air pollution in urban environments, impairs vasomotor function and endogenous fibrinolysis [Mills, *et al.* 2005]. The fundamental mechanisms underlying these detrimental vascular endothelial effects remain poorly understood. Furthermore, the exact components of air pollution responsible for these effects have not been defined, although it is proposed that airborne particulate matter is likely to be the major arbiter [Miller, *et al.* 2007].

Endothelin-1 (ET-1), an endogenous vasoconstrictor 100-fold more potent that norepinephrine [Levin 1995], is a 21-amino acid peptide produced by the

vascular endothelium in response to stress. It is produced initially as preproendothelin-1, which is processed to form big ET-1, before being cleaved by endothelin converting enzyme (ECE) into ET-1. The actions of ET-1 are mediated by two G-protein coupled receptors; the ET_A and ET_B receptors. Stimulation of either the ET_A or ET_B receptor causes vasoconstriction, although the ET_B receptor is also expressed on endothelial cells where it releases nitric oxide. The endothelin system plays a major role in cardiovascular and renal physiology [Goddard, *et al.* 2004], and although its actions are complex, ET-1 contributes to the maintenance of basal vascular tone and blood pressure in man [Haynes and Webb 1994; Haynes, *et al.* 1996].

Recent work has suggested that plasma ET-1 concentrations are increased by exposure to air pollution. Rats raised with daily exposure to diesel exhaust particles and urban particulate matter have increased blood pressure, plasma ET-1 concentrations [Vincent, *et al.* 2001], and ET-1 expression in cardiac tissue [Ito, *et al.* 2008]. In children from Mexico City, plasma ET-1 concentrations correlated with the degree of air pollution exposure [Calderón-Garcidueñas, *et al.* 2007]. Peretz *et al* recently demonstrated elevated plasma ET-1 concentrations in a heterogeneous population of healthy volunteers and patients with the metabolic syndrome, 3 hours after a controlled 2-hour resting exposure to diesel exhaust [Peretz, *et al.* 2008].

The aims of this study were to assess the effect of diesel exhaust inhalation on plasma ET-1 and big-ET-1 concentrations, ET-1 mediated vasoconstriction, and the contribution of ET-1 to basal vascular tone

4.3 Methods

4.3.1 Subjects

Fifteen healthy male volunteers were recruited between February and March 2008 at the University Hospital, Umeå, Sweden. All subjects had normal lung function, were non-smokers and took no regular medication. Those with a significant occupational exposure to air pollution and those with an intercurrent illness were excluded. The trial was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of each participant.

4.3.2 Study design

Subjects attended for two consecutive days on four occasions at least one week apart. In a double-blind randomised cross-over study, subjects were exposed to either filtered air or dilute diesel exhaust at 300 $\mu g/m^3$ for 1 hour in a specially built diesel exposure chamber as described previously [Salvi, *et al.* 1999]. During the exposure, subjects performed 15-min periods of exercise on a bicycle ergometer (minute ventilation of 20 $L/min/m^2$) alternated with 15-min periods of rest.

Based on previous studies [Mills, *et al.* 2005], vascular assessments and intra-arterial infusions were commenced 2 hours after the exposure. All subjects abstained from alcohol for 24 hours, caffeine-containing drinks for at least 8

hours and fasted for at least 4 hours before commencement of the vascular study. All subjects remained indoors at rest between the exposure and the vascular assessment to minimise additional exposure to air pollution.

A validated ambulatory blood pressure monitor (Model 90217, Spacelabs Healthcare, Washington, USA) was applied to the right arm 2 hours prior to the start of the exposure, and monitoring was continued for a total of 24 hours.

4.3.3 Diesel exposure

Diesel exhaust emissions were generated using an idling Volvo (Volvo TD45, 4.5L, 4-cylinders, 680 rpm) diesel engine. Over 90% of the exhaust was shunted away, and the remaining part diluted with air and fed into the exposure chamber at steady state concentration. During the exposure, air was sampled in the breathing zone of the subjects and monitored for nitrogen oxides, particle number and total hydrocarbons (measured as propane). Filter samples were collected and analysed for mass concentration. The exposures were standardised by keeping the particle (diameter <10 μm) mass concentration at ~300 $\mu g/m^3$. Temperature and humidity in the chamber were controlled at 22˚C and 50% respectively.

4.3.4 Vascular studies

All subjects underwent brachial artery cannulation in the non-dominant arm using a 27-gauge steel needle under controlled conditions. After a 30-min baseline infusion of 0.9% saline, subjects received either a 60-min infusion of endothelin-1 (American Peptide, CA, USA) at 5 pmol/min [Haynes, *et al.* 1991] or infusion of BQ-123 (an ET_A receptor antagonist, American Peptide) at 10 nmol/min for 60 min [Verhaar, *et al.* 1998] followed by co-infusion of BQ-123 (10 nmol/min) and BQ-788 (an ET_B receptor antagonist, American Peptide; 1 nmol/min) [Ishikawa, *et al.* 1994; Strachan, *et al.* 2000] for a further 60 min.

Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography with mercury-in-silicone elastomer strain gauges as described previously [Wilkinson and Webb 2001]. Supine heart rate and blood pressure were determined in the non-infused arm at intervals throughout the study using the ambulatory blood pressure monitor.

Venous cannulae (17-gauge) were inserted into large subcutaneous veins in the antecubital fossae of both arms. Blood (10 mL) was drawn simultaneously from each arm at the end of the baseline saline infusion, and at the end of each 60-min infusion period. Blood samples were also obtained

by separate venepuncture at baseline, immediately, 6 and 24 hours after the exposure.

4.3.5 Biochemical analyses

Blood samples taken at baseline, 6 and 24 hours after the exposure were analysed for total and differential cell counts by an autoanalyser. Plasma samples were collected into ethylene diamine tetra-acetic acid and kept on ice until centrifuged at 3000 rpm for 30 min. Plasma samples were immediately frozen and stored at -80 °C. Plasma ET-1 and big-ET-1 concentrations were measured according to an acetic acid extraction technique using a commercial radioimmunoassay with rabbit anti-human ET-1 or big-ET-1 (Peninsular Laboratories Europe Ltd, St Helens, UK) as described previously [Adam, *et al.* 2001].

4.3.6 Data and statistical analysis

Nursing staff at the clinical research facility at University Hospital, Umeå, Sweden, randomised exposure type and the associated vascular study protocol. The investigators were blinded to the exposure received. Plethysmography data were analysed as described previously [Newby, *et al.* 1999]. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical analyses were performed using paired Student's *t*-tests and two-way analysis-of-variance (ANOVA) with repeated measures including time and exposure as variables where appropriate. Statistical

significance was taken at the 5% level. All analyses were performed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA) on a Macintosh personal computer.

4.4 Results

Thirteen subjects, median age 23 years (Table 4.1), completed the study (Figure 4.1). The diesel exposure generated a mean particle mass concentration of 331 ± 13 μg/m^3 and this was associated with concentrations of NO_2 of 1.0 ± 0.04 ppm, NO of 3.3 ± 0.13 ppm and total hydrocarbons of 1.4 ± 0.04 ppm. There were no differences in 24-hour mean systolic or diastolic blood pressure or mean heart rate after exposure to air or diesel exhaust (P>0.05 for all) although there was a slightly lower nocturnal diastolic blood pressure following diesel exhaust exposure (62 ± 1 *vs.* 65 ± 1 mmHg; P<0.01). There was a rise in blood pressure following exercise on both study visits although there was no difference between the two exposures (data not shown, P>0.05).

Table 4.1. Baseline characteristics of the 13 subjects who completed the study. Mean ± standard error of the mean (SEM) unless otherwise stated.

Parameter	**n=13**
Age, years (median, range)	23 (21-28)
Height, cm	181 ± 2
Weight, kg	79 ± 3
BMI, kg/m^2	24 ± 1
Haemoglobin concentration, g/L	153 ± 3
White cell count, x10^9/L	5.1 ± 0.4
Neutrophil count, x10^9/L	2.5 ± 0.3
Lymphocyte count, x10^9/L	2.0 ± 0.1
Monocyte count, x10^9/L	0.4 ± 0.03
Platelet count, x10^9/L	209 ± 11

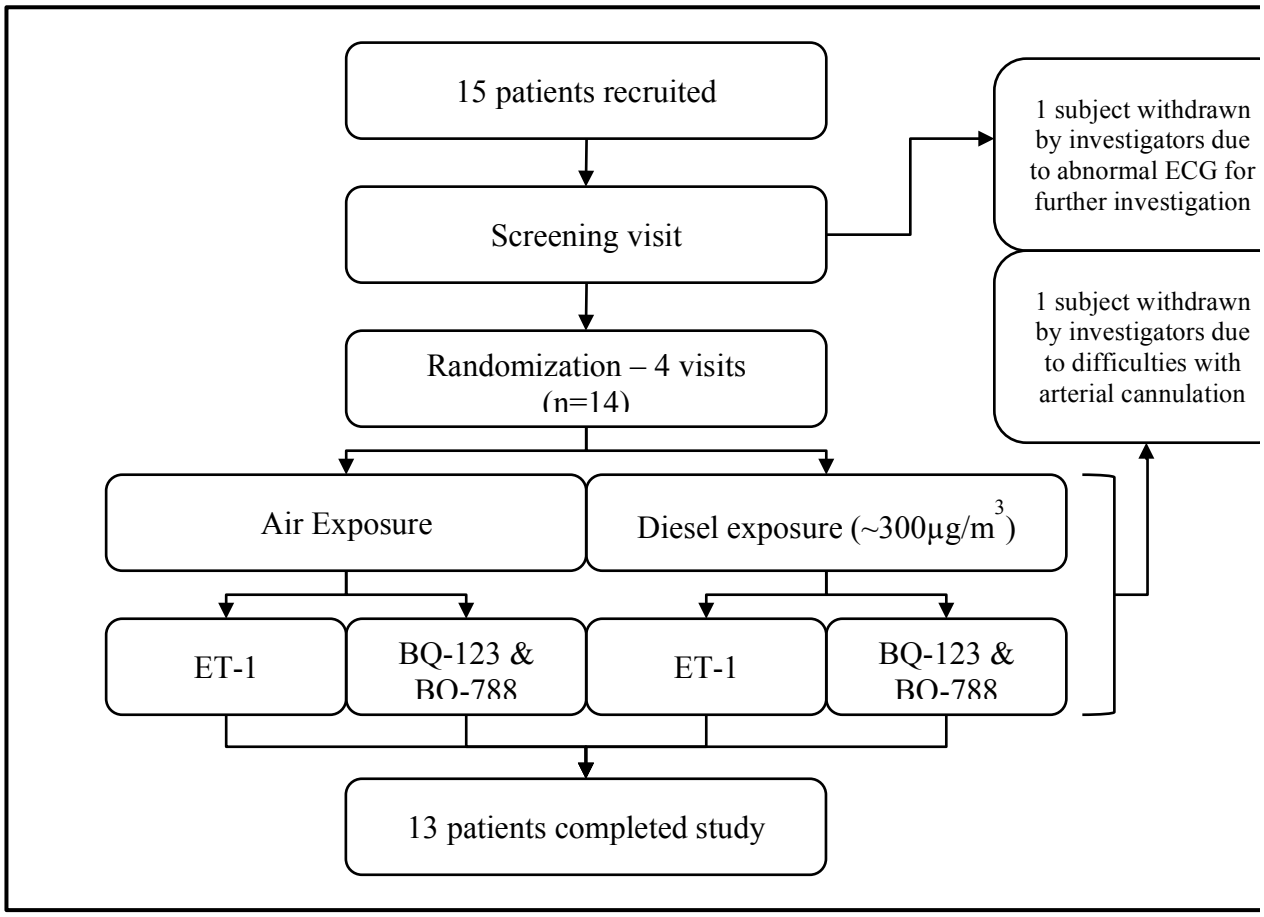


Figure 4.1. Consort flow chart of study participants.

Infusion of ET-1 caused a slow onset vasoconstriction following diesel exhaust inhalation (17 ± 10 % peak reduction in blood flow) although there was little effect following filtered air (Figure 4.2; ANOVA, $P<0.001$ for exposure effect). Infusion of the endothelin receptor antagonists, BQ-123 and BQ-788, caused a slow onset vasodilatation (77 ± 14 % peak increase in blood flow after filtered air; Figure 4.3). This vasodilatation was greater following filtered air compared with the diesel exhaust exposure (Figure 4.3; ANOVA,

P<0.001). The difference was greatest at 60 min but, after infusion of the BQ-788, there was little difference in blood flow by 120 min (P>0.05).

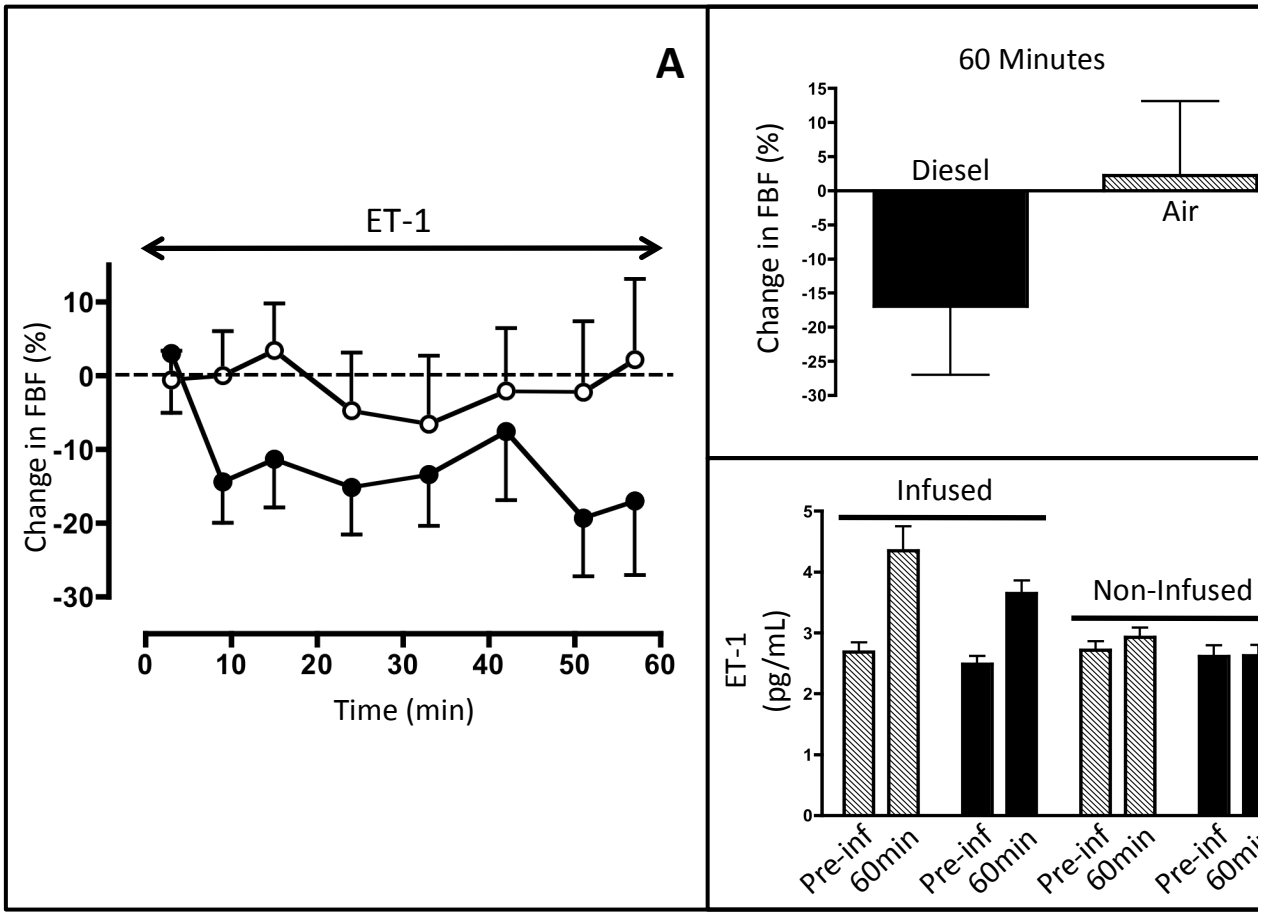


Figure 4.2. (A) Forearm blood flow (FBF) during infusion of ET-1 (5pmol/min) after exposure to air (○) and diesel exhaust (●) (ANOVA, P<0.001). (B) Maximal effect at 60 minutes and (C) comparison of plasma ET-1 concentrations in infused and non-infused arms before and at the end of the forearm vascular study following air (hashed bars; P<0.001 for infused, P>0.05 for non-infused) and diesel exhaust inhalation (solid bars; P<0.0001 for infused, P>0.05 for non-infused). Paired Student's *t*-test of air *vs.* diesel.

Plasma ET-1 and big-ET-1 concentrations were unchanged at all time points following diesel exhaust or filtered air exposure (P>0.05 for both).

Comparison of the infused and non-infused arm plasma ET-1 concentrations confirmed that the ET-1 infusion increased local plasma ET-1 concentrations by 58±9 % (Figure 4.2; P<0.01 for infused arms, and P>0.05 for non-infused arms for both exposures).

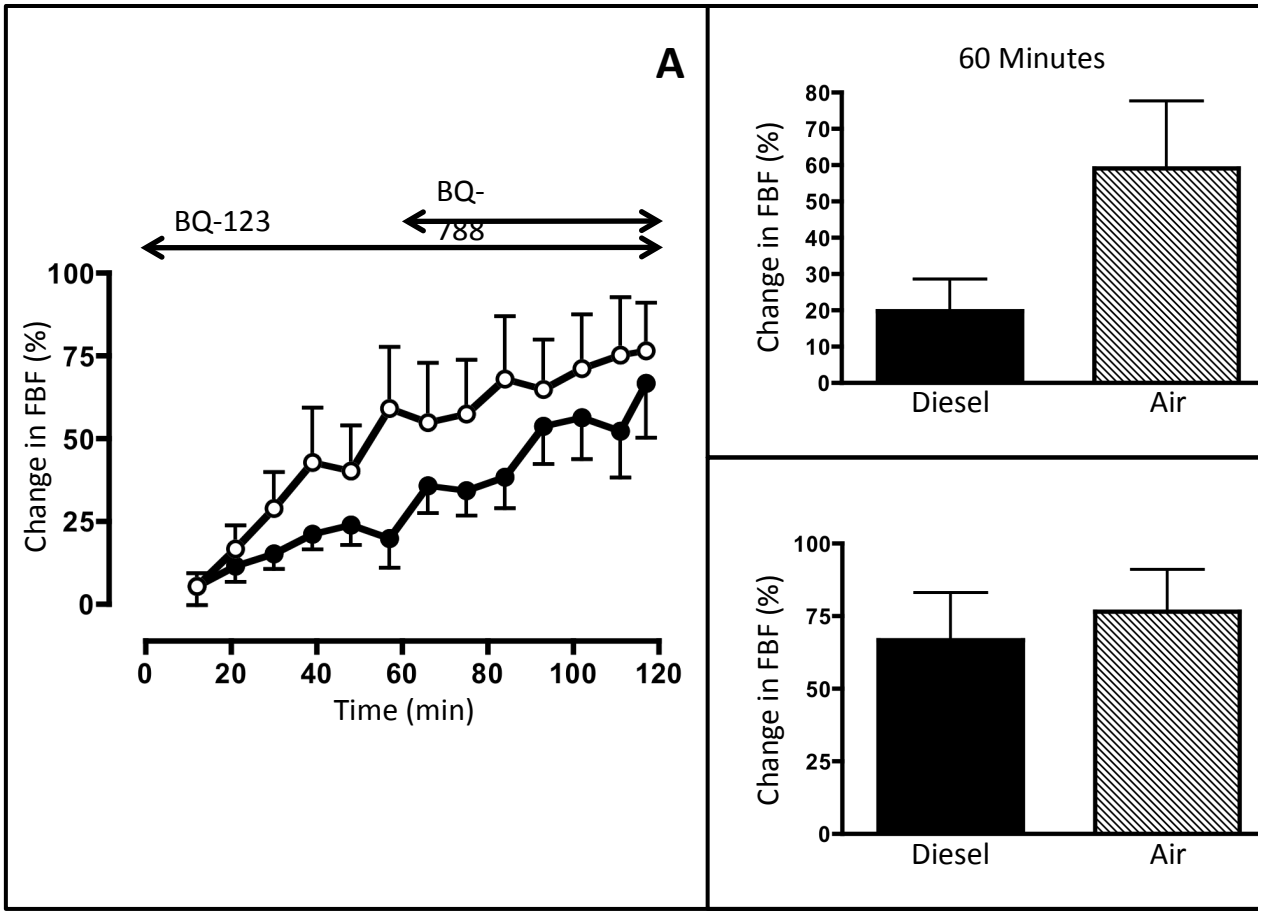


Figure 4.3. (A) Forearm blood flow (FBF) during infusion of BQ-123 (10nmol/min) and BQ-788 (1nmol/min) after exposure to air (○) and diesel exhaust (●) (ANOVA, P<0.001). (B) Maximal effect after BQ-123 infusion alone and (C) after dual endothelin receptor antagonism.

4.5 Discussion

Although diesel exhaust inhalation had no effect on plasma ET-1 or big ET-1 concentrations, there was an increase in vascular sensitivity to ET-1 associated with a reduced ET_A induced vasodilatation. These apparently contradictory findings can be explained by impaired ET-1 induced nitric oxide release and are consistent with pre-clinical evidence of nitric oxide mediated alterations in vascular reactivity to ET-1 [Knuckles, *et al.* 2008]. We conclude that diesel exhaust inhalation, at levels commonly encountered in the urban environment, does not affect plasma ET-1 concentrations but alters vascular reactivity to ET-1 probably through effects on nitric oxide release and bioavailability.

4.5.1 Endothelin-1

We did not demonstrate any change in plasma concentrations of endothelin-1 or its immediate precursor, big-endothelin-1, following exposure to filtered air or diesel exhaust. Whilst this is consistent with our own previous work [Mills, *et al.* 2005], it is at odds with other reports. In rodent studies, plasma ET-1 (and ET-3) concentrations were up regulated following exposure to diesel exhaust and concentrated urban particles [Ito, *et al.* 2008; Vincent, *et al.* 2001]. It is possible that there are species differences in the response to diesel exhaust inhalation, and up regulation of ET-1 in rats may not translate into man. However, Peretz *et al* studied a heterogeneous group of individuals comprising of patients with metabolic syndrome and healthy volunteers

[Peretz, *et al.* 2008], and showed an increase in plasma ET-1 concentrations 3 hours after diesel exhaust exposure. Their study was not designed specifically to look at ET-1 and was limited by missing data, and small numbers in a heterogeneous population in whom vascular endothelial function may not be equivalent [Melikian, *et al.* 2008]. In contrast, our study was specifically designed to address the endothelin hypothesis, and employed a robust cross-over study design, in a homogenous group of healthy volunteers, with samples optimally collected to assess plasma ET-1 and big-ET-1 concentrations [Adam, *et al.* 2001]. Taken together with our previous study, our experience represents the largest sample size to date (n=43). We therefore think it is unlikely that diesel exhaust inhalation causes major changes in plasma endothelin concentrations.

We recognise that plasma ET-1 concentrations may not reflect the activity of the endothelin system since 90% of ET-1 synthesised by the vascular endothelium is secreted abluminally and acts locally on vascular smooth muscle in a paracrine manner [Levin 1995]. Therefore, in addition to measuring plasma ET-1 concentrations, we assessed the effects of endothelin agonism and antagonism on peripheral vascular tone.

4.5.2 Endothelin Agonism

We demonstrated increased vasoconstriction following exposure to diesel exhaust, but little effect following exposure to filtered air, suggesting an

increased vascular sensitivity to ET-1. We were surprised to see little vasoconstriction with ET-1 following exposure to filtered air having previously reported approximately 30-40% reductions in forearm blood flow during infusions of 5 pmol/min [Ferro, *et al.* 1997; Haynes and Webb 1994]. In the present study, we used an alternative preparation of ET-1 and suggest that the disparity in vascular effects is attributable to differing potencies of the preparations. Because of this, we measured plasma ET-1 concentrations in both forearms and demonstrated a selective 60% increase in plasma ET-1 concentrations in the infused arm. Assuming a forearm blood flow of 25 mL/min, we achieved an end-organ concentration approximately a tenth of that anticipated. However, this simple calculation does assume that there is no clearance or extraction of ET-1 across the forearm. However, we do not believe that the modest increase in venous ET-1 concentrations can be solely accounted for by clearance and conclude that it reflects a reduced activity of the infused peptide preparation. Although the reduced activity of ET-1 limits comparisons with other studies, this was perhaps fortuitous since it enabled us to assess the vasoreactivity to ET-1 at the threshold for vasoconstriction, and to observe an alteration in ET-1 sensitivity.

4.5.3 Endothelin A Receptor Antagonism

The reduction in vasodilatation to BQ-123 infusion following diesel exhaust inhalation has several potential explanations. First, this may relate to reduced production or increased clearance of active ET-1 from the

vasculature. However, this seems unlikely given that plasma endothelin-1 and big-endothelin-1 concentrations were unchanged although we acknowledge that an effect on the abluminal release of endothelin-1 cannot be excluded. Second, a reduced sensitivity of the vascular smooth muscle ET_A receptor could have occurred but this is at odds with the increased ET-1 vasoconstriction and is therefore unlikely. We believe that there is third more likely explanation.

Looking closely at ET_A receptor antagonism, it is clear that the mechanism of vasodilatation is complex. This reflects the distribution and basal activity of both the ET_A and ET_B receptors. Both receptors contribute to the maintenance of basal vascular tone but have differing actions and vascular distributions: ET_A receptors are present on vascular smooth muscle cells only and mediate vasoconstriction, whereas ET_B receptors are present on both vascular smooth muscle and endothelial cells where they mediate vasoconstriction and vasodilatation respectively. Moreover, selective ET_A receptor antagonism leads not only to inhibition of the ET_A receptor but potentially hyperstimulation of the ET_B receptor. Indeed, we have demonstrated that BQ-123 induced vasodilatation can be markedly attenuated by concomitant blockade of nitric oxide release [Verhaar, *et al.* 1998] suggesting that selective ET_A receptor antagonism does indeed lead to significant ET_B receptor mediated vasodilatation through endothelial nitric oxide release. Given the central role of nitric oxide in modulating and balancing the effects of the

endothelin system, we propose that changes in the L-arginine-NO pathway offer the most plausible hypothesis for the impaired vasodilatation to BQ-123. This would also explain the enhanced vasoconstriction to ET-1 with a reduction in the opposing vasodilatory actions of nitric oxide. Moreover, we have previously shown that diesel exhaust impairs nitric oxide bioavailability [Miller, *et al.* 2009; Mills, *et al.* 2005] and suggest that the observed effects on ET-1 vasoreactivity can be explained by the reduction in endothelin induced nitric oxide release and bioavailability.

The finding of reduced vasodilatation in response to ET_A receptor blockade is at odds with previous studies demonstrating enhanced response in patients with conditions such as hypertension and hypercholesterolaemia who have pre-existing endothelial dysfunction mediated by reduced nitric oxide bioavailability [Cardillo, *et al.* 2002b; Cardillo, *et al.* 2000]. The reason for this discrepancy is unclear but here we have induced an acute and brief episode of endothelial dysfunction in an otherwise healthy population of volunteers. Chronic dysfunctional states are likely to invoke compensatory mechanisms that may result in important differences in these vascular responses.

4.5.4 Combined Endothelin A and B Receptor Antagonism

Previous data, including our own work [Verhaar, *et al.* 1998], would suggest that combined ET_A/ET_B receptor antagonism should produce less vasodilatation, and have less of an effect on systemic haemodynamics [Leslie, *et al.* 2005], than selective ET_A receptor antagonism. We were therefore surprised to observe the continued further modest vasodilatation when ET_B receptor antagonism was superimposed on ET_A receptor antagonism. We believe that the explanation for this observation is three-fold. First, vasodilatation to endothelin agonism and antagonism is of slow onset and offset. In our own hands, BQ-123 induced vasodilatation appears to reach a peak effect by 60 min [Verhaar, *et al.* 1998] but may take up to 90 min [Haynes and Webb 1994]. The continued vasodilatation may therefore reflect further and more complete ET_A receptor antagonism. Second, we chose this study design to minimise the number of visits given the invasive nature of the studies. We attempted to assess both ET_A receptor antagonism and combined ET_A/ET_B receptor antagonism on the same visit. This approach has been used once before by Cardillo and colleagues [Cardillo, *et al.* 2002a] in a small subgroup of patients with diabetes mellitus. Here, they demonstrated a brisk vasodilatation to BQ-123 of around 65% with maximal vasodilatation by 60 minutes. Importantly, the predicted "tailing-off" of the response when BQ-788 was added did not occur and the vasodilatation plateaued rather than fell. This is consistent with the findings in our study. Finally, there may be an interaction when ET_A receptor antagonism precedes

combined ET_A/ET_B receptor antagonism. This may reflect alterations in ET_B receptor expression on both the endothelium and vascular smooth muscle cells in the face of ET_A receptor antagonism. Indeed, there is considerable cross-talk between the receptors as we have previously described [Mickley, *et al.* 1997]. Thus the differing profile of responses may reflect the dynamic interaction of the two receptors over the course of the study.

This altered profile of vasodilatation does not detract from the comparison between the filtered air and diesel exhaust exposure. Combined ET_A and ET_B receptor antagonism appears to be unaffected by diesel exhaust exposure whereas selective ET_A receptor antagonism is impaired. This is likely to reflect the greater and marked dependence of ET_A receptor antagonism on nitric oxide release in comparison to combined ET_A and ET_B receptor antagonism.

4.5.5 Conclusions

Our data demonstrate that the previously documented impairment of endothelium-dependent vasodilatation following a one-hour exposure to combustion-derived air pollutants is not mediated by an upregulation of the endothelin system. Furthermore we have shown that diesel exhaust inhalation has no effect on plasma ET-1 concentrations or systemic blood pressure. Our data are consistent with the hypothesis that the diesel exhaust-

induced vascular effects are predominantly driven by reduced endothelial nitric oxide bioavailability. However, we cannot exclude a role for other vasoactive mediators, such endothelium-derived hyperpolarising factor, and further studies are warranted to investigate the L-arginine:nitric oxide and other pathways in more detail.

4.5.6 Perspectives

Air pollution exposure is associated with increased cardiovascular morbidity and mortality, and is thought to lead to around 3 million deaths worldwide each year. Understanding the underlying mechanism for these detrimental effects is crucial in trying to reduce this significant disease burden. In this study, we show that the well-established adverse vascular endothelial effects demonstrated following inhalation of diesel exhaust are not directly mediated through the endothelin system. We propose instead that these may be driven by changes in nitric oxide bioavailability. Further studies are warranted to investigate this hypothesis in more detail.

CHAPTER 5

Altered nitric oxide bioavailability contributes to diesel exhaust inhalation-induced vascular dysfunction in man

Under submission for publication by Langrish JP, Unosson J, Bosson J, Barath S, Muala A, Blackwell S, Söderberg S, Pourazar J, Megson IL, Treweeke A, Sandström T, Newby DE, Blomberg A, Mills NL. Altered nitric oxide bioavailability contributes to diesel exhaust inhalation-induced cardiovascular dysfunction in man. *J Am Heart Ass* 2012;***under submission***.

5.1 SUMMARY

Diesel exhaust inhalation causes cardiovascular dysfunction including impaired vascular reactivity, increased blood pressure and arterial stiffness. We investigated the role of nitric oxide (NO) bioavailability in mediating these effects.

In a randomized double-blind crossover study, healthy non-smokers were exposed to diesel exhaust or filtered air. Bilateral forearm blood flow was measured during intrabrachial infusions of acetylcholine (ACh; 5-20 μg/min) and sodium nitroprusside (SNP; 2-8 μg/min) in the presence of the NO clamp (NO synthase inhibitor N^G-monomethyl-L-arginine, L-NMMA, 8 μg/min co-infused with the NO donor SNP at 90-540 ng/min to restore basal blood flow).

Following diesel exhaust inhalation, plasma nitrite concentrations were increased (68±48 *vs* 41±32 nM; P=0.006) despite similar L-NMMA-induced reductions in basal blood flow (-20.6±14.7 *vs* -21.1±14.6 %; P=0.559) compared to air. In the presence of the NO clamp, ACh and SNP caused dose-dependent vasodilatation that was not affected by diesel exhaust inhalation (P>0.05 for both).

Diesel exhaust inhalation disturbs normal vascular homeostasis with enhanced NO generation unable to compensate for excess consumption. We

suggest the adverse cardiovascular effects of air pollution are, in part, mediated through reduced NO bioavailability.

5.2 Introduction

Exposure to air pollution increases cardiovascular morbidity and mortality [Anderson, *et al.* 1996; Dockery, *et al.* 1993; Miller, *et al.* 2007; Pope, *et al.* 2002], and is associated with the triggering of acute myocardial infarction [Peters, *et al.* 2004]. Recent controlled exposure studies in man have shown that inhalation of dilute diesel exhaust, at environmentally relevant concentrations, leads to an impairment of both vascular vasomotor and fibrinolytic function [Mills, *et al.* 2005]. Similarly, exposure to concentrated ambient particles causes acute arterial vasoconstriction [Brook, *et al.* 2002] and an increase in diastolic blood pressure [Urch, *et al.* 2005]. Although providing plausible explanations for the increase in cardiovascular events [Heitzer, *et al.* 2001], the mechanisms of how air pollution induces these cardiovascular abnormalities remain to be established.

Inhalation of fine particulate matter causes inflammation within the lungs [Nightingale, *et al.* 2000; Salvi, *et al.* 1999] in part through local oxidative stress [Baulig, *et al.* 2003; Behndig, *et al.* 2006]. Vascular oxidative stress may subsequently develop as a consequence of the systemic response to pulmonary inflammation and oxidative stress, or through reactive oxygen species generation within the vascular wall following translocation of particles into the circulation [Nemmar, *et al.* 2002; Nemmar, *et al.* 2004]. Irrespective of the site of generation, it has been proposed that this oxidative stress has a direct influence on the function of the vascular endothelium

[Lum, *et al.* 2001] as well as reducing the vascular bioavailability of nitric oxide (NO) [Ikeda, *et al*. 1998; Mills, *et al*. 2005].

NO is a powerful endogenous vasodilator and plays a crucial role in the maintenance of basal vascular tone [Moncada, *et al.* 1993]. In patients with pre-existing vascular endothelial dysfunction, such as those with hypercholesterolemia, stimulated endothelium-dependent NO-mediated vasodilatation is impaired, and this is thought to be mediated through oxidative stress-mediated depression of NO bioavailability [Verma, *et al.* 2002]. In rats exposed to inhaled particles, the generation of oxygen-centred free radicals leads to the scavenging of NO, thereby reducing its bioavailability [Nurkiewicz, *et al.* 2009].

In this study, we investigated the hypothesis that changes in NO bioavailability contribute to the cardiovascular dysfunction induced by diesel exhaust inhalation.

5.3 Methods

5.3.1 Subjects

Eighteen healthy non-smoking volunteers were recruited from the Department of Medicine, Division of Respiratory Medicine and Allergy, University Hospital, Umeå. Subjects taking regular medication (except the oral contraceptive pill), with a significant occupational exposure to air pollution or those with intercurrent illness were excluded. All subjects were free of symptoms of respiratory tract infection for at least 6 weeks before the study. The trial was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of each participant.

5.3.2 Diesel exhaust exposure

Diesel exhaust was generated using an idling Volvo diesel engine (Volvo TD40 GJE, 4.0 L, four cylinders, 680 rpm). Over 90 % of the exhaust was shunted away, the remaining part diluted with HEPA filtered air and fed into the exposure chamber at steady state concentration. During the exposure, air was sampled in the breathing zone of the subjects for oxides of nitrogen, particle mass concentration and total hydrocarbons (measured as propane). Filter samples were collected and analysed gravimetrically to determine mass concentration. The exposures were standardised based on particulate matter (particles with a mean aerodynamic diameter ≤10 μm [PM_{10}]) concentrations and a mean airborne particle concentration of 313 ± 11

μg/m^3 was generated. The particle exposure was associated with concentrations of nitrogen dioxide (NO_2) of 0.9 ± 0.1 ppm, NO of 3.1 ± 0.2 ppm and total hydrocarbons of 2.5 ± 0.2 ppm. Temperature and humidity in the chamber were controlled at 22°C and 50% respectively.

5.3.3 Study design

Subjects attended on two occasions at least one week apart. In a randomized double-blind crossover study, subjects were exposed to either filtered air or dilute diesel exhaust at ~300 μg/m^3 in a specially built diesel exposure chamber for 1 hour as described previously [Rudell, *et al.* 1996]. During the exposures, subjects performed 15-min periods of exercise on a bicycle ergometer, to achieve a mean minute ventilation of 20 L/min/m^2 as determined by a pre-study exercise test, alternating with 15 min of rest.

Based on previous studies, vascular assessments were commenced 2 h after the exposure [Langrish, *et al.* 2009; Mills, *et al.* 2005]. All subjects abstained from alcohol for 24 h, caffeinated drinks for 8 h and food for 4 h prior to the cardiovascular assessments. All subjects remained indoors between the exposure and assessments to minimise additional exposure to air pollution. Venous blood samples were collected for measurement of plasma nitrite concentration, endogenous NOS inhibitors and total and differential cell counts. Lung function was determined by spirometry at baseline and 2 h following the exposure (Jaeger Masterlab, Jaeger AG, Würzburg, Germany)

recording forced vital capacity (FVC), forced expiratory volume in one second (FEV_1) and slow vital capacity (VC).

5.3.4 Vascular assessments

Forearm blood flow was determined using forearm venous occlusion plethysmography and detected using mercury-in-silastic strain gauges placed around each forearm as described previously [Wilkinson, *et al.* 2001]. All subjects underwent unilateral brachial artery cannulation using a 27-gauge steel needle (Coopers Needle Works Ltd., Birmingham, UK) under controlled conditions. After a 30-min baseline infusion of 0.9% saline, subjects received an infusion of the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA; Clinalfa® basic, Bachem, Weil an Rhein, Germany) at incremental doses of 2, 4 and 8 μmol/min. The infusion of L-NMMA was continued at 8 μmol/min for the remainder of the study to fully inhibit all basal NOS activity [Japp, *et al.* 2008; Stroes, *et al.* 1997]. Sodium nitroprusside (SNP), an NO donor, was then co-infused with L-NMMA at doses of 90 to 540 ng/min and titrated to restore basal blood flow. Once basal blood flow was restored, the SNP infusion was continued for the remainder of the study to produce an "NO clamp" as described previously [Japp, *et al.* 2008]. This permits the assessment of vascular function in the absence of endogenous NO generation but without the potential confounding effects of basal vasoconstriction induced by isolated NO synthase inhibition.

The dose-response relationship of the vasodilators acetylcholine (5, 10 and 20 μg/min [Mills, *et al.* 2005]; endothelium-dependent NO donor) and SNP (2, 4 and 8 μg/min [Mills, *et al.* 2005]; endothelium-independent NO donor) were assessed in the presence of the clamp. The vasodilators were given in a random order and were separated by an infusion of 0.9% saline for 20 min. The combined total infusion rate of the parallel infusions was kept constant at 1 mL/min throughout the study.

5.3.5 Biochemical analyses

Blood samples were analysed for total and differential cell counts by an auto analyser (Sysmex XE2100, Sysmex Europe GmbH, Hamburg, Germany). Plasma samples were collected into ethylene diamine tetra-acetic acid and kept on ice until centrifuged at 2000 *g* for 30 min at 4°C. Serum samples were collected and left to clot on melting ice for 60 min before being centrifuged at 2000 *g* for 10 min at 4°C. Samples were immediately frozen and stored at -80°C prior to subsequent analysis.

Plasma was analysed for the endogenous NOS inhibitors asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) as well as the NO precursors L-arginine and L-homoarginine using a high performance liquid chromatography (HPLC) method as described previously [Blackwell, *et al.* 2009]. Briefly, arginine, homoarginine, ADMA

and SDMA were extracted from plasma using Isolute PRS cation exchange solid phase extraction columns (1 mL/50 mg; Kinesis Ltd., Epping, UK). The columns were consecutively washed with borate buffer (1 mL), water (3 mL) and methanol (3 mL) before analytes were eluted with 3 mL of a solution containing 50% methanol and 10% concentrated ammonia in water. The eluent was then evaporated to dryness at 80^{o}C under air. The dried extract was then dissolved in 0.1 mL water and 0.1 mL of the derivization reagent (10 mg of ortho-phthaldialdehyde dissolved in 0.2 mL methanol followed by the addition of 1.8 mL of 200 nM borate buffer and 10 µL of 3-mercaptopropionic acid, then diluted 5-fold using 200 nM borate buffer immediately before use) before being transferred to autosampler vials, maintained at 10^{o}C, and 20 µL was injected into the HPLC analytical column for chromatography. Following separation, arginine, homoarginine, ADMA and SDMA were measured fluorimetrically (Waters 2475, Waters, Watford UK) and quantified using single level calibration using an internal standard (80 µL of 5 µM monoethylarginine) added before sample extraction.

Blood samples obtained for the measurement of plasma nitrite were collected into lithium heparin, immediately transferred to Eppendorf® tubes prewashed with Milli-Q de-ionised water and immediately centrifuged at 5000 *g* for 1 min. Plasma was then transferred into a dark coloured Eppendorf® tube containing 100 µL of a solution containing 1 mM

diethylenetriamine-pentaacetic acid and 62.5 mM N-ethylmaleimide before being snap-frozen on dry ice and stored at -80^{o}C prior to further analysis.

Plasma nitrite samples were defrosted on ice before 100 μL was injected into a reaction vial containing glacial acetic acid and iodide [Nagababu, *et al.* 2007]. NO generated in the reaction chamber was driven off by a continual stream of oxygen-free nitrogen and detected in the exhaust gas using a Sievers NOA 280i chemiluminescent analyzer (Analytix, Co Durham, UK). Analyses were performed in triplicate. The limit of detection of this assay was ~30 nM with a co-efficient of variation of 3.1% for a 250 nM standard.

5.3.6 Data analysis and statistics

Plethysmography data were analysed as described previously [Newby, *et al.* 1999; Petrie, *et al.* 1998]. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. Statistical analyses were performed using Student's paired *t*-tests and 2-way analysis-of-variance (ANOVA) with repeated measures where appropriate. Statistical significance was taken as two-sided $P<0.05$. All analyses were performed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA) on a Macintosh personal computer.

5.4 Results

Sixteen subjects, median age 23 years (Table 5.1), completed the study protocol: one subject failed to attend scheduled visits and one was withdrawn for technical reasons (failed cannulation). The study was well tolerated with no significant adverse events.

There were no changes in any indices of lung function (FEV_1, FVC and VC), although the study was powered for the vascular outcomes and as such may be underpowered to see a small difference, or in haemoglobin concentration, white cell count (or differential cell counts) or platelet count ($P>0.05$ for all; data not shown).

Table 5.1. Baseline characteristics of study participants.

Baseline Characteristics		**(n=16)**
Age, years (median, range)		23 (21 to 27)
Male / Female		9 (56%) / 7 (44%)
Use of oral contraceptive (female)		5 (72%)
Height, cm	All	176 ± 9
	Male	181 ± 7
	Female	169 ± 6
Weight, kg	All	72 ± 13
	Male	80 ± 11
	Female	60 ± 4
BMI, $kg.m^{-2}$	All	23.1 ± 2.8
	Male	24.6 ± 2.8
	Female	21.2 ± 1.0

Data expressed as mean ± standard deviation unless otherwise stated

5.4.1 Plasma Nitrite and Arginine Concentrations

Two hours following exposure to diesel exhaust, plasma nitrite concentrations were higher as compared to the filtered air exposure (68±48 *vs* 41±32 nM; P=0.006). In contrast, there were no differences in plasma concentrations of the NO precursor L-homoarginine, or in the endogenous NOS inhibitors ADMA and SDMA at 2 or 6 h following exposures (Table 5.2). The plasma concentration of L-arginine was lower throughout the study day during the diesel exposures, but this is present in vast molar excess in the circulation and as such cannot account for the changes in NO seen.

Table 5.2. Plasma concentrations of NOS substrates and endogenous NOS inhibitors.

Parameter	Exposure	2 h	6 h	P-Value	
				Time	*Exposure*
L-Arginine µmol/L	Air	61.6 ± 17.8	59.3 ± 10.6	0.471	0.043
	Diesel	58.2 ± 10.0	54.6 ± 10.3		
L-Homoarginine µmol/L	Air	1.66 ± 0.48	1.70 ± 0.51	0.768	0.727
	Diesel	1.63 ± 0.48	1.69 ± 0.53		
ADMA µmol/L	Air	0.44 ± 0.09	0.51 ± 0.07	0.017	0.756
	Diesel	0.44 ± 0.06	0.50 ± 0.07		
SDMA µmol/L	Air	0.41 ± 0.07	0.43 ± 0.05	0.257	0.808
	Diesel	0.41 ± 0.05	0.43 ± 0.05		

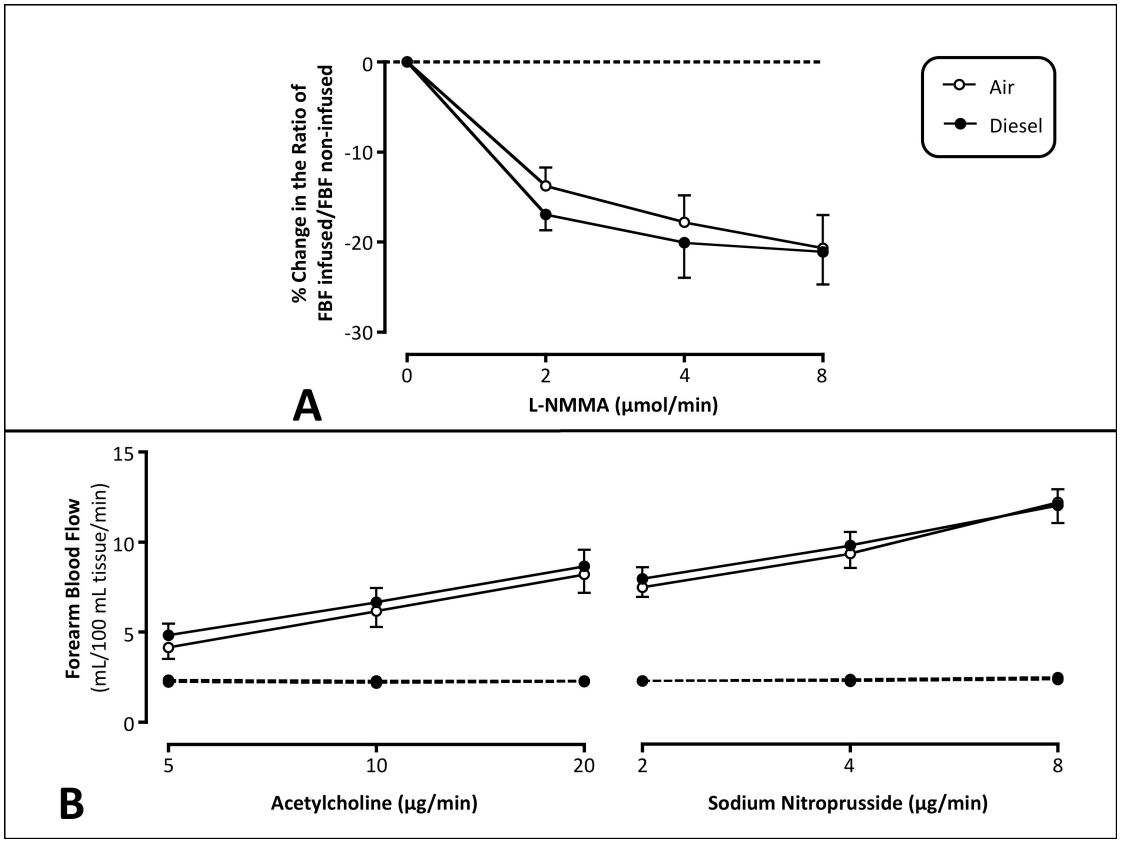
Plasma concentrations of endogenous NOS inhibitors and NOS substrates at baseline, 2 and 6 hr after the exposure during Study 1 (Local L-NMMA administration. All data expressed as mean ± standard deviation. P-values from 2-way repeated measures ANOVA or Student's paired t-tests.

5.4.2 Vascular vasomotor responses

Blood pressure, heart rate and baseline forearm blood flow were not different following either exposure (P>0.05 for all; data not shown).

Infusion of the NOS inhibitor, L-NMMA, resulted in a similar dose-dependent vasoconstriction following both exposures (P=0.559; Figure 5.1A). After establishing the NO clamp by restoring basal blood flow with SNP co-infusion (208±33 and 236±26 ng/min following air and diesel exhaust exposure respectively; P=0.453), both acetylcholine and SNP caused dose-dependent vasodilatation (P<0.01 for both) that was attenuated in comparison to previous studies [Mills, *et al.* 2005]. However, this vasodilatation was similar following both exposures (dilute diesel exhaust *vs* filter air: P=0.209 for acetylcholine and P=0.613 for sodium nitroprusside; Figure 5.1B).

Figure 5.1. *Panel A:* forearm blood flow response during infusion of L-NMMA (2-way repeated measures ANOVA: P=0.559 for exposure). *Panel B:* forearm blood flow during infusion of the vasodilators acetylcholine and sodium nitroprusside (2-way repeated measures ANOVA: P=0.209 and P=0.613 respectively for exposure). Data expressed as mean ± S.E.M



5.5 Discussion

We have demonstrated that inhalation of dilute diesel exhaust increases plasma nitrite concentrations suggesting an increase in basal NO release. However, local NO synthase inhibition causes similar degrees of vasoconstriction following both diesel exhaust and filtered air exposure, suggesting a balanced increase in basal NO generation and consumption that attempts to maintain basal peripheral resistance vessel tone. This is consistent with our observation that diesel exhaust inhalation-induced vasomotor dysfunction is no longer demonstrable in the presence of the NO clamp.

5.5.1 Plasma nitrite concentrations

We demonstrated higher basal venous plasma nitrite concentrations following diesel exhaust exposure. These data are consistent with the up-regulation of vascular NO generation, which we suggest may represent a homeostatic feedback loop to compensate for increased consumption by local oxidative stress in order to maintain basal vascular tone and blood pressure. We also observed no changes in plasma concentrations of the NO precursors and endogenous NOS inhibitors, such as ADMA. This is not surprising given that the precursors, L-arginine and L-homoarginine, are present in vast molar excess and short-term modest increases in NO generation are unlikely to affect their plasma concentrations or indeed those of the naturally occurring endogenous NOS inhibitors.

We have previously demonstrated in isolated rat aortic rings that diesel exhaust particles attenuate endothelium-derived vasorelaxation. This effect is reversed by co-incubation with superoxide dismutase thereby reducing oxygen-centred free radical production [Miller, *et al.* 2009]. We have also shown that diesel exhaust particles themselves reduce the concentrations of bioavailable NO from the NO donor DEA/NO (2-(N,N-diethylamino)-diazenolate-2-oxide) [Verma, *et al.* 2002]. Nurkiewicz and colleagues have reported that, following inhalation of titanium dioxide nanoparticles, local NO was scavenged by oxygen-centred free radicals [Nurkiewicz, *et al.* 2009]. This reduction in NO bioavailability appears to be driven by uncoupling of endothelial NOS by diesel exhaust particles or by local oxidative stress [Knuckles, *et al.* 2008]. However, whilst reflective of production and consumption of NO across the vascular bed, plasma nitrite concentrations do not definitively describe the activity of the NOS enzymes and their isoforms, and it remains feasible that some of the increase in plasma nitrite is in fact due to absorption from the higher oxides of nitrogen during the exposure itself. Knuckles and colleagues have recently reported that following diesel exhaust exposure, rats had increased plasma NO_x (nitrate and nitrite combined) concentrations that appeared to be predominantly as a result of absorption of NO from the exposure itself, although they demonstrate significant increase in eNOS expression consistent with increased NO production [Knuckles, *et al.* 2011]. Controlled exposures to NO and nitrogen

dioxide in man with subsequent measurement of plasma nitrite concentrations may help to define this further.

5.5.2 Vascular vasomotor function

Individuals with hypercholesterolemia have impaired vascular vasomotor dysfunction: the classic paradigm of vascular endothelial dysfunction as a result of changes in the L-arginine-NO pathway [Chowienczyk, *et al.* 1992; Creager, *et al.* 1990]. However, these subjects have normal responses to NOS inhibition with L-NMMA, but reduced vasodilatation to endothelium-dependent vasodilators such as acetylcholine and serotonin [Stroes, *et al.* 1995]. In the present studies, we demonstrate the same pattern of vascular dysfunction following the inhalation of dilute diesel exhaust. Indeed in the presence of the NO clamp, and the absence of endogenous NO release, the previously demonstrated attenuation in vascular vasomotor responses following diesel exhaust inhalation [Mills, *et al.* 2005] is abolished. These findings are consistent with the suggestion that basal NO generation is already increased and therefore further stimulation may be proportionately less effective, and that the NO released may be consumed more rapidly by local oxidative stress. Furthermore, given that the responses to stimulation with acetylcholine are similar in the absence of endogenous NO release, this would suggest that the other key vasodilator mechanisms, such as endothelium-derived hyperpolarising factor (EDHF) or prostaglandins, are intact and unaffected by diesel exhaust inhalation. We recognise a limitation

of our study design is that we have not reproduced the findings of vascular vasomotor impairment prior to investigating the nitric oxide pathways in depth. The findings of vascular vasomotor function are however robust and repeatable and we have repeatedly demonstrated this finding in more than 6 studies in over 100 subjects, and we have no reason to suppose that on this occasion this will not have occurred.

Whilst we suggest that changes in NO bioavailability are consistent with our findings, a similar effect may be seen with other mechanisms, such as activation of the autonomic nervous system. Indeed changes in autonomic nervous system activation may underlie the acute vasoconstriction [Brook*, et al.* 2002] and changes in heart rate variability previously reported [Gold*, et al.* 2000]. Further studies are warranted to evaluate the contribution of the autonomic nervous system in these important vascular effects.

5.5.3 Conclusions

In these studies we have demonstrated for the first time in man that inhalation of dilute diesel exhaust results in higher basal plasma nitrite concentrations. This is consistent with homeostatic regulation in the presence of increased NO consumption due to local oxidative stress. Similarly, we have shown that in the presence of the NO clamp, and the absence of endogenous NO production, diesel exhaust inhalation does not result in further vascular impairment. We conclude that the vascular dysfunction

associated with diesel exhaust inhalation is mediated predominantly by reduced NO bioavailability.

CHAPTER 6

Cardiovascular effects of reducing exposure to particulate air pollution with a simple facemask in healthy volunteers

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6.1 SUMMARY

Exposure to air pollution is an important risk factor for cardiovascular morbidity and mortality, and is associated with increased blood pressure, reduced heart rate variability, endothelial dysfunction and myocardial ischaemia. Our objectives were to assess the cardiovascular effects of reducing air pollution exposure by wearing a facemask.

In an open-label cross-over randomised controlled trial, 15 healthy volunteers (median age 28 years) walked on a predefined city centre route in Beijing in the presence and absence of a highly efficient facemask. Personal exposure to ambient air pollution and exercise was assessed continuously using portable real-time monitors and global positional system tracking respectively. Cardiovascular effects were assessed by continuous 12-lead electrocardiographic and ambulatory blood pressure monitoring.

Ambient exposure ($PM_{2.5}$ 86±61 *vs* 140±113 μg/m^3; particle number 2.4±0.4 *vs* 2.3±0.4 x 10^4 particles/cm^3), temperature (29±1 *vs* 28±3 °C) and relative humidity (63±10 *vs* 64±19%) were similar (P>0.05 for all) on both study days. During the 2-hour city walk, systolic blood pressure was lower (114±10 *vs* 121±11 mmHg, P<0.01) when subjects wore a facemask, although heart rate was similar (91±11 *vs* 88±11 /min; P>0.05). Over the 24-hour period heart rate variability increased (SDNN 65.6±11.5 *vs* 61.2±11.4 ms, P<0.05; LF-power 919±352 *vs* 816±340 ms^2, P<0.05) when subjects wore the facemask.

Wearing a facemask appears to abrogate the adverse effects of air pollution on blood pressure and heart rate variability. This simple intervention has the potential to protect susceptible individuals and prevent cardiovascular events in cities with high concentrations of ambient air pollution.

6.2 Introduction

Air pollution, and especially traffic-derived particulate matter [Laden, *et al.* 2000], is now established as a major cause of cardiorespiratory morbidity and mortality [Anderson, *et al.* 1996; Dockery, *et al.* 1993; Pope, *et al.* 2002]. Epidemiological studies have shown that chronic air pollution exposure is associated with the degree of atherosclerosis [Hoffmann, *et al.* 2007; Kunzli, *et al.* 2005], and the risk of cardiovascular events [Miller, *et al.* 2007]. Acute exposure causes exacerbation of existing cardiorespiratory conditions leading to an increase in hospital admissions [von Klot, *et al.* 2005] and deaths [Peters, *et al.* 2004].

The mechanisms of these associations are unclear but recent controlled exposure studies have demonstrated that air pollution causes vascular endothelial dysfunction [Mills, *et al.* 2005], arterial vasoconstriction [Brook, *et al.* 2002], increased blood pressure [Urch, *et al.* 2005] and myocardial ischaemia [Mills, *et al.* 2007]. Observational studies have also suggested that air pollution exposure impairs regulation of the autonomic nervous system and reduces heart rate variability [Chuang, *et al.* 2007; Gold, *et al.* 2000]. A combination of these effects is likely to account for the increase in cardiovascular events seen following exposure to air pollution. There is therefore a need to consider approaches that can reduce ambient air pollution exposure on both a personal and societal level.

In Beijing China, particulate matter (particle diameter <10 μm; PM_{10}) air pollution averages around 150 $\mu g/m^3$ and, in 2006, levels exceeded the World Health Organisation recommended national standards (PM_{10} concentration <50 $\mu g/m^3$) on 241 out of 365 days [UNEP, 2008]. Despite considerable efforts to improve air quality, pollution remains the single largest environmental and public health issue affecting Beijing. The extensive use of coal and the growing number of motor vehicles (estimated 3.3 million vehicles on the roads in August 2008) have contributed to air pollution. In addition, the city's geographical location exacerbates the problem with the surrounding mountain ranges impeding air circulation and dispersion of pollutants.

Increasing concern relating to the health effects of air pollution has led many individuals to use facemasks to reduce personal exposure. The efficiency of these masks and the potential cardiovascular benefits on people exposed to urban air pollution has yet to be established. The aims of this study were to assess the efficacy of facemasks in removing potentially hazardous particulate air pollution and to determine the potential cardiovascular benefits of a simple facemask in a polluted urban environment.

6.3 Methods

6.3.1 Subjects

Fifteen healthy volunteers were recruited from the Fuwai Hospital, Beijing in August 2008. All subjects were non-smokers, received no regular medication, and had no intercurrent illnesses. All subjects gave their written consent to participate in the study, which was reviewed and approved by the local ethics committee, in accordance with the Declaration of Helsinki.

6.3.2 Assessment of mask efficacy

Masks designed for use by cyclists, pedestrians and occupational settings were tested for penetrance of fresh diesel exhaust particulate. Diesel engine exhaust was generated from the idling (1500 rpm) engine (F3M2011, Deutz Ag, Köln, Germany) of a 35KVA generator (Bredenoord, Apeldoorn, Netherlands). The exhaust was diluted with filtered air to obtain a mass concentration of 75±12 $\mu g/m^3$ (as measured by gravimetric analysis) and a particle number concentration of 500,000 particles/cm^3 (condensation particle counter [CPC] model 3022, TSI Instruments, High Wycombe, UK). Sections of each mask filter were mounted in a filter holder. After 5 min of baseline measurements, filters were introduced between the exhaust and the CPC that sampled at a flow rate of 1.5 L/min. Particle number was recorded for 5 min and penetrance defined as the percentage of particles passing through the filter compared to baseline.

6.3.3 Study design

All 15 subjects attended the Fuwai Hospital on two occasions, each at least one week apart, during August 2008. In a randomised open-label controlled cross-over study, subjects were randomised to wear no mask or a highly efficient facemask filter (Dust Respirator 8812, 3M, St Paul USA). When randomised to wear the facemask, subjects were asked to wear the mask for 24 hours prior to the study day and 24 hours of the study day. Subjects were asked to wear the mask at all times when outside, and as much as possible whilst indoors. On the study day, subjects were asked to walk for 2 hours in a city centre location (Figure 6.1) along the inner ring road in Beijing between 8 and 10 am.

6.3.4 Pollution and activity monitoring

Personal exposure to air pollutants was monitored using a collection of portable monitoring equipment mounted in a backpack. Particle mass concentration (particle diameter <2.5 μm; $PM_{2.5}$) was measured in using a light-scattering nephelometric method using a DataRAM monitor (pDR-1500, Thermo Scientific, Franklin, USA). Particle number was measured using a handheld condensation particle counter (CPC 3007, TSI Instruments Ltd, High Wycombe, UK). Ambient temperature and relative humidity were recorded using a sensor on the outside of the backpack (Omegaette® HH-314, Omega Engineering Ltd, Connecticut, USA). Gaseous pollutants were measured using a multigas analyser (X-am 7000, Dräger Safety, Pittsburgh,

USA) measuring carbon monoxide (CO), nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) using electrochemical sensors with a sensitivity of 1 part per million.

Figure 6.1. City centre route chosen in central Beijing. A representative recording from the GPS device contained in the monitoring backpack is shown. The walk goes from the Fuwai Hospital (A), along the inner ring road (B) and towards the city centre (C) before turning back. Image courtesy of Google™ Earth.

Physical activity was assessed using a portable global positioning system (GPS) monitor secured to the outside of the bag (eTrex Summit HC, Garmin, USA). This recorded the route taken by volunteers, their total distance travelled, and average speed. This information was used, along with baseline

anthropometric measurements, to calculate the energy expended during the walk in kilocalories and metabolic equivalents (METS).

6.3.5 Holter monitoring

Subjects were fitted with a 12-lead continuous electrographic Holter monitor (Lifecard 12, Spacelabs, UK) at the beginning of the study day for 24 hours. Holter electrographic traces were analysed using DelMar Reynolds proprietary software packages by two blinded observers. The quality of the electrocardiographic trace was manually inspected before arrhythmias were automatically detected using the Pathfinder software package. Identified arrhythmias were then individually inspected, verified or deleted as appropriate. Average heart rate and heart rate variability in both time and frequency domains were analysed using the HRV Tools software package, with identified arrhythmias excluded from this analysis.

6.3.6 Ambulatory blood pressure

Subjects were fitted with an ambulatory blood pressure monitor (Model 90217, Spacelabs, UK) at the beginning of the study day. Blood pressure was recorded at the left brachial artery every 15 minutes during the 2-hour walk, every 30 minutes for the rest of the daytime (07:00 to 22:00), and every hour overnight (22:00 – 07:00).

6.3.7 Symptom questionnaire

Subjects were asked to complete a symptom questionnaire using a visual analogue scale at the beginning of the study day, after the 2-hour walk and at the 24-hour visit. They were asked to record any physical symptoms, as well as report a perception of the degree of pollution and the tolerability of the mask.

6.3.8 Data analysis and statistical methods

Subjects were randomised to wearing a mask on their first or second visit using a random number generator. All data are expressed as mean (95% confidence interval [CI]) unless otherwise stated. The symptom questionnaire was based on a visual analogue scale. Scores were converted into a percentage, and analysed using 2-way analysis of variance (ANOVA) with repeated measures using time and the mask intervention as variables. The occurrences of arrhythmias during the 24-hour monitoring period were compared using the Wilcoxon matched pairs method. All other parameters were evaluated using paired Student's *t*-tests. Statistical significance was taken at the 5% level. All data were analysed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA) on a Macintosh personal computer.

6.4 Results

6.4.1 Mask efficiency

Mask penetrance was highly dependent on mask type (Figure 6.2). The 3M Dust Respirator (Model 8812, 3M, St Pauls, USA) was selected for the intervention study as it provided good filtration performance and was extremely efficient and comfortable to wear.

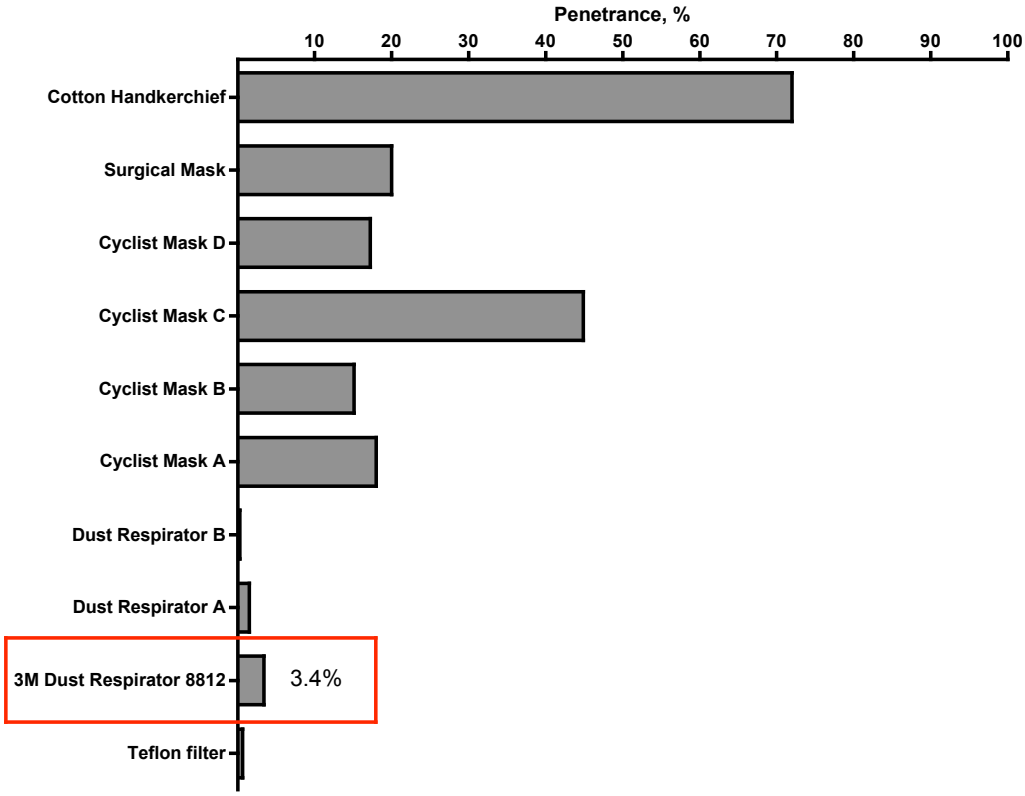

Figure 6.2. Penetrance of commercially available filters: 3M Dust Respirator 8812, Dust Respirators A and B, Cyclist Masks A to D. The Teflon filter is an industry standard filter for aerosol studies included as a control. Cotton handkerchiefs and surgical masks are often seen worn in public areas in parts of Asia.

6.4.2 Intervention study

Fifteen subjects (20-45 years) completed the study. Subjects were predominantly female (13:2) with a mean height of 164 cm (95% CI, 160 – 167), weight of 55 kg (95% CI, 50 – 60) and body mass index of 20.5 kg/m^2 (95% CI, 19.3 – 21.7). There were no differences (P>0.05 for all parameters) in ambient pollution exposure during the 2-hour walk between the two visits (Table 6.1). Based on the measured penetrance of 3.4%, assuming a perfect facial fit and similar flow rates, we predict that the particle count to which subjects were exposed when wearing a mask was reduced to just 795 (95% CI, 726 – 864) particles/cm^3.

Table 6.1. Exposure characteristics during city centre walks.

	Without Mask	With Mask
$PM_{2.5}$, µg/m^3	86 (52 – 120)	140 (77 – 203)
Particle count, number/cm^3	24184 (22061 – 26306)	23379 (21350 – 25409)
CO, number of peaks	6.2 (3.2 – 9.3)	3.3 (1.3 – 5.2)
NO_2, number of peaks	Nil	Nil
SO_2, number of peaks	Nil	Nil
Temperature, ^oC	29.2 (28.6 – 29.8)	28.1 (26.3 – 29.9)
Relative humidity, %	63 (58 – 68)	64 (53 – 74)

Data expressed as mean (95% confidence interval).

P>0.05 compared to control (without mask) day for all, Student's paired t*-test.*

There were no differences in 24-hour average heart rate or blood pressure during the two study days (Table 6.2). Holter analysis revealed an increased SDNN (65.6±11.5 *vs* 61.2±11.4 ms, P<0.05) and LF-power (919±352 *vs* 816±340 ms^2, P<0.05) over the 24 hours when subjects wore the mask. There were no clinically relevant arrhythmias recorded in any subject (Table 6.3).

During the 2-hour walk, there was no difference in exercise intensity in the presence or absence of the facemask (Table 6.4) although subjects had a lower systolic blood pressure (114±10 *vs* 121±11 mmHg; P<0.01) when wearing a mask. This was not associated with a change in diastolic blood pressure, heart rate or in heart rate variability measurements. As expected, parameters of HRV changed during exercise as compared to the 24 hour period consistent with immediate withdrawal of vagal tone.

Subjects reported only very minor symptoms (Table 6.5) during the study period. The mask was generally well tolerated with an average score of 24.8% (95% CI, 16.2 – 33.3%); 0% being completely tolerable and 100% being intolerable.

Table 6.2. 24-hour ambulatory blood pressure monitoring and Holter analysis for heart rate variability with each visit.

		Without Mask	With Mask
24 hour	SBP, mmHg	106 (100 – 112)	106 (101 – 111)
	DBP, mmHg	69 (66 – 73)	70 (67 – 73)
	MAP, mmHg	82 (79 – 86)	82 (78 – 86)
	Heart rate, bpm	74 (70 – 77)	72 (68 – 76)
Night	SBP, mmHg	100 (93 – 107)	101 (95 – 106)
	DBP, mmHg	63 (60 – 67)	64 (61 – 67)
	MAP, mmHg	76 (73 – 79)	75 (71 – 79)
	Heart rate, bpm	64 (61 – 67)	61 (58 – 65)
Day	SBP, mmHg	110 (104 – 116)	109 (104 – 114)
	DBP, mmHg	73 (69 – 76)	73 (70 – 76)
	MAP, mmHg	85 (81 – 88)	85 (81 – 89)
	Heart rate, bpm	79 (74 – 84)	78 (73 – 82)
Heart rate variability	Data validity, %	95.9	95.0
	Average NN interval, ms	829 (789 – 869)	850 (805 – 896)
	pNN50, %	15.9 (10.7 – 21.0)	17.9 (14.2 – 21.6)
	RMSSD, ms	35.1 (29.2 – 41.0)	37.1 (32.2 – 42.0)
	SDNN, ms	**61.2 (54.9 – 67.5)**	**65.6*** **(59.0 – 72.2)**
	Triangular Index	12.9 (11.9 – 13.9)	13.8 (13.0 – 14.5)
	LF-power, ms^2	**816 (628 – 1004)**	**919*** **(717 – 1122)**
	HF-power, ms^2	460 (325 – 595)	485 (400 – 569)
	LFn, ms	62.8 (56.7 – 68.9)	64.5 (60.6 – 68.4)
	HFn, ms	29.2 (25.5 – 32.8)	30.0 (27.0 – 33.1)
	HF/LF ratio	0.738 (0.507 – 0.970)	0.680 (0.519 – 0.842)

*All data expressed as mean (95% confidence interval). *P<0.05 compared to control (no mask) day, Student's paired* t*-test.*

Table 6.3. Arrhythmia analysis from 24-hour Holter electrocardiograms.

	Without Mask	With Mask
Pause	0	0
Dropped beat	0	0
Ventricular tachycardia	0	0
Salvo	0	0
Triplet	0	0
Couplet	0	0
Bradycardia (≤50bpm)	71	227
Supraventricular tachycardia	0	0
Bigeminy	57	157
Trigeminy	4	7
"R on T"	0	0
Premature aberrant	3246	4698
Isolated aberrant	18	3
Premature normal	11	17
Maximum heart rate	134 (126 – 143)	128 (120 – 137)
Minimum heart rate	51 (48 – 54)	49 (46 – 53)

Data shown are total number of events recorded in each condition over all subjects. P>0.05 for all (Wilcoxon matched pairs test). Maximum and minimum heart rates shown as mean (95% confidence intervals), P>0.05 for both, Student's paired t*-test.*

Table 6.4. Exercise performed and physiological parameters during 2-hour walk.

		Without Mask	With Mask
Activity	Energy expenditure, kcals	340 (314 – 367)	364 (304 – 426)
	Energy expenditure, METS	3.33 (3.09 – 3.57)	3.61 (3.12 – 4.10)
Ambulatory blood pressure	Systolic blood pressure, mmHg	**121 (115 – 127)**	**114*** **(108 – 120)**
	Diastolic blood pressure, mmHg	81 (75 – 87)	79 (74 – 83)
	Mean arterial pressure, mmHg	94 (89 – 99)	90 (86 – 94)
	Heart rate, bpm	88 (82 – 94)	91 (85 – 97)
Heart rate variability	Data validity, %	99.1	97.8
	Average NN interval, ms	594 (562 – 627)	613 (571 – 655)
	pNN50, %	3.3 (0.8 – 5.7)	2.1 (-0.1 – 4.4)
	RMSSD, ms	17.2 (13.4 – 21.0)	20.0 (15.5 – 24.6)
	SDNN, ms	45.8 (36.8 – 54.8)	54.8 (42.5 – 67.0)
	Triangular Index	10.7 (9.1 – 12.4)	11.4 (9.4 – 13.3)
	LF-power, ms^2	313 (170 – 455)	414 (233 – 595)
	HF-power, ms^2	76.5 (33.6 – 120.0)	116.8 (52.6 – 181.0)
	LFn, ms	68.2 (60.9 – 75.5)	67.9 (61.9 – 73.9)
	HFn, ms	16.1 (11.9 – 20.3)	16.0 (12.5 – 19.4)
	HF/LF ratio	0.259 (0.173 – 0.344)	0.247 (0.180 – 0.314)

All data expressed as mean (95% confidence interval).

**P<0.01 compared to control (without mask) day, paired Student's* t*-test.*

P>0.05 for all other parameters compared to control (without mask) day.

Table 6.5. Symptom questionnaire.

Symptoms assessed by visual analogue scale (0 – 100)							
	Without mask			With mask			P
	Before walk	After walk	24 hours after walk	Before walk	After walk	24 hours after walk	
Headache	0 (0-1)	0 (0-2)	0 (0-2)	0 (0-1)	0 (0-1)	0 (0-1)	n/s
Dizziness	0 (0-0)	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-1)	0 (0-1)	n/s
Tiredness	0 (0-1)	2 (0-15)	1 (0-16)	0 (0-2)	5 (0-10)	0 (0-2)	n/s
Sickness	0 (0-0)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-2)	n/s
Cough	0 (0-1)	1 (0-1)	0 (0-1)	0 (0-1)	0 (0-20_	0 (0-1)	n/s
Difficulty in breathing	0 (0-0)	0 (0-1)	0 (0-1)	0 (0-2)	1 (0-5)	0 (0-2)	<0.05
Irritation of the eyes	0 (0-0)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-1)	n/s
Irritation of the throat	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-0)	0 (0-1)	0 (0-1)	n/s
Irritation of the nose	0 (0-1)	1 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)	n/s
Unpleasant smell	0 (0-1)	1 (0-1)	0 (0-2)	0 (0-1)	0 (0-1)	0 (0-1)	n/s
Bad taste	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-1)	0 (0-2)	n/s
Difficulty walking		7 (0-24)			16 (4-28)		n/s
Perception of pollution		16 (6-25)			9 (2-22)		n/s

All data expressed as median (interquartile range). "Difficulty walking" and "Perception of pollution" tested using Wilcoxon signed rank test. All other variables tested using 2-way ANOVA with repeated measures including time and mask intervention as variables. P value shown is the effect of the mask intervention.

6.5 Discussion

In this study, we have shown for the first time that a simple well-tolerated personal intervention to reduce exposure to airborne particulate air pollution leads to a reduction in systolic blood pressure during exercise and an increase in heart rate variability. If translated into a susceptible population, our findings would suggest that wearing a simple facemask has the potential to reduce the incidence of acute cardiovascular events in cities with high levels of air pollution, and could influence the advice given to patients with chronic cardiovascular diseases.

We tested a range of facemasks that differed widely in their efficiency as particle filters. In general, those masks designed to reduce occupational exposure to dusts were more efficient than those marketed as personal protection to cyclists and pedestrians in an environmental setting. The choice of the mask used in this study was influenced by efficiency and comfort. The chosen mask was very well tolerated by subjects as demonstrated by the visual analogue score, and was predicted to reduce the exposure to particulate matter dramatically. When wearing the masks, the subjects did report slightly greater difficulty breathing whilst walking although this did not reduce the level of exercise undertaken by the subjects. This increased resistance to respiration is unlikely to affect the main study findings since such stresses would be predicted to increase blood pressure rather than reduce it.

Recent studies have confirmed a link between blood pressure and exposure to air pollution. Population-based studies have shown increases in both systolic blood pressure and pulse pressure [Auchincloss, *et al.* 2008; Ibald-Mulli, *et al.* 2001] with increasing levels of ambient pollution exposure. Controlled exposure studies to concentrated ambient particles and ozone have demonstrated an increase in diastolic blood pressure during a two-hour exposure [Urch, *et al.* 2005]. Although there were no differences in blood pressure over the whole 24-hour period, we observed a marked difference in systolic blood pressure with exercise. In both groups, blood pressure increased during exercise compared to the 24-hour average, although this increase was less when wearing a facemask. This, in combination with previous controlled exposure studies [Urch, *et al.* 2005], suggests that particulate air pollution may augment exercise-induced increases in blood pressure, and that the use of a simple facemask can abrogate this. Exercise induced increases in systolic blood pressure have been linked to myocardial infarction [Mundal, *et al.* 1996] as well as stroke [Kurl, *et al.* 2001], and increased blood pressure is an established major risk factor for the development of both atherosclerosis and cardiovascular mortality [Hippisley-Cox, *et al.* 2008; Wilson, *et al.* 1998]. The reduction in systolic blood pressure seen in this study is similar to that seen with many antihypertensive agents, which have been shown to reduce major cardiovascular events. Therefore we predict that the use of a facemask in a

susceptible population has the potential to reduce the incidence of acute cardiovascular events as well as myocardial ischaemia [Chuang, *et al.* 2008; Mills, *et al.* 2007].

Heart rate variability is a measure of the variation in the R-R interval on the electrocardiogram. A balance of the parasympathetic and sympathetic nervous systems controls the heart rate in order to maintain a constant cardiac output at rest or to respond to increased demands during exercise. A reduction in heart rate variability occurs in various pathophysiological conditions including hypertension [Singh, *et al.* 1998], heart failure [Saul, *et al.* 1988] and diabetes mellitus [Singh, *et al.* 2000], and predicts cardiovascular outcomes [Nolan, *et al.* 1998]. Previous studies have demonstrated a reduction in measures of heart rate variability, particularly the robust and simple time-domain measurement SDNN following exposure to air pollution [Chang, *et al.* 2007; Chuang, *et al.* 2007; Devlin, *et al.* 2003; Gold, *et al.* 2000; Liao, *et al.* 1999; Magari, *et al.* 2001; Park, *et al.* 2008; Pope, *et al.* 1999]. In our study we report an increase in overall heart rate variability (SDNN) when subjects wore a mask, suggesting that wearing a mask can, at least in part, prevent the adverse effects of air pollution exposure on heart rate variability.

LF-power also increased with the use of a mask to prevent exposure to air pollution although interpreting this change is more challenging. LF-power is associated with changes in sympathetic tone, and an increase might suggest

an increased contribution of the sympathetic nervous system to basal heart rate control. However, simply wearing the facemask may have had a small effect on the measures of heart rate variability described. As previously discussed, subjects did report an increased resistance to breathing when wearing the facemask that may have increased subject anxiety. This in turn could have increased sympathetic nervous system tone and hence lead to a small increase in LF-power. This is a limitation of our study, and the use of a sham facemask in a blinded fashion would have helped minimise the effect of anxiety on these sensitive outcome measures. Furthermore, the improvement in parameters of HRV when wearing the mask was small and the physiological significance of this change is unclear in healthy volunteers. We recognise that the menstrual cycle of the female volunteers may have small effects on vascular and autonomic function and this may be a confounding factor in our findings.

Our study has a number of important public health messages. First we have demonstrated that exposure to ambient air pollution has direct and measurable effects on cardiovascular physiological parameters, even young healthy individuals habitually exposed to such elevated levels. Second we have shown that wearing a facemask can abrogate some of these effects in a short period of time. Particle traps are increasingly being fitted to new vehicles to reduce the emissions of particulate matter, both by mass and number concentrations, and this may well go some way to offsetting the

associated health effects. Currently, patients with chronic respiratory and cardiovascular conditions are advised to limit their exposure outdoors on days when ambient air pollution levels are high [COMEAPS, 2000; British Heart Foundation, 2008; Brook, *et al.* 2004]. We have shown that wearing a simple inexpensive and well-tolerated facemask can provide an alternative that may lead to reduced cardiovascular morbidity and mortality. We believe that this intervention now needs to be tested in patients with pre-existing coronary heart disease to define its potential role in reducing the burden of cardiovascular disease in polluted environmental settings.

Our study has a number of important limitations. We recruited young healthy volunteers rather than those most susceptible to the effects of air pollution exposure, such as those with coronary heart disease. Whilst it is likely that our findings will be transferrable to this population, further studies are required to confirm our findings. In addition, it was not possible to assess accurately the efficacy of the mask filter when worn by the subjects. Leaks around the facemask will lead to a reduction in the efficacy of particle filtration [Yang, *et al.* 2007; Zhuang, *et al.* 2005] and therefore our predicted exposures during application of the facemask are likely to be an underestimate. However, despite this, we were still able to demonstrate beneficial cardiovascular effects during their use.

6.5.1 Conclusions

Air pollution exposure is associated with increased cardiovascular morbidity and mortality, and adverse effects on the cardiovascular system. We have shown for the first time that a wearing a facemask appears to abrogate the adverse effects of air pollution on blood pressure and heart rate variability. This simple intervention has the potential to protect susceptible individuals and prevent cardiovascular events in cities with high concentrations of ambient air pollution.

CHAPTER 7

Reducing particulate air pollution exposure in patients with coronary heart disease

Published by Langrish JP, Li X, Wang S, Lee MMY, Barnes GD, Lei GG, Miller MR, Cassee FR, Boon NA, Donaldson K, Li J, Mills NL, Newby DE and Jiang L. Reducing personal exposure to particulate air pollution improves cardiovascular health in patients with coronary heart disease. *Environ Health Perspect.* 2012;120:367-72.

7.1 SUMMARY

Air pollution exposure increases cardiovascular morbidity and mortality, and is a major global public health concern. We investigated the benefits of reducing personal exposure to urban air pollution in patients with coronary heart disease.

In an open randomised crossover trial, 98 patients with coronary heart disease walked on a pre-defined route in central Beijing, China in the presence and absence of a highly efficient facemask. Symptoms, exercise, personal air pollution exposure, blood pressure, heart rate and 12-lead electrocardiography were monitored throughout the 24-hour study period.

Ambient air pollutants were dominated by fine and ultrafine particles that were present at high levels (74 $\mu g/m^3$ for particulate matter <2.5 μm). Consistent with traffic-derived sources, this particulate matter contained organic carbon and polycyclic aromatic hydrocarbons, and were highly oxidising generating large amounts of free radicals. The facemask was well tolerated, decreased symptoms ($P<0.05$), and reduced maximal ST segment depression (-142 *vs* -156 μV, P=0.046) over the 24-hour period. Mean arterial pressure was lower (93±10 *vs* 96±10 mmHg; P=0.025) and heart rate variability increased (HF-power 54 *vs* 40 ms^2, P=0.005; HFn 23.5 *vs* 20.5 ms, P=0.001; RMSSD 16.7 *vs* 14.8 ms, P=0.007) during the prescribed walk, whilst heart rate and energy expenditure were similar ($P>0.05$).

Reducing personal exposure to air pollution using a highly efficient facemask appears to reduce symptoms, and improve a range of measures of cardiovascular health in patients with coronary heart disease. Such approaches have the potential to reduce the incidence of cardiovascular events in this highly susceptible population.

7.2 INTRODUCTION

Air pollution exposure is an established risk factor for cardiovascular morbidity and mortality [Brook, *et al.* 2010], especially that derived from traffic and industrial sources [Laden, *et al.* 2000; Lall, *et al.* 2011; Lipfert, *et al.* 2006; Pope, *et al.* 2002; Sarnat, *et al.* 2008]. Acute exposure to combustion-derived particulate matter is associated with the onset of myocardial infarction, admissions to hospital in survivors of myocardial infarction, and is proposed as a trigger for acute cardiovascular events [Klot, *et al.* 2005; Peters, *et al.* 2004]. Although estimates vary, chronic exposure to air pollution increases all-cause mortality by between 2 and 4 % per 10 $\mu g/m^3$ increase in particulate matter [Dockery, *et al.* 1993; Pope, *et al.* 2002], with the majority of deaths due to cardiovascular disease [Miller, *et al.* 2007]. The World Health Organisation estimates that outdoor urban air pollution results in around 800,000 deaths worldwide each year [UNEP 2006].

In controlled exposure studies, inhalation of particulate air pollution causes changes in blood pressure, abnormalities in vascular function, coagulation and myocardial ischaemia [Mills, *et al.* 2009]. These responses provide a plausible mechanism to explain the observed increase in acute cardiovascular events and increased cardiovascular mortality following exposure to particulate air pollution. However, whilst acute exposure induces these adverse effects, it is unclear whether improvements in cardiovascular health can be achieved by interventions targeted to reduce

exposure in those living and working in highly polluted urban environments.

Major environmental health policy interventions can have a substantial impact on the health of populations, as can be observed following the banning of bituminous coal in Dublin, Ireland in 1990 [Clancy, *et al.* 2002], and more recently with the restriction of smoking in public places [Pell, *et al.* 2008], where there were major reductions in cardiovascular events. Such environmental interventions are particularly challenging to deliver in rapidly developing countries where economic growth is dependent on road traffic and heavy industry [Smith, *et al.* 2009]. More practical solutions to reduce individuals' exposure and protect susceptible persons are urgently required. Therefore, we investigated the effects of a simple facemask intervention to reduce particulate air pollution exposure on measures of cardiovascular health in patients with coronary heart disease.

7.3 METHODS

7.3.1 Subjects

One hundred and two patients were recruited from the Fuwai Hospital, Beijing in March 2009. All patients were non-smokers and had a history of coronary heart disease. Exclusion criteria were a history of arrhythmia, severe coronary artery disease without revascularisation, resting conduction abnormality, digoxin therapy, uncontrolled hypertension, renal or hepatic failure or an acute coronary syndrome within three months. All subjects gave their written informed consent and the study was reviewed and approved by the local research ethics committee.

7.3.2 Study design

Subjects attended the Fuwai Hospital or the ChaoYang Hospital in Beijing on two occasions, each at least a week apart (median time between visits was 9 days), between March and May 2009. Each subject attended the same hospital on each visit. In a prospective randomised open blinded endpoint (PROBE) crossover study, subjects walked for two hours along a prescribed city centre route (Figure 7.1) in Beijing between 9 and 11 am, in the presence and absence of a highly efficient facemask (Dust Respirator 8812, 3M, St Paul, USA). This mask consists of a lightweight polypropylene filter, which is effective at removing airborne particles without an effect on ambient gases, with an expiration valve, and complies with EN149:2001 FFP1 European Standard and has an assigned protection factor of 4 (i.e. it can be worn in

atmospheres containing up to 4 times the workplace exposure limit [WEL for respirable carbon particles, carbon black, is 3.5 mg/m^3 over an 8 hour time weighted average] as defined by the UK Health and Safety Executive [http://www.hse.gov.uk/]). The mask was worn on one of the two visits using balanced computer-generated randomisation incorporating concealment of treatment order. In order to maximise the difference in particulate air pollution exposure, subjects wore the mask for 24 hours prior to, and 24 hours during, the study day, and were given instructions to wear the mask at all times whilst outdoors and as much as possible when indoors.

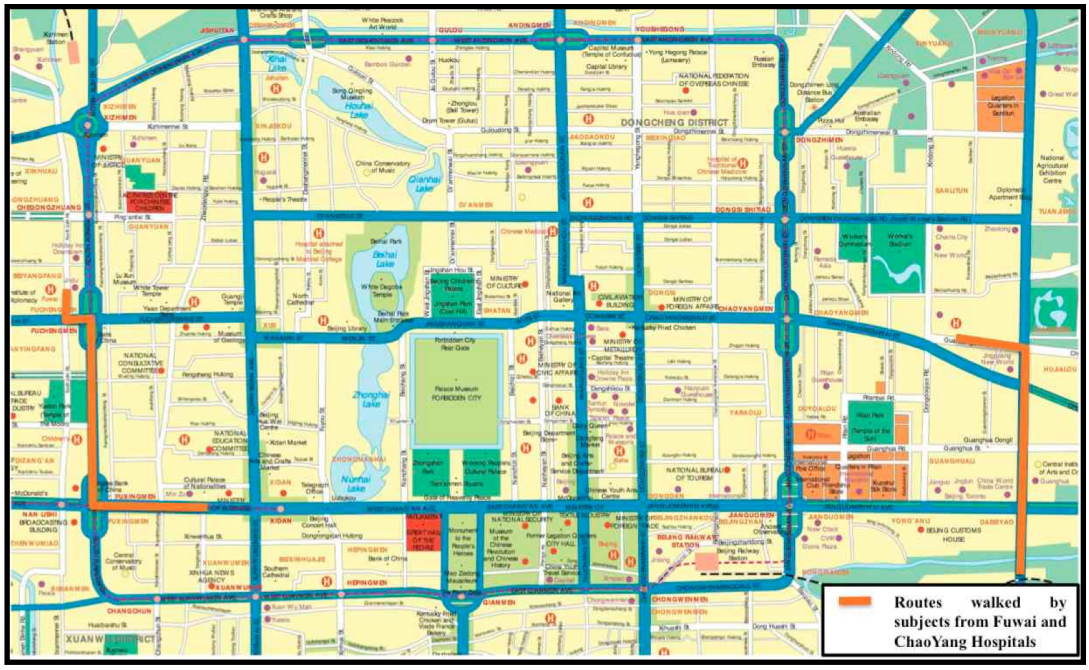

Figure 7.1. Prescribed walks from the Fuwai Hospital (west of city centre in Xicheng District) and ChaoYang Hospital (east of city centre in ChaoYang District). Map freely available online from Weller Cartographic Services Ltd. at http://www.mapmatrix.com/tmhtm/mapcat.html#Asia%20Catalogue

7.3.3 Personal pollution exposure and activity monitoring

Personal air pollutant exposure was determined using monitoring equipment contained within a backpack. Particle mass concentration ($PM_{2.5}$; particle diameter <2.5 μm) was determined using a DataRAM monitor (pDR-1500, Thermo Scientific, Franklin, MA, USA), and particle number measured using a condensation particle counter (CPC 3007, TSI Instruments Ltd, High Wycombe, UK). Ambient temperature and relative humidity were recorded using an external sensor (Omegaette® HH-314, Omega Engineering Ltd, Stamford, CT, USA). Gaseous pollutants were measured using a multi-gas analyser with electrochemical sensors for carbon monoxide, sulphur dioxide and nitrogen dioxide (X-am 7000, Dräger Safety, Lübeck, Germany). Physical activity was measured using global positioning system (GPS) tracking (eTrex Summit HC, Garmin, Olathe, KS, USA), and energy expenditure estimated using activity data and anthropometric data as described previously [Langrish, *et al*. 2009].

7.3.4 Background pollution monitoring

Background exposure was recorded from permanent monitoring stations in the same district as the patients' walk (http://www.bjepb.gov.cn/air2008/olympic.aspx). Airborne particulate matter was collected onto Teflon filters (Pall Corp., Ann Arbor, MI, USA) in three size fractions: coarse (mean aerodynamic diameter 2.5-10 μm), fine

(0.18-2.5 μm) and ultrafine (<0.18 μm) using a MOUDI cascade impactor (MSP Corp., Shoreview, MN, USA).

Particulate mass was determined gravimetrically for each size fraction from the above filters after temperature and humidity conditioning, and subsequently analysed for elemental and organic carbon fractions, metals and cations, nitrate and sulphate anions and organic matter.

7.3.5 Chemical and toxicological analysis of collected particulate matter

Collected particulate samples were analysed for total carbon content, as well as elemental and organic carbon fractions, using the Sunset method (NIOSH 5040; http://www.cdc.gov/niosh/docs/2003-154/pdfs/5040.pdf). Metals and cations were determined using inductively-coupled plasma mass spectrometry (ICP-MS) following pre-treatment with nitric acid. Nitrate and sulphate anions were determined following extraction with water using LC-ICP-MS (liquid chromatography paired with ICP-MS). Organic matter was extracted from filters by ultrasonification with toluene and analysed using gas chromatography-mass spectrometry (GC-MS).

Oxidative potential of particles was assessed using electron paramagnetic resonance (EPR) [Miller, *et al.* 2009]. EPR was used to establish oxygen-centred free radical generation from the collected particulate matter in the

absence of tissue. Filters for all size fractions were pooled and sonicated in phosphate buffered saline to give a final concentration of particles of 1 mg/mL. Solutions were incubated with the spin-trap 1-hydroxyl-2,2,6,6,-tetramethyl-4-oxo-piperidine (Tempone-H; 1 mM), loaded into a capillary tube and assessed at 37 ^{o}C in an X-band EPR spectrometer (Magnettech MS-200, Berlin, Germany) as described previously [Miller, *et al.* 2009]. Pyrogallol (100 μM) was used as a positive control, and samples were compared to the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) standard reference materials: urban dust (SRM-1649a; 1 mg/mL) and diesel exhaust particles (SRM-2975; 10 μg/mL).

7.3.6 Electrocardiographh and blood pressure monitoring

Continuous 12-lead electrocardiography (Lifecard 12, Spacelabs Healthcare, UK) was assessed using the Pathfinder automated arrhythmia analysis package (DelMar Reynolds, UK). Identified arrhythmias were individually inspected and verified or deleted as appropriate, and heart rate variability was assessed using the HRV Tools software package (DelMar Reynolds, UK) as described previously [Langrish, *et al.* 2009]. ST segment analysis was performed at the J-point +80 ms in three representative leads (II, V2 and V5) that were analysed separately for each subject. Maximal ST segment depression and ischaemic burden (product of the change in ST segment amplitude and the duration of the recording) were determined for each lead and as a composite [Mills, *et al.* 2007].

Ambulatory blood pressure (Model 90217, Spacelabs Healthcare, UK) was measured every 15 min during the 2-hour walk, then every 30 min during the day and every hour overnight (22:00 to 07:00).

7.3.7 Symptom questionnaire

Subjects completed a symptom questionnaire using a visual analogue scale at the beginning of the study day, following the 2-hour walk and at the 24-hour visit. They were asked to report physical symptoms, their perception of the pollution, their perceived workload and the tolerability of the mask.

7.3.8 Data analysis and statistical methods

In our previous study of healthy volunteers, we demonstrated a difference in systolic blood pressure of 7 mmHg with a standard deviation of 5 mmHg [Langrish, *et al.* 2009]. Based on the assumption that the effect size would be considerably smaller, we powered the study to detect a 2 mmHg difference in systolic blood pressure, giving a sample size of 101 at 80% power and two-sided $P<0.05$.

Blood pressure and electrocardiographic endpoints were analysed by investigators blinded to treatment allocation. Data were locked before undertaking statistical analysis. All data are expressed as median (interquartile range) or mean ± standard deviation unless otherwise stated.

Treatment-period interactions were assessed as described previously [Hills, *et al.* 1979], before data were compared using paired Student's *t*-tests or Wilcoxon matched pairs signed rank test as appropriate. Occurrence of arrhythmias, reported symptoms and ST segment event frequency were compared using the Chi-squared analysis. All data were analysed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA). Statistical significance was taken as a two-sided $P<0.05$.

7.4 RESULTS

7.4.1 Subjects and Facemask Intervention

Ninety-eight patients completed the study protocol (Figure 7.2 and Table 7.1). All subjects tolerated the mask intervention well, scoring the comfort of the mask as 0.64±1.06 on a 0-10 scale (0 represents completely comfortable and 10 intolerable). The mask intervention reduced general symptoms, and patients' perceived effort of work (Figure 7.3; P<0.05 for all), as well their perception of the level of ambient air pollution (2.46±1.67 *vs.* 2.73±1.64 on the 0-10 visual analogue scale; P=0.03). There were no significant treatment-period interactions for any outcome measure.

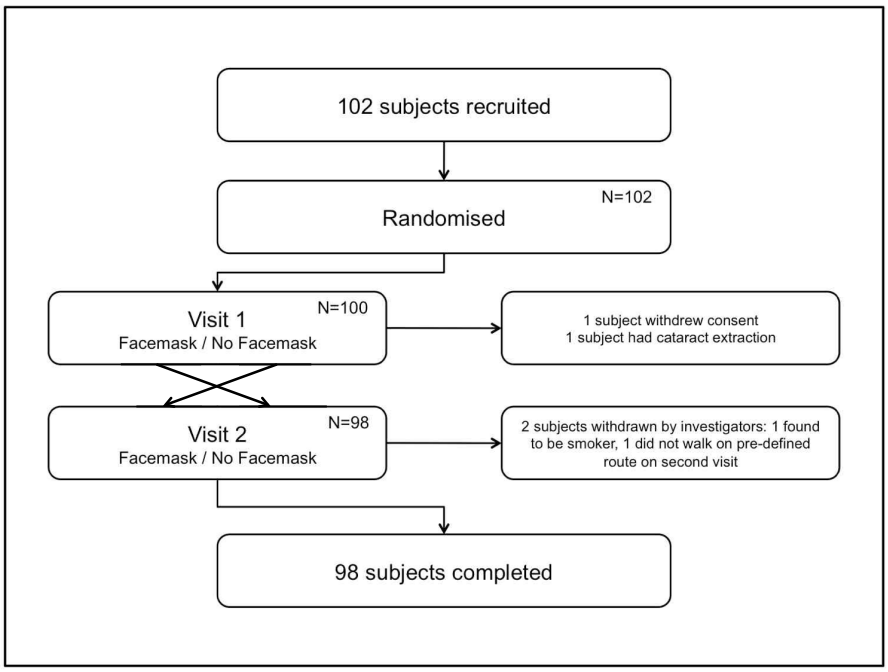

Figure 7.2. Study consort flowchart

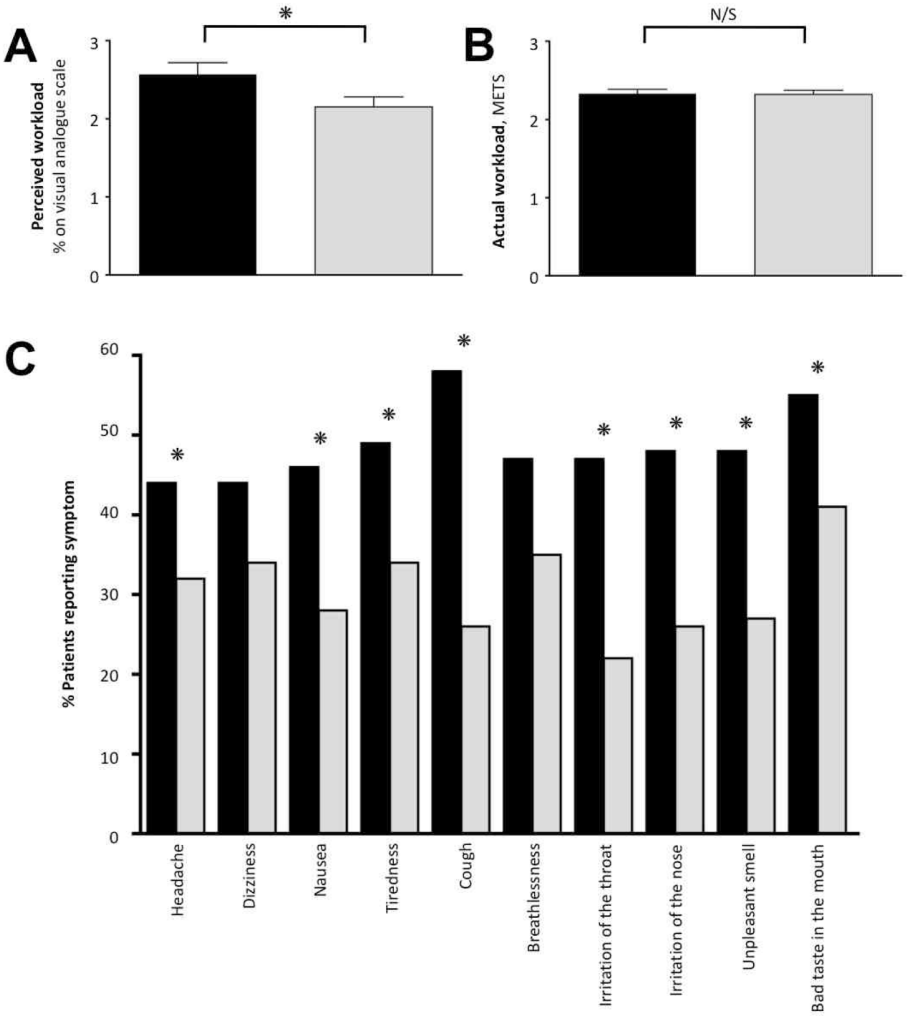

Figure 7.3. (A) Perceived effort of work and (B) actual work performed during the prescribed walk, and (C) symptoms of well-being in the presence (grey bars) or absence (black bars) of the facemask. Data expressed as mean ± SEM or frequency. ∗P<0.05.

Table 7.1. Baseline characteristics of subjects completing the study

Baseline Characteristics, n=98		
Age, yrs		62 ± 7
Male / Female		85 (87%) / 13 (13%)
Height, cm		169 ± 6
Weight, kg		74.5 ± 10.4
BMI, kg/m²		25.9 ± 2.9
Risk Factors	Hypertension	79 (81%)
	Diabetes Mellitus	45 (46%)
	Stroke	15 (15%)
	Peripheral Vascular Disease	5 (5%)
	Previous Myocardial Infarction	68 (69%)
	Previous PCI	60 (61%)
	Previous CABG	38 (39%)
	LV Ejection Fraction (n=31), %	62 ± 9
Angina Status	CCSC Class I	67 (68%)
	CCSC Class II	31 (32%)
	Seattle Angina Score (max 500)	387 ± 34
Clinical Biochemistry	Random glucose, mmol/L	5.5 ± 1.8
	Triglycerides, mmol/L	1.52 ± 0.69
	Cholesterol, mmol/L	3.9 ± 0.9
	HDL Cholesterol, mmol/L	1.2 ± 0.3
	LDL Cholesterol, mmol/L	2.0 ± 0.8
Medication Use	Aspirin	92 (94%)
	Clopidogrel	17 (17%)
	Warfarin	1 (1%)
	ACE inhibitor or ATII receptor antagonist	54 (55%)
	Beta-blocker	73 (74%)
	Calcium channel blocker	42 (43%)
	Statin, Fibrate or Ezetimibe	83 (85%)
	Nitrate	45 (46%)
	Other anti-anginal	5 (5%)
	Diabetic medication	42 (43%)
	Traditional Chinese medicine	19 (19%)

Data expressed as mean ± standard deviation or number (%). BMI=body mass index; PCI=percutaneous coronary intervention; CABG=coronary artery bypass graft; LV=left ventricle; CCS=Canadian Carsiovascular Society; HDL=high density lipoprotein; LDL=low density lipoprotein; ACE=angiotensin converting enzyme; ARB=angiotensin II receptor blocker.

7.4.2 Air Quality and Pollutants

Ambient levels of air pollutants were similar on both study days (Table 7.2) although we predict from previous studies of filter efficacy (97% reduction in particle number) that the particle exposure in the presence of the mask would be reduced to ~2 μg/m^3 and ~1200 particles/cm^3 [Langrish*, et al.* 2009]. Temperature (17.3 vs 16.8 ^oC) and ambient relative humidity (30 vs 35%) were similar on both visits ($P>0.05$ for both). Airborne particulate matter was predominantly (>99% by particle number) in the fine and ultrafine fractions (Figure 7.4) and contained a large amount of organic carbon and high concentrations of polycyclic aromatic hydrocarbons, nitrates, hopanes and steranes suggesting that much of this is combustion-derived and related to traffic sources (Appendix 1). These collected particles were highly oxidising and generated large amounts of free radicals as detected by EPR, exceeding the signal seen with both the standard reference NIST urban dust material at an equivalent concentration and the intense free radical generating oxidant, pyrogallol (Figure 7.4).

Table 7.2. Personal pollution exposure during study.

Parameter		Mask	No Mask
Personal monitoring	$PM_{2.5}$ exposure, $\mu g/m^3$	61 (20-88)	89 (25-170)
	Particle count, $x10^4$ particles/cm^3	4.19 ± 1.29	4.39 ± 1.45
	Temperature, ^oC	17.3 ± 5.2	16.8 ± 5.8
	Relative humidity, %	30.4 ± 14.0	34.8 ± 18.2
	Peaks of NO_2 >1 ppm, number	None	None
	Peaks SO_2 >1 ppm, number	None	None
	Peaks CO >1 ppm, number	5 (2-7.5)	4 (2-8)
Background monitoring	PM_{10} exposure, $\mu g/m^3$	92 (70-117)	103 (83-180)
	SO_2 exposure, ppb	38 (29-53)	54 (32-77)
	NO_2 exposure, ppb	36 (29-42)	36 (32-47)

Data expressed as mean ± standard deviation or median (interquartile range). Personal monitoring data collected using portable monitoring equipment during the 2–hour walk, background data from permanent monitoring stations for the whole 24–hour period. Estimated particulate exposure is calculated based on filter efficacy studies where 97% of fresh diesel exhaust particles were removed.[18] $PM_{2.5}$=particulate matter with a mean aerodynamic diameter of <2.5 µm; NO_2=nitrogen dioxide; SO_2=sulphur dioxide; CO=carbon monoxide; PM_{10}=particulate matter with a mean aerodynamic diameter of <10 µm; ppm=parts per million; ppb=parts per billion.

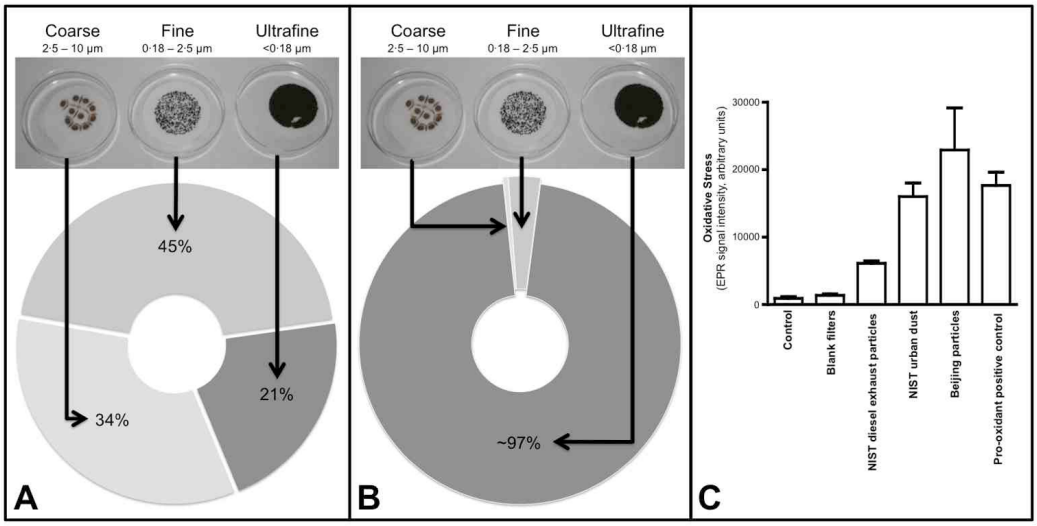

Figure 7.4. (A) Representative filter samples collected showing contribution by mass of the three size fractions averaged over 3 days. (B) Estimated contribution of each size fraction collected on filters by particle number. (C) Oxidative potential of the collected Beijing particles (1 mg/mL) using electron paramagnetic resonance (EPR) to assess oxygen-centred free radical generation, as compared to NIST (National Institute of Standards and Technology) diesel exhaust particles (10 µg/mL) and urban dust (1 mg/mL) and the pro-oxidant positive control (pyrogallol 100 µM) as described previously [Miller, *et al.* 2009]. Data expressed as mean ± standard deviation (n=2–4).

7.4.3 Effect of the Facemask on Cardiovascular Health

Over the 24-hour period, the maximal ST segment depression recorded was lower (-142 (-179 to -110) *vs* -156 (-202 to -123) µV; P=0.046) in the presence of the facemask. However, myocardial ischaemic burden was similar on both visits during the 2-hour walk and over the entire 24-hour period (P>0.05 for both, Table 7.3).

During the 2-hour prescribed city centre walks, subjects walked a comparable distance, at a similar average speed, and expended the same amount of energy (P>0.05 for all, Table 7.4) in the presence and absence of

the facemask. Despite this similar workload, mean ambulatory arterial blood pressure was lower (93±10 mmHg *vs* 96±10 mmHg; P=0.025) in the presence of the facemask (Table 7.5) although heart rate was similar (P>0.05). During the 2-hour walk, heart rate variability (HF-power, HFn, HF/LF ratio and RMSSD; P<0.01 for all) was higher when wearing the facemask (Table 7.6). There were no differences in overall 24-hour ambulatory blood pressure or heart rate variability (Table 7.5, Table 7.6). There was no difference in the incidence of arrhythmias on the two visits (P>0.05 for all; Table 7.7).

Where subjects were not wearing a facemask $PM_{2.5}$ mass concentration did not correlate significantly with measures of cardiovascular health (Table 7.8).

Table 7.3. Myocardial ischaemia measured as ischaemic burden, in each individual territory and as a composite. Data expressed as median (interquartile range). P>0.05 for all using Wilcoxon signed rank test.

Parameter		**Walk**		**24-hour**	
		Mask	*No Mask*	*Mask*	*No Mask*
Ischaemic Burden, mVs	*Inferior (II) territory*	-66 (-118 to -26)	-52 (-149 to -21)	-641 (-767 to -504)	-615 (-820 to -473)
	Anterior (V_2) territory	-66 (-142 to -16)	-50 (-124 to -13)	-597 (-859 to -435)	-632 (-905 to -489)
	Lateral (V_5) territory	-37 (-104 to -8)	-43 (-85 to -18)	-604 (-811 to -429_	-586 (-790 to -412)
	Sum (II + V_2 + V_5)	-189 (-382 to -90)	-188 (-340 to -112)	-1930 (-2306 to -1541)	-1934 (-2391 to -1575)

Table 7.4. Exercise performed and energy expended during the 2 controlled city centre walks. Data expressed as mean ± standard deviation. P>0.05 for all parameters using paired Student's *t*-tests.

Parameter	**Mask**	**No Mask**
Distance travelled, km	6.37 ± 1.44	6.40 ± 1.51
Average moving speed, km/h	4.25 ± 0.96	4.27 ± 1.01
Energy expenditure, Kcal	345 ± 87	347 ± 93
Energy expenditure, METS	2.32 ± 0.52	2.33 ± 0.55

Table 7.5. Blood pressure and heart rate during the 2-hour urban walk and the 24-hour study period. Data expressed as mean ± standard deviation. P values from 2-way ANOVA with repeated measures.

Parameter	**Walk**			**24 hours**		
	Mask	***No Mask***	***P-value***	***Mask***	***No Mask***	***P-value***
Systolic blood pressure, mmHg	126.9 ± 15.9	128.1 ± 16.5	N/S	121.2 ± 11.9	120.8 ± 12.4	N/S
Diastolic blood pressure, mmHg	78.0 ± 9.3	79.5 ± 8.6	N/S	73.8 ± 7.2	74.0 ± 7.3	N/S
Mean arterial pressure, mmHg	93.3 ± 9.7	95.7 ± 10.0	P=0.025	89.8 ± 7.5	90.0 ± 7.9	N/S
Heart rate, bpm	81.5 ± 8.7	81.5 ± 10.1	N/S	77.6 ± 11.3	76.7 ± 11.1	N/S

Table 7.6. Heart rate variability during the 2-hour urban walk and the 24-hour study period. Data expressed as median (interquartile range). *P<0.01 using Wilcoxon matched pairs signed rank test: mask vs no mask.

Parameter	Walk		24 hours	
	Mask	*No Mask*	*Mask*	*No Mask*
Data validity, %	98.9 (96.7-100.0)	99.2 (96.2-100.0)	60.7 (54.2-66.7)	58.4 (54.5-66.0)
LF-power, ms²	133 (68-97)	136 (52-227)	81 (40-172)	93 (46-208)
HF-power, ms²	54 * (27-108)	40 (20-69)	27 (11-77)	31 (11-68)
LFn, ms	58.4 * (45.6-69.1)	62.9 (51.1-75.5)	67.2 (55.5-78.0)	71.1 (59.4-81.1)
HFn, ms	23.5 * (18.0-32.4)	20.5 (13.5-27.9)	21.4 (15.0-31.6)	20.9 (12.7-30.1)
HF/LF ratio	0.418 * (0.258-0.712)	0.328 (0.207-0.573)	0.301 (0.190-0.554)	0.306 (0.161-0.492)
Average heart rate, bpm	81 (76-88)	81 (75-88)	77 (71-85)	78 (68-83)
pNN50, %	1.2 (0.2-2.8)	0.7 (0.0-2.3)	0.5 (0.0-3.1)	0.6 (0.0-2.6)
RMSSD, ms	16.7 * (13.2-22.5)	14.8 (10.9-19.6)	15.5 (11.0-22.6)	14.4 (10.3-20.3)
SDNN, ms	59.8 (46.4-79.1)	60.1 (41.0-79.3)	45.6 (30.8-70.4)	48.2 (30.0-66.3)
Triangular index	8.9 (7.3-11.5)	8.5 (6.7-10.6)	8.9 (6.8-11.5)	8.2 (6.3-10.6)

Table 7.7. Cardiac arrhythmias during the 24-hour electrocardiographic monitoring period. Data expressed as number of patients or as median (interquartile range). P>0.05 for all using Chi-squared (number of patients) and Mann-Whitney U tests (number of events). Bradycardia defined as heart rate < 50bpm; VT=ventricular tachycardia; SVT=supraventricular tachycardia.

n=98	**Number of patients**		**Events per patient**	
Arrhythmia	***Mask***	***No Mask***	***Mask***	***No Mask***
Dropped beat	2	2	1 (1-1)	1 (1-1)
VT	1	2	2 (2-2)	1 (1-1)
Salvo	1	2	4 (4-4)	2 (1-3)
Bigeminy	15	18	4 (1-33)	6 (2-36)
Triplet	1	0	11 (11-11)	0 (0-0)
Couplet	9	4	2 (1-5)	2 (1-10)
Bradycardia	20	19	52 (3-275)	89 (9-347)
SVT	2	5	1 (1-1)	1 (1-1)
Trigeminy	3	4	12 (6-134)	10 (6-230)
Premature aberrant	79	77	17 (3-122)	22 (3-181)
Isolated aberrant	52	54	3 (1-16)	5 (1-25)
Premature normal	81	84	12 (3-56)	12 (3-42)

Table 7.8. Correlation between cardiovascular outcome measures and particulate air pollution exposure ($PM_{2.5}$ mass concentration) during the 2-hour city centre walk on the "no mask" visit alone.

	Parameter	Spearman's correlation co-efficient	P Value
Hemodynamics	Systolic blood pressure	-0.038	0.712
	Diastolic blood pressure	0.010	0.926
	Mean arterial pressure	0.027	0.790
	Heart rate	0.054	0.607
Heart rate variability	LF power	-0.042	0.884
	HF power	0.199	0.054
	HF/LF ratio	0.100	0.334
	pNN50	-0.005	0.959
	RMSSD	-0.047	0.654
	SDNN	-0.088	0.398
ial	Maximal ST segment deviation	-0.174	0.093
	Total ischemic burden	-0.034	0.746

LF=low frequency; HF=high frequency; pNN50=percentage of successive RR intervals that differ by more than 50 ms; RMSSD=root mean square differences of successive differences; SDNN=standard deviation of RR intervals. LF and SDNN reflect mainly sympathetic nervous stimulation; HF, pNN50 and RMSSD reflect parasympathetic tone.

7.5 DISCUSSION

Particulate air pollution is a major public health concern and is associated with increases in cardiovascular morbidity and mortality. In this study we have demonstrated, for the first time, that in patients with coronary heart disease reducing personal exposure to urban airborne particulate matter by means of a simple facemask appears to be associated with a reduction in symptoms, and objective improvements in myocardial ischaemia, blood pressure and heart rate variability. Reducing personal exposure to particulate air pollution has the potential to reduce the incidence of cardiovascular events in patients with coronary heart disease living and working in industrialized or urban environments.

Using a robust PROBE design, we have conducted the first randomised controlled trial to assess the impact of reducing personal air pollution exposure in patients with coronary heart disease in a polluted urban environment. Through the use of portable monitoring devices and sample collection, we have undertaken a very detailed and sophisticated characterisation of air pollutant exposure that has highlighted the remarkably complex and toxic composition of ambient air particulate with its extremely high pro-oxidative potential. We have combined this individualised pollution monitoring with a comprehensive cardiovascular assessment that incorporates haemodynamic and electrophysiological monitoring in conjunction with GPS tracking. Despite only reducing

exposure for a 48-hour period in patients chronically exposed to a polluted urban environment, we have shown consistent beneficial effects on a range of biomarkers of cardiovascular health following the introduction of this simple but highly efficient facemask intervention.

7.5.1 Myocardial ischaemia

In a cohort of 20 men with stable asymptomatic coronary disease, we demonstrated greater exercise-induced maximum ST segment depression during exposure to diesel exhaust [Mills, *et al.* 2007]. However, although acute air pollution exposure exacerbates myocardial ischaemia, many people around the world are chronically exposed to high levels of air pollution and it is unknown whether interventions targeted at reducing exposure will decrease myocardial ischaemia.

In the present study, we have shown that in patients with coronary heart disease decreasing personal exposure to ambient air pollution reduces maximal ST segment depression over a 24-hour period. The significance of silent myocardial ischaemia is still debated but it is associated with increases in major cardiac events in the general population. Moreover in patients with recent myocardial infarction or unstable angina, the occurrence of silent ischaemia is a poor prognostic factor and is associated with around a three-fold increase in major cardiac events and death [Cohn, *et al.* 2003]. It seems plausible therefore that the modest reduction in silent myocardial ischaemia

seen in this study is likely to be of benefit and may, if sustained, result in significant reductions in major cardiac events and cardiovascular mortality.

7.5.2 Blood pressure

Chronic exposure to air pollution is associated with increases in blood pressure in large epidemiological studies [Auchincloss, *et al.* 2008]. Similarly controlled exposure to concentrated ambient particles and ozone in healthy volunteers results in an acute increase in diastolic blood pressure [Urch, *et al.* 2005]. Hypertension is a major risk factor for the development of atherosclerosis and acute increases in blood pressure may trigger plaque rupture leading to an acute cardiovascular event. Consistent with this, exercise-related increases in blood pressure are predictive of the incidence of myocardial infarction [Mundal, *et al.* 1996], stroke [Kurl, *et al.* 2001], and cardiovascular mortality [Kikuya, *et al.* 2000].

In healthy volunteers (chapter 6), reducing personal particulate air pollution exposure with a facemask reduced exercise-related systolic blood pressure by 7 mmHg [Langrish, *et al.* 2009]. This is consistent with the current findings, although the lower workload of this older population with heart disease and the modifying effects of antihypertensive medication use are likely to have contributed to the lower reduction in pressure of 3 mmHg [Barclay, *et al.* 2009]. However, if replicated on a population level, such modest changes in blood pressure will be associated with fewer major

cardiovascular events, as seen in many interventional trials of blood pressure reduction.

7.5.3 Heart rate variability

Heart rate variability is a reflection of the autonomic control (a balance of the sympathetic and parasympathetic nervous systems) of the heart and is a measure of the variation in the RR intervals on a continuous electrocardiogram. A reduction in heart rate variability has been demonstrated in a variety of pathophysiological conditions including hypertension, heart failure, and diabetes mellitus. Indeed, reduced heart rate variability has been linked to increased cardiovascular mortality [Nolan, *et al.* 1998]. There are now a large number of studies linking exposure to air pollutants with a reduction in heart rate variability [Brook, *et al.* 2010].

Here, we have shown that reducing personal exposure to particulate air pollution in patients with coronary heart disease is associated with an improvement in heart rate variability during exercise in both general measures of variability and in specific frequency bands. In this study, the changes demonstrated were predominantly in the HF-power (high-frequency power) band, which is associated with changes in parasympathetic tone, and an improvement may suggest an increased contribution of vagal tone to heart rate control. In this study we demonstrated that the high-frequency components of HRV were actually

higher during exercise than over the 24-hour period. This is an unusual finding, and we would expect the HF components to be reduced during exercise as the physiological response to increased physical exercise if vagal withdrawal. This may be a reflection of the lower quality data from the 24 hour recording limiting the comparison here.

In our previous healthy volunteer study [Langrish, *et al.* 2009], heart rate variability increased following the facemask intervention although changes were seen predominantly in the LF-power band – suggesting effects on sympathetic nervous system control. We suggest that this difference (HF-power *vs.* LF-power changes) arises as a result of the high use of beta-blocker therapy (74% of patients), which is likely to modify any changes in sympathetic tone. The significance of acute changes in heart rate variability is not clear, although it has been demonstrated that the higher the variability, the lower the cardiovascular mortality [Kikuya, *et al.* 2000]. We suggest that a sustained improvement in heart rate variability has the potential to improve patients' prognosis and reduce the impact of air pollution on cardiovascular morbidity and mortality.

7.5.4 Symptoms

Patients perceived an increase in general well-being, a reduction in effort of work, and a decline in the background pollution levels when they wore the facemask. Whilst we observed no change in the occurrence of anginal

symptoms, this is perhaps not surprising given that we recruited a highly selected population with stable coronary disease, without significant clinical angina, who were maintained on optimal medical therapy.

7.5.5 Limitations

We chose a PROBE study design because we wanted to determine the acceptability of wearing a facemask, as well as its potential beneficial effects on both symptoms and objective measures of cardiovascular health. We recognise that a double-blind approach incorporating a sham mask has the benefits of reducing the potential for subjective bias and being more scientifically robust [Smith, *et al.* 2007] In addition we acknowledge that such an intervention may be more readily accepted in Chinese and Asian societies where the wearing of a facemask is more common-place due to concern over airborne diseases, pollution and even fashion, and furthermore that this may have affected patients' reporting of symptom improvement. However, even a sham mask will filter air pollutants to some degree [Langrish, *et al.* 2009] and true blinding is difficult to achieve given that large differences in mask efficiency would be readily apparent to trial participants and alterations in mask design obvious to investigators. It would also be anticipated that the greater effort of breathing through a mask during exercise would lead to an increase in blood pressure rather than the reverse.

We have assessed an acute intervention and it remains to be seen whether wearing a facemask for more prolonged periods would have sustained benefits that could impact on clinical outcomes.

7.5.6 Conclusions

In this randomised controlled crossover intervention trial, we have shown for the first time that reducing personal exposure to particulate air pollution appears to be associated with small but consistent objective improvements in myocardial ischaemia, exercise-related increases in blood pressure and heart rate variability in patients with coronary heart disease. Strategies to reduce urban air pollution exposure have the potential to reduce the incidence of acute cardiovascular events as well as improving patients' general well-being.

CHAPTER 8

Conclusions and Future Directions

8.1 Conclusions

Air pollution is emerging as a major risk factor for cardiovascular disease, and in epidemiological studies exposure to particulate air pollution is consistently associated with small increases in cardiovascular morbidity and mortality [Brook, *et al.* 2010]. Acute exposure to air pollution, and particularly that derived from vehicle exhaust has been linked to the triggering of myocardial infarction [Bhaskaran, *et al.* 2011; Peters, *et al.* 2004]. Indeed, a recent meta-regression of factors causing the triggering of myocardial infarction suggests that on a population level, given the ubiquitous nature of exposure, inhalation of traffic-derived air pollution has the highest population-attributable fraction [Nawrot, *et al.* 2011] and is certainly on a par with well-recognised risk factors such as physical exertion [Newby 2010].

Controlled exposure studies in humans are ideal for investigating the acute effects of exposure to air pollutants [Langrish, *et al.* 2010]. Dilute diesel exhaust has been used as a model exposure as it is simple to generate, can be carefully controlled and is directly relevant to everyday exposures as it has been shown that in the urban environment up to 40% of the airborne particulate matter is derived from diesel exhaust engines [Zheng, *et al.* 2007]. Previous work has demonstrated that following inhalation of diesel exhaust there is an increase in arterial vasoconstriction [Peretz, *et al.* 2008] and an increase in central arterial stiffness [Lundback, *et al.* 2009]. During the

exposure there is an increase in blood pressure [Urch, *et al.* 2005] and in myocardial ischaemia measured using electrocardiography [Mills, *et al.* 2007]. Following exposure, changes in vascular endothelial function have been demonstrated, with impairment of vascular vasomotor function and endogenous fibrinolysis [Mills, *et al.* 2005]. Furthermore, exposure to dilute diesel exhaust leads to an activation of platelets and an increase in overall thrombotic tendency [Lucking, *et al.* 2008].

Whilst these observations provide mechanisms whereby exposure to air pollutants may result in acute cardiovascular events, many unanswered questions remain. Here we have investigated the role of different air pollutants, the mechanisms underlying the changes in vascular endothelial function and begun to address the important public health question of how we can protect the vulnerable from the adverse effects of air pollution exposure.

8.1.1 Exposure to nitrogen dioxide is not associated with vascular dysfunction in man

Air pollution is a complex mixture of gaseous, volatile and particulate matter and the contribution of each individual component in the adverse effects of exposure are unknown. Using a robust randomised double-blind controlled crossover design 10 healthy volunteers were exposed to filtered air and nitrogen dioxide (4 ppm) for 1 hour with intermittent exercise. We have

demonstrated for the first time that exposure to nitrogen dioxide, a principal gaseous pollutant associated with combustion-derived air pollution, is not associated with any vascular vasomotor or fibrinolytic dysfunction. The adverse cardiovascular effects of combustion-derived air pollution appear to be mediated via components other than nitrogen dioxide, and it is plausible that these vascular effects are rather driven by the fine and ultrafine particle fractions [Langrish, *et al.* 2010].

8.1.2 Contribution of endothelin-1 to the vascular effects of diesel exhaust inhalation in humans

The vascular mechanisms underlying the endothelial dysfunction demonstrated following dilute diesel exhaust exposure remain unclear. In this study we investigated the role of the endogenous vasoconstrictor endothelin-1 in these effects. 13 health volunteers were exposed to dilute diesel exhaust and filtered air in a double-blind randomised controlled crossover study. Diesel exhaust exposure increased vascular sensitivity to endothelin-1 and reduced vasodilatation to endothelin-A receptor antagonism despite unchanged plasma endothelin concentrations. These data demonstrate that the previously documented impairment of endothelium-dependent vasodilatation following a one-hour exposure to combustion-derived air pollutants is not mediated by an upregulation of the endothelin system. Given the tonic interaction between the endothelin and nitric oxide systems, our data are consistent with the hypothesis that the

diesel exhaust-induced vascular effects are predominantly driven by reduced endothelial nitric oxide bioavailability [Langrish, *et al.* 2009].

8.1.3 Altered nitric oxide bioavailability contributes to diesel exhaust inhalation-induced vascular dysfunction in man

In our subsequent study, we addressed the hypothesis that nitric oxide bioavailability is central to the adverse vascular effects of diesel exhaust inhalation. We demonstrated that plasma nitrite, a marker of vascular nitric oxide, is increased after diesel exhaust inhalation consistent with homeostatic regulation in the presence of increased nitric oxide consumption due to local oxidative stress. In the presence of the nitric oxide clamp and the absence of endogenous nitric oxide production, diesel exhaust inhalation does not result in further vascular impairment. We conclude that the vascular dysfunction associated with diesel exhaust inhalation is predominantly mediated by reduced nitric oxide bioavailability.

8.1.4 Cardiovascular effects of reducing exposure to particulate air pollution with a simple facemask in healthy volunteers

Air pollution is something to which we are all exposed on a daily basis, and as the evidence mounts as to the detrimental effects of exposure on the cardiovascular and respiratory systems, the question of how best to protect the vulnerable remains. Increasing concern relating to the health effects of air pollution has led many individuals to use facemasks to reduce personal

exposure, although the efficacy if such a strategy remains unclear. In this study we demonstrate for the first time that in health volunteers wearing a facemask appears to abrogate the adverse effects of air pollution on blood pressure and heart rate variability. This simple intervention has the potential to protect susceptible individuals and prevent cardiovascular events in cities with high concentrations of ambient air pollution [Langrish, *et al.* 2009].

8.1.5 Reducing particulate air pollution exposure in patients with coronary heart disease

We addressed the potential for facemasks to reduce personal exposure to particulate air pollution and improve cardiovascular health in patients with coronary heart disease, a population thought to be vulnerable to the adverse effects of air pollution exposure. The need for such studies was highlighted in the recent American Heart Association statement on particulate air pollution and cardiovascular disease [Brook, *et al.* 2010].

In an open randomised crossover trial, 98 patients with coronary heart disease walked on a pre-defined route in central Beijing, China in the presence and absence of a highly efficient facemask.

We demonstrated for the first time that in patients with coronary heart disease reducing personal exposure to urban airborne particulate matter by means of a simple facemask appears to be associated with a reduction in

symptoms, and objective improvements in myocardial ischaemia, blood pressure and heart rate variability. We conclude that reducing personal exposure to particulate air pollution has the potential to reduce the incidence of cardiovascular events in patients with coronary heart disease living and working in industrialized or urban environments as well as improving patients' general well-being.

8.2 Future directions

A number of important questions have arisen from the results and peer review of our studies that we plan to explore in future work.

8.2.1 How do inhaled particles influence the vascular endothelium?

It is now well established that inhalation of particulate air pollution results in acute vascular effects and in our studies we have addressed a number of the vascular endothelial mechanisms that may underlie these effects. A fundamental question of how these particles influence the vascular endothelium however remains: namely whether inhaled particles cause a local inflammation within the lung and then secondary inflammatory mediators cross into the bloodstream and affect the vascular endothelium, or whether the particles themselves can access the bloodstream and have a direct influence [Mills, *et al.* 2009]. Previous attempts to address this fundamental question have been inconclusive, predominantly due to the

difficulties of labelling particles [Mills, *et al.* 2006; Möller, *et al.* 2008; Nemmar, *et al.* 2002].

We plan to investigate this further in man using a model particle exposure. Twelve healthy male volunteers will be exposure to an aerosol of gold nanoparticles generated in a spark generator for 2 hours during intermittent exercise. Gold particles have been chosen as they are of a similar size to air pollution particles, are relatively inert, can easily be measured in blood and urine and are currently under development as a clinical imaging contrast agent and for drug delivery [Thakor, *et al.* 2011]. Blood samples will be taken over the 24 hours after exposure, and urine collected for 24 hours and analysed for the presence of gold using high-resolution inductively-coupled plasma mass spectroscopy.

The results from this study will clarify the fundamental mechanism by which particles enter the body and exert their influence on the cardiovascular system. In order to protect the general population, particularly those in high-risk groups, from the adverse effects of exposure to airborne particulate matter it is crucial to understand this step. If particles themselves are capable of crossing into the bloodstream, then pharmacological interventions, or alterations in the surface chemicals adsorbed onto the particles are unlikely to have a big impact on limiting the harmful effects, thus focussing attention again on reducing individuals' exposure.

The findings from this study not only have relevance in the study of atmospheric air pollution, but also within the continually expanding field of nanotechnology. Engineered nanoparticles are already being employed in a staggering array of products and their use is rapidly growing. Occupational exposure to engineered nanoparticles is therefore a concern, and is likely to affect a growing number of the population in the future. Understanding the risks associated with such exposures, and the fundamental mechanisms underlying the risk, is key to protecting the workforce and limiting harm.

8.2.2 If particles do translocate into the bloodstream, is this via the lungs or the gastrointestinal tract?

Following on from the above study, if we are able to identify that particles can be detected in blood and urine following exposure, the route of uptake still remains unclear. Although most particles will be inhaled deep into the respiratory tree and are retained there for a long period of time [Möller, *et al.* 2008], many particles will also be swallowed into the gastrointestinal tract by collecting in saliva or by transport via the mucociliary escalator.

Ten healthy male volunteers will be recruited and in a similar design to the previous study will be asked to drink a suspension of gold nanoparticles generated as previously but collected in distilled water, before blood samples

and a 24 hour urine collection are performed to determine whether particles can be detected in the bloodstream or not.

The results from this study will clarify whether the main route of access to the body of airborne particulate matter is through inhalation or by ingestion of particles trapped in saliva or respiratory tract secretions. To date, studies have focussed on inhalation alone as the route of exposure of patients and all interventions have been targeted within the lungs or using respiratory protection. Should particles be capable of accessing the body through the gastrointestinal tract then this would provide another avenue of study and another potential target for limiting the adverse effects of air pollutants. As with the proposed gold inhalation study, the findings from this study not only have relevance in the study of atmospheric air pollution, but also within the continually expanding field of nanotechnology.

8.2.3 What is the role of nitric oxide bioavailability on systemic haemodynamics following diesel exhaust exposure?

Following on from our studies investigating the effects of local nitric oxide synthase inhibition, we plan to study the effect of nitric oxide inhibition on systemic haemodynamics. Sixteen healthy volunteers will be recruited for a randomised double-blind crossover study and will be exposure to dilute diesel exhaust and filtered air during intermittent exercise. After the exposure, continuous measurements of blood pressure will be recorded by

means of a radial artery catheter inserted under aseptic conditions and central arterial stiffness measured using peripheral arterial tonometry. Cardiac output and systemic vascular resistance will be determined using thoracic bioimpedance (Hotman®, HemoSapiens, USA). Recordings will be taken with subjects lying quietly at rest before the intravenous infusion of L-NMMA at 3mg/kg over 5 mins followed by normal saline for 10 mins and nor-epinephrine at 50 ng/kg/min for 15 mins in a randomised double-blind fashion. Recordings will be performed during the infusions and for the following 45 minutes, at which time we anticipate responses will have returned to baseline.

The vascular endothelium is critical in ensuring the health of our cardiovascular system. Changes in endothelial function precede the development of clinically relevant atherosclerotic diseases and acute cardiovascular events. Understanding the effect of exposure to air pollution on nitric oxide bioavailability is crucial both in explaining the current observations, but also in designing interventions to protect the population against adverse effects in the future.

8.2.4 What is the role of the autonomic nervous system in the adverse vascular effects of air pollution exposure?

Exposure to particulate air pollution is consistently associated with changes in heart rate variability, an indirect marker of sympathetic and

parasympathetic nervous system activity [Brook, *et al.* 2010]. Furthermore, acute exposure to particulate air pollution and dilute diesel exhaust is associated with increases in blood pressure [Langrish, *et al.* 2009; Urch, *et al.* 2005], increased vascular tone [Brook, *et al.* 2002; Peretz, *et al.* 2008], central arterial stiffness [Lundbäck, *et al.* 2009] and myocardial ischaemia [Mills, *et al.* 2007], all of which may be mediated by autonomic nervous system activity. Furthermore, our observations that the vascular endothelial effects are driven predominantly by changes in nitric oxide bioavailability also highlight the possible contribution of the autonomic nervous system. The nitric oxide system within the vasculature is intimately linked to the autonomic nervous system and dysregulation of either influences the other [Zanzinger 1999].

We therefore propose to investigate the role of the autonomic nervous system in the adverse cardiovascular effects of air pollution exposure directly. Sixteen healthy male volunteers will be recruited and exposed to dilute diesel exhaust or filtered air during intermittent exercise as previously. Direct measurement of sympathetic nervous system activity will be performed using microneurography and measurement of muscle sympathetic nerve activity from the peroneal nerve during intermittent autonomic stress by means of the cold pressor test, valsalva manoeuvre, deep breathing techniques and lower-body negative pressure as described previously [Burke, *et al.* 2011]. Simultaneous continuous blood pressure,

systemic vascular resistance and cardiac output will be recorded using a Portapres® (Finapres Medical Systems, Amsterdam, Netherlands) and blood and urine samples collected for measurement of catecholamines. 3-lead continuous electrocardiography will be performed for measurement of heart rate variability.

The autonomic nervous system plays an important role in vascular haemostasis and is known to be upregulated in states such as cardiac failure. The adverse effects of the autonomic nervous system are readily treated with simple pharmacological agents (i.e. beta-blockers) currently used in clinical practice. The utility of such agents in limiting harm from exposure is unclear, although it is recognised that the effect of exposure is generally lower in patients maintained on such medications [Barclay, *et al.* 2009]. Understanding the role of the autonomic nervous system will both further the understanding of the fundamental mechanisms underlying the adverse effects of exposure, but also lead the way for intervention trials in the future.

8.2.5 Does inhalation of dilute diesel exhaust exacerbate symptoms of angina and markers of myocardial ischaemia in patients with stable angina pectoris?

Inhalation of dilute diesel exhaust has been shown to increase sub-clinical myocardial ischaemia as measured using 12-lead continuous electrocardiography in patients with stable asymptomatic coronary heart

disease [Mills, *et al.* 2007]. The question of whether this will have an important functional consequence on patients with coronary heart disease remains. Twenty patients with stable angina pectoris and documented coronary heart disease will be recruited from the Royal Infirmary of Edinburgh cardiology department. In a randomised double-blind crossover study, subjects will attend on 4 separate occasions and will be exposed to filtered air, and three concentrations of dilute diesel exhaust (30, 100 and 300 $\mu g/m^3$) for 1 hour during intermittent exercise as previously. Continuous 12-lead electrocardiography will be performed, and subjects will complete a detailed symptom questionnaire. Immediately following the exposure, subjects will complete a symptom limited submaximal treadmill exercise stress test using the standard BRUCE protocol, and time to symptoms and time to 1 mm ST segment depression recorded. Blood samples will be collected and analysed for highly sensitive biomarkers of ischaemia including ischaemically-modified albumin and ultra-sensitive troponin I (Singulex, Alameda, CA, USA).

The acute and chronic effects of exposure, whilst still incompletely understood, are now well-documented and help to explain the epidemiological associations with cardiovascular morbidity and mortality. However, the key for patients with coronary heart disease is whether these effects result in changes in the clinical syndrome of angina pectoris. Understanding this important element will enable us to better advise

patients with pre-existing cardiovascular conditions, and tailor our treatments more effectively. It may enable the targeted use of interventions such as facemasks to those at highest risk to limit the clinically apparent effects of exposure.

8.2.6 Does inhalation of dilute diesel exhaust reduce myocardial perfusion?

Following on from the above question, twenty subjects with ischaemic heart disease will be recruited from the Royal Infirmary of Edinburgh cardiology department. In a randomised double-blind crossover study, subjects will be exposed to filtered air and dilute diesel exhaust during intermittent exercise. Following the exposure, myocardial perfusion will be measured using CT-PET and dobutamine stress as previously described [Di Carli*, et al.* 2007].

The electrocardiographic changes during exposure to particulate air pollution are well-documented [Mills, *et al.* 2007] and suggest an increase in acute myocardial ischaemia. Similarly, there are documented effects on vascular vasomotor function that may explain the mechanism of reduced coronary artery flow. It remains to be shown however, whether exposure can indeed reduce myocardial blood flow and alter perfusion, factors clearly important both to the individual patient and in advising patients with pre-existing disease as to their future activity.

8.3 Perspectives

In these studies, we have demonstrated that the adverse vascular effects of exposure to air pollution are driven predominantly by airborne particulate matter, and result in vascular endothelial effects which appear to be predominantly driven by changes in nitric oxide bioavailability. Furthermore, we have demonstrated that by reducing personal exposure to particulate air pollution we can reduce the adverse effects on blood pressure, autonomic control of the heart, symptoms and myocardial ischaemia. These findings help us to understand the pathophysiology underlying the epidemiological observations and provide potential therapeutic targets. The demonstration of early and important benefits of reducing exposure to particulate exposure, alongside epidemiological observations demonstrating the effectiveness of interventions to reduce combustion-derived air pollution [Clancy, *et al.* 2002] and tobacco smoke [Meyers, *et al.* 2009; Pell, *et al.* 2008] (which shares many physical and chemical similarities with particulate air pollution) exposure on deaths from cardiovascular disease lend weight to the growing lobby to change environmental health policy and improve air quality for all. These findings are important worldwide, but perhaps more so in countries with developing economies where the rapid expansion of industry, road transport and domestic power supplies is driving the economic expansion. Air quality in these developing countries is often poor, and the need for economic growth understandably is seen to take priority over improving air quality in major cities.

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APPENDIX 1

Particle characteristics and chemical composition

from Beijing, China

Appendix 1. Particle characteristics and chemical composition for particulate matter collected in Beijing China in March 2009. Data is expressed as mean concentrations (< represents result below lower limit of detection). Comparator data is shown for 2 European cities (Amsterdam and Rome) collected and analysed employing the same techniques in Spring 2009 [courtesy of Dr Miriam Gerlofs, National Institute for Public Health and the Environment, Bilthoven, Netherlands].

Particle Characteristics in Beijing and 2 European Comparators		**Beijing** *17-18th March 2009*			**Amsterdam** *Spring 2009*		**Rome** *Spring 2009*	
		Coarse	**Fine**	**Ultrafine**	**Coarse**	**Fine**	**Coarse**	**Fine**
		$PM_{2.5-10}$	$PM_{0.18-2.5}$	$PM_{<0.18}$	$PM_{2.5-10}$	$PM_{0.18-2.5}$	$PM_{2.5-10}$	$PM_{0.18-2.5}$
Mass, µg/m³ (% total mass)		114 (34%)	151 (45%)	72 (21%)	10	6	18	15
Carbon	Total carbon (µg/mg dust)	166	497	571	Not Tested			
	Elemental carbon (µg/mg dust)	12	91	99				
	Organic carbon (µg/mg dust)	154	445	472				
	EC : OC ratio	0·08	0·21	0·21				
Anions	Nitrates (µg/mg dust)	93	590	510	121	222	82	106
	Sulphates (µg/mg dust)	77	217	183	52	133	32	176

16 US EPA Priority Polycyclic Aromatic Hydrocarbons (PAHs)	Napthalene (ng/g dust)	2599	3015	4265	5400	2300	220	2400
	Acenaphthylene (ng/g dust)	2107	1771	7315	1100	900	<	<
	Acenaphthene (ng/g dust)	3540	2882	3886	15200	<	4000	<
	Flourene (ng/g dust)	3234	2558	3399	8500	800	2600	<
	Phenanthrene (ng/g dust)	1959	3004	3502	21400	22100	9300	7600
	Anthracene (ng/g dust)	1423	1100	1553	1300	3100	800	1700
	Fluoranthene (ng/g dust)	1649	17858	16071	24500	44800	14600	12700
	Pyrene (ng/g dust)	1780	16689	14485	18500	38800	14200	13600
	Benzo[a]anthracene (ng/g dust)	1728	9501	7343	1600	8600	1500	7400
	Chrysene (ng/g dust)	2323	22107	21533	3500	20700	3300	13600
	Benzo[b]fluoranthene (ng/g dust)	2235	27478	29031	1900	24200	1200	26200
	Benzo[k]fluoranthene (ng/g dust)	1321	22251	18664	900	6200	900	4800
	Benzo[a]pyrene (ng/g dust)	1781	18099	17762	2400	10300	900	13300
	Ideno[123–cd]pyrene (ng/g dust)	1573	16378	15061	800	9300	700	10500
	Dibenzo[ah]anthracene (ng/g dust)	2108	3510	3359	200	2200	<	1700
	Benzo[ghi]perylene (ng/g dust)	1624	24488	24829	1000	13800	1300	17800

Hopanes	17α(H),21β(H)22,29,30–Trisnorhopane (ng/mg dust)	1·9	2·8	17·1	4·7	7·4	2·8	4·7
	17α(H),21β(H)–Hopane (ng/mg dust)	4·4	3·7	10·3	4·7	12·7	8·8	19·7
Steranes	20R–5α(H),14β(H),17β(H)–Cholestane (ng/mg dust)	3·2	1·8	24·7	1·0	3·2	1·4	3·4
	20R–5α(H),14α(H),17α(H)–Cholestane (ng/mg dust)	0·0	1·3	14·7	1·1	0·9	0·7	0·9
	20R–5α(H),14β(H),17β(H)–24S–methycholestane (ng/mg dust)	0·0	1·2	12·2	0·6	2·3	1·0	3·4
	20R–5α(H),14β(H),17β(H)–24R–Ethylcholestane (ng/mg dust)	1·9	1·5	15·4	5·4	3·5	2·1	6·8

Heavy Metals	Aluminium (ng/mg dust)	18733	4093	7486	5954	3564	32514	15764
	Antimony (ng/mg dust)	64	71	75	173	96	145	121
	Barium (ng/mg dust)	2403	678	968	267	117	350	124
	Cadmium (ng/mg dust)	8	30	27	3	11	2	10
	Calcium (ng/mg dust)	64357	12854	20119	18562	3810	47683	13370
	Cerium (ng/mg dust)	50	12	17	11	3	46	21
	Chromium (ng/mg dust)	78	60	58	74	42	93	56
	Copper (ng/mg dust)	303	292	313	638	279	518	272
	Iron (ng/mg dust)	38976	11045	14001	20488	7638	28820	12880
	Lanthanum (ng/mg dust)	25	5	9	7	3	21	9
	Lead (ng/mg dust)	228	1364	1277	111	324	106	364
	Magnesium (ng/mg dust)	18799	3791	6293	23384	10736	15581	6936
	Manganese (ng/mg dust)	847	629	665	359	232	440	243
	Neodymium (ng/mg dust)	20	4	6	3	<	18	7
	Nickel (ng/mg dust)	85	57	70	78	204	47	82
	Potassium (ng/mg dust)	8211	17556	17246	7007	2837	6878	5190
	Silicon (ng/mg dust)	2396	1659	2669	11137	<	59812	28108
	Sodium (ng/mg dust)	9475	5060	5679	22517	4687	7031	2540
	Strontium (ng/mg dust)	326	85	125	122	38	50	29
	Sulphur (ng/mg dust)	13060	33606	32504	Not Tested			
	Titanium (ng/mg dust)	970	326	402	234	58	760	344

APPENDIX 2

Patient symptom questionnaire

Symptom Score Sheet – Before Walk

Mask over past 24 hours? Yes ❑ No ❑

Draw a cross on the lines below…..

	None at all	*Worst ever*
Headache:	⊢——————————⊣	
Dizziness:	⊢——————————⊣	
Sickness:	⊢——————————⊣	
Tiredness:	⊢——————————⊣	
Cough:	⊢——————————⊣	
Difficulty in breathing:	⊢——————————⊣	
Irritation of the eyes:	⊢——————————⊣	
Irritation of the throat:	⊢——————————⊣	
Irritation of the nose:	⊢——————————⊣	
Unpleasant smell:	⊢——————————⊣	
Bad taste in the mouth:	⊢——————————⊣	

Working scale – how hard has walking today been?

Very easy ⊢——————————⊣ Impossible

Perception of pollution – how bad do you think it is today?

None at all ⊢——————————⊣ Worst ever

Symptom Score Sheet – After Walk

Mask during the walk? Yes ❑ No ❑

Draw a cross on the lines below…..

	None at all	*Worst ever*
Headache:	⊢——————————————————⊣	
Dizziness:	⊢——————————————————⊣	
Sickness:	⊢——————————————————⊣	
Tiredness:	⊢——————————————————⊣	
Cough:	⊢——————————————————⊣	
Difficulty in breathing:	⊢——————————————————⊣	
Irritation of the eyes:	⊢——————————————————⊣	
Irritation of the throat:	⊢——————————————————⊣	
Irritation of the nose:	⊢——————————————————⊣	
Unpleasant smell:	⊢——————————————————⊣	
Bad taste in the mouth:	⊢——————————————————⊣	

Working scale – how hard has walking today been?

Very easy ⊢——————————————————⊣ Impossible

Perception of pollution – how bad do you think it is today?

None at all ⊢——————————————————⊣ Worst ever

Symptom Score Sheet – 24 hours

Mask over past 24 hours? Yes ❑ No ❑

Draw a cross on the lines below…..

	None at all	*Worst ever*
Headache:	⊢——————————————————⊣	
Dizziness:	⊢——————————————————⊣	
Sickness:	⊢——————————————————⊣	
Tiredness:	⊢——————————————————⊣	
Cough:	⊢——————————————————⊣	
Difficulty in breathing:	⊢——————————————————⊣	
Irritation of the eyes:	⊢——————————————————⊣	
Irritation of the throat:	⊢——————————————————⊣	
Irritation of the nose:	⊢——————————————————⊣	
Unpleasant smell:	⊢——————————————————⊣	
Bad taste in the mouth:	⊢——————————————————⊣	

Working scale – how hard has walking today been?

Very easy ⊢——————————————————⊣ Impossible

Perception of pollution – how bad do you think it is today?

None at all ⊢——————————————————⊣ Worst ever

How tolerable did you find wearing the mask (*if applicable*)?

Very easy ⊢——————————————————⊣ Impossible

APPENDIX 3

Publications arising from this thesis

Publications arising from or relevant to this thesis

1. Barath S, Mills N, Törnqvist H, Lucking A, **Langrish JP**, Söderberg S, Boman C, Westerholm R, Löndahl J, Donaldson K, Sandström T, Newby D and Blomberg A. Impaired vascular function after exposure to diesel exhaust generated at urban transient running conditions. *Part Fibre Toxicol*. 2010;7:19.

2. **Langrish JP**, Frampton M and Blomberg A. 11. Human exposure studies. *in* Cardiovascular effects of inhaled ultrafine and nanosized particles. F. Cassee, N. Mills and D. Newby (eds). 2010. J Wiley & Sons Inc. Hoboken, New Jersey.

3. **Langrish JP**, Lundback M, Barath S, Soderberg S, Mills NL, Newby DE, Sandstrom T and Blomberg A. Exposure to nitrogen dioxide is not associated with vascular dysfunction in man. *Inhal Toxicol*. 2010;22:192-198.

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5. **Langrish JP**, Mills NL, Chan JK, Leseman DL, Aitken RJ, Fokkens PH, Cassee FR, Li J, Donaldson K, Newby DE and Jiang L. Beneficial cardiovascular effects of reducing exposure to particulate air pollution with a simple facemask. *Part Fibre Toxicol*. 2009;6:8.

6. **Langrish JP**, Mills NL, Donaldson K and Newby DE. Response to Peter Joseph. *Heart*. 2010;96:472-3.

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PARTICLE AND FIBRE TOXICOLOGY

RESEARCH Open Access

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

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Abstract

Background: Traffic emissions including diesel engine exhaust are associated with increased respiratory and cardiovascular morbidity and mortality. Controlled human exposure studies have demonstrated impaired vascular function after inhalation of exhaust generated by a diesel engine under idling conditions.

Objectives: To assess the vascular and fibrinolytic effects of exposure to diesel exhaust generated during urban-cycle running conditions that mimic ambient 'real-world' exposures.

Methods: In a randomised double-blind crossover study, eighteen healthy male volunteers were exposed to diesel exhaust (approximately 250 μg/m^3) or filtered air for one hour during intermittent exercise. Diesel exhaust was generated during the urban part of the standardized European Transient Cycle. Six hours post-exposure, vascular vasomotor and fibrinolytic function was assessed during venous occlusion plethysmography with intra-arterial agonist infusions.

Measurements and Main Results: Forearm blood flow increased in a dose-dependent manner with both endothelial-dependent (acetylcholine and bradykinin) and endothelial-independent (sodium nitroprusside and verapamil) vasodilators. Diesel exhaust exposure attenuated the vasodilatation to acetylcholine ($P < 0.001$), bradykinin ($P < 0.05$), sodium nitroprusside ($P < 0.05$) and verapamil ($P < 0.001$). In addition, the net release of tissue plasminogen activator during bradykinin infusion was impaired following diesel exhaust exposure ($P < 0.05$).

Conclusion: Exposure to diesel exhaust generated under transient running conditions, as a relevant model of urban air pollution, impairs vasomotor function and endogenous fibrinolysis in a similar way as exposure to diesel exhaust generated at idling. This indicates that adverse vascular effects of diesel exhaust inhalation occur over different running conditions with varying exhaust composition and concentrations as well as physicochemical particle properties. Importantly, exposure to diesel exhaust under ETC conditions was also associated with a novel finding of impaired of calcium channel-dependent vasomotor function. This implies that certain cardiovascular endpoints seem to be related to general diesel exhaust properties, whereas the novel calcium flux-related effect may be associated with exhaust properties more specific for the ETC condition, for example a higher content of diesel soot particles along with their adsorbed organic compounds.

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Background

Increasing attention has been directed towards the adverse health effects associated with particulate matter (PM) air pollution in terms of both respiratory and cardiovascular morbidity and mortality [1-3]. Changes in air pollution levels over previous decades account for as much as 17% of the change in life expectancy over this period [4]. Several studies have pointed towards traffic-related air pollution as being of particular concern. Living or going to school in proximity to major thoroughfares is associated with reduced lung growth in children and increased risk of asthma, as well as cardiovascular events including increased atherosclerosis and left ventricular hypertrophy in adult populations [5-10].

In a pivotal study, Peters and co-workers demonstrated that patients admitted with myocardial infarction were three times more likely to have been in traffic in the hours prior to the onset of symptoms, suggesting a causal link to the triggering of acute coronary events. The authors identified an association between traffic exposure and the onset of chest pain immediately and six hours prior to the event, indicating that more than one mechanism is likely to be involved [11].

Emissions from motor vehicles, in particular combustion-derived particles from diesel engines, contribute to up to 40% of urban PM and are thought to play a major role in the adverse effects of PM. Experimental exposure studies in healthy and asthmatic individuals have demonstrated oxidative stress, airway inflammation and worsening of asthma, as well as acute cardiovascular events [12-17]. Recent studies have confirmed that diesel exhaust (DE), generated during idling engine conditions, causes endothelial dysfunction, arterial stiffness, decreased fibrinolytic capacity, increased platelet activation and increased *ex-vivo* thrombus formation in the first 24 hours following exposure [12-14,18]. Furthermore, patients with stable coronary heart disease experienced more ST-segment depression with exercise during exposure to diesel exhaust as compared to filtered air [19]. Additionally, diesel exhaust inhalation impaired endogenous fibrinolytic capacity in these patients with reduced endothelial release of tissue plasminogen activator (t-PA) six hours following exposure. The time course of these adverse vascular and prothrombotic effects is consistent with the observation of an immediate and delayed association between traffic exposure and the onset of acute myocardial infarction [20].

Diesel exhaust particles (DEP) generated under different engine running conditions vary in size and chemical composition. It is therefore of interest to elucidate the associations between physicochemical properties of the DEP and health effects caused by exposure to DE generated under various engine running conditions. In general, the mass of emitted DEP is often dominated by carbonaceous agglomerated soot particles, typically 50-300 nm, while the number concentration is dominated by smaller particles, below 50 nm, mainly composed of volatile organic material and sulphur compounds. Adsorbed organic compounds, as well as small amounts of sulphate, nitrate, metals and other trace elements are generally also associated with the diesel exhaust. Further, a fraction of fuel and lube oil generally escape oxidation in the engine and are emitted as exhaust PM, consisting of different organic compounds including polycyclic aromatic hydrocarbon (PAH) compounds [21,22].

People living in urban areas are mainly exposed to particulate emissions generated during acceleration and deceleration conditions rather than from idling engines. We have therefore designed a human exposure system using the standardized European Transient Cycle (ETC) urban sequence that better reflects the exposures generated in ambient urban settings. The aim of the present study was to determine whether inhalation of dilute diesel exhaust generated during the ETC urban sequence would affect vasomotor function and fibrinolytic capacity in healthy human subjects, in a similar or different way as following exposure to diesel exhaust generated under idling conditions.

Methods

Subjects

Eighteen healthy non-smoking males mean age 27 years (range 21-30 years) with normal lung function were included in the study, following a standardized clinical examination. All were free from symptoms of respiratory tract infections for at least 6 weeks prior to and during the study. Female subjects were not included due to the potential for cyclical hormones to affect vascular responses. The study was approved by the local ethics committee. All volunteers gave their written informed consent and the study was performed in accordance to the Declaration of Helsinki.

Study design

The subjects were exposed to filtered air and diesel exhaust for one hour according to a randomized, double-blind, cross-over design with an interval of 42 (22-62) days between visits [15,23]. During each exposure, the subjects performed moderate exercise (average minute ventilation, 20 L/min/m^2 body surface) on a bicycle ergometer alternating with rest at 15-minute intervals.

Based on previous exposure studies exploring respiratory and cardiovascular effects of diesel exhaust [12,13,15,17], vascular assessments using venous forearm plethysmography were performed 6-hours after diesel and filtered air exposure. All subjects refrained from alcohol for 24-hours and from food and caffeine-containing

beverages for at least 4-hours before each vascular study. They were not allowed to carry out strenuous exercise during the day of exposure. The studies were carried out in a quiet, temperature-controlled room maintained at 22°C to 24°C, with subjects in the supine position.

Diesel exhaust exposure

The exposures were performed in a specially built and validated human exposure chamber that has been described in detail elsewhere [23]. Diesel exhaust (DE) was generated by a diesel engine from 1991 (Volvo TD40 GJE, 4.0 L, four cylinders) connected to an engine dynamometer and running under control of a computer program according to the European Transient Cycle (ETC). The ETC is a well-established running condition for standardized tests of engines and vehicles and is designed to mimic real life running conditions for vehicles in an urban environment. As such, it is based on authentic recordings of accelerations and retardations, with variations in engine load as well as periods with constant speed. The running of the engine was controlled adjusting the actions of the injection system and an engine dynamometer that varied the load of the engine. The ETC test cycle has been introduced together with the European Stationary Cycle (ESC), for emission certification of heavy-duty diesel engines in Europe (Directive 1999/96/EC). The ETC comprises three different driving conditions, including urban, rural and motorway. In this study we used only the urban driving part, representing city driving with a maximum speed of 50 km/h, frequent starts, stops, and idling, i.e. transient engine running conditions (Figure 1).

The diesel fuel used was Statoil class 1; cetane number 54; aromatics, 4% volume; polycyclic aromatic hydrocarbons, < 0.02 vol-%; sulphur < 1ppm. The initial boiling point was 195°C and 95% volume boiling point was 280°C. More than 90% of the exhaust was shunted away and the remaining part was accumulated in an approximately 1 m^3 container in order to smooth the concentration variation during the transient running conditions. A partial flow of DE was continuously drawn from the container, diluted with filtered air and fed into the exposure chamber, giving a particle mass concentration of approximately 250 $\mu g/m^3$ in the exposure chamber.

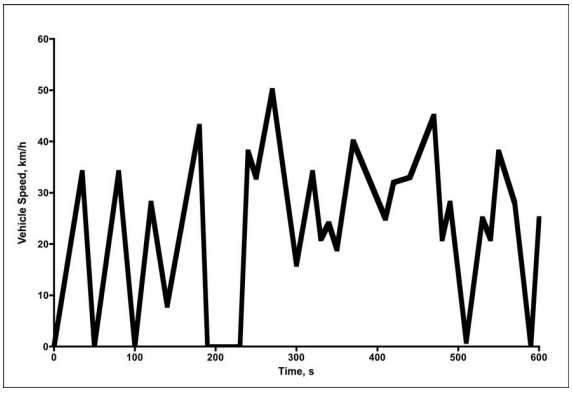

Figure 1 The urban sequence of the European Transient Cycle applied in the present study.

The particle mass concentration (as PM_{10}) of diesel exhaust particles (DEP) in the chamber during the exposures was measured gravimetrically employing standard glass fibre filter sampling as well as monitored with a Tapered Element Oscillating Microbalance (TEOM 1400) instrument (at 50°C). Measurements of NO_x, NO and NO_2 were performed with standard instruments using a chemiluminescence technique (ECO Physics CLD 700 AL Med, Switzerland). Total hydrocarbon in the exposure aerosol was measured using a flame ionization detection method (Hydrocarbon Analyzer, Model 3-300, JUM Engineering Co, Oakland, California, USA). Data on exposure characteristics are given in Table 1.

Extensive physicochemical characterisation of the DEP in the chamber was performed on a single occasion after the campaign, but under the same conditions as during the exposures. The aerodynamic particle mass size distribution in the range of 0.03-10 μm was determined using a 13 stage low-pressure cascade impactor (Dekati Ltd, Tampere, Finland). As seen in Figure 2, fine particles (< 1 μm) totally dominated the PM mass in the chamber and the fine mode aerodynamic mass median diameter (MMDa) was determined to be 116 nm (Table 1).

A scanning mobility particle sizer (SMPS) was used to classify the particles regarding their number, size distribution and concentration within 0.013 to 0.380 μm (equivalent mobility diameter). The system consisted of a differential mobility analyzer (DMA) (TSI model 3071A, TSI, St Paul, Minnesota, USA) and a condensation particle counter (CPC) (TSI model 3010, TSI, St Paul, Minnesota, USA). Relatively long scan times, i.e. 120 s up and 40 s down, were used to enable purging of the system between the scans. Aerosol flow was set to 1 L/min and sheath air flow to 7 L/min. The average total number concentration of fine particles in the chamber (normalized to a mass concentration of 300 $\mu g/m^3$) and average count median (equivalent mobility) diameter (CMDm) are shown in Table 1 and Figure 3.

The DEP were characterized chemically regarding carbon fractionation (organic/elemental carbon), PAH and trace elements. The fraction of organic and elemental carbon of the PM in the chamber was determined by thermal-optical carbon analysis (Method NIOSH 5040) using quartz and teflon+quartz filters in parallel according to standard procedures [24]. "Elemental carbon" is

Table 1 Exposure characteristics of the present study during European Transient Cycle urban conditions compared to earlier studies [12,14] during idling engine conditions.

	ETC urban present study	Idling previous study
Exposure campaigns		
Diesel engine	Volvo TD40, 4.0 L, four cylinders	Volvo TD45, 4.5 L four cylinders
Diesel fuel	Diesel class I	Diesel class III
PM_{10} mass concentration (filter), μg/m^3	254 ± 36	330 ± 12 [(3)]
PM_{10} mass concentration (TEOM), μg/m^3	228 ± 19	-
NO_x, ppm	7.5 ± 0.3	2.8 ± <0.1 [(3)]
NO_2, ppm	0.9 ± 0.1	0.6 ± <0.1 [(3)]
NO, ppm	6.6 ± 0.3	2.2 ± <0.1 [(3)]
Total hydrocarbons (THC), ppm	1.2 ± 0.2	1.6 ± 0.2 [(3)]
Complementary measurements [(1)]		
Particle number concentration, #/cm^3	1.2×10^5	9.5×10^5 [(4)]
Particle count median size ($CMD_{mobility}$), nm	129	55 [(4)]
Particle mass median size ($MMD_{aerodynamic}$) nm	116	199 [(4)]
Organic carbon fraction (OC/TC[(2)]), %	12	94.5 [(4)]
Elemental carbon fraction (EC/TC[(2)]), %	88	5.5 [(4)]
Total PAH concentration, μg/m^3	0.96	3.5 [(4)]
Semi-volatile PAH concentration, μg/m^3	0.69	3.4 [(4)]
PM-associated PAH concentration, μg/m^3	0.27	0.16 [(4)]

[(1)] Particle number and PAH concentrations normalized to a PM mass concentration of 300 μg/m^3 to enable a comparison between ETC and idling conditions.
[(2)] TC = Total carbon content in PM mass
[(3)] Exposure protocol data from a previously reported study [14]
[(4)] Un-published DEP characteristics data associated with the exposure conditions during the previously reported studies [12,14]
For the exposure campaign data, mean values are given mean ± std.dev.

in principle a measure of the soot fraction of samples and the result therefore implies that the mass of DEP during the present ETC conditions was dominated by soot particles (Table 1).

PAH were sampled by glass fiber filter sampling (diameter 47 mm) for the particulate fraction followed by a polyurethane foam (PUF) plug (diameter 75 mm × 50 mm) for the semi-volatile fraction. Forty-nine PAH compounds were analyzed by gas chromatography-mass spectrometry (GC-MS) for quantification in the particulate and semi-volatile fractions, respectively, according to procedures described in more detail elsewhere [25-27]. A total PAH concentration (normalized to a mass concentration of 300 μg/m^3) of 0.96 μg/m^3 was

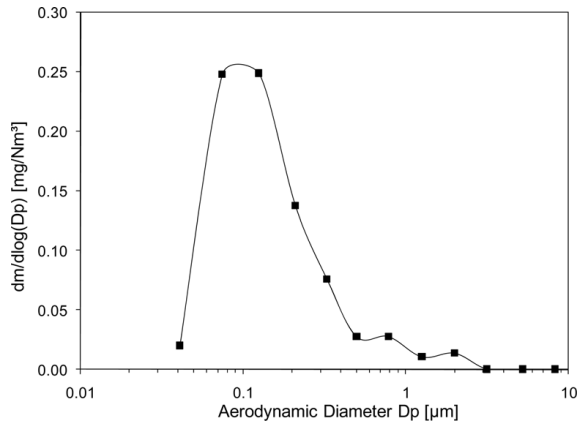

Figure 2 Mass size distribution of diluted diesel exhaust particles in the exposure chamber during the urban running part of the European Trancient Cycle. MMD_a for the fine mode was 0.116 μm.

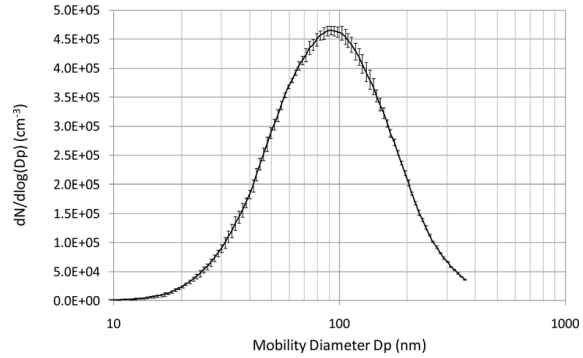

Figure 3 Number size distribution of diluted diesel exhaust particles in the exposure chamber during the urban running part of the European Transient Cycle. Standard deviations for 3 scans are shown as error bars. A log-normal fitting calculation showed that the size distribution comprised in principal only one single mode.

determined in the chamber distributed as 0.69 μg/m^3 in the semi-volatile fraction and 0.27 μg/m^3 as particulate associated (Table 1). This implies that PAH constituted < 1% of the total DEP mass in the chamber. During the idling engine conditions, the PAHs contributing most to the atmosphere in the exposure chamber were phenanthrene and methyl derivatives of phenanthrene, equivalent to more than 80% of the PAHs determined. The corresponding value for the transient engine load (urban part of ETC) was more than 75%.

Determination of oxidative properties of particles

DEP were collected using a high volume cascade sampler with a multi-stage round slit nozzle impactor, within the exposure chamber under both idling and transient (ETC urban part) engine conditions. Coarse and fine diesel particles were collected onto polyurethane foams by impaction [28] and extracted from the collection substrate with methanol using extensive vortexing and sonication, as described previously [29]. PM oxidative activity was assessed in a synthetic respiratory tract lining fluid (RTLF) on an equal mass basis (50 μg/mL). This involved establishing the capacity of fine ($PM_{0.1-2.5}$) and coarse ($PM_{2.5-10}$) samples to deplete ascorbate (AA), glutathione (GSH) and urate (UA), each at a starting concentration of 200 μM, over a 4-h incubation period (37°C). After the 4-h incubation period, samples were either acidified with metaphosphoric acid to a final concentration of 5% (w/v) for UA andAA measurement, or diluted into 100 mM phosphate buffer for the determination of glutathione (GSH). Methodological details for these antioxidant determinations have been described previously [30]. The final PM oxidative potentials (OP) were expressed as the percentage loss of either AA (OP^{AA}) or GSH (OP^{GSH}) over the 4-h incubation, relative to the particle free control, expressed per μg of extracted PM. The OP values associated with the coarse and fine fraction for each antioxidant were combined to derive an expression for $PM_{0.1-10}$. Urate was not lost from the synthetic RTLF with any of the PM samples tested.

Vascular studies

Forearm blood flow (FBF) was measured during unilateral brachial artery infusion of endothelial-dependent and -independent vasodilators using venous occlusion plethysmography with mercury-in-silicone elastomer strain gauges, as described previously [12]. Briefly, the brachial artery of one arm was cannulated with 27-standard wire gauge steel needle and 17 gauge venous cannulae were inserted in large veins in the antecubital fossa of both arms. Following a 30-minute baseline saline infusion, acetylcholine 5, 10, and 20 μg/min (endothelium-dependent vasodilator which does not release tissue plasminogen activator [t-PA]; Merck Biosciences); bradykinin at 100, 300, and 1,000 pmol/min (endothelium-dependent vasodilator which releases t-PA; Merck Biosciences); and sodium nitroprusside (SNP) 2, 4, and 8 μg/min (endothelium-independent vasodilator which does not release t-PA; David Bull Laboratories) were infused for 6 minutes at each dose with FBF measured for the last 3 minutes of each infusion. The infusions of the three vasodilators were given in a random order and separated by 20-minute saline infusions. Verapamil at 10, 30, and 100 μg/min (endothelium-and NO-independent vasodilator which does not release t-PA) was infused at the end of the study protocol due to its prolonged action. Blood pressure and heart rate were measured during the forearm study using a validated semi-automated oscillometric sphygmanometer.

Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during infusion of each dose of bradykinin and collected into acidified buffered citrate (Stabilyte tubes, Biopool International) for tissue plasminogen activator (t-PA) assay. The samples were kept on ice until centrifuged at 2,000 *g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen concentrations were determined by ELISA (TintElize t-PA, Biopool EIA). Hematocrit was determined by capillary tube centrifugation at baseline and at the end of the study protocol.

Markers of systemic inflammation

Peripheral blood samples were taken immediately before and 2 and 6 hours post-exposure and were analyzed for total and differential cell counts by an autoanalyzer in the local clinical biochemistry reference laboratory (Umeå University Hospital, Umeå, Sweden). Serum and plasma were prepared for measurement of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), soluble P-selectin, soluble intracellular adhesion molecule-1 (ICAM-1) and CD40 ligand using commercially available ELISAs (Quantikine, R&D Systems). Serum C-reactive protein (CRP) was measured using an immunonephelometric assay (Behring BN II nephelometer).

Data analysis and statistics

Plethysmographic data were analyzed as described previously [31]. The net release of t-PA 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 [31]. Data were analysed by 2-way ANOVA with repeated measures or 2-tailed Student's *t*-tests, where appropriate, using GraphPad Prism (GraphPad Software, Version 4 for Macintosh) and SPSS (SPSS inc. Chicago, IL, USA,

version 15). All data are expressed as mean ± SEM. Statistical significance was taken at $p < 0.05$.

Results

Exposure characteristics

Compared to the idling situation, exposure data from the ETC urban running condition revealed that the particles were larger but lower in number, despite similar mass concentration. During ETC, the fraction of organic carbon/elemental carbon was reduced, indicating higher soot content, and the total PAH concentration was reduced compared to the idling situation. Furthermore, NO_x levels were increased under ETC, although the oxidative potential of the diesel particles was reduced (Table 1).

Oxidative potential

Determination of oxidative potential (OP) of the PM samples demonstrated that DEP generated under the present ETC urban engine conditions were less oxidizing ($PM_{0.1\text{-}10}$ OP^{GSH} = 0.15 ± 0.12%/μg, $PM_{0.1\text{-}10}$ OP^{AA} = 0.25 ± 0.13%/μg) than DEP generated under presently studied idling engine conditions ($PM_{0.1\text{-}10}$ OP^{GSH} = 0.43 ± 0.11%/μg, $PM_{0.1\text{-}10}$ OP^{AA} = 0.43 ± 0.16%/μg, ETC *versus* idling engine). The oxidative properties observed from both these experimental tail-pipe emissions were minor compared with those observed in a previous study of ambient PM samples collected from roadside sites (OP^{GSH} = 0.60 ± 0.45 and OP^{AA} = 1.26 ± 0.37) [32].

Vascular and fibrinolytic function

There were no differences in resting heart rate, blood pressure, or baseline FBF ($P > 0.05$ for all) after exposure to diesel exhaust or filtered air (Table 2). All infusions of vasoactive drugs increased blood flow in a dose dependent manner ($P < 0.001$). Diesel exhaust exposure significantly attenuated the increase in forearm blood flow during infusions of both endothelium-dependent and -independent vasodilators, as compared with filtered air exposure ($P < 0.05$ for bradykinin, $P < 0.001$ for acetylcholine, $P < 0.05$ for sodium nitroprusside and $P < 0.001$ for verapamil(Figure 4).The net release of t-PA following bradykinin infusion was reduced ($P < 0.05$) after exposure to diesel exhaust compared with filtered air (Figure 5).

Table 2 Baseline haemodynamic parameters after filtered air and ETC urban diesel exhaust exposures.

	Filtered air	Diesel exhaust
Heart rate, bpm	63 ± 1.7	63 ± 2.5
Systolic blood pressure, mmHg	142 ± 3.2	142 ± 2.7
Diastolic blood pressure, mmHg	68 ± 2.9	68 ± 1.8
FBF in infused arm, ml/100 ml tissue/min	3.1 ± 0.3	2.8 ± 0.3
FBF in non-infused arm, ml/100 ml tissue/min	2.5 ± 0.2	2.9 ± 0.3

Data expressed as mean ± SEM. $P > 0.05$ for all using paired Student's *t*-test

Systemic inflammation

Peripheral blood leukocyte, neutrophil and platelet counts, as well as plasma concentrations of TNF-α, IL-6, soluble P-selectin, soluble ICAM-1, CD40L and CRP did not differ ($P > 0.05$ for all) between diesel exhaust and filtered air exposures (Table 3).

Discussion

We demonstrate that short-term exposure to diesel exhaust generated under urban transient engine running conditions, mimicking a 'real-world' urban environment, impairs vascular vasomotor function and endogenous fibrinolytic function in human subjects. These vascular effects are consistent with those reported in studies employing a similar study protocol, but using an idling engine. This suggests that different diesel engine running conditions induce similar adverse vascular responses.

The ETC urban running condition was selected for this study as it is based on actual recordings from vehicles, and therefore provides a highly relevant model of ambient air pollution exposures in urban settings. Whilst the vascular responses were similar after ETC urban running conditions and idling, it was found that the chemical and physical properties differed between exposure situations. The particles generated under the transient running setting were slightly larger (according to the mobility diameter, related to the number concentration) and had a higher elemental carbon to organic carbon ratio, i.e. more soot and less organics, than those generated from the idling diesel engine. The PAH profiles in the present study were also in good agreement with previous diesel PAH exhaust studies [26,33]. Furthermore, when comparing the PAH profiles from the idling and the transient running conditions, they appeared similar, but with the former having more than 4 times greater concentrations of sum PAHs (ng/m^3) in the exposure chamber, mainly related to higher idling gaseous PAH concentrations. In contrast, PM-associated PAH concentrations were similar, but differed in profile, with higher concentrations of phenanthrene, fluoranthene and pyrene under transient load and speed conditions (Figure 6). PM-associated PAHs present under idling conditions were distributed as a higher number of compounds, including heavier PAH (4-rings) species (Figure 7). This can be explained by the fact that the engine's combustion temperature is at its lowest value when operating at idling mode. The relatively larger hydrocarbon emissions at idling reflect unburned fuel

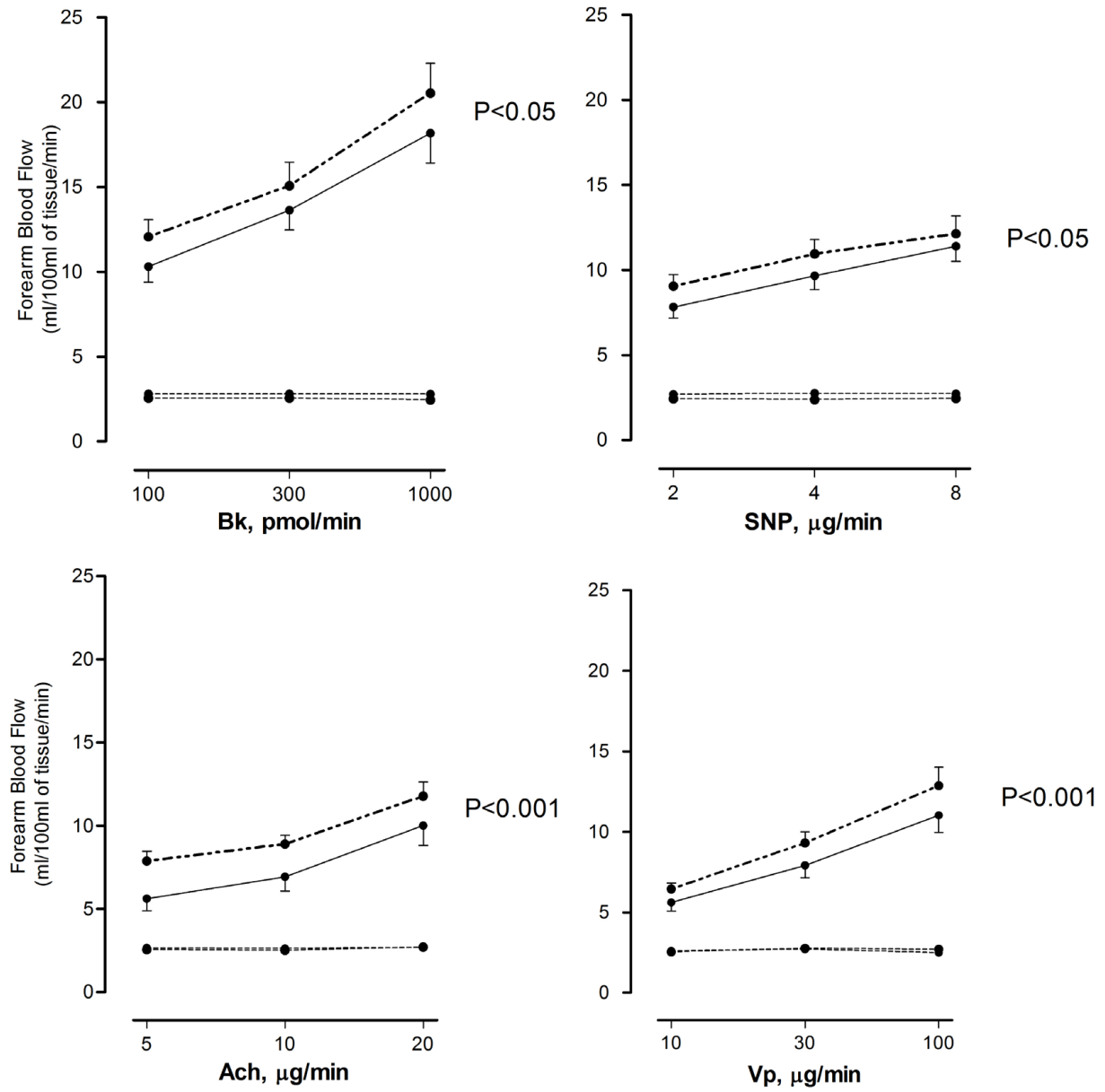

Figure 4 Forearm blood flow measured six hours after diesel exhaust (solid line) and filtered air (dashed line) exposure during unilateral intra-brachial infusion of bradykinin (Bk), acetylcholine (Ach), sodium nitroprusside (SNP) and verapamil (Vp) in infused and non-infused (dotted lines) arms. Effect of exposure compared with using 2-way ANOVA with repeated measures. Data plotted as mean ± SEM. P-values are given for diesel exhaust exposure vs. air.

constituents, indicating that the major part of the PAHs in the exhaust originates from PAHs originally in the diesel fuel. It has previously been shown that PAHs in the diesel fuel represent a significant source for exhaust PAHs, besides the pyrosynthesis of exhaust PAH [33,34].

The present study demonstrates that inhalation of diesel exhaust generated under urban ETC running conditions, causes a reduction in endothelium-dependent and endothelium-independent vasodilatation, together with an impairment of t-PA release from the vascular endothelium. Perturbations of vascular homeostasis are thought to be important in the triggering of acute myocardial infarction and stroke [8,35]. The vascular assessment in the present study was identical to that used in previously reported studies employing diesel exhaust generated under idling conditions [12,13] and was

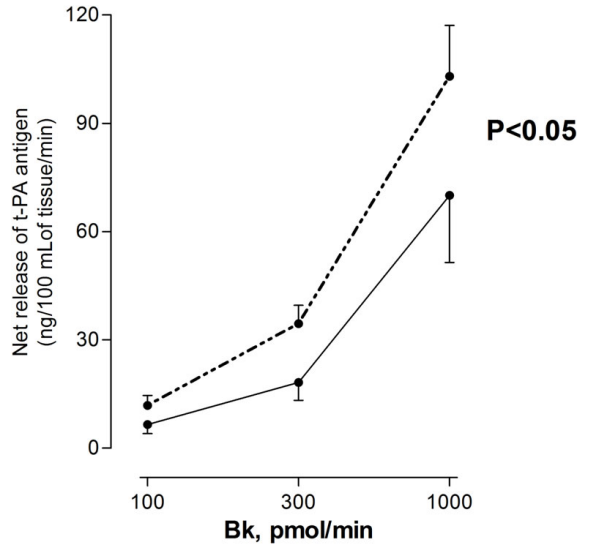

Figure 5 Net release of t-PA antigen in the infused arm during unilateral intra-brachial infusion of bradykinin (Bk) after diesel (solid line) and air (dashed line) exposures. Effect of exposure compared using 2-way ANOVA with repeated measures. Data plotted as mean ± SEM.

intended to enable comparisons between studies. The mass concentration of diesel exhaust particles within the exposure chamber differed slightly between the ETC and idling exposures (254 versus 330 $\mu g/m^3$), as did the particle properties. Irrespective of a slightly lower PM mass, difference in particle size and composition, as well as a lower concentration of PAHs in the present study, we demonstrated similar adverse vascular responses following exposure to diesel exhaust generated by an engine under the ETC running conditions compared to the idling situation.

Interestingly, the present investigation demonstrated that vasodilatation in response to intra-arterial verapamil was impaired following diesel exhaust generated under transient running conditions. This suggests that the vascular smooth muscle also demonstrates a calcium flux-dependent vasomotor function disturbance, which is not endothelium-dependent. This has not previously been shown in studies with idling diesel exhaust that has a higher content of gaseous (semi volatile) PAH and contains a lower fraction of soot particles than the exhaust generated under ETC urban conditions. It can thus be speculated that the higher diesel-related soot content and its associated (adsorbed) organic material in exhaust generated under transient running conditions may cause additional vascular smooth muscle effects, but this issue will need further investigation.

The oxidative capacity of diesel exhaust particles generated under idling conditions has previously been confirmed by analysis of bronchoalveolar lavage and bronchial mucosal biopsies sampled from human subjects, as well as *in vitro* studies [30]. Of note, the oxidative potential of diesel exhaust particles generated in the present study was less pronounced compared to the previous idling situation. Whilst certain differences in particle size, organic carbon/elemental carbon-ratio, PAH-content and oxidative potential exist between the two exposure situations, the cardiovascular effects were generally similar following ETC running conditions although the verapamil-induced vasodilatation was at variance to our previous studies. One could speculate that other exhaust components (particle associated or gaseous) may be the causal link between the exposure and the different adverse vascular effects. In this context, Xia *et al* suggest that organic substances such as quinones may be important in mediating some of the cardiovascular effects of diesel exhaust [36]. Quinones and semi-quinones generated during combustion or cell metabolism have strong oxidative capacity and may interact with mitochondria and affect membrane potential and cell breathing [36,37]. This could be mechanistically linked to diesel exhaust-induced vascular events, as well as the increase in ST-segment depression, identified

Table 3 Peripheral blood parameters after filtered air and diesel exhaust exposures.

	Before air exposure	6 hours after air exposure	Before diesel exposure	6 hours after diesel exposure
Leukocytes (× 10^9 cells/L)	5.6 ± 0.3	5.9 ± 0.3	6.0 ± 0.3	5.5 ± 0.2
Neutrophils (× 10^9 cells/L)	2.4 ± 0.2	3.6 ± 0.3	2.6 ± 0.2	3.1 ± 0.2
Platelets (× 10^9 cells/L)	226 ± 8	214 ± 8	229 ± 10	214 ± 8
TNF-α (pg/ml)	0.92 ± 0.21	0.89 ± 0.17	1.03 ± 0.17	0.80 ± 0.17
IL-6 (pg/ml)	0.68 ± 0.22	2.58 ± 0.82	0.60 ± 0.14	2.17 ± 0.76
CRP (mg/l)	1.32 ± 0.52	1.30 ± 0.46	2.03 ± 0.56	1.85 ± 0.56
CD40L (pg/ml)	95.4 ± 9.2	88.8 ± 10.8	98.4 ± 11.1	85.6 ± 11.1
P-Selectin (ng/ml)	52.0 ± 5.7	58.4 ± 7.1	57.4 ± 6.7	54.1 ± 7.4
ICAM-1 (ng/ml)	260.0 ± 13.9	273.1 ± 24.4	267.8 ± 14.2	252.8 ± 17.4

Data expressed as mean ± SEM. P > 0.05 for all using 2-way ANOVA with repeated measures.

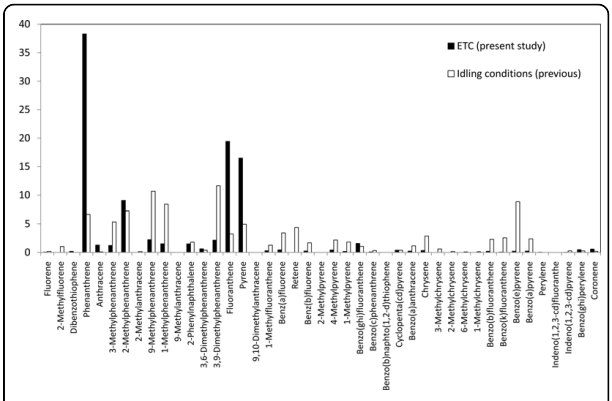

Figure 6 Relative distribution (in % of total PAH) for specific particulate associated PAH compounds for the present ETC running conditions in comparison with idling conditions used in previous studies [12,14].

in patients with stable coronary disease [19]. Accordingly, it is important to further elucidate the role of quinones in mediating the adverse effects of combustion-related air pollution.

There was a difference in NO_x concentration and the ratio NO/NO_2 between the present and previous study employing diesel exhaust exposure at idling. The published literature strongly suggests that the vascular effects present in this and previous experimental exposure studies are due to the presence of combustion-derived particles in diesel exhaust [8]. It has also been recently shown that exposure to NO_2 does not affect vascular function [38], which further implies the importance of a particulate-related source for the vascular effects following exposure to diesel exhaust. However, further studies employing particle concentrator technologies or filtration systems are necessary to clarify the specific role of the particulate and gaseous components, other than NO_x, in diesel exhaust.

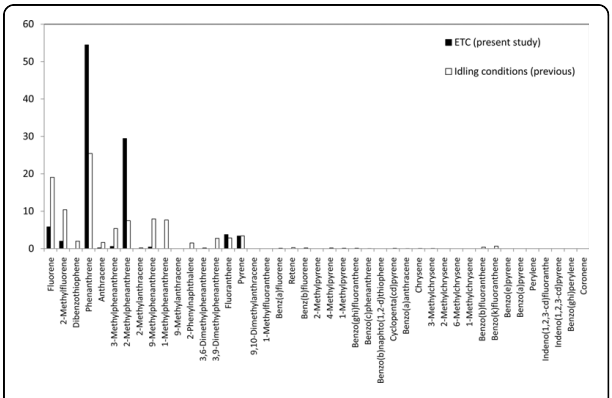

Figure 7 Relative distribution (in % of total PAH) for specific semi-volatile PAH compounds for the present ETC running conditions in comparison with idling conditions used in previous studies [12,14].

Diesel exhaust induces a complex response in the human lungs, including oxidative stress, activation of redox-sensitive transcription factors, inflammatory cell influx, cell activation and secretion of a range of pro-inflammatory components [15,17,32,39]. It has been suggested that this pulmonary inflammation would "spill-over" to the circulation, thereby causing systemic effects. Systemic inflammation has indeed been implicated as an important component in atherosclerosis, vasomotor dysfunction and coronary events [30]. However, in the present investigation we were unable to demonstrate any increased systemic inflammation as reflected by CRP, CD40L, sP-selectin, sICAM-1, IL-6 and TNF-alpha levels in blood at 6 hours post-exposure. This is in line with our previous experiences from diesel exhaust exposures in humans that the presence of major inflammatory events reflected in the blood is not present during the early stages after exposure, but in the later stage of 18-24 hours coinciding with the peak inflammatory bronchoalveolar response [13,17]. This suggests that the diesel exhaust-induced vascular responses described here and in previous diesel exhaust studies using an idling engine are not related to a systemic inflammatory response.

It has been implied that inhaled diesel exhaust particles might directly elicit adverse effects outside the lungs, as inhaled particles are believed to have the potential to translocate from the alveoli to the vascular system and subsequently cause effects in the vasculature, heart and other organs. However, in humans, particle translocation has not yet been consistently proven [40-42]. Small numbers of diesel exhaust particles with their complex highly reactive surfaces, or soluble components from these particles, may well reach the circulation, affecting the endothelium in the pulmonary and peripheral vasculature.

Apart from the running conditions, the exposure protocol was the same as in previous studies and a similar particle mass concentration was intended to enable comparisons. It is recognised that a direct comparison would benefit from having the different engines and running modes included within the same study. This was not possible due to practical issues, and would have demanded even more extensive resources with dual engines mounted, as well as multiple exposures and invasive vascular measurements in the same individuals.

It is concluded that exposure to diesel exhaust generated during urban transient running conditions, a highly relevant model of urban air pollution, impairs vasomotor function and endogenous fibrinolysis. These findings are consistent with previous studies employing an idling engine and indicate that adverse vascular effects of diesel exhaust inhalation occur over different running

conditions with varying gaseous and particle composition and concentrations as well as physicochemical particle properties. Of note, exposure to diesel exhaust under urban running conditions was also associated with a disturbance of calcium channel-dependent vasomotor function, not previously demonstrated. This may suggest that certain cardiovascular endpoints following exposure to diesel exhaust are related to exhaust properties in common between the two studied engine running conditions, whereas the novel calcium flux-related effect seems to be associated with exhaust properties more specific for transient load and speed (ETC) conditions, for example a higher content of diesel soot particles and their adsorbed organic compounds.

List of abbreviations

AA: Ascorbic Acid; Ach: Acetylcholine; Bk: Bradykinin; Bpm: Beats per minute; DE: Diesel exhaust; DEP: Diesel exhaust particles; EC: Elemental carbon; ETC: European transient cycle; FBF: Forearm blood flow; GSH: Reduced glutathione; OP: Oxidative potential; NO: Nitric oxide; NO_x: Oxides of nitrogen; NO_2: Nitrogen dioxide; OC: Organic carbon; PAH: Polycyclic Aromatic Hydrocarbons; PM: Particulate matter; SNP: Sodium nitroprusside; TC: Total carbon; t-PA: Tissue plasminogen activator; Vp: Verapamil.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SB, AB, NM, KD, TS and DN coordinated and were responsible for the planning of the study. NM, AL and SB were responsible for the diesel exhaust exposures. CB and JB were responsible for the exposure characteristics. IM carried out and interpreted data on oxidative potential and made input on manuscript. RW performed the PAH characteristics. SB and NM analysed data and performed statistical analysis. The manuscript was written by SB, NM and AB and then read, corrected and approved by all authors.

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CHAPTER 11

HUMAN EXPOSURE STUDIES

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INTRODUCTION

The epidemiological links between exposure to air pollution and cardiorespiratory morbidity and mortality are well established, and are described in detail earlier in this book (Chapters 2 and 3). These well-designed and carefully controlled observational studies, while of undeniable importance, are limited to describing associations between air pollution exposure and cardiorespiratory disease, and, by definition, cannot prove causation. At best, these studies provide the basis for speculation and hypothesis generation regarding potential pathophysiological mechanisms. Moreover, fundamental uncertainties remain as to the components of air pollution that might cause the observed biological effects.

Human exposure studies have been performed to study the biological and pathophysiological mechanisms underlying the observed health effects.

"REAL-WORLD" STUDIES

One approach to human experimental studies has used controlled exposures to ambient air pollution in city center locations (McCreanor et al., 2007; Langrish et al., 2009). In these studies, human subjects have been studied during and after a walk in a busy city center environment, and have thus been exposed to "normal" ambient air pollution—at "real-world" concentrations and composition. The exposure has been monitored as closely as possible near the breathing zone of the subject using portable monitoring equipment contained in a handcart (McCreanor et al., 2007) or a backpack (Langrish et al., 2009). Exposures in the breathing zone may be much higher than that measured at background monitoring stations (Watt et al., 1995; Buzzard et al., 2009).

The major advantage of such studies is that the exposures are real, and therefore, the results can be extended to similar exposure scenarios. However, these studies may be limited by the day-to-day variability in ambient pollutant

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concentration and chemical composition, depending on traffic density, activity of nearby industry, weather conditions, and a multitude of other factors. As in epidemiological and panel studies, exposures are to complex mixtures, and attributing effects to specific pollutants may not be possible. Furthermore, it is impossible to control for environmental factors other than pollutants that may affect the outcome measures, such as differences in noise levels or visual cues.

Therefore, an alternative experimental design is required, which provides a more stable and predictable exposure, to identify the biological and pathophysiological mechanisms underlying the hypothesized health effects.

GENERAL PRINCIPLES

Requirements for Human Exposure Studies

Because of the inherent variability and difficulties in "real-world" exposures described above, to study the biological and pathophysiological effects of inhaled air pollutants in man, facilities are needed that enable the carefully controlled exposure of human subjects to commonly encountered air pollutants. Such systems need to be highly adjustable to produce the required "test atmosphere" on each occasion. They must also produce a stable and predictable exposure to prevent excessive exposure and potential toxicity.

Any human study needs to be performed within a comfortable environment for the study participant. Balancing the need for space and comfort with the need to maintain an airtight seal around the exposure "chamber" and retain an ability to carefully control the exposure (more difficult in a larger volume of air) and provide continuous monitoring of the actual exposure delivered. Such exposures have been carried out in both large room-sized facilities, as in Umeå, Sweden (Salvi et al., 1999); Rochester, NY (Utell et al., 1984; Morrow et al., 1988); and Seattle, WA (Gould et al., 2008), and in a modified body box as in the mobile exposure facility described by Mills et al. (2008).

Monitoring of Exposures

The key to providing a stable and predictable test environment lies in careful and continuous monitoring of the exposure variables. There is really no limit in the variables that can be measured, although in general, these can be divided into measurements of the particulate components, gaseous components, and volatile hydrocarbons. When measuring exposure variables, the key is to sample the air from the breathing zone of the subject. In large room-sized exposure facilities, sampling inlets are placed in front of the subject, as close to the nose and mouth as practical. In the modified body box, the test "atmosphere" is delivered to the subject through an inlet situated immediately in front of the face, and sampling is carried out from the inlet tube just before it passes into the body box.

Particle Monitoring Particle mass concentration can be measured in "real time" using a number of techniques. The most commonly used method is the tapered

element oscillated microbalance (TEOM). A TEOM provides a nearly real-time measurement of mass concentration by recording the continuous measurement of inertial mass. The sampled aerosol is passed through a hollow tapered tube that is fixed at its wide end. The narrow end holds a filter cartridge, and the whole tube/filter unit acts as a harmonic oscillator. The frequency of the oscillations varies with the mass deposited on the filter, and thus, by recording the frequency changes, mass can be extrapolated following calibration. This can be expressed as mass concentration as the volume of air sampled is known. In this system, the aerosol is heated to around 50°C to prevent any condensation and to provide a standard sampling environment. This also removes any semivolatile components in the aerosol, to ensure that only the particulate phase of the air pollutant is measured (Moosmüller et al., 2001).

2 Nephelometric monitors, such as the DataRAM (Thermo Scientific), work by measuring light scattering. Incident aerosol is passed through a near-infrared light (780 nm), which is deflected by airborne particulate matter. The subsequent scattering of light is a function of particle size and refractive index, but is well described. The nephelometer detects light scattered at 90° and extrapolates mass density, following calibration using a standardized test dust. As particle size and refractive index may not be constant during an exposure, the nephelometric measurements are less accurate than those provided by the TEOM. However, they are extremely sensitive and offer excellent time resolution.

Whichever method is employed to measure mass concentration, exposures are usually also characterized using a gravimetric method. This may be achieved by the use of a micro-orifice uniform deposit impactor (MOUDI). This takes the form of
3 a series of conventional impactors (see Section 1.3), with each stage having a different cutoff point. Each stage then collects particles of a certain size distribution onto a filter media. If those filters are preweighed, and if the volume of air sampled is known, the mass concentration (broken down into different size fractions) can be determined by weighing the filters again at the end of the test run. As well as being used to gravimetrically determine mass concentration, these samples can also be collected and used to chemically assess the composition of the particles.

The size distribution of the airborne particulate can also be determined in real time using a scanning mobility particle sizer (SMPS). With this device, particles are separated into size fractions before being counted using a condensation particle counter (CPC). The aerosol is sampled at a low flow rate (around 1 L/min) from the breathing zone of the subject. It is then passed through an aerosol neutralizer. This consists of a radioactive source of krypton^{85} gas, which generates a high concentration of positively and negatively charged ions, and thus "neutralizes" the electrical charge of the incident particles to a Fuchs' equilibrium charge distribution (Fuchs, 1963). The next step is to pass the aerosol through a differential mobility analyzer (DMA). The DMA consists of two concentric cylinders: the outer one kept at ground potential and the inner one having a precise negative charge applied. The flow of particles is then altered by the electrical charge, with negatively charged particles being repelled from the inner electrode and positively charged particles attracted. By changing the charge applied, only particles of a certain charge (and therefore size distribution) pass through the DMA and onto the next stage. By ramping the

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charge across the DMA, different size fractions can be collected in sequence. After passing through the DMA, the next stage is a CPC. The CPC simply counts the number of particles passing though the sensor (and in combination with the SMPS, creates a size distribution profile). The aerosol is passed into the CPC where it passes through a heated saturator containing butanol (or in some models, water). The aerosol and alcohol vapor pass together into a cooled condenser, where the alcohol vapor condenses around the airborne particles—thus increasing the size of each particle in the sample making it easier to be detected. The saturated aerosol is then passed through a focused laser in a very fine jet, and as the particles pass through the light source, light is deflected to a photodetector at 90° to the laser source, and the particle is thus counted. The exhaust stream consisting of the saturated particles is discarded after being passed through a charcoal filter to remove the butanol.

Whether or not the size distribution of the particles is measured using an SMPS, a CPC is usually used in combination with a measure of mass concentration in the monitoring of the particle exposure. This is important as the smallest particles carry the least weight and vice versa, and thus, a simple mass concentration may not reflect the airborne particle load by itself.

In addition to size distribution, mass concentration, and particle number offered by the SMPS, TEOM/RAM, and CPC in turn, surface area can also be determined. Ultrafine and nanoparticles in the aerosol carry very little mass, although these can be present in very large quantities—especially in fresh combustion-derived particulate matter such as diesel exhaust. Although these particles have little mass, they have a very large surface area and thus offer a very large reactive surface. There is evidence that this ultrafine fraction may be more toxic than the larger particles, perhaps mediated by their ability to penetrate deeply into the lungs where they provide a large reactive surface for interaction (Donaldson et al., 1998). Thus, particle surface area offers additional information that both helps in determining the size of the particles encountered and also their potential toxicity.

Surface area may be measured using a nanoparticle surface area monitor (NSAM). The NSAM samples the incident aerosol at a constant rate of 2.5 L/min before splitting the flow into two streams. One stream passes directly into a mixing chamber. The second stream passes through a carbon filter and then a HEPA filter (highly efficient particle filter) to provide filtered clean air. This clean airstream is then passed across an ionizer where it collects a charge of positive ions before being passed into the mixing chamber. In the mixing chamber, it encounters the incident unfiltered aerosol from earlier, and, by diffusion charging, the particles are brought into a defined and electrically charged state. After the removal of excess ions in a trap, these charged particles are then drawn through an electrometer, where their charge is measured. The degree of charge held by the particles is closely associated to their surface area, and thus, an estimate of total surface area can be made.

The chemical composition of the particles in the aerosol can be measured by collecting particles on a filter. Particles can then be removed from the filter and assessed using mass spectrometry and other methods. An alternative is to use an aerosol time-of-flight mass spectrometer (ATOFMS) to measure single-particle characteristics at the time of sampling (Freney et al., 2006). The ATOFMS samples the aerosol and collimates the particles using a nozzle passing into a low-pressure

airstream. The aerodynamic size of the incident particle is measured by the time delay for the particle to pass through two sequential continuous-wave lasers, and the appropriate delay for firing the Nd:YAG ablation/ionization laser (266-nm wavelength) is determined. As the particle passes through the ablation region, it is ionized by the laser, and the resulting positive and negative ions are accelerated in opposite directions into time-of-flight mass analyzers, giving a positive and negative ion spectra for each individual particle. In this way, "real-time" chemical composition of the incident particles can be determined. This is of specific importance when assessing "real-world" ambient particles that, by their nature, can vary significantly (see Section 1.3).

Monitoring Gaseous and Volatile Organic Copollutants The gaseous components of the incident aerosol can be determined by continuous gas analyzers, sampling from the breathing zone of the subject. The most abundant gaseous components of particulate matter air pollution such as diesel exhaust, are carbon monoxide, nitrogen dioxide (and other oxides of nitrogen), and oxides of sulfur. Real-time continuous monitors are available to assess all these gaseous copollutants, and these can separate the different oxides of nitrogen into nitric oxide (NO), nitrogen dioxide (NO_2), and total oxides of nitrogen (NO_x).

Volatile organics are usually assessed as total hydrocarbons and measured in real-time using a flame ionization detector (FID) (Salvi et al., 1999). The FID works by passing the aerosol through a hydrogen flame that is burning in hydrocarbon-free air. As the hydrocarbons present in the incident aerosol pass through the flame, they are combusted producing CH fragments. These are rapidly oxidized by oxygen in the air to form CHO^+ ions, which can be detected as an electrical charge. The total electrical charge is proportional to the total hydrocarbon content in the aerosol.

CONCENTRATED AMBIENT PARTICLES (CAPs)

Ambient air pollution concentrations vary widely depending on location and environmental conditions, and the chemical composition of the ambient fine-particulate matter fraction is also highly variable. This makes controlled exposures to ambient air pollution fraught with difficulty, as any exposure can be unpredictable, and adequate control of the different fractions (gaseous/particulate/volatile) cannot be achieved. In addition, while chronic exposure to low levels of particulate air pollution is associated with increased cardiovascular risk (Miller et al., 2007), and acute exposure is related to the triggering of acute myocardial infarction (Peters et al., 2004), in general, ambient concentrations are too low to study the pathophysiological and biological effects in any mechanistic fashion (Cuddihy and McClellan, 1989). Therefore, to study the effects of ambient air pollution, specifically designed exposure systems are required that can increase an individual's exposure while introducing systems to control exposure to the "unwanted" components.

The strongest epidemiological association between exposure to air pollution and cardiovascular health outcomes is with the fine particulate fraction ($PM_{2.5}$; particulate matter with a mean aerodynamic diameter of 2.5 µm or less). As discussed

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previously, to study the biological and pathophysiological effects of particulate as opposed to gaseous ambient air pollution, it is necessary to find a way to concentrate these particles suspended in the air, without changing the chemical characteristics of the particles or concentrations of the gaseous components, prior to introducing them into a human exposure facility (Sioutas et al., 1995).

Two systems have been employed to concentrate ambient particulate matter: the *virtual impactor system* and the *versatile aerosol concentration enrichment system* (VACES), of which the virtual impactor system was the first to be developed (Sioutas et al., 1995).

Virtual Impactor Systems

A virtual impactor is a device used to separate airborne particulate matter according to its aerodynamic size (Sioutas et al., 1995, 1997; Kim et al., 2000). Simply, a virtual impactor works by drawing air across an inlet (Fig. 11.1) at a high flow rate. As the particle-laden air passes through the inlet, a fraction of that flow is allowed to pass straight through into a collection nozzle. This is termed the "minor flow." Larger particles, which have the most mass and therefore the most momentum, travel across the collection inlet into the minor flow, but smaller particles, with little inertia, get diverted with the deflected air jet—the "major flow." The major flow is discarded as waste, while the minor flow (typically 10–50% of the total flow) is collected for the next stage of the process. Particles in the minor flow are concentrated by a factor of Q_{total}/Q_{minor}, where Q_{total} is the total flow entering the impactor and Q_{minor} is the

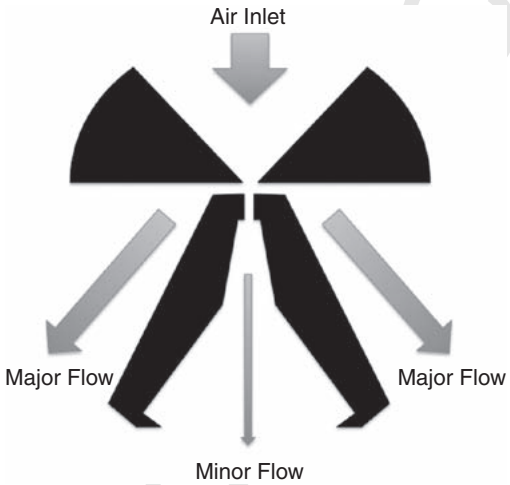

Figure 11.1 Schematic diagram of a virtual impactor nozzle. Air is drawn through an inlet nozzle, thus accelerating the flow. A small fraction of the air that passes through the nozzle continues straight into a collecting nozzle along with the particles with the most mass (minor flow). The remaining air is diverted around the collection nozzle with the smaller and lighter particles (major flow). By adjusting the size of the slits in the impactor, and the flow though it, the particles of interest can be collected.

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minor flow rate. Therefore, with a minor flow rate of 20%, particles within the minor flow are concentrated by a factor of 5.

The use of virtual impactors to concentrate airborne fine particulate matter is well described (Sioutas et al., 1999). In general, these impactors are used in series, with each stage concentrating the particle concentration yet further.

Exposure systems designed to deliver concentrated ambient particulate matter to human volunteers based on virtual impactor technology have been used at the Environmental Protection Agency in Chapel Hill, NC (Ghio et al., 2000); at the University of Southern California, Los Angeles (Gong et al., 2003, 2008); and the Environmental Health Unit in Toronto, Ontario (Brook et al., 2002).

The system used at Chapel Hill is described here in detail. The first stage of this system is a "filter" to remove all the particles larger than those of interest. In the large epidemiological studies into the health effects of air pollution, the primary focus has been on fine particulate matter with a mean diameter of 2.5 µm or less ($PM_{2.5}$), and therefore, the "filter" stage is set with a 50% cutoff of 2.5 µm. However, the use of a simple size selective filter requires a low sampling rate to maximize efficiency, and this is suboptimal when wanting to concentrate particles further using virtual impactors, which, by their nature, further reduce the volume of air in which particles are suspended.

To sample at the required flow rates, a conventional inertial impactor is used to filter the particles above 2.5 µm from the sampled air prior to delivery to the concentrator stage. This, in effect, is similar to the virtual impactor described above. Air is drawn through the impactor at a high flow rate and accelerated through the inlet nozzle. The shape and size of the nozzle can be altered, based on the well-described Navier–Stokes equation (Marple and Liu, 1974), to produce a jet with predictable flow characteristics. The jet is directed at an impaction plate, usually coated with an adhesive surface, to trap particles that hit the impaction plate. Again, particles with the most mass and hence inertia continue straight on with the jet and become trapped, and smaller particles with less inertia become diverted around the impaction plate with the deflected air. By altering the inlet nozzle as described above, the cutoff point for the particles that collide with the plate can be altered. The air passing through the impactor/filter stage now contains predominantly particles with a mean diameter less than 2.5 µm ($PM_{2.5}$) and passes to the next stage for concentration.

The concentration of particles is then performed using a series of virtual impactors. At Chapel Hill, a three-stage impactor is used (Ghio et al., 2000). The first stage is composed of five virtual impactors in parallel, each operating at 1000 L/min with a minor flow rate of 20% and a slit 50% cutoff of 0.1 µm. The second stage of a single impactor again works at 1000 L/min and has a minor flow rate of 20% and a cutoff of 0.1 µm. The final stage is a single impactor working at 200 L/min with a minor flow rate of 40% and a cutoff of 0.1 µm resulting in 80 L/min of minor flow containing the concentrated particles. This is then mixed in a conditioning chamber with 120 L/min of fresh filtered air before being introduced into a human exposure chamber at 200 L/min. Theoretically, the first stage and second stage of the concentrator should each concentrate the ambient particulate matter by a factor of 5, and the final stage by a factor of 2.5, although, in practice, concentrations are

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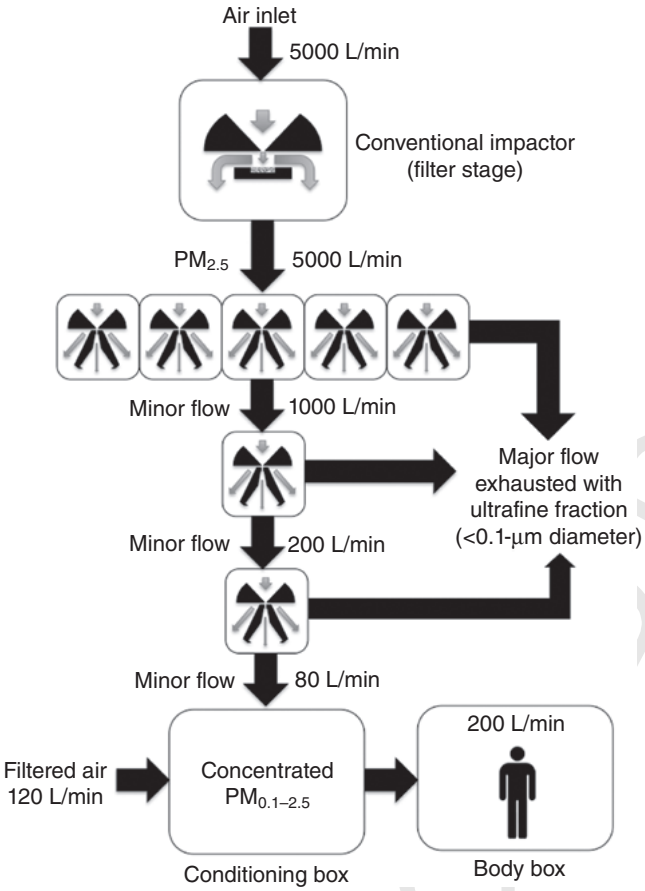

Figure 11.2 Schematic diagram of the virtual impactor concentrator at Chapel Hill, NC. The first stage consists of a conventional impactor working as a filter stage to remove large particles, and then three concentration stages employing virtual impactors to increase the concentration of collected particles.

increased by around half of this. In all, the three-stage concentrator allows a 6- to 10-fold concentration of ambient particulate matter at the inlet to the exposure chamber (Fig. 11.2). Concentrators used in Southern California and Toronto use only a two-stage concentration process.

VACES

One disadvantage of virtual impactor technology is that the concentrators cannot collect and concentrate the ultrafine particulate component of ambient air. These particles, smaller than 0.1 μm in diameter, have virtually no mass and cannot achieve sufficient momentum to escape from the deflected flow in the virtual impactor to be collected and concentrated. Epidemiological studies have suggested that it is the fine particulate matter ($PM_{\leq 2.5}$) associated with combustion and traffic sources that is

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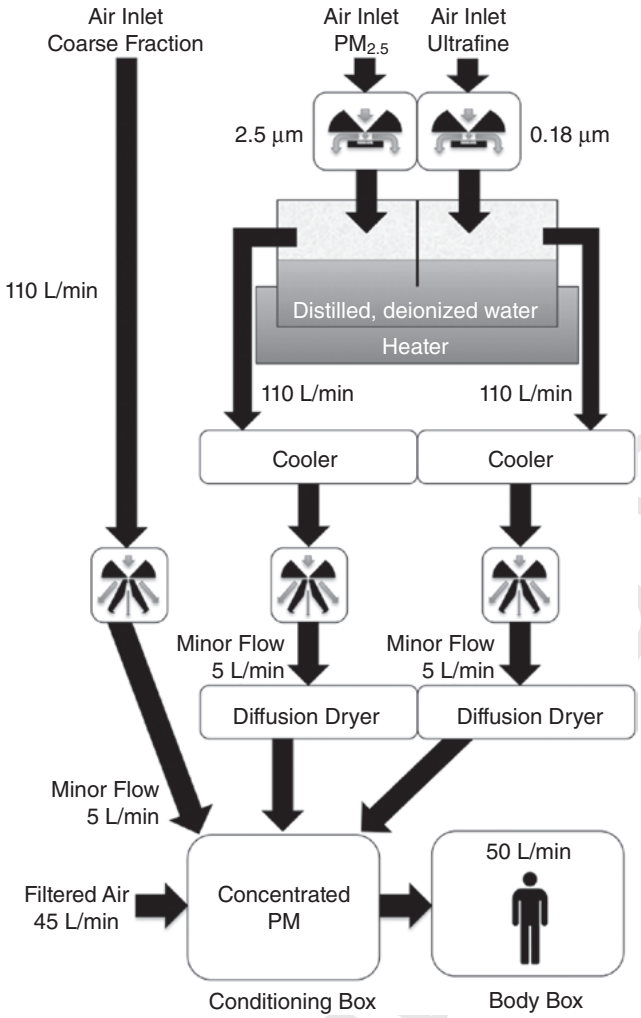

Figure 11.3 Schematic diagram of the versatile aerosol concentration enrichment system (VACES).

most closely associated with adverse health outcomes (Laden et al., 2000). The majority of particles (by number) actually fall within the ultrafine fraction (until they aggregate), and there is evidence that the ultrafine fraction may actually be more toxic than the larger particles (Peters et al., 1997). It is therefore desirable to concentrate both the fine and ultrafine fractions of ambient particulate air pollution when addressing air pollution-induced health effects in a mechanistic fashion.

The VACES has been developed to enable the concentration of all the different fractions of ambient air pollution simultaneously (Kim et al., 2001) and is shown in schematic form in Figure 11.3. Briefly, air is drawn through the system using a powerful vacuum pump operating at 330 L/min. Ambient air is drawn through three inlets, two of which are attached to a conventional inertial impactor to remove larger

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particulate matter, as described previously. One is set to remove particles larger than 2.5 µm in diameter, leaving behind the $PM_{2.5}$ or fine fraction, the other to remove particles larger than 0.18 µm in diameter, leaving the ultrafine fraction. The third outlet inlet does not have an impactor but instead collects total suspended particles, or the coarse fraction.

The coarse fraction is concentrated using a virtual impactor as described previously, with a 50% cutoff at 1.5 µm. Coarse particles can be concentrated by as much as a factor of 33 in this sampling line. The smaller fractions are also concentrated using virtual impactors, but to concentrate these smaller fractions, the particles in the sampling line are processed first. They are passed through a 10-L stainless steel container that is partially filled with warm, distilled, and deionized water. This saturates the aerosol with water vapor. The aerosol is then passed through aluminum tubes embedded in a saline–ice mixture, causing condensation and supersaturation. This causes all particles to grow to around 3-µm droplets. The aerosol is then passed through a virtual impactor with a 50% cutoff at 1.5 µm as previously, and the now enlarged and heavier particles pass into the minor flow and are hence concentrated. The concentrated aerosol is then passed through a diffusion dryer to remove the excess water vapor from the particles and return them to their original size distribution. Any of the fractions can then be diluted with filtered air in a conditioning box before being introduced into the exposure chamber.

Gaseous and Volatile Components

Particle concentrators based on virtual impactor technology do not alter the gaseous or volatile components in the concentrated flow, which is one of the strengths of the technology. However, it is possible to remove the gaseous and volatile components within the concentrated flow prior to delivery into the body box to allow a "pure" particle exposure.

Gaseous and semivolatile organic components can be removed using a simple denuder (Cheng et al., 2009). These denuders work by passing the aerosol across an active surface onto which the organic matter is adsorbed. This may consist of an activated carbon surface or a polystyrene-divinylbenzene (XAD) resin. The activated carbon denuders consist either of impregnated foam or a series of carbon tubes through which the aerosol is passed. They are highly efficient, removing close to 100% of gaseous organic material, and can be operated at up to 40 L/min for a few months before needing to be replaced (Eatough et al., 1999). XAD denuders are less efficient. They consist of either a series of glass tubes coated in XAD resin or an impregnated honeycomb disk over which the aerosol is passed. To achieve a close to 100% efficiency for the carbon-based denuders, lower flow rates (up to a maximum of 16.7 L/min) need to be used, and the active surface needs to be replaced every 10–20 h of operation (Fan et al., 2003).

Denuders can also be used to remove gaseous components from the aerosol before delivery to the body box. Small but highly efficient denuders have been developed (Koutrakis et al., 1993) that pass the aerosol across a large number of glass hexagonal tubes in a honeycomb arrangement coated with sodium carbonate/glycerol to remove basic gases and citric acid/glycerol to remove acidic gases.

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Advantages and Disadvantages of Using CAPs The key strength of using CAPs is also its major weakness. CAPs are "real-world" particles, collected from ambient air and concentrated prior to delivery to the exposure chamber. It therefore allows assessment of the toxicological effect of particles encountered in peoples' normal daily lives, which are the particles proposed to cause the increased cardiorespiratory morbidity and mortality observed in epidemiological studies. The particle concentrators enable the delivery of concentrated particles without significantly altering the chemical composition of the particles themselves (Sioutas et al., 1997), or increasing the concentration of gaseous and volatile pollutants, which is important from a toxicological perspective. Indeed, with the addition of gaseous and semivolatile denuders, the concentration of chemicals in the gaseous phase can actually be reduced prior to delivery into the exposure chamber.

However, there are problems inherent in the use of "real-world" particles. The composition of the airborne particulate matter is dependent on collection location and time, and reflects the relative contribution of different sources such as traffic, combustion, road and brake wear, dust, and pollen. Indeed, in one recently reported study in which no vascular endothelial effects were demonstrated after exposure to concentrated ambient particulate matter in Edinburgh, UK, detailed analysis of the particulate matter revealed that the major chemical component of the particles was in fact inert sodium chloride, reflective of the study's maritime location (Mills et al., 2008). Airborne particulate load is highly dependent on weather conditions such as wind speed, wind direction, and precipitation. This means that during the course of a clinical study, the exposure given to individual patients may vary considerably in both particle concentration and composition. This fact can limit the interpretation of the study results and may also present problematic questions regarding an appropriate statistical approach during analysis.

The concentrators themselves are extremely sensitive to ambient conditions, a fact that can prove problematic in the conduct of clinical studies. In wet and very humid conditions, particles can adhere to the concentrator inlet slits rather than pass through, thus reducing or eliminating the ability to concentrate particles effectively. As a consequence, studies cannot be performed in rain (Ghio and Huang, 2004).

DIESEL EXHAUST EXPOSURE

The epidemiological links with combustion-derived particulate matter and cardiorespiratory morbidity and mortality are well established. The biological and pathological mechanisms behind this observation can be assessed in controlled exposure studies, but, as discussed above, simply using concentrated ambient particulate matter to assess the effects of combustion-derived particulates presents a number of difficulties. The inherent variability of the exposure characteristics and possibility for low concentrations of combustion-derived particles within the concentrated aerosol have resulted in search for a more stable and predictable model.

In the urban environment, up to 40% of airborne particulate matter is derived from diesel exhaust, a fraction that is likely to increase with new restrictions on industry and the current drive for "green" taxation on road vehicles linked to carbon

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dioxide production. Diesel engines produce a cloud of carbon-centered nanoparticulate that includes unburnt fuel, lube oil, polycyclic aromatic hydrocarbons, metals, and sulfates (Scheepers and Bos, 1992; Sydbom et al., 2001). Although diesel engines produce less carbon monoxide and carbon dioxide than gasoline engines, they produce more nitrogen oxides and aldehydes, and the particle emissions are up to 100 times greater—of which >80% (by particle number) are within the ultrafine fraction (<100 nm in diameter), proposed to be the most closely associated with adverse health effects.

Exposure to diluted diesel exhaust has been used as a stable and predictable model of pure combustion-derived air pollution in the study of the health effects of particulate air pollution in human subjects (Nightingale et al., 2000; Sydbom et al., 2001; Mills et al., 2005; Törnqvist et al., 2007; Gould et al., 2008; Peretz et al., 2008).

Briefly, fresh diesel exhaust is produced by a commercial diesel engine under either idling conditions, or during a European city cycle. More than 90% of the exhaust is vented externally, with a small fraction collected and delivered to a conditioning chamber where it is mixed with clean HEPA-filtered air. The conditioning chamber allows complete mixing and the maintenance of a stable temperature and humidity within the aerosol. This is then drawn through an airtight exposure chamber in which the subject is situated. The air within the chamber is sampled from the breathing zone of the subject and monitored as described previously. The concentration of airborne particulate is normally maintained at 200–300 $\mu g/m^3$ and can be adjusted by altering the amount of exhaust drawn into the conditioning chamber. Control exposures to filtered air are generated in the same facility, without adding any diesel exhaust to the chamber. To "blind" the subject to the exposure situation, the diesel engine is running during both exposures to create a similar noise at the two different exposure events (Fig. 11.4).

Idling Engine versus European City Cycle

The characteristics of the diesel exhaust generated from the engine differ depending on a variety of conditions. The initial studies looking at the effects of diesel exhaust inhalation used an unloaded idling engine (Salvi et al., 1999; Mills et al., 2005). In these studies, the engine was allowed to reach a steady-state temperature before the study commenced, as the particle characteristics change with increasing temperature and increasing combustion efficiency. Other studies have been performed using a city cycle, designed to mimic the emissions from a diesel vehicle travelling through an urban area.

The European city cycle is characterized by low vehicle speed, low engine load, and low exhaust gas temperature, and the cycle is repeated throughout the study period (Fig. 11.5, Table 11.1). Using the city cycle alters the characteristics of the particles produced, more closely mimicking the actual exposure to combustion-derived air pollution encountered in urban environments. In general, as the engine's speed and load increase, the particulate mass produced increases exponentially—thus, the bulk of the particle mass is produced at higher speed and load. Nitrogen oxides also increase with engine speed and load, although the growth rate slows at

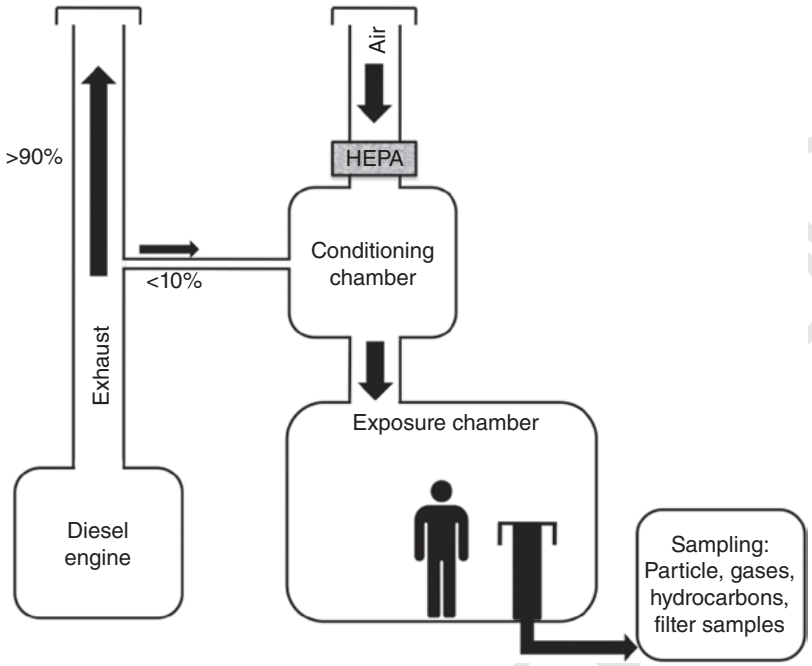

Figure 11.4 Schematic diagram of a diesel exhaust exposure facility.

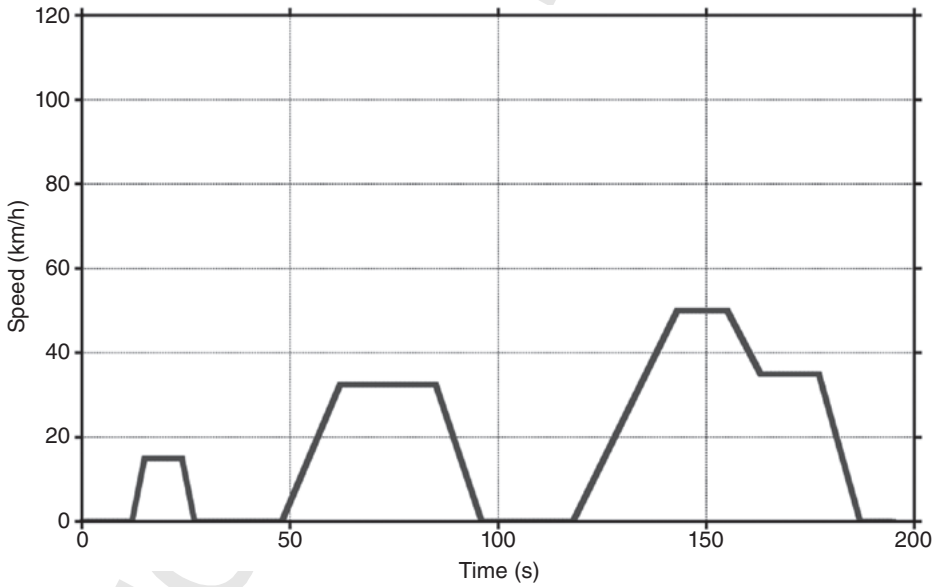

Figure 11.5 The European city cycle (ECE 15). Data points obtained from http://www.dieselnet.com/standards/cycles/ece_eudc.html.

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TABLE 11.1 European City Cycle (ECE 15) Characteristics

Characteristics	Unit	ECE 15
Distance	km	4.052
Duration	s	780
Average speed	km/h	18.7
Maximum speed	km/h	50

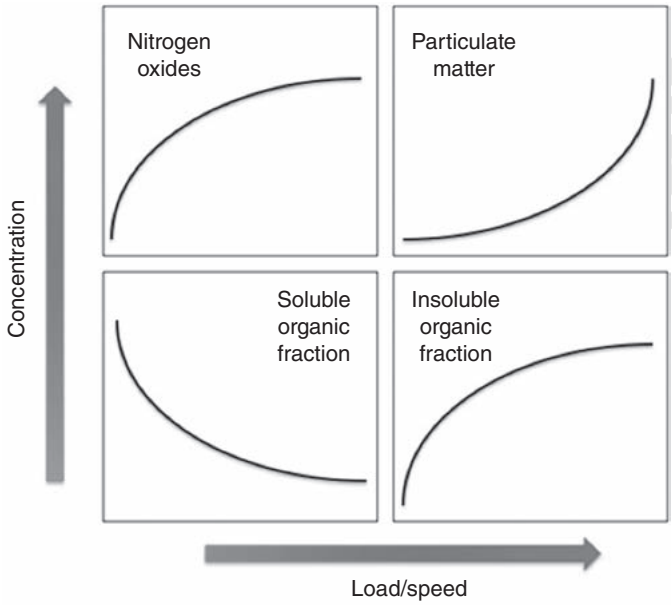

Figure 11.6 Changing composition of diesel exhaust emissions under different loading conditions.

higher velocities. The chemical composition of the particles produced also changes (Fig. 11.6): as the engine speed increases, the proportion of soluble organics decreases and the proportion of insoluble organics increases (Tan et al., 2004).

Exploring New Fuels: Biodiesel and Fuel Additives

A diesel exhaust exposure setup also offers the perfect opportunity to study alternative fuels. There is a current drive to move away from reliance on fossil fuels toward more sustainable fuels such as biodiesel. While it is presently true that commercially available biodiesel is generally mixed with standard diesel fuel as well as suggested that the adverse health effects are driven predominantly by the standard diesel fuel, the possible health effects of biodiesel are unknown (Swanson et al., 2007). To the authors' knowledge, no studies have yet been published on the effect of biodiesel (or commercial fuel additive) exposure on humans, although initial experiments in animal models are described (Finch et al., 2002).

Advantages and Disadvantages of the Diesel Exhaust Exposure

Using a predictable model of exposure such as diesel exhaust offers many advantages. To ensure consistency of approach, exposures can be performed identically on different study days, thus making interpretation of the results much easier. The inherent variability of chemical composition and particulate load seen with CAPs is not an issue. The technology required to produce a diesel exposure does not rely on complex impactors that are sensitive to environmental and weather conditions, meaning that studies can be carried out on any particular day—a definite advantage in scheduling clinical experiments involving human subjects.

However, for all these advantages, diesel exhaust exposures will always be open to criticism. Diesel exhaust is used as a model of ambient combustion-derived air pollution. While it is true that diesel exhaust can make up a significant proportion of an individual's exposure to air pollution in urban areas, exposure to pure diesel exhaust (or indeed pure combustion-derived particulate) is artificial. Therefore, extrapolating the results from experimental diesel exhaust exposure chamber studies to the ambient exposure situation is fraught with uncertainty. However, to gain important mechanistic information on air pollution-induced health effects, experimental exposure chamber studies are essential and complementary to "real-world" exposures.

GASOLINE EXPOSURES

Interest in the potential health effects of gasoline exhaust has been limited in the literature. This is largely due to the observation that adverse health effects closely correlate with combustion-derived particulate air pollution. Although gasoline engines do emit particulate matter, this amount is fairly insignificant compared with commercial diesel engines, which can emit over 100 times as many particles. In addition, since the introduction of lead-free petrol and catalytic converters, the environmental focus in reducing air pollution associated with vehicles has shifted toward diesel engines.

Several controlled exposure studies looking at the potential adverse health effects of gasoline exhaust have been performed in animals, and these were elegantly reviewed by McDonald et al. (2007). To the authors' knowledge, no human exposure studies are described. Human exposures to gasoline exhaust are limited by the characteristics of gasoline exhaust itself. Although gasoline produces fewer particulate emissions, the gaseous components are more abundant. The predominant gaseous emissions are carbon monoxide and carbon dioxide, which can reach relatively high concentrations. In the animal studies described by McDonald et al., the average carbon monoxide concentration in the exposure chambers was around 50–80 ppm (McDonald et al., 2007). As carbon monoxide is a toxic gas with a strong affinity to hemoglobin, exposure to high concentrations in both animals and man can cause permanent health effects and even death (Ilano and Raffin, 1990; Penney, 1990). Although controlled exposure studies to pure carbon monoxide

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have been performed in a variety of patient groups and healthy volunteers, at concentrations up to 150 ppm (Allred et al., 1991), human exposure studies to carbon monoxide must balance the known risks with any benefits of the knowledge to be gained. The current workplace exposure guidelines restrict an individual's exposure to an 8-h average of 30 ppm in the United Kingdom (HSE, 2007) and 50 ppm in the United States (OSHA, 1999), levels below those that might be encountered in a gasoline exposure facility.

The exposure setup used in the animal experiments is very similar to the diesel exposure chamber described above. Fresh gasoline exhaust is generated using a commercial "mid-mileage" gasoline engine using standard "national average" fuel during a defined control cycle (similar to the European city cycle, ECE 15, described above). Exhaust is immediately diluted in the exhaust pipe using fresh HEPA-filtered air before entering the exposure chamber. The exposure variables are once again measured in the breathing zone of the animal (McDonald et al., 2008).

PURE AND ENGINEERED NANOPARTICLES

Diesel exhaust and CAPs both consist of a complex mixture of gases, volatile agents, and particulate matter. Indeed, within each exposure, the chemical composition of the particle phase can vary significantly. The exact components of this complex "soup" that are responsible for the demonstrated health effects are still debated. Combustion-derived particulate matter consists of a carbon core onto which a complex mixture of organic compounds and transition metals is adsorbed. To tease out the particle-specific effect (rather than that of the adsorbed agents), there has been some interest into the use of pure engineered particles in inhalation studies, as well as exposures to the gaseous components alone (discussed in Section 1.9).

Human exposures to pure ultrafine carbon particles have been performed. In these studies, human subjects are exposed to particles of elemental carbon delivered via a mouthpiece while they wear a nose clip. The carbon particles are generated in a pure argon environment (to prevent oxidation by ambient oxygen) using an electrical spark discharge between two graphite rods in a commercially available generator. The generated particles are then deionized and mixed with HEPA-filtered air to reach the required concentration before being delivered to the subject (Chalupa et al., 2002; Shah et al., 2008; Zareba et al., 2008).

Similarly, some groups have performed human exposures to radiolabeled ultrafine carbon particles to track the fate of those particles and to identify whether or not inhaled nanoparticles can translocate from the lungs to the systemic circulation (Nemmar et al., 2004; Mills et al., 2006). Although these studies do not reach a consensus view on the translocation of particles, the exposure system used was identical and in line with well-established clinical protocols (Cook and Clarke, 1992): ^{99m}Technetium-labeled carbon particles can be used as a clinical tool in nuclear medicine, that is, in the detection of pulmonary embolism. ^{99m}Technetium-labeled carbon particles (Technegas) can be generated in a commercially available generator, before being delivered to the subject via a mouthpiece in a similar fashion to the carbon ultrafine particles described above (Mills et al., 2006). The

important difference with these studies is that this system only allows a brief exposure to concentrated labeled particles, and not a prolonged exposure as described previously.

HUMAN INSTILLATION STUDIES

Inhalation exposures in humans have been widely employed due to the physiological nature, and ease, of the exposure. Animal studies are more complicated however, and the inhalation route is fraught with difficulty. In many animals, especially large rodents such as rats, the nasal cavity is more complex due to the fact that the animals rely much more heavily on the sense of smell. As a result, the nasal cavity is a much more efficient filter for inhaled particulate material, and actual pulmonary exposure to inhaled particulate is only a fraction of that inhaled and can be difficult to predict. For this reason, toxicological studies in animals have predominantly employed the technique of tracheal instillation to ensure a pulmonary load of particulate matter.

In humans, while most of the fine inhaled particles do pass into the lungs, some of the larger material is filtered out by the nasal cavity. For this reason, mouth breathing is encouraged by asking patients to exercise while inside the chamber. In addition to encouraging mouth breathing, this also increases ventilation rate and therefore exposure to the ambient test atmosphere.

Some studies have been performed in humans in a similar fashion to the rodent studies. While intratracheal exposures are clearly difficult and could be hazardous, bronchoscopy has been used to deliver particles in solution directly into specific lung segments (Ghio and Devlin, 2001; Schaumann et al., 2004). The advantage of these studies, as with the rodent studies, is the fact that the exact pulmonary exposure to particulate matter is known. In both these studies, the aim was to identify the inflammatory potential of a fixed mass of particles collected from different sites at different times—clearly something difficult to do if the added unpredictability of exercise, respiratory rate, and ratio of mouth : nose breathing is taken into account.

While this approach has some advantages in terms of identifying the toxicological potential of different inhaled particles, the delivery route is not physiologically relevant—large boluses of particles administered to one area of the lung directly—and the generalizability of any results can be questioned. The invasive nature of this method makes human studies difficult to perform and reduces the acceptability to potential volunteers.

EXPOSURE TO POLLUTANT GASES

Air pollution is a complex mixture of gaseous, volatile, and particulate components. Driven by the strong epidemiological data, much of the research interest has recently focused on the biological effects of the particulate phase of air pollution. However, there are also epidemiological associations between exposure to nitrogen dioxide

and cardiovascular morbidity and mortality (Anderson et al., 1996; Hoek et al., 2001; Rosenlund et al., 2008). While some have attributed this association to the fact that nitrogen oxides are key copollutants in combustion-derived air pollution, and emissions are therefore closely related to the concentration of fine and ultrafine particulate matter (Sarnat et al., 2001), others have demonstrated important effects on airway inflammatory responses and the incidence of respiratory morbidities (Mostardi et al., 1981; Love et al., 1982; Blomberg et al., 1997).

As for particulate air pollution, controlled exposure studies employing nitrogen dioxide have been performed in human subjects (Frampton and Utell, 1998; Frampton et al., 2002). Briefly, these exposures were performed in a large airtight exposure chamber, through which HEPA-filtered air was drawn at a constant flow rate of 30 m^3/h. Pure nitrogen dioxide (NO_2), provided in compressed bottled form, was then added to the ventilation duct just before it reached the exposure chamber (to allow adequate mixing), its flow rate controlled by a mass flow controller and flowmeter. NO_2 at high concentrations is known to be highly toxic, and inhalation of high concentrations (above 150 ppm) can be rapidly fatal (Last et al., 1994); therefore, air was sampled in the breathing zone of the subject and monitored continually for oxides of nitrogen, and used as a feedback control to maintain a constant (and safe) concentration of nitrogen dioxide within the chamber (Blomberg et al., 1997).

Similarly, studies have been performed using other gaseous pollutants (ATS, 1996; Frampton and Utell, 2007). Human exposures to sulfur dioxide have been performed using a head-dome exposure system, through which air was drawn at 120 L/min. Bottled sulfur dioxide was once again introduced just prior to entry into the exposure chamber, and carefully controlled by digital mass flow controllers to maintain a constant concentration of 200 ppb (Routledge et al., 2006). Ozone has been used at concentrations between 80 and 600 ppb (Krishna et al., 1997; Torres et al., 1997; Frampton et al., 1999; Olin et al., 2001; Samet et al., 2001; Brook et al., 2002; Mudway and Kelly, 2004). The ozone exposures were carried out in large exposure chambers (Olin et al., 2001; Brook et al., 2002), using a head dome (Mudway and Kelly, 2004) and a face mask (Krishna et al., 1997). In the exposure chambers, ozone was generated in a commercial on-site arc generator and introduced into the ventilation duct for the chamber during constant airflow, as described previously. During the face-mask exposure, medical compressed air was used to generate the ozone, and the ozone was delivered along with a constant flow of medical air to maintain constant concentrations. In all experiments, ozone concentrations were measured continuously within the breathing zone of the subjects and used as a negative feedback control.

Although fraught with more difficulty, as discussed above in Section 1.5, carbon monoxide—another major gaseous pollutant generated by combustion sources—has been used in human clinical studies in both healthy volunteers and patient groups. The exposures themselves were carried out in similar exposure facilities as described above, using bottled pure carbon monoxide to produce the test atmosphere along with HEPA-filtered air (Allred et al., 1991). As mentioned previously in Section 1.6, concentrations up to 150 ppm have been used for 1 h. Interestingly, in some of these patient studies, the actual exposure has been moni-

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tored by measuring repeated blood samples from patients given the variable uptake of carbon monoxide between individuals, and the carbon monoxide concentration titrated to achieve a carboxyhemoglobin concentration of 2–4%, thus reducing the anxiety of possible carbon monoxide toxicity (Allred et al., 1991).

EXPOSURE TO ALTERNATIVE FUELS: BIOMASS

Most research to date has focused on outdoor air pollution and modeling this exposure in a controlled fashion to study the pathophysiological effects. However, across the world, the predominant burden of air pollution exposure arises from indoor air pollution. Indeed, some three-quarters of all global particulate air pollution occurs indoors in the developing world (Fullerton et al., 2008). Indoor air pollution consists predominantly of smoke from biomass fuels (wood, charcoal, dung, and crop residues) burned for cooking, heating, and lighting. Similar to urban air pollution exposure, exposure to indoor air pollution is associated with the increased incidence of cardiorespiratory conditions including respiratory infections and cardiac events (Boman et al., 2003).

Although biomass smoke is now increasingly recognized as an important influence on health worldwide, there are few reported studies into the potential adverse health effects in man. Recently, a number of studies have been reported that have used controlled exposures to wood smoke (Sallsten et al., 2006; Barregard et al., 2008). These studies have employed a very similar exposure setup for the diesel exhaust exposures. Smoke is generated using a mixture of hardwood and softwood burned in a cast-iron stove. A fraction of the smoke is diverted from the chimney into a conditioning chamber and mixed with HEPA-filtered air, and then fed into the human exposure chamber. It takes around 1 h to reach a steady-state concentration of particulate within the chamber due to the more unpredictable nature of wood smoke as compared with diesel exhaust. Again, the exposure is closely monitored from the breathing zone of the subject.

SUMMARY

The negative health effects associated with air pollution have been demonstrated in elegant and consistent large-scale epidemiological studies. To experimentally prove these associations by demonstrating causality and identify the pathophysiological mechanisms, a variety of controlled exposure studies have been performed. Specifically designed exposure facilities allow controlled exposure of human subjects to ambient and concentrated levels of individual air pollutants including complex mixtures of ambient particles and gases, model test atmospheres such as diesel exhaust fumes, and individual components such as pure carbon-centered particles and copollutant gases. Further studies employing such facilities will help to identify the pathophysiological mechanisms underlying the strong epidemiological link between air pollution and cardiorespiratory health, and will hopefully lead to improvements in both patient care and environmental health policy.

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RESEARCH ARTICLE

Exposure to nitrogen dioxide is not associated with vascular dysfunction in man

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Abstract

Background: Exposure to air pollution is associated with increased cardiorespiratory morbidity and mortality. It is unclear whether these effects are mediated through combustion-derived particulate matter or gaseous components, such as nitrogen dioxide.

Objectives: To investigate the effect of nitrogen dioxide exposure on vascular vasomotor and six fibrinolytic functions.

Methods: Ten healthy male volunteers were exposed to nitrogen dioxide at 4 ppm or filtered air for 1 h during intermittent exercise in a randomized double-blind crossover study. Bilateral forearm blood flow and fibrinolytic markers were measured before and during unilateral intrabrachial infusion of bradykinin (100–1000 pmol/min), acetylcholine (5–20 µg/min), sodium nitroprusside (2–8 µg/min), and verapamil (10–100 µg/min) 4 h after the exposure. Lung function was determined before and after the exposure, and exhaled nitric oxide at baseline and 1 and 4 h after the exposure.

Results: There were no differences in resting forearm blood flow after either exposure. There was a dose-dependent increase in forearm blood flow with all vasodilators but this was similar after either exposure for all vasodilators ($p > .05$ for all). Bradykinin caused a dose-dependent increase in plasma tissue-plasminogen activator, but again there was no difference between the exposures. There were no changes in lung function or exhaled nitric oxide following either exposure.

Conclusion: Inhalation of nitrogen dioxide does not impair vascular vasomotor or fibrinolytic function. Nitrogen dioxide does not appear to be a major arbiter of the adverse cardiovascular effects of air pollution.

Keywords: *Air pollution; endothelial function; fibrinolysis; nitrogen dioxide; NO_2*

Introduction

Exposure to air pollution is a major public health problem, and is associated with an increased risk of cardiorespiratory morbidity and mortality. Epidemiological studies have consistently shown a strong association of cardiorespiratory illness and fine particulate matter (Dockery et al., 1993; Miller et al., 2007; Pope et al., 2002), although similar positive associations have been shown for long-term exposure to nitrogen dioxide (Anderson et al., 1996; Hoek et al., 2001; Rosenlund et al., 2008). Nitrogen dioxide and other oxides of nitrogen are major constituents of combustion-derived air pollution, such as diesel exhaust, and the associations of nitrogen dioxide with adverse outcomes have usually been attributed to the close association of fine particulate with nitrogen dioxide concentrations (Sarnat et al., 2001). However, isolated real life exposures to nitrogen dioxide have been linked to increases in respiratory illness and susceptibility to airway infection (Guidotti, 1978; Mostardi et al., 1981; Love et al., 1982). Controlled exposure to nitrogen dioxide induces airway inflammation and modifies antioxidants in the respiratory tract lining fluid (Kelly et al., 1996).

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The cardiovascular effects of inhaled diesel exhaust are well documented. We have demonstrated that controlled exposures to diesel exhaust, as a model of combustion-derived fine particulate air pollution, causes acute vascular endothelial effects (Mills et al., 2005; Törnqvist et al., 2007). In these models, the actual exposure is a complicated mixture of carbon-centered particulate matter, volatile organic compounds, and gaseous pollutants (Scheepers & Bos, 1992). The predominant gaseous components are nitrogen dioxide and other oxides of nitrogen, with NO_x concentrations reaching around 4 ppm in previous controlled exposures to diesel exhaust (Mills et al., 2005; Salvi et al., 1999; Behndig et al., 2006). These models have consistently demonstrated an impairment of vascular endothelial function and endogenous fibrinolysis following exposure to diesel exhaust that appears to be mediated through increased oxidative stress and reduced nitric oxide bioavailability (Mills et al., 2005; Miller et al., 2009). Although particulate matter appears to be a major arbiter of these adverse vascular effects, the question remains as to the independent effect of a pure exposure to nitrogen dioxide. Nitrogen dioxide is itself a powerful oxidizing species, and this is proposed to underlie the airway inflammatory and antioxidant responses, as well as the vascular dysfunction previously described (Blomberg et al., 1997; Patel & Block, 1986).

The aim of this study was to explore the effect of a pure exposure to 4 ppm of nitrogen dioxide on vascular endothelial and fibrinolytic function, and to test the hypothesis that the previously demonstrated adverse effects are driven by nitrogen dioxide.

Methods

Subjects

Ten healthy male volunteers were recruited into the trial. Women were not included to avoid the potential confounding influence of cyclical changes in estrogen on vascular endothelial function (Ganz, 2002). One subject developed a respiratory tract infection during the study and was excluded and replaced. All subjects were nonsmokers, had no intercurrent illness, took no regular medication, and all had normal lung function. All subjects had been free of symptoms of upper airway infection for at least 6 weeks prior to the study. All subjects gave their written informed consent, and the trial was approved by the local research ethics committee in accordance with the Declaration of Helsinki.

Exposure protocol

The exposures to nitrogen dioxide or filtered air took place at the Medical Division of the National Institute for Working Life in Umeå as described previously (Kelly et al., 1996). Briefly, ambient air was drawn continuously through a large exposure chamber at a constant rate of 30 m^3/h, which was maintained at a temperature of 20°C and relative humidity of 50%. Nitrogen dioxide (AGA Special Gas, Lidingö, Sweden) was passed through a mass flow controller (Model 5850TR, Brooks Instrument B.V., Veenendaal, Netherlands) and a flow meter (Rota, Hannover, Germany) to control the gas flow, and was introduced to the ventilating duct just before reaching the chamber. Air was sampled in the breathing zone of the subjects and analyzed continuously for nitrogen dioxide, nitric oxide, and total NO_x using a CSI 1600 oxides of nitrogen analyzer (Columbia Scientific Industries, Austin, TX, USA). The concentration of nitrogen dioxide within the chamber was maintained at 4 ppm, a concentration selected to match that of NO_x concentrations seen in controlled exposures to diesel exhaust as described previously (Mills et al., 2005; Salvi et al., 1999; Behndig et al., 2006). Filtered air exposures were performed by HEPA filtration of air prior to introduction into the exposure chamber without introduction of additional nitrogen dioxide.

Study design

Subjects attended the clinical research facility at Umeå University Hospital on two occasions, each at least 1 week apart. In a randomized double-blind crossover trial, subjects were exposure to either filtered air or nitrogen dioxide at 4 ppm for 1 h with intermittent exercise. Subjects performed exercise on a bicycle ergometer while in the chamber at a predesignated workload to achieve a mean ventilation rate of 25 L/min, based on a prestudy screening exercise ventilation stress test. Subjects then returned to the clinical research facility for forearm vascular plethysmography studies with active drug infusions commencing 4 h after the exposure.

Outcome measures

Vascular assessments

Subjects underwent forearm venous occlusion plethysmography as described previously (Wilkinson & Webb, 2001). The brachial artery of the nondominant arm was cannulated with a 27-gauge steel needle under aseptic technique and local anaesthesia. After a 30-min baseline saline infusion, forearm blood flow was recorded during infusion of the endothelial- dependent (acetylcholine, 5, 10, and 20 μg/min; bradykinin, 100, 300, and 1000 pmol/min) and -independent (sodium nitroprusside, 2, 4, and 8 μg/min; verapamil, 10, 30, and 100 μg/min) vasodilators. The vasodilators were infused for 6 min at each dose, with the blood flow determined for last 3 min of the infusion. Vasodilators were administered in a random order, except for verapamil, which was always administered last given its prolonged vascular action, and were separated by a washout period of 20 min during which saline was infused.

Biochemical analyses

Peripheral venous blood samples were obtained at baseline and at 4 and 6 h after exposure for total and differential cell counts. Analysis was performed on an autoanalyzer by the local hematology reference laboratory (Department of Clinical Chemistry, University Hospital, Umeå). Blood samples were obtained during the forearm vascular study from indwelling 17-gauge venous cannulae inserted into a large antecubital vein on both arms before and after each dose of bradykinin. Bradykinin is an endothelial-dependant

vasodilator that also stimulates the release of stored tissue-plasminogen activator (t-PA) from the vascular endothelium (Brown et al., 1999, 2000). Samples were collected into acidified buffered citrate (Stabilyte, Biopool International) for analysis of t-PA and into citrate (BD Vacutainer) for plasminogen-activator inhibitor type 1 (PAI-1) assays to assess the activity of the endogenous fibrinolytic pathway.

t-PA and PAI-1 antigen concentrations were determined by commercially available enzyme-linked immunosorbent assays (ELISAs) (t-PA combi Actibind, Technoclone, Vienna, Austria; Elitest PAI-1, Hyphen BioMed, Neuville-sue-Oise, France). As much of the t-PA present in the circulation is bound to PAI-1, thus rendering both inactive and leading to hepatic clearance (Chandler et al., 1997), both t- PA and PAI-1 activity were assessed using enzymatic assays (t-PA combi Actibind, Zymutest PAI-1) as described previously (Mills et al., 2005).

Exhaled nitric oxide and lung function

The standardized fractional exhaled nitric oxide (FENO) was measured in duplicate at expiratory flow rates of 10, 50, 100, and 250 ml/s at baseline, 1 h, and 4 h after both exposures using a chemiluminescence analyzer (NiOX; Aerocrine AB, Stockholm, Sweden) (ATS/ERS 2005). Lung function was measured using simple spirometry (Vitalograph, Bucks, UK) at baseline and after the exposure, recording forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and slow vital capacity (VC).

Statistical analysis

We calculate, based on previous studies (Mills et al., 2005), that a sample size of 10 gives an 80% power of detecting a difference in forearm blood flow responses of 20% at a two-sided statistical significance level of 5%. All investigators were blinded to the exposure received. Plethysmography data were analyzed as described previously (Newby et al., 1999). Data were analysed using two-way analysis-of-variance (ANOVA) with repeated measures. The variables included in the ANOVA were dose and exposure for the forearm blood flow data, and time and exposure for the other outcome measures. All data are expressed as mean ± standard deviation unless otherwise stated. Statistical significance was taken as a two-sided p value of .05. Data were analyzed using GraphPad Prism (Version 4 for Macintosh; GraphPad Software, San Diego, USA) on a Macintosh personal computer.

Results

Ten subjects, median age 24 years, completed the study (Table 1). There were no changes in any indices of lung function seen following the nitrogen dioxide exposure (Table 2). Exhaled nitric oxide (FENO), measured as a surrogate of airway inflammation, was unchanged at all time points following either nitrogen dioxide or filtered air exposure (Table 2).

The forearm vascular studies revealed a dose-dependent increase in blood flow in the infused arm compared to the noninfused arm in all subjects after each infused vasodilator. There was no difference in the vascular responses to any of the vasodilators (endothelium-dependent or -independent) following air or nitrogen dioxide exposure (Figure 1). Bradykinin stimulated a dose-dependent increase in release of plasma tissue-plasminogen activator (t-PA),

Table 1. Baseline characteristics of the 10 subjects completing the study.

Age, years (median, range)	24 (22–28)
Male sex, %	100%
Height, cm	181 ± 5
Weight, kg	79 ± 10
BMI, kg m^{-2}	24 ± 2
FEV_1, L	4.95 ± 0.33
FVC, L	5.99 ± 0.52
VC, L	5.91 ± 0.47
Systolic blood pressure, mm Hg	136 ± 11
Diastolic blood pressure, mm Hg	67 ± 8
Heart rate, bpm	60 ± 12
Hemoglobin, g/L	146 ± 7
White cell count, $\times 10^9$/L	4.9 ± 1.5
Platelet count, $\times 10^9$/L	224 ± 53
PAI-1 antigen, ng/mL	6.87 ± 4.04
PAI-1 activity, U/mL	0.27 ± 0.22
t-PA antigen, ng/mL	2.82 ± 1.85
t-PA activity, U/mL	1.25 ± 0.54

Note: Data expressed as mean ± standard deviation unless otherwise stated. VC, Slow vital capacity; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity. Plasminogen activator type 1 (PAI-1) and tissue plasminogen activator (t-PA) concentrations measured during forearm study on control air day before infusion of bradykinin.

Table 2. Basic spirometry and exhaled nitric oxide (FENO) at flow rates of 10, 50, 100 and 250 ml/s after 1 h of nitrogen dioxide (4 ppm) or filtered air exposure with intermittent exercise.

				p values	
	Time	NO_2	Air	Time	Exposure
VC, L	Pre-exposure	5.82 ± 0.41	5.91 ± 0.47	.9586	.1525
	Post-exposure	5.88 ± 0.44	5.87 ± 0.48		
FEV1, L	Pre-exposure	4.95 ± 0.24	4.95 ± 0.33	.9758	.9200
	Post-exposure	4.95 ± 0.33	4.94 ± 0.33		
FVC, L	Pre-exposure	5.93 ± 0.47	5.99 ± 0.52	.8824	.5704
	Post-exposure	5.93 ± 0.49	5.92 ± 0.54		
FENO 250 ml/s, ppm	Pre-exposure	6.28 ± 2.77	6.49 ± 2.36	.7960	.4244
	1 hour	5.80 ± 2.31	6.14 ± 2.10		
	4 hours	6.27 ± 1.69	6.83 ± 2.53		
FENO 100 ml/s, ppm	Pre-exposure	10.23 ± 5.62	10.53 ± 3.22	.8667	.8194
	1 hour	10.33 ± 5.45	10.52 ± 5.32		
	4 hours	11.28 ± 4.44	11.44 ± 5.43		
FENO 50 ml/s, ppm	Pre-exposure	16.55 ± 9.67	15.71 ± 9.02	.8628	.7003
	1 hour	16.94 ± 9.71	16.34 ± 8.32		
	4 hours	18.25 ± 9.01	17.81 ± 9.48		
FENO 10 ml/s, ppm	Pre-exposure	51.15 ± 32.65	51.87 ± 32.59	.8292	.9924
	1 hour	55.74 ± 32.00	54.28 ± 33.16		
	4 hours	58.57 ± 27.16	59.48 ± 35.41		

Note: Data shown as mean ± standard deviation. p values shown from two-way ANOVA with repeated measures showing effect of time and exposure. VC, Slow vital capacity; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity, FVC.

although the response was similar following each exposure (Table 3). Plasma plasminogen-activator inhibitor 1 (PAI-1) concentrations were unchanged ($p > .05$ for both infused and noninfused arms (data not shown) following infusion of bradykinin, with no differences following either exposure ($p > .05$ for all, data not shown).

There was no difference in white cell count, differential cell count, or platelet count through the study following either exposure (Table 4). We did observe a small fall in systemic hemoglobin concentrations through the study period consistent with repeated venesection, although there was no difference between the study exposures (Table 4).

Discussion and conclusions

We have demonstrated for the first time that direct exposure to nitrogen dioxide is not associated with vascular vasomotor or fibrinolytic dysfunction. This suggests there are components other than nitrogen dioxide that are responsible for the previously documented adverse cardiovascular effects of air pollution.

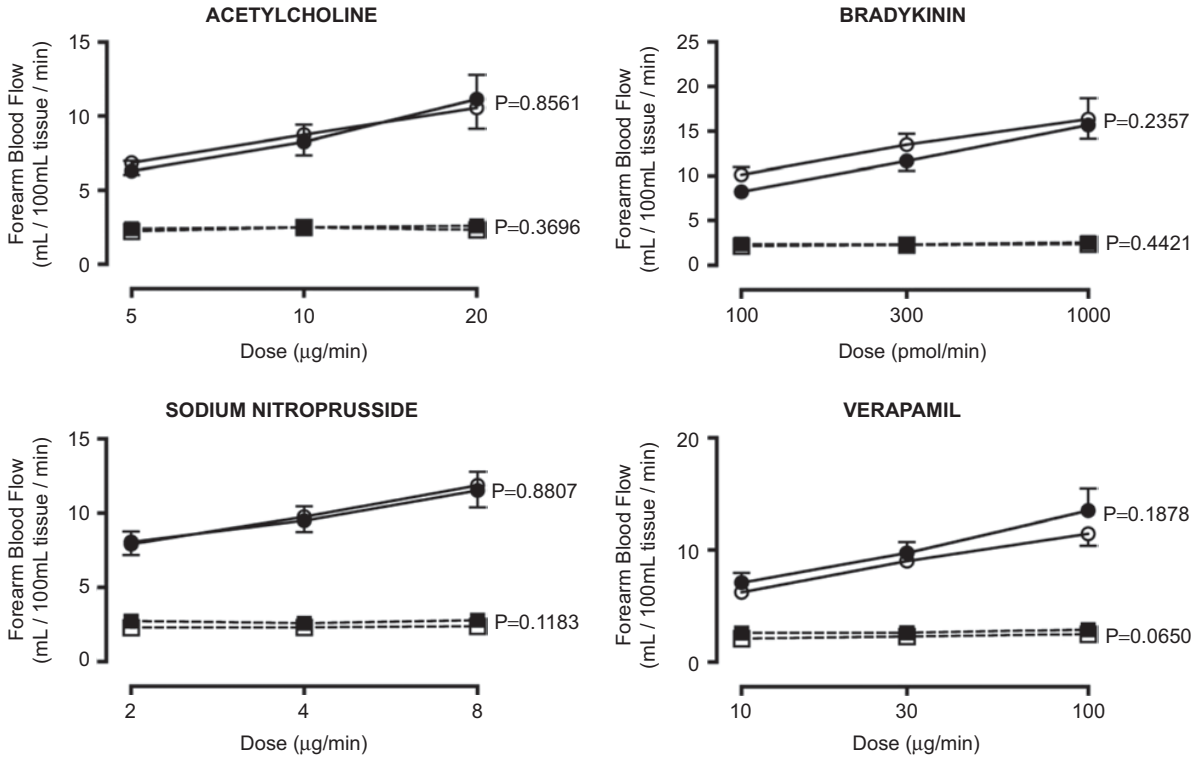

Figure 1. Forearm venous occlusion plethysmography with intra-arterial infusion of vasodilators performed 4 h after exposure. Data plotted as mean and T-bars show standard error of the mean. Solid lines show infused arm, dotted lines show noninfused arm after exposure to air (open symbols) or nitrogen dioxide (solid symbols) at 4 ppm. *p* values shown from two-way ANOVA with repeated measures showing effect of exposure for both infused and noninfused arms.

Table 3. Plasma tissue-plasminogen activator (t-PA) antigen concentrations and activity and estimated net t-PA release following infusion of bradykinin after 1 hour of nitrogen dioxide (4 ppm) or filtered air exposure with intermittent exercise.

		Air				NO_2				*p* Value	
Bradykinin (pmol/min)		Baseline	100	300	1000	Baseline	100	300	1000	Time	Exp.
t-PA antigen ng/ml	Infused arm	2.82±1.85	2.46±1.26	4.22±2.78	8.52±4.74	3.01±1.98	2.67±3.32	3.32±2.42	5.98±3.10	**.0005**	.0982
	Noninfused arm	2.50±1.54	1.81±1.01	2.08±1.16	2.24±1.23	2.82±1.88	1.99±1.44	2.17±1.35	2.36±1.51	.6724	.2562
	Estimated net t-PA release (ng/100ml tissue/min)	0.43±0.67	3.78±1.87	16.54±13.90	54.27±30.52	0.33±0.64	2.93±4.59	8.89±12.29	36.71±25.87	**<.0001**	.0833
t-PA activity U/ml	Infused arm	1.25±0.54	1.81±0.55	2.60±0.72	3.48±1.57	1.25±0.43	1.82±0.77	2.11±1.11	2.95±1.01	**<.0001**	.2130
	Noninfused arm	0.98±0.49	1.08±0.32	1.20±0.36	1.54±0.49	1.00±0.34	1.04±0.44	1.13±0.42	1.53±0.54	**.0009**	.8191
	Estimated net t-PA activity release (U/100ml tissue/min)	0.43±0.42	4.82±2.66	12.24±6.59	17.29±10.14	0.39±0.26	3.97±3.23	7.60±5.73	15.09±10.20	**<.0001**	.2158

Note: Data shown as mean ± standard deviation. *p* values shown from two-way ANOVA with repeated measures showing effect of time and exposure (Exp.).

Table 4. Differential cell count and hemoglobin concentrations before and after a 1-hour nitrogen dioxide (4 ppm) or filtered air exposure with intermittent exercise.

				p values	
	Time	NO_2	Air	Time	Exposure
Haemoglobin (g/L)	Pre-exposure	148 ± 8	146 ± 7	.0124	.2842
	4 hours	142 ± 8	143 ± 7		
	6 hours	139 ± 7	137 ± 7		
White cell count ($\times 10^9$/L)	Pre-exposure	4.8 ± 0.9	4.9 ± 1.5	.2434	.4340
	4 hours	5.6 ± 1.5	5.7 ± 1.6		
	6 hours	6.2 ± 2.1	5.7 ± 1.5		
Platelet count ($\times 10^9$/L)	Pre-exposure	209 ± 44	224 ± 53	.8358	.2365
	4 hours	214 ± 55	226 ± 50		
	6 hours	209 ± 46	209 ± 45		
Neutrophils ($\times 10^9$/L)	Pre-exposure	2.37 ± 0.75	2.34 ± 1.14	.1580	.2961
	4 hours	3.14 ± 1.10	3.15 ± 1.21		
	6 hours	3.57 ± 1.78	3.00 ± 1.22		
Lymphocytes ($\times 10^9$/L)	Pre-exposure	1.89 ± 0.34	1.91 ± 0.47	.6226	.6763
	4 hours	1.91 ± 0.55	1.92 ± 0.44		
	6 hours	2.12 ± 0.54	2.04 ± 0.48		
Monocytes ($\times 10^9$/L)	Pre-exposure	0.39 ± 0.11	0.40 ± 0.10	.6687	.9647
	4 hours	0.41 ± 0.15	0.43 ± 0.13		
	6 hours	0.45 ± 0.16	0.43 ± 0.12		

Note: Data shown as mean ± standard deviation. *p* values shown from two-way ANOVA with repeated measures showing effect of time and exposure.

Vascular effects

In this study we have employed a robust and well-validated technique to assess vascular endothelial function following a short-term exposure to nitrogen dioxide. Exposure to nitrogen dioxide, a major component of combustion-derived air pollution, has been linked to cardiovascular morbidity and mortality in epidemiological studies. Why then did we not observe adverse vascular effects? Many workers have attributed the epidemiological link with nitrogen dioxide to a bystander association or epi-phenomenon rather than a casual relationship. Nitrogen dioxide is a copollutant that correlates tightly with fine and ultrafine particulate matter (Sarnat et al., 2001). Our current and previous findings are in agreement with this hypothesis. Using controlled exposures to dilute diesel exhaust, we have previously demonstrated marked adverse effects on vascular endothelial function (Mills et al., 2005) that are present 2 and 6 h after the exposure but have largely resolved by 24 h (Törnqvist et al., 2007). In the present study, performed 4 h after the exposure and well within the time window of the previously demonstrated adverse vascular effects, we aimed to separate the effects of the major gaseous copollutant, nitrogen dioxide, by using a pure gaseous exposure.

It is important to note that the nitrogen dioxide concentration employed in this study has been previously demonstrated to cause airway inflammatory responses (Blomberg et al., 1997; Sandstrom et al., 1991). In homes with gas stoves and in certain industries, nitrogen dioxide concentrations may peak at 1–2 ppm (Samet et al., 1987). Alongside busy roads, nitrogen dioxide concentrations may reach 0.6 ppm (WHO, 1999). Whereas the concentration of nitrogen dioxide chosen for this study was matched to the overall concentration of oxides of nitrogen from previous diesel exhaust exposure studies (Mills et al., 2005; Salvi et al., 1999), in these studies nitrogen dioxide concentrations reached an average of 1.6 ppm. Therefore, even at levels above those encountered in dilute diesel exposures, when pronounced adverse vascular effects were demonstrated, we have clearly demonstrated that short-term exposure to pure nitrogen dioxide is not associated with vascular endothelial dysfunction in healthy young male subjects, although we cannot rule out a small effect in women or elderly people with comorbidities who may be more sensitive to environmental pollutants.

Nitrogen dioxide is a powerful oxidizing species, and we have demonstrated that its inhalation is associated with mild airway inflammation (Blomberg et al., 1997) and changes in airway antioxidant responses (Kelly et al., 1996; Blomberg et al., 1997). Moreover, after exposure to nitrogen dioxide in in vitro studies, porcine pulmonary artery and aortic endothelial cells have been shown to suffer a significant oxidant injury, with lipid peroxidation and impaired cell membrane function (Patel & Block, 1986). We have suggested that the vascular effects of air pollution exposure are mediated by oxidative stress (Miller et al., 2008), so why has inhalation of nitrogen dioxide failed to have an effect on vascular function? Nitrogen dioxide is relatively insoluble, and does not easily diffuse across to the bloodstream from the lungs. In fact, its penetration of the alveolar space is further inhibited by the normal respiratory tract lining fluid (Postlethwait et al., 1991). Therefore it is likely that inhaled nitrogen dioxide is confined mainly to the lungs where it exerts a localized and specific pulmonary inflammatory stimulus (Blomberg et al. 1997), without any significant vascular or systemic oxidative insult. In light of these findings, we suggest that the vascular endothelial effects we have previously demonstrated following exposure to diesel exhaust are driven by exposure to fine and ultrafine particulate matter, rather than any gaseous component.

Thrombotic effects

Thrombosis plays a central role in coronary heart disease. Formation of thrombus on disrupted atherosclerotic plaques may cause acute vessel occlusion, and acute coronary syndromes. Exposure to air pollution is proposed to be procoagulant. Exposure to ambient nitrogen dioxide and carbon monoxide has been associated with increased plasma fibrinogen (Pekkanen et al., 2000) and a reduced prothrombin time (Baccarelli et al., 2007), and ambient sulfur dioxide with plasma viscosity (Peters et al., 1997), but again the question of whether exposure to nitrogen dioxide and carbon monoxide is a surrogate for exposure to combustion-derived particulate matter arises. We have previously demonstrated that short-term exposure to dilute diesel exhaust results in increased platelet activation and enhanced thrombus formation (Lucking et al., 2008), and at the same time impairs vascular release of tissue-plasminogen activator (t-PA), an endogenous fibrinolytic enzyme (Mills et al., 2005) responsible for local dissolution of formed blood clot.

In this study we have not shown impaired release of tissue-plasminogen activator, or any increase in its endogenous inhibitor plasminogen-activator inhibitor type 1 (PAI-1), following exposure to nitrogen dioxide. Therefore we propose that the previously demonstrated procoagulant and antifibrinolytic effects of air pollution exposure are primarily driven by exposure to fine and ultrafine particulate matter rather than the gaseous copollutants.

Pulmonary effects

Our study demonstrated no change in exhaled nitric oxide (FENO) or simple spirometry following exposure to nitrogen dioxide. Spirometry provides a basic measure of lung function, assessing airway restriction and obstruction, and forms a critical part of the diagnosis of chronic lung conditions such as asthma and chronic obstructive pulmonary disease. Small effects on lung function have been demonstrated in patients with asthma following exposure to inhaled ambient air pollution (McCreanor et al., 2007). In light of this, we chose to assess changes in airway reactivity using simple spirometry in this group of healthy volunteers. Our study findings of no change in spirometry indices are in concordance with similar studies performed after exposure to diesel exhaust in healthy volunteers (Nightingale et al., 162).

Exhaled nitric oxide is proposed as a marker of airway inflammation, especially in asthmatic patients (ATS/ERS, 2005), and is closely correlated with eosinophilic inflammation (Lim & Mottram, 2008). As a marker, it is helpful in obtaining a diagnosis of asthma (Dupont et al., 2003) and may be used to track response to treatment (Yates et al., 1995). Although FENO has been used in other airway conditions, such as chronic obstructive pulmonary disease, interstitial lung diseases, and allergic rhinitis (2005), its usefulness as a marker of airway inflammation in healthy volunteers is unclear. Although exposure to the strong oxidative air pollutant ozone does not affect exhaled nitric oxide concentrations at an ozone dose known to induce a pronounced neutrophilic airway inflammation (Olin et al., 2001), we have recently demonstrated a significant increase in FENO following a 1-h exposure to dilute diesel exhaust in young healthy volunteers (Barath et al., 2007) and for this reason we chose FENO as a marker of airway inflammation in this study. In healthy volunteers we have previously demonstrated a mild airway inflammatory response, with increases in interleukin-8 (IL-8) concentrations in bronchial washings 90 min after a 4-h exposure to nitrogen dioxide (2 ppm). At 6 h after the exposure, we demonstrated increased IL-8 concentrations and neutrophil counts within bronchial washings but no signs of inflammatory cell recruitment into the endobronchial mucosa (Blomberg et al., 1997). We therefore believe that our measurements of FENO were performed within the established timeframe of an early airway inflammatory response.

Allowing for the limitations of FENO as a sensitive marker of airway inflammation as a result of air pollution exposure, we suggest that our study provides no strong evidence for an early marked airway inflammatory response following short-term nitrogen dioxide exposure. However, we also acknowledge that measurements of FENO can be highly variable (Olin et al., 2001), and therefore that our small study may be underpowered to detect a clinically significant change. We therefore suggest that further investigation into the ability of short-term high-dose nitrogen dioxide to cause an inflammatory response is warranted.

Using a robust controlled study design, we have demonstrated for the first time that exposure to nitrogen dioxide is not associated with any vascular vasomotor or fibrinolytic dysfunction. The adverse cardiovascular effects of combustion-derived air pollution appear to be mediated via components other than nitrogen dioxide, and it is plausible that these vascular effects are rather driven by the fine and ultrafine particle fractions.

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***Trial Registration*:** www.ClinicalTrials.gov; NCT00774514.

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Contribution of Endothelin 1 to the Vascular Effects of Diesel Exhaust Inhalation in Humans

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Contribution of Endothelin 1 to the Vascular Effects of Diesel Exhaust Inhalation in Humans

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Abstract—Diesel exhaust inhalation impairs vascular function, and, although the underlying mechanism remains unclear, endothelin (ET) 1 and NO are potential mediators. The aim of this study was to identify whether diesel exhaust inhalation affects the vascular actions of ET-1 in humans. In a randomized, double-blind crossover study, 13 healthy male volunteers were exposed to either filtered air or dilute diesel exhaust (331 ± 13 $\mu g/m^3$). Plasma concentrations of ET-1 and big-ET-1 were determined at baseline and throughout the 24-hour study period. Bilateral forearm blood flow was measured 2 hours after the exposure during infusion of either ET-1 (5 pmol/min) or the ET_A receptor antagonist, BQ-123 (10 nmol/min) alone and in combination with the ET_B receptor antagonist, BQ-788 (1 nmol/min). Diesel exhaust exposure had no effect on plasma ET-1 and big-ET-1 concentrations ($P>0.05$ for both) or 24-hour mean blood pressure or heart rate ($P>0.05$ for all). ET-1 infusion increased plasma ET-1 concentrations by 58% ($P<0.01$) but caused vasoconstriction only after diesel exhaust exposure (−17% versus 2% after air; $P<0.001$). In contrast, diesel exhaust exposure reduced vasodilatation to isolated BQ-123 infusion (20% versus 59% after air; $P<0.001$) but had no effect on vasodilatation to combined BQ-123 and BQ-788 administration ($P>0.05$). Diesel exhaust inhalation increases vascular sensitivity to ET-1 and reduces vasodilatation to ET_A receptor antagonism despite unchanged plasma ET-1 concentrations. Given the tonic interaction between the ET and NO systems, we conclude that diesel exhaust inhalation alters vascular reactivity to ET-1 probably through its effects on NO bioavailability. (***Hypertension*. 2009;54:910-915.**)

Key Words: air pollution ■ particulate matter ■ endothelial function ■ endothelin receptor antagonists ■ ET-1 ■ endothelin-1 ■ blood pressure

Exposure to combustion-derived fine particulate air pollution is a recognized risk factor for cardiorespiratory mortality and morbidity.[1,2] There is a strong relationship between acute exposure to traffic-derived particulate matter and the incidence of acute myocardial infarction[3] and hospital readmission in survivors of myocardial infarction.[4] The World Health Organization estimates that annually ≈3 million deaths worldwide can be attributable to air pollution.[5]

Recent controlled exposure studies have demonstrated that inhalation of concentrated ambient particles and ozone causes acute arterial vasoconstriction 2 hours after the exposure.[6] Inhalation of diesel exhaust, a major component of fine particulate air pollution in urban environments, impairs vasomotor function and endogenous fibrinolysis.[7] The fundamental mechanisms underlying these detrimental vascular endothelial effects remain poorly understood. Furthermore, the exact components of air pollution responsible for these effects have not been defined, although it is proposed that airborne particulate matter is likely to be the major arbiter.[2]

Endothelin (ET) 1, an endogenous vasoconstrictor 100-fold more potent that norepinephrine,[8] is a 21-amino acid peptide produced by the vascular endothelium in response to stress. It is produced initially as preproendothelin-1, which is processed to form big-ET-1, before being cleaved by ET-converting enzyme into ET-1. The actions of ET-1 are mediated by 2 G protein–coupled receptors, the ET_A and ET_B receptors. Stimulation of either the ET_A or ET_B receptor causes vasoconstriction, although the ET_B receptor is also expressed on endothelial cells where it releases NO. The ET system plays a major role in cardiovascular and renal physiology,[9] and although its actions are complex, ET-1 contributes to the maintenance of basal vascular tone and blood pressure in humans.[10,11]

Recent work has suggested that plasma ET-1 concentrations are increased by exposure to air pollution. Rats raised with daily exposure to diesel exhaust particles and urban particulate matter have increased blood pressure, plasma ET-1 concentrations,[12] and ET-1 expression in cardiac tissue.[13] In children from Mexico City, Mexico, plasma ET-1 concentrations correlated with the degree of air pollution exposure.[14] Peretz et al[15] recently demonstrated elevated

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This trial has been registered at www.clinicaltrials.gov (identifier NCT00745693).

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plasma ET-1 concentrations in a heterogeneous population of healthy volunteers and patients with the metabolic syndrome 3 hours after a controlled 2-hour resting exposure to diesel exhaust.

The aims of this study were to assess the effect of diesel exhaust inhalation on plasma ET-1 and big-ET-1 concentrations, ET-1–mediated vasoconstriction, and the contribution of ET-1 to basal vascular tone.

Methods

Subjects

Fifteen healthy male volunteers were recruited between February and March 2008 at Umeå University Hospital. All of the subjects had normal lung function, were nonsmokers, and took no regular medication. Those with a significant occupational exposure to air pollution and those with an intercurrent illness were excluded. The trial was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki, and with the written informed consent of each participant.

Study Design

Subjects attended for 2 consecutive days on 4 occasions ≥1 week apart. In a double-blind, randomized crossover study, subjects were exposed to either filtered air or dilute diesel exhaust at 300 $\mu g/m^3$ for 1 hour in a specially built diesel exposure chamber, as described previously.[16] During the exposure, subjects performed 15-minute periods of exercise on a bicycle ergometer (minute ventilation: 25 L/min per meter squared) alternated with 15-minute periods of rest.

On the basis of previous studies,[7] vascular assessments and intra-arterial infusions were commenced 2 hours after the exposure. All of the subjects abstained from alcohol for 24 hours and caffeine-containing drinks for ≥8 hours and fasted for ≥4 hours before commencement of the vascular study. All of the subjects remained indoors at rest between the exposure and the vascular assessment to minimize additional exposure to air pollution. A validated ambulatory blood pressure monitor (model 90217, SpaceLabs Healthcare) was applied to the right arm 2 hours before the start of the exposure, and monitoring was continued for a total of 24 hours.

Diesel Exposure

Diesel exhaust emissions were generated using an idling Volvo (Volvo TD45, 4.5 L, 4 cylinders, 680 rpm) diesel engine. More than 90% of the exhaust was shunted away, and the remaining part was diluted with air and fed into the exposure chamber at steady-state concentration. During the exposure, air was sampled in the breathing zone of the subjects and monitored for nitrogen oxides, particle number, and total hydrocarbons (measured as propane). Filter samples were collected and analyzed for mass concentration. The exposures were standardized by keeping the particle (diameter: <10 μm) mass concentration at ≈ 300 $\mu g/m^3$. Temperature and humidity in the chamber were controlled at 22°C and 50%, respectively.

Vascular Studies

All of the subjects underwent brachial artery cannulation in the nondominant arm using a 27-gauge steel needle under controlled conditions. After a 30-minute baseline infusion of 0.9% saline, subjects received either a 60-minute infusion of ET-1 (American Peptide) at 5 pmol/min[17] or infusion of BQ-123 (an ET_A receptor antagonist, American Peptide) at 10 nmol/min for 60 minutes,[18] followed by coinfusion of BQ-123 (10 nmol/min) and BQ-788 (an ET_B receptor antagonist, American Peptide; 1 nmol/min)[19,20] for a further 60 minutes.

Forearm blood flow was measured in the infused and noninfused arms by venous occlusion plethysmography with mercury-in-silicone elastomer strain gauges, as described previously.[21] Supine heart rate and blood pressure were determined in the noninfused arm at intervals throughout the study using the ambulatory blood pressure monitor.

Venous cannulae (17-gauge) were inserted into large subcutaneous veins in the antecubital fossae of both arms. Blood (10 mL) was drawn simultaneously from each arm at the end of the baseline saline infusion and at the end of each 60-minute infusion period. Blood samples were also obtained by separate venipuncture at baseline, immediately, and 6 and 24 hours after the exposure.

Table. Baseline Characteristics of the 13 Subjects Who Completed the Study

Parameter	Data (N=13)
Age, median (range), y	23 (21 to 28)
Height, cm	181±2
Weight, kg	79±3
Body mass index, kg/m^2	24±1
Hemoglobin concentration, g/L	153±3
White blood cell count, $\times 10^9$/L	5.1±0.4
Neutrophil count, $\times 10^9$/L	2.5±0.3
Lymphocyte count, $\times 10^9$/L	2.0±0.1
Monocyte count, $\times 10^9$/L	0.40±0.03
Platelet count, $\times 10^9$/L	209±11

Data show the mean±SEM unless otherwise stated.

Biochemical Analyses

Blood samples taken at baseline and 6 and 24 hours after the exposure were analyzed for total and differential cell counts by an autoanalyzer. Plasma samples were collected into ethylene diamine tetra-acetic acid and kept on ice until centrifuged at 3000 rpm for 30 minutes. Plasma samples were immediately frozen and stored at −80°C. Plasma ET-1 and big-ET-1 concentrations were measured according to an acetic acid extraction technique using a commercial radioimmunoassay with rabbit antihuman ET-1 or big-ET-1 (Peninsular Laboratories Europe Ltd), as described previously.[22]

Data and Statistical Analysis

Nursing staff at the clinical research facility at the Umeå University Hospital randomized exposure type and the associated vascular study protocol. The investigators were blinded to the exposure received. Plethysmography data were analyzed as described previously.[23] Data are expressed as mean±SEM unless otherwise stated. Statistical analyses were performed using paired Student *t* tests and 2-way ANOVA with repeated measures, including time and exposure as variables, where appropriate. Statistical significance was taken at the 5% level. All of the analyses were performed using GraphPad Prism (version 4 for Macintosh, GraphPad Software) on a Macintosh personal computer.

Results

Thirteen subjects, with a median age of 23 years (Table), completed the study (Figure 1). The diesel exposure generated a mean particle mass concentration of 331±13 $\mu g/m^3$, and this was associated with concentrations of NO_2 of 1.0±0.04 ppm, NO of 3.3±0.13 ppm, and total hydrocarbons of 1.4±0.04 ppm. There were no differences in 24-hour mean systolic or diastolic blood pressures or mean heart rate after exposure to air or diesel exhaust ($P>0.05$ for all), although there was a slightly lower nocturnal diastolic blood pressure after diesel exhaust exposure (62±1 versus 65±1 mm Hg; $P<0.01$). There was a rise in blood pressure after exercise on both study visits, although there was no difference between the 2 exposures (data not shown; $P>0.05$).

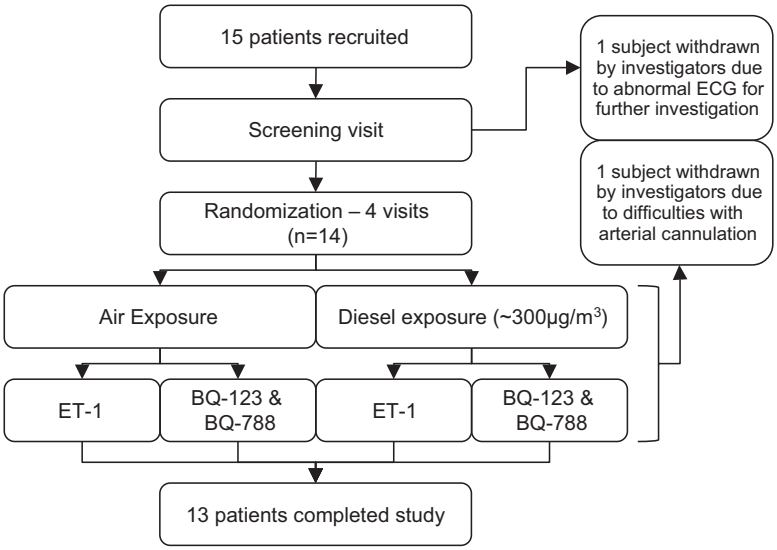

Figure 1. Consort flowchart of study participants.

Infusion of ET-1 caused a slow-onset vasoconstriction after diesel exhaust inhalation (17±10% peak reduction in blood flow), although there was little effect after filtered air (Figure 2; ANOVA, $P<0.001$ for exposure effect). Infusion of the ET receptor antagonists, BQ-123 and BQ-788, caused a slow-onset vasodilatation (77±14% peak increase in blood flow after filtered air; Figure 3). This vasodilatation was greater after filtered air compared with the diesel exhaust exposure (Figure 3; ANOVA, $P<0.001$). The difference was greatest at 60 minutes, but, after infusion of the BQ-788, there was little difference in blood flow by 120 minutes ($P>0.05$).

Plasma ET-1 and big-ET-1 concentrations were unchanged at all of the time points after diesel exhaust or filtered air exposure ($P>0.05$ for both). Comparison of the infused and noninfused arm plasma ET-1 concentrations confirmed that the ET-1 infusion increased local plasma ET-1 concentrations by 58±9% (Figure 2; $P<0.01$ for infused arms and $P>0.05$ for noninfused arms for both exposures).

Discussion

Although diesel exhaust inhalation had no effect on plasma ET-1 or big-ET-1 concentrations, there was an increase in vascular sensitivity to ET-1 associated with a reduced ET_A induced vasodilatation. These apparently contradictory findings can be explained by impaired ET-1–induced NO release and are consistent with preclinical evidence of NO-mediated alterations in vascular reactivity to ET-1.[24] We conclude that diesel exhaust inhalation, at levels commonly encountered in the urban environment, does not affect plasma ET-1 concentrations but alters vascular reactivity to ET-1 probably through effects on NO release and bioavailability.

Endothelin 1

We did not demonstrate any change in plasma concentrations of ET-1 or its immediate precursor, big-ET-1, after exposure to filtered air or diesel exhaust. Although this is consistent with our own previous work,[7] it is at odds with other reports.

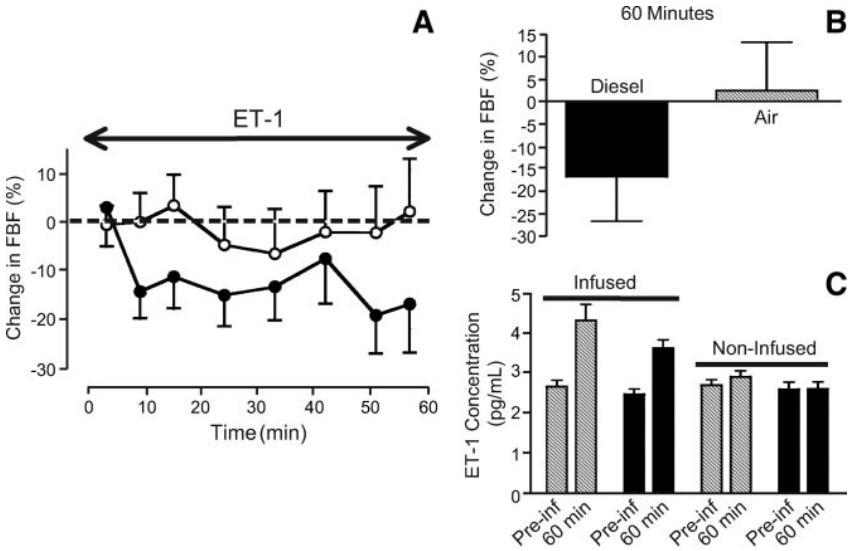

Figure 2. A, Forearm blood flow (FBF) during infusion of ET-1 (5 pmol/min) after exposure to air (○) and diesel exhaust (●; ANOVA, $P<0.001$). B, Maximal effect at 60 minutes and (C) comparison of plasma ET-1 concentrations in infused and noninfused arms before and at the end of the forearm vascular study after air (▨; $P<0.001$ for infused and $P>0.05$ for noninfused) and diesel exhaust inhalation (■; $P<0.0001$ for infused and $P>0.05$ for noninfused). Data are from a paired Student *t* test of air vs diesel.

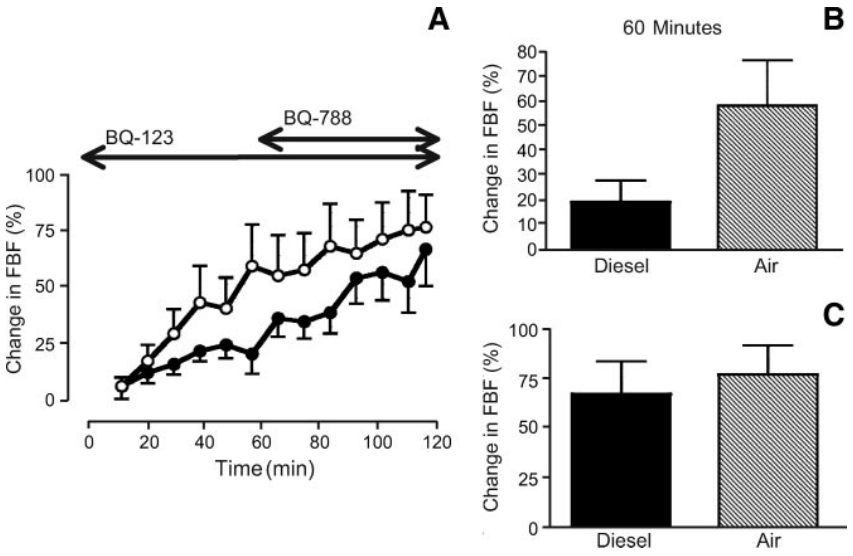

Figure 3. A, Forearm blood flow (FBF) during infusion of BQ-123 (10 nmol/min) and BQ-788 (1 nmol/min) after exposure to air (○) and diesel exhaust (●; ANOVA, $P<0.001$). B, Maximal effect after BQ-123 infusion alone and (C) after dual endothelin receptor antagonism.

In rodent studies, plasma ET-1 (and ET-3) concentrations were upregulated after exposure to diesel exhaust and concentrated urban particles.[12,13] It is possible that there are species differences in the response to diesel exhaust inhalation, and upregulation of ET-1 in rats may not translate into humans. However, Peretz et al[15] studied a heterogeneous group of individuals composed of patients with metabolic syndrome and healthy volunteers and showed an increase in plasma ET-1 concentrations 3 hours after diesel exhaust exposure. Their study was not designed specifically to look at ET-1 and was limited by missing data and small numbers in a heterogeneous population in whom vascular endothelial function may not be equivalent.[25] In contrast, our study was specifically designed to address the ET hypothesis and used a robust crossover study design, in a homogenous group of healthy volunteers, with samples optimally collected to assess plasma ET-1 and big-ET-1 concentrations.[22] Taken together with our previous study, our experience represents the largest sample size to date (n=43). Therefore, we think it is unlikely that diesel exhaust inhalation causes major changes in plasma ET concentrations.

We recognize that plasma ET-1 concentrations may not reflect the activity of the ET system because 90% of ET-1 synthesized by the vascular endothelium is secreted abluminally and acts locally on vascular smooth muscle in a paracrine manner.[8] Therefore, in addition to measuring plasma ET-1 concentrations, we assessed the effects of ET agonism and antagonism on peripheral vascular tone.

ET Agonism

We demonstrated increased vasoconstriction after exposure to diesel exhaust, but little effect after exposure to filtered air, suggesting an increased vascular sensitivity to ET-1. We were surprised to see little vasoconstriction with ET-1 after exposure to filtered air, having previously reported ≈30% to 40% reductions in forearm blood flow during infusions of 5 pmol/min.[10,26] In the present study, we used an alternative preparation of ET-1 and suggest that the disparity in vascular effects is attributable to differing potencies of the preparations. Because of this, we measured plasma ET-1 concentrations in both forearms and demonstrated a selective 60% increase in plasma ET-1 concentrations in the infused arm. Assuming a forearm blood flow of 25 mL/min, we achieved an end-organ concentration approximately one tenth of that anticipated. However, this simple calculation does assume that there is no clearance or extraction of ET-1 across the forearm, but we do not believe that the modest increase in venous ET-1 concentrations can be solely accounted for by clearance, and conclude that it reflects a reduced activity of the infused peptide preparation. Although the reduced activity of ET-1 limits comparisons with other studies, this was perhaps fortuitous, because it enabled us to assess the vasoreactivity to ET-1 at the threshold for vasoconstriction and to observe an alteration in ET-1 sensitivity.

ET_A Receptor Antagonism

The reduction in vasodilatation to BQ-123 infusion after diesel exhaust inhalation has several potential explanations. First, this may relate to reduced production or increased clearance of active ET-1 from the vasculature. However, this seems unlikely given that plasma ET-1 and big-ET-1 concentrations were unchanged, although we acknowledge that an effect on the abluminal release of ET-1 cannot be excluded. Second, a reduced sensitivity of the vascular smooth muscle ET_A receptor could have occurred, but this is at odds with the increased ET-1 vasoconstriction and is, therefore, unlikely. We believe that there is a third, more likely, explanation.

Looking closely at ET_A receptor antagonism, it is clear that the mechanism of vasodilatation is complex. This reflects the distribution and basal activity of both the ET_A and ET_B receptors. Both receptors contribute to the maintenance of basal vascular tone but have differing actions and vascular distributions: ET_A receptors are present on vascular smooth muscle cells only and mediate vasoconstriction, whereas ET_B receptors are present on both vascular smooth muscle and endothelial cells, where they mediate vasoconstriction and vasodilatation, respectively. Moreover, selective ET_A receptor antagonism leads not only to inhibition of the ET_A

receptor but potentially to hyperstimulation of the ET_B receptor. Indeed, we have demonstrated that BQ-123–induced vasodilatation can be markedly attenuated by concomitant blockade of NO release,[18] suggesting that selective ET_A receptor antagonism does indeed lead to significant ET_B receptor–mediated vasodilatation through endothelial NO release. Given the central role of NO in modulating and balancing the effects of the ET system, we propose that changes in the L-arginine-NO pathway offer the most plausible hypothesis for the impaired vasodilatation to BQ-123. This would also explain the enhanced vasoconstriction to ET-1 with a reduction in the opposing vasodilatory actions of NO. Moreover, we have shown previously that diesel exhaust impairs NO bioavailability[7,27] and suggest that the observed effects on ET-1 vasoreactivity can be explained by the reduction in ET-induced NO release and bioavailability.

The finding of reduced vasodilatation in response to ET_A receptor blockade is at odds with previous studies demonstrating enhanced response in patients with conditions such as hypertension and hypercholesterolemia who have preexisting endothelial dysfunction mediated by reduced NO bioavailability.[28,29] The reason for this discrepancy is unclear, but here we have induced an acute and brief episode of endothelial dysfunction in an otherwise healthy population of volunteers. Chronic dysfunctional states are likely to invoke compensatory mechanisms that may result in important differences in these vascular responses.

Combined ET_A and ET_B Receptor Antagonism

Previous data, including our own work,[18] would suggest that combined ET_A/ET_B receptor antagonism should produce less vasodilatation than selective ET_A receptor antagonism. We were, therefore, surprised to observe the continued further modest vasodilatation when ET_B receptor antagonism was superimposed on ET_A receptor antagonism. We believe that the explanation for this observation is 3-fold. First, vasodilatation to ET agonism and antagonism is of slow onset and offset. In our own hands, BQ-123–induced vasodilatation appears to reach a peak effect by 60 minutes[18] but may take up to 90 minutes.[10] The continued vasodilatation may, therefore, reflect further and more complete ET_A receptor antagonism. Second, we chose this study design to minimize the number of visits given the invasive nature of the studies. We attempted to assess both ET_A receptor antagonism and combined ET_A/ET_B receptor antagonism on the same visit. This approach has been used once before by Cardillo et al[30] in a small subgroup of patients with diabetes mellitus. Here, they demonstrated a brisk vasodilatation to BQ-123 of ≈65% with maximal vasodilatation by 60 minutes. Importantly, the predicted "tailing off" of the response when BQ-788 was added did not occur, and the vasodilatation plateaued rather than fell. This is consistent with the findings in our study. Finally, there may be an interaction when ET_A receptor antagonism precedes combined ET_A/ET_B receptor antagonism. This may reflect alterations in ET_B receptor expression on both the endothelium and vascular smooth muscle cells in the face of ET_A receptor antagonism. Indeed, there is considerable cross-talk between the receptors, as we have described previously.[31] Thus, the differing profile of responses may reflect the dynamic interaction of the 2 receptors over the course of the study.

This altered profile of vasodilatation does not detract from the comparison between the filtered air and diesel exhaust exposure. Combined ET_A and ET_B receptor antagonism appears to be unaffected by diesel exhaust exposure, whereas selective ET_A receptor antagonism is impaired. This is likely to reflect the greater and marked dependence of ET_A receptor antagonism on NO release in comparison with combined ET_A and ET_B receptor antagonism.

Conclusions

Our data demonstrate that the previously documented impairment of endothelium-dependent vasodilatation after a 1-hour exposure to combustion-derived air pollutants is not mediated by an upregulation of the ET system. Furthermore, we have shown that diesel exhaust inhalation has no effect on plasma ET-1 concentrations or systemic blood pressure. Our data are consistent with the hypothesis that the diesel exhaust–induced vascular effects are predominantly driven by reduced endothelial NO bioavailability. However, we cannot exclude a role for other vasoactive mediators, such as endothelium-derived hyperpolarizing factor, and further studies are warranted to investigate the L-arginine:NO and other pathways in more detail.

Perspectives

Air pollution exposure is associated with increased cardiovascular morbidity and mortality and is thought to lead to ≈3 million deaths worldwide each year. Understanding the underlying mechanism for these detrimental effects is crucial in trying to reduce this significant disease burden. In this study, we show that the well-established adverse vascular endothelial effects demonstrated after inhalation of diesel exhaust are not directly mediated through the ET system. We propose instead that these may be driven by changes in NO bioavailability. Additional studies are warranted to investigate this hypothesis in more detail.

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Disclosures

None.

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Research

Beneficial cardiovascular effects of reducing exposure to particulate air pollution with a simple facemask

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Abstract

Background: Exposure to air pollution is an important risk factor for cardiovascular morbidity and mortality, and is associated with increased blood pressure, reduced heart rate variability, endothelial dysfunction and myocardial ischaemia. Our objectives were to assess the cardiovascular effects of reducing air pollution exposure by wearing a facemask.

Methods: In an open-label cross-over randomised controlled trial, 15 healthy volunteers (median age 28 years) walked on a predefined city centre route in Beijing in the presence and absence of a highly efficient facemask. Personal exposure to ambient air pollution and exercise was assessed continuously using portable real-time monitors and global positional system tracking respectively. Cardiovascular effects were assessed by continuous 12-lead electrocardiographic and ambulatory blood pressure monitoring.

Results: Ambient exposure ($PM_{2.5}$ 86 ± 61 *vs* 140 ± 113 μg/m^3; particle number 2.4 ± 0.4 *vs* 2.3 ± 0.4 × 10^4 particles/cm^3), temperature (29 ± 1 *vs* 28 ± 3°C) and relative humidity (63 ± 10 *vs* 64 ± 19%) were similar (P > 0.05 for all) on both study days. During the 2-hour city walk, systolic blood pressure was lower (114 ± 10 *vs* 121 ± 11 mmHg, P < 0.01) when subjects wore a facemask, although heart rate was similar (91 ± 11 *vs* 88 ± 11/min; P > 0.05). Over the 24-hour period heart rate variability increased (SDNN 65.6 ± 11.5 *vs* 61.2 ± 11.4 ms, P < 0.05; LF-power 919 ± 352 *vs* 816 ± 340 ms^2, P < 0.05) when subjects wore the facemask.

Conclusion: Wearing a facemask appears to abrogate the adverse effects of air pollution on blood pressure and heart rate variability. This simple intervention has the potential to protect susceptible individuals and prevent cardiovascular events in cities with high concentrations of ambient air pollution.

Introduction

Air pollution, and especially traffic-derived particulate matter [1], is now established as a major cause of cardiorespiratory morbidity and mortality [2-4]. Epidemiological studies have shown that chronic air pollution exposure is associated with the degree of atherosclerosis [5,6], and the risk of cardiovascular events [7]. Acute exposure causes exacerbation of existing cardiorespiratory conditions leading to an increase in hospital admissions [8] and deaths [9].

The mechanisms of these associations are unclear but recent controlled exposure studies have demonstrated that air pollution causes vascular endothelial dysfunction [10], arterial vasoconstriction [11], increased blood pressure [12] and myocardial ischaemia [13]. Observational studies have also suggested that air pollution exposure impairs regulation of the autonomic nervous system and reduces heart rate variability [14,15]. A combination of these effects is likely to account for the increase in cardiovascular events seen following exposure to air pollution. There is therefore a need to consider approaches that can reduce ambient air pollution exposure on both a personal and societal level.

In Beijing China, particulate matter (particle diameter < 10 μm; PM_{10}) air pollution averages around 150 μg/m^3 and, in 2006, levels exceeded the World Health Organisation recommended national standards (PM_{10}concentration < 50 μg/m^3) on 241 out of 365 days [16]. Despite considerable efforts to improve air quality, pollution remains the single largest environmental and public health issue affecting Beijing. The extensive use of coal and the growing number of motor vehicles (estimated 3.3 million vehicles on the roads in August 2008) have contributed to air pollution. In addition, the city's geographical location exacerbates the problem with the surrounding mountain ranges impeding air circulation and dispersion of pollutants.

Increasing concern relating to the health effects of air pollution has led many individuals to use facemasks to reduce personal exposure. The efficiency of these masks and the potential cardiovascular benefits on people exposed to urban air pollution has yet to be established. The aims of this study were to assess the efficacy of facemasks in removing potentially hazardous particulate air pollution and to determine the potential cardiovascular benefits of a simple facemask in a polluted urban environment.

Methods

Subjects

Fifteen healthy volunteers were recruited from the Fuwai Hospital, Beijing in August 2008. All subjects were non-smokers, received no regular medication, and had no intercurrent illnesses. All subjects gave their written consent to participate in the study, which was reviewed and approved by the local ethics committee, in accordance with the Declaration of Helsinki.

Assessment of mask efficacy

Masks designed for use by cyclists, pedestrians and occupational settings were tested for penetrance of fresh diesel exhaust particulate. Diesel engine exhaust was generated from the idling (1500 rpm) engine (F3M2011, Deutz Ag, Köln, Germany) of a 35 KVA generator (Bredenoord, Apeldoorn, Netherlands). The exhaust was diluted with filtered air to obtain a mass concentration of 75 ± 12 μg/m^3 (as measured by gravimetric analysis) and a particle number concentration of 500,000 particles/cm^3 (condensation particle counter [CPC] model 3022, TSI Instruments, High Wycombe, UK). Sections of each mask filter were mounted in a filter holder. After 5 min of baseline measurements, filters were introduced between the exhaust and the CPC that sampled at a flow rate of 1.5 L/min. Particle number was recorded for 5 min and penetrance defined as the percentage of particles passing through the filter compared to baseline.

Study design

All 15 subjects attended the Fuwai Hospital on two occasions, each at least one week apart, during August 2008. In a randomised open-label controlled cross-over study, subjects were randomised to wear no mask or a highly efficient facemask filter (Dust Respirator 8812, 3 M, St Paul USA). When randomised to wear the facemask, subjects were asked to wear the mask for 24 hours prior to the study day and 24 hours of the study day. Subjects were asked to wear the mask at all times when outside, and as much as possible whilst indoors. On the study day, subjects were asked to walk for 2 hours in a city centre location (Figure 1) along the inner ring road in Beijing between 8 and 10 am.

Pollution and activity monitoring

Personal exposure to air pollutants was monitored using a collection of portable monitoring equipment mounted in a backpack. Particle mass concentration (particle diameter < 2.5 μm; $PM_{2.5}$) was measured in using a light-scattering nephelometric method using a DataRAM monitor (pDR-1500, Thermo Scientific, Franklin, USA). Particle number was measured using a handheld condensation particle counter (CPC 3007, TSI Instruments Ltd, High Wycombe, UK). Ambient temperature and relative humidity were recorded using a sensor on the outside of the backpack (Omegaette® HH-314, Omega Engineering Ltd, Connecticut, USA). Gaseous pollutants were measured using a multigas analyser (X-am 7000, Dräger Safety, Pittsburgh, USA) measuring carbon monoxide (CO),

Figure 1
City centre route chosen in central Beijing. A representative recording from the GPS device contained in the monitoring backpack is shown. The walk goes from the Fuwai Hospital (A), along the inner ring road (B) and towards the city centre (C) before turning back. Image courtesy of Google™ Earth.

nitrogen dioxide (NO_2) and sulphur dioxide (SO_2) using electrochemical sensors with a sensitivity of 1 part per million.

Physical activity was assessed using a portable global positioning system (GPS) monitor secured to the outside of the bag (eTrex Summit HC, Garmin, USA). This recorded the route taken by volunteers, their total distance travelled, and average speed. This information was used, along with baseline anthropometric measurements, to calculate the energy expended during the walk in kilocalories and metabolic equivalents (METS).

Holter monitoring

Subjects were fitted with a 12-lead continuous electrographic Holter monitor (Lifecard 12, Spacelabs, UK) at the beginning of the study day for 24 hours. Holter electrographic traces were analysed using DelMar Reynolds proprietary software packages by two blinded observers. The quality of the electrocardiographic trace was manually inspected before arrhythmias were automatically detected using the Pathfinder software package. Identified arrhythmias were then individually inspected, verified or deleted as appropriate. Average heart rate and heart rate variability in both time and frequency domains were analysed using the HRV Tools software package, with identified arrhythmias excluded from this analysis.

Ambulatory blood pressure

Subjects were fitted with an ambulatory blood pressure monitor (Model 90217, Spacelabs, UK) at the beginning of the study day. Blood pressure was recorded at the left brachial artery every 15 minutes during the 2-hour walk, every 30 minutes for the rest of the daytime (07:00 to 22:00), and every hour overnight (22:00 – 07:00).

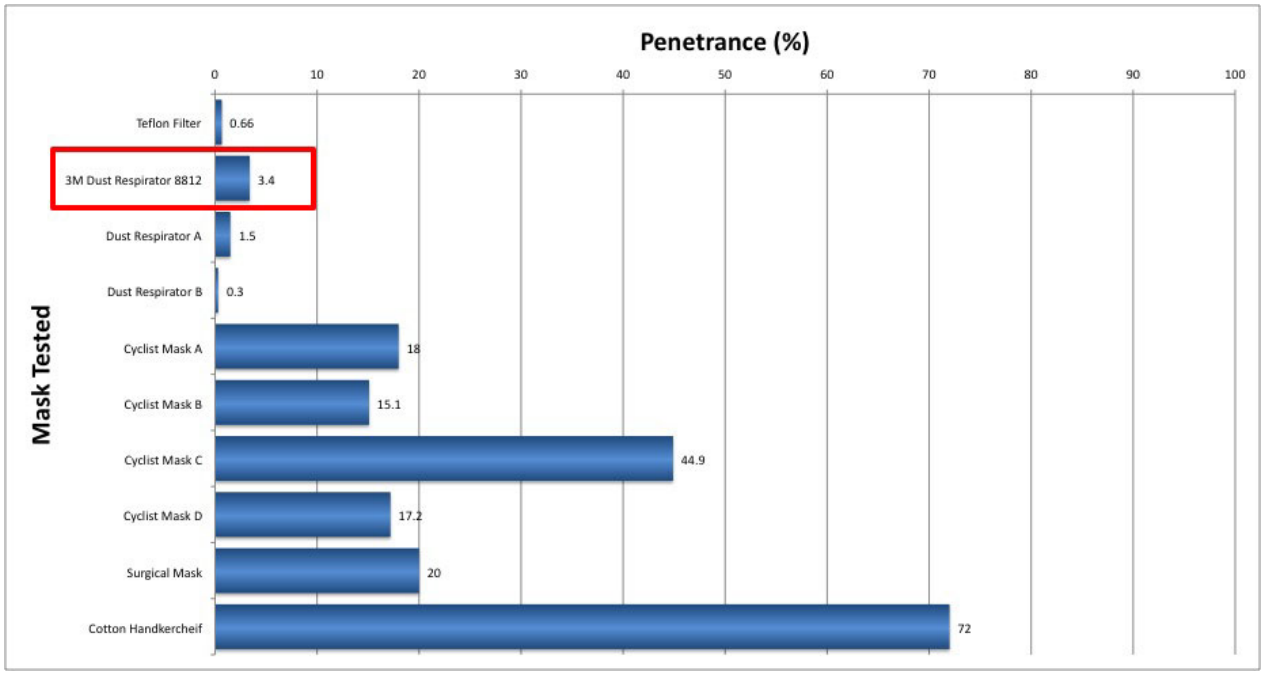

Figure 2
Penetrance of commercially available filters: 3 M Dust Respirator 8812, Dust Respirators A and B, Cyclist Masks A to D. The Teflon filter is an industry standard filter for aerosol studies included as a control. Cotton handkerchiefs and surgical masks are often seen worn in public areas in parts of Asia.

Symptom questionnaire

Subjects were asked to complete a symptom questionnaire using a visual analogue scale at the beginning of the study day, after the 2-hour walk and at the 24-hour visit. They were asked to record any physical symptoms, as well as report a perception of the degree of pollution and the tolerability of the mask.

Table 1: Exposure characteristics during city centre walks.

	Without Mask	With Mask
$PM_{2.5}$, μg/m^3	86 (52 – 120)	140 (77 – 203)
Particle count, number/cm^3	24184 (22061 – 26306)	23379 (21350 – 25409)
CO, number of peaks	6.2 (3.2 – 9.3)	3.3 (1.3 – 5.2)
NO_2, number of peaks	Nil	Nil
SO_2, number of peaks	Nil	Nil
Temperature, °c	29.2 (28.6 – 29.8)	28.1 (26.3 – 29.9)
Relative humidity, %	63 (58 – 68)	64 (53 – 74)

Data expressed as mean (95% confidence interval).
P > 0.05 compared to control (without mask) day for all, Student's paired t-test.

Data analysis and statistical methods

Subjects were randomised to wearing a mask on their first or second visit using a random number generator. All data are expressed as mean (95% confidence interval [CI]) unless otherwise stated. The symptom questionnaire was based on a visual analogue scale. Scores were converted into a percentage, and analysed using 2-way analysis of variance (ANOVA) with repeated measures using time and the mask intervention as variables. The occurrences of arrhythmias during the 24-hour monitoring period were compared using the Wilcoxon matched pairs method. All other parameters were evaluated using paired Student's *t*-tests. Statistical significance was taken at the 5% level. All data were analysed using GraphPad Prism (Version 4 for Macintosh, GraphPad Software, San Diego, USA) on a Macintosh personal computer.

Results

Mask efficiency

Mask penetrance was highly dependent on mask type (Figure 2). The 3 M Dust Respirator (Model 8812, 3 M, St Pauls, USA) was selected for the intervention study as it provided good filtration performance and was extremely efficient and comfortable to wear.

Table 2: 24-hour ambulatory blood pressure monitoring and Holter analysis for heart rate variability with each visit.

		Without Mask	With Mask
24 hour	SBP, mmHg	106 (100 – 112)	106 (101 – 111)
	DBP, mmHg	69 (66 – 73)	70 (67 – 73)
	MAP, mmHg	82 (79 – 86)	82 (78 – 86)
	Heart rate, bpm	74 (70 – 77)	72 (68 – 76)
Night	SBP, mmHg	100 (93 – 107)	101 (95 – 106)
	DBP, mmHg	63 (60 – 67)	64 (61 – 67)
	MAP, mmHg	76 (73 – 79)	75 (71 – 79)
	Heart rate, bpm	64 (61 – 67)	61 (58 – 65)
Day	SBP, mmHg	110 (104 – 116)	109 (104 – 114)
	DBP, mmHg	73 (69 – 76)	73 (70 – 76)
	MAP, mmHg	85 (81 – 88)	85 (81 – 89)
	Heart rate, bpm	79 (74 – 84)	78 (73 – 82)
Heart rate variability	Data validity, %	95.9	95.0
	Average NN interval, ms	829 (789 – 869)	850 (805 – 896)
	pNN50, %	15.9 (10.7 – 21.0)	17.9 (14.2 – 21.6)
	RMSSD, ms	35.1 (29.2 – 41.0)	37.1 (32.2 – 42.0)
	SDNN, ms	61.2 (54.9 – 67.5)	65.6* (59.0 – 72.2)
	Triangular Index	12.9 (11.9 – 13.9)	13.8 (13.0 – 14.5)
	LF-power, ms^2	816 (628 – 1004)	919* (717 – 1122)
	HF-power, ms^2	460 (325 – 595)	485 (400 – 569)
	LFn, ms	62.8 (56.7 – 68.9)	64.5 (60.6 – 68.4)
	HFn, ms	29.2 (25.5 – 32.8)	30.0 (27.0 – 33.1)
	HF/LF ratio	0.738 (0.507 – 0.970)	0.680 (0.519 – 0.842)

All data expressed as mean (95% confidence interval). *P < 0.05 compared to control (no mask) day, Student's paired t-test.

Intervention study

Fifteen subjects (20–45 years) completed the study. Subjects were predominantly female (13:2) with a mean height of 164 cm (95% CI, 160 – 167), weight of 55 kg (95% CI, 50 – 60) and body mass index of 20.5 kg/m^2 (95% CI, 19.3 – 21.7). There were no differences (P > 0.05 for all parameters) in ambient pollution exposure during the 2-hour walk between the two visits (Table 1). Based on the measured penetrance of 3.4%, assuming a perfect facial fit and similar flow rates, we predict that the particle count to which subjects were exposed when wearing a mask was reduced to just 795 (95% CI, 726 – 864) particles/cm^3.

There were no differences in 24-hour average heart rate or blood pressure during the two study days (Table 2). Holter analysis revealed an increased SDNN (65.6 ± 11.5 *vs* 61.2 ± 11.4 ms, P < 0.05) and LF-power (919 ± 352 *vs* 816

Table 3: Arrhythmia analysis from 24-hour Holter electrocardiograms.

	Without Mask	With Mask
Pause	0	0
Dropped beat	0	0
Ventricular tachycardia	0	0
Salvo	0	0
Triplet	0	0
Couplet	0	0
Bradycardia (=50 bpm)	71	227
Supraventricular tachycardia	0	0
Bigeminy	57	157
Trigeminy	4	7
"R on T"	0	0
Premature aberrant	3246	4698
Isolated aberrant	18	3
Premature normal	11	17
Maximum heart rate	134 (126 – 143)	128 (120 – 137)
Minimum heart rate	51 (48 – 54)	49 (46 – 53)

Data shown are total number of events recorded in each condition over all subjects.
P > 0.05 for all (Wilcoxon matched pairs test). Maximum and minimum heart rates shown as mean (95% confidence intervals), P > 0.05 for both, Student's paired t-test.

± 340 ms^2, $P < 0.05$) over the 24 hours when subjects wore the mask. There were no clinically relevant arrhythmias recorded in any subject (Table 3).

During the 2-hour walk, there was no difference in exercise intensity in the presence or absence of the facemask (Table 4) although subjects had a lower systolic blood pressure (114 ± 10 *vs* 121 ± 11 mmHg; $P < 0.01$) when wearing a mask. This was not associated with a change in diastolic blood pressure, heart rate or in heart rate variability measurements.

Subjects reported only very minor symptoms (Table 5) during the study period. The mask was generally well tolerated with an average score of 24.8% (95% CI, 16.2 – 33.3%); 0% being completely tolerable and 100% being intolerable.

Discussion

In this study, we have shown for the first time that a simple well-tolerated personal intervention to reduce exposure to airborne particulate air pollution leads to a reduction in systolic blood pressure during exercise and an increase in heart rate variability. If translated into a susceptible population, our findings would suggest that wearing a simple facemask has the potential to reduce the incidence of acute cardiovascular events in cities with high levels of air pollution, and could influence the advice given to patients with chronic cardiovascular diseases.

We tested a range of facemasks that differed widely in their efficiency as particle filters. In general, those masks designed to reduce occupational exposure to dusts were more efficient than those marketed as personal protection to cyclists and pedestrians in an environmental setting. The choice of the mask used in this study was influenced by efficiency and comfort. The chosen mask was very well tolerated by subjects as demonstrated by the visual analogue score, and was predicted to reduce the exposure to particulate matter dramatically. When wearing the masks, the subjects did report slightly greater difficulty breathing whilst walking although this did not reduce the level of exercise undertaken by the subjects. This increased resistance to respiration is unlikely to affect the main study findings since such stresses would be predicted to increase blood pressure rather than reduce it.

Recent studies have confirmed a link between blood pressure and exposure to air pollution. Population-based studies have shown increases in both systolic blood pressure and pulse pressure [17,18] with increasing levels of ambient pollution exposure. Controlled exposure studies to concentrated ambient particles and ozone have demonstrated an increase in diastolic blood pressure during a two-hour exposure [12]. Although there were no differences in blood pressure over the whole 24-hour period, we observed a marked difference in systolic blood pressure with exercise. In both groups, blood pressure increased during exercise compared to the 24-hour average, although this increase was less when wearing a facemask. This, in combination with previous controlled exposure studies [12], suggests that particulate air pollution may augment exercise-induced increases in blood pressure, and that the use of a simple facemask can abrogate this. Exercise induced increases in systolic blood pressure have been linked to myocardial infarction [19] as well as stroke [20], and increased blood pressure is an established major risk factor for the development of both atherosclerosis and cardiovascular mortality [21,22]. The reduction in systolic blood pressure seen in this study is similar to that seen with many antihypertensive agents, which have been shown to reduce major cardiovascular events. Therefore we predict that the use of a facemask in a susceptible population has the potential to reduce the incidence of acute cardiovascular events as well as myocardial ischaemia [13,23].

Heart rate variability is a measure of the variation in the R-R interval on the electrocardiogram. A balance of the parasympathetic and sympathetic nervous systems controls the heart rate in order to maintain a constant cardiac output at rest or to respond to increased demands during exercise. A reduction in heart rate variability occurs in various pathophysiological conditions including hypertension [24], heart failure [25] and diabetes mellitus [26],

Table 4: Exercise performed and physiological parameters during 2-hour walk.

		Without Mask	With Mask
Activity	Energy expenditure, kcals	340 (314 – 367)	364 (304 – 426)
	Energy expenditure, METS	3.33 (3.09 – 3.57)	3.61 (3.12 – 4.10)
Ambulatory blood pressure	Systolic blood pressure, mmHg	121 (115 – 127)	114* (108 – 120)
	Diastolic blood pressure, mmHg	81 (75 – 87)	79 (74 – 83)
	Mean arterial pressure, mmHg	94 (89 – 99)	90 (86 – 94)
	Heart rate, bpm	88 (82 – 94)	91 (85 – 97)
Heart rate variability	Data validity, %	99.1	97.8
	Average NN interval, ms	594 (562 – 627)	613 (571 – 655)
	pNN50, %	3.3 (0.8 – 5.7)	2.1 (-0.1 – 4.4)
	RMSSD, ms	17.2 (13.4 – 21.0)	20.0 (15.5 – 24.6)
	SDNN, ms	45.8 (36.8 – 54.8)	54.8 (42.5 – 67.0)
	Triangular Index	10.7 (9.1 – 12.4)	11.4 (9.4 – 13.3)
	LF-power, ms^2	313 (170 – 455)	414 (233 – 595)
	HF-power, ms^2	76.5 (33.6 – 120.0)	116.8 (52.6 – 181.0)
	LFn, ms	68.2 (60.9 – 75.5)	67.9 (61.9 – 73.9)
	HFn, ms	16.1 (11.9 – 20.3)	16.0 (12.5 – 19.4)
	HF/LF ratio	0.259 (0.173 – 0.344)	0.247 (0.180 – 0.314)

All data expressed as mean (95% confidence interval).
*P < 0.01 compared to control (without mask) day, paired Student's t-test.
P > 0.05 for all other parameters compared to control (without mask) day.

and predicts cardiovascular outcomes [27]. Previous studies have demonstrated a reduction in measures of heart rate variability, particularly the robust and simple time-domain measurement SDNN following exposure to air pollution [14,15,28-33]. In our study we report an increase in overall heart rate variability (SDNN) when subjects wore a mask, suggesting that wearing a mask can, at least in part, prevent the adverse effects of air pollution exposure on heart rate variability.

LF-power also increased with the use of a mask to prevent exposure to air pollution although interpreting this change is more challenging. LF-power is associated with changes in sympathetic tone, and an increase might suggest an increased contribution of the sympathetic nervous system to basal heart rate control. However, simply wearing the facemask may have had a small effect on the measures of heart rate variability described. As previously discussed, subjects did report an increased resistance to breathing when wearing the facemask that may have increased subject anxiety. This in turn could have increased sympathetic nervous system tone and hence lead to a small increase in LF-power. This is a limitation of our study, and the use of a sham facemask in a blinded fashion would have helped minimise the effect of anxiety on these sensitive outcome measures.

Our study has a number of important public health messages. First we have demonstrated that exposure to ambient air pollution has direct and measurable effects on cardiovascular physiological parameters, even young healthy individuals habitually exposed to such elevated levels. Second we have shown that wearing a facemask can abrogate some of these effects in a short period of time. Particle traps are increasingly being fitted to new vehicles to reduce the emissions of particulate matter, both by mass and number concentrations, and this may well go some way to offsetting the associated health effects. Currently, patients with chronic respiratory and cardiovascular conditions are advised to limit their exposure outdoors

Table 5: Symptom questionnaire.

Symptoms assessed by visual analogue scale (0 – 100)							
	Without mask			With mask			P
	Before walk	After walk	24 hours after walk	Before walk	After walk	24 hours after walk	
Headache	4.00 ± 14.13	2.53 ± 5.55	1.13 ± 2.26	3.47 ± 12.06	0.73 ± 1.03	1.13 ± 2.10	n/s
Dizziness	0.40 ± 0.91	1.07 ± 2.22	0.67 ± 1.35	0.47 ± 0.99	0.80 ± 1.57	0.47 ± 0.92	n/s
Tiredness	1.40 ± 4.12	8.47 ± 12.14	9.60 ± 14.78	2.07 ± 5.35	7.40 ± 9.37	2.13 ± 4.10	n/s
Sickness	0.40 ± 0.91	1.07 ± 2.22	0.67 ± 1.35	0.53 ± 0.99	0.87 ± 1.51	0.80 ± 1.32	n/s
Cough	1.07 ± 3.08	1.80 ± 4.80	0.80 ± 1.61	0.73 ± 1.49	1.00 ± 1.73	0.60 ± 1.18	n/s
Difficulty in breathing	0.40 ± 0.91	0.67 ± 0.90	1.13 ± 2.83	3.87 ± 9.23	3.80 ± 8.10	1.60 ± 3.70	<0.05
Irritation of the eyes	1.00 ± 3.09	1.40 ± 3.60	1.13 ± 2.83	1.00 ± 2.59	1.67 ± 3.27	0.87 ± 1.69	n/s
Irritation of the throat	1.00 ± 2.83	1.47 ± 4.07	1.73 ± 4.56	0.73 ± 1.87	1.07 ± 2.63	1.40 ± 2.77	n/s
Irritation of the nose	1.00 ± 2.56	1.53 ± 3.78	1.27 ± 3.58	0.67 ± 1.23	1.07 ± 1.91	0.67 ± 1.35	n/s
Unpleasant smell	0.40 ± 0.74	0.93 ± 1.22	1.00 ± 1.69	0.67 ± 1.23	0.60 ± 0.91	0.80 ± 1.15	n/s
Bad taste	0.53 ± 1.13	0.73 ± 0.96	0.60 ± 0.83	0.40 ± 0.74	0.60 ± 1.18	0.93 ± 1.49	n/s
Difficulty walking		12.53 ± 13.24			15.13 ± 11.51		n/s
Perception of pollution		19.80 ± 18.37			11.60 ± 10.44		n/s

All data expressed as mean ± standard deviation. "Difficulty walking" and "Perception of pollution" tested using paired Student's t-tests. All other variables tested using 2-way ANOVA with repeated measures including time and mask intervention as variables. P value shown is the effect of the mask intervention.

on days when ambient air pollution levels are high [34-36]. We have shown that wearing a simple inexpensive and well-tolerated facemask can provide an alternative that may lead to reduced cardiovascular morbidity and mortality. We believe that this intervention now needs to be tested in patients with pre-existing coronary heart disease to define its potential role in reducing the burden of cardiovascular disease in polluted environmental settings.

Our study has a number of important limitations. We recruited young healthy volunteers rather than those most susceptible to the effects of air pollution exposure, such as those with coronary heart disease. Whilst it is likely that our findings will be transferrable to this population, further studies are required to confirm our findings. In addition, it was not possible to assess accurately the efficacy of the mask filter when worn by the subjects. Leaks around the facemask will lead to a reduction in the efficacy of particle filtration [37,38] and therefore our predicted exposures during application of the facemask are likely to be an underestimate. However, despite this, we were still able to demonstrate beneficial cardiovascular effects during their use.

Conclusion

Air pollution exposure is associated with increased cardiovascular morbidity and mortality, and adverse effects on the cardiovascular system. We have shown for the first time that a wearing a facemask appears to abrogate the adverse effects of air pollution on blood pressure and heart rate variability. This simple intervention has the potential to protect susceptible individuals and prevent cardiovascular events in cities with high concentrations of ambient air pollution.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JL, NM, KD and DN conceived and participated in the design of the study. JC, JL and LJ carried out the clinical studies and collected the data. JL and NM performed the data analysis and statistical analysis. PF, DL, RA and FC performed the assessment of mask filter efficacy. JL drafted the manuscript, and all authors read and approved the final manuscript.

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Response to Peter Joseph

J P Langrish, N L Mills, K Donaldson, et al.

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Response to Bhaskaran *et al*'s "Effects of air pollution on the incidence of myocardial infarction"

To the Editor: I am writing to comment on the excellent article by Bhaskaran *et al* in *Heart*,[1] and especially on the editorial by David Newby[2] who commented on this paper. In my opinion, Dr Newby has missed the most important conclusion of Bhaskaran *et al*'s paper.

Dr Newby's editorial was focused mainly on the cardiac effects of fine particulate pollution and included a graph of data from Beijing, China.

However, it seems to me that a far more important contribution of the Bhaskaran paper was the observation of an "ozone protective effect" in several parts of the world. The authors pointed out that this effect is probably due to some pollutant that is negatively correlated with ozone and cited my previous paper,[3] suggesting that the effect could be due to unsuspected methyl nitrite (MN) in the air. This hypothesis has a high degree of plausibility because it is known that MN, unlike ozone, is rapidly destroyed by sunlight and so would be negatively correlated with ozone. To the best of my knowledge, this ozone protective effect is totally inexplicable unless an unknown pollutant, such as MN, is present.

If Dr Newby's point of view is to be accepted, then presumably he must argue that fine particulate pollution is also the cause of the ozone protective effect. However, that hypothesis has been thoroughly discredited in another paper.[4] In that paper, I showed that fine particulate matter cannot explain the negative ozone associations in any parts of the world in which there is published evidence for the negative effects. I particularly showed that explanation is extremely unlikely in Hong Kong, China.

Hence, I feel that the most important contribution of the Bhaskaran paper is to alert the world to the possible existence of a very important toxic pollutant whose presence has escaped attention. Most desperately needed is funding for research to identify MN in engine exhaust. To date, such funding has not been available in the USA.

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4. **Joseph PM.** Can fine particulate matter explain the paradoxical ozone associations? *Environ Int* 2008;**34**:1185–91.

Response to Peter Joseph

The authors' reply: We would like to thank Dr Joseph for his interest and comments on our editorial [1] accompanying the excellent systematic review by Bhaskaran *et al*.[2] As Dr Joseph points out, one of the systematic review's findings was a weak negative association between ozone exposure and the incidence of myocardial infarction.

As Bhaskaran *et al* indicate, a reduction in ozone concentrations is likely to be a marker of increased combustion-derived fine particulate matter ($PM_{2.5}$) from vehicle exhaust. Sarnat similarly found that ambient concentrations of O_3, NO_2, CO and SO_2 serve as "surrogates for personal exposure to $PM_{2.5}$ alone."[3] Fine combustion-derived particulate matter is robustly and consistently associated with increased cardiovascular morbidity and mortality.[4 5] Indeed, this was the major finding of the Bhaskaran systematic review[2] and has many plausible underlying mechanisms.[6]

The production of methyl nitrite in vehicle exhaust is suggested by Dr Joseph[7] to explain the cardiovascular toxicity of air pollution and the apparent protective effect of ozone. Its generation requires additives to "oxygenate" the fuel and to increase its octane content. Such additives are being phased out in the USA (prohibited in some states), never seen in Australia and used in minute quantities in Europe.[8] Thus, methyl nitrite is not a major current or future public health concern.

Dr Joseph has argued that the paradoxical relationship between cardiorespiratory morbidity and ozone cannot be explained by $PM_{2.5}$ because they are positively correlated.[9]

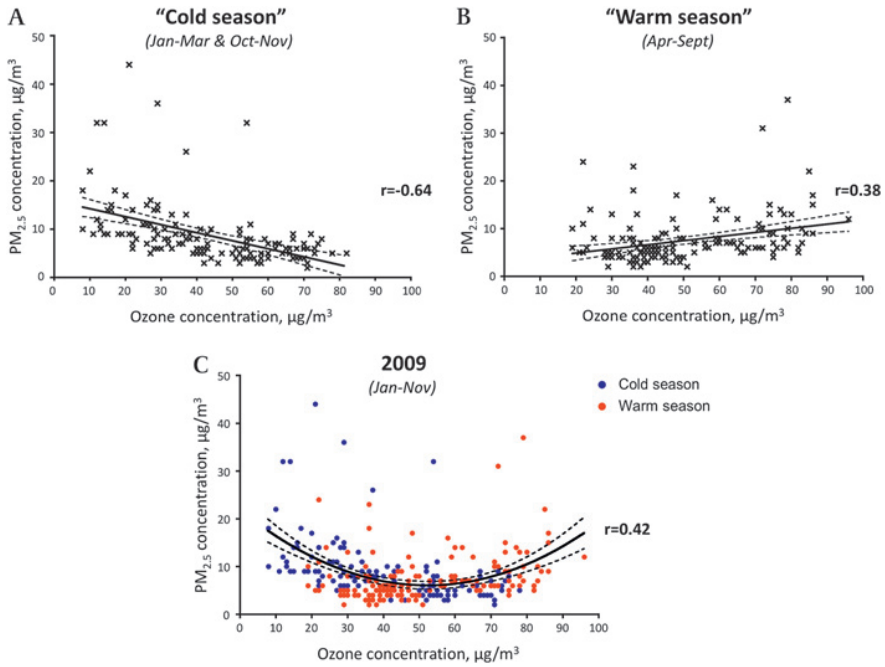

Figure 1 Daily mean $PM_{2.5}$ and ozone concentrations (µg/m^3) in Edinburgh, UK, from January to November 2009. Data for the (A) first and fourth quarters and (B) second and third quarters. Linear regression lines shown (95% CI) with Spearman's rank correlation coefficients. (C) Composite data for 2009 with the non-linear regression line (95% CI) and correlation coefficient.

However, this is based on analyses restricted to the summer months. In Dr Joseph's previous paper,[9] there is a negative correlation in the winter months: a time when most myocardial infarctions occur. We have reproduced these findings using local data (figure 1). As Dr Joseph points out, many epidemiological studies perhaps incorrectly use a simple linear model. Used here over the whole data set, there is a weak negative correlation ($r=-0.1417$, $p=0.0145$), suggesting that as ozone concentrations increase, $PM_{2.5}$ concentrations fall. However, these data best fit with a parabolic second-order non-linear regression, and we believe there is a complex interrelationship between PM and ozone that may explain some of the weak paradoxical relationships described.

We concur with Bhaskaran *et al* and maintain that it is the fine combustion-derived particulate matter that mediates the adverse cardiovascular effects demonstrated.

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Response to Peter Joseph

The authors' reply: We thank Professor Joseph for his thoughtful comments on our study. As his letter points out, our review identified a number of studies that showed an apparently reduced risk of myocardial infarction associated with higher ambient ozone levels.[1] Similar short-term 'protective' associations with ozone have been observed in studies of other outcomes—for example, cardiovascular admissions[2] and general practitioner consultations for upper respiratory diseases.[3] On the other hand, a recent review of mortality studies suggested broadly detrimental health associations with ozone.[4] Protective associations were largely restricted to the cold season when levels are characteristically low, suggesting confounding may play a role.[5]

These inconsistencies clearly require some explanation; a causally protective effect of ozone seems implausible for any outcome. As we discussed in our review, various theories have been put forward. For example, the correlation between ozone and sunlight means that ozone levels could in some settings be associated with time spent indoors, and therefore exposure to indoor pollutants; outdoor ozone levels may also be an unreliable proxy for personal exposure. However, the most commonly suggested explanation is that a negative correlation between ozone and some other harmful pollutant explains apparent protective effects. The studies that our review highlighted did not consider the effects of ozone in multipollutant models, leaving open the possibility that exposure to particulate matter or nitrogen oxides—for example, could explain the findings. But studies of other outcomes suggest that adjustment for the commonly measured pollutants does not remove the protective effect,[2 3] and Professor Joseph's recent work suggests that fine particulates would not explain the effect in some parts of the world.[6]

As we mentioned in our paper, methyl nitrite is a potential candidate pollutant. Sunlight tends to increase ozone levels and destroy methyl nitrite, leading to a negative correlation with ozone. However, disentangling individual pollution effects in population-based studies is problematic. Experimental exposure studies into the health effects of individual pollutants—including methyl nitrites—are likely to be needed.

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INTERNAL MEDICINE JOURNAL

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EDITORIAL

Air pollution: the new cardiovascular risk factor

In August of this year, the XXIXth Olympic Games held in Beijing China brought the hazards of air pollution exposure into the public eye. International headlines stressed the poor air quality and the potential health effects that might have an adverse impact on the performance of athletes, as well as on those with chronic cardiorespiratory diseases. At least one high-profile marathon runner declared that he would not run in Beijing because of the poor air quality and on arrival to Beijing, the US cycling team wore close-fitting face masks designed to reduce personal exposure to airborne particles. So was this international concern justified?

Epidemiological studies have confirmed the link between exposure to air pollution and increased cardiorespiratory mortality.[1–3] Chronic exposure to traffic-derived fine-particulate air pollution is associated with the degree of coronary atherosclerosis and coronary artery calcium scores assessed using electron-beam computed tomography.[4] Consistent with this and animal models of atherosclerosis,[5,6] human exposure to $PM_{2.5}$ (particles with a mean diameter of 2.5 μm or less) correlates to carotid intima-medial thickness, a measure of subclinical atherosclerosis.[7] In the largest study to date (n = 65 893), Miller *et al.* showed that in postmenopausal women without pre-existing vascular disease, each 10 μg/m^3 increase in background fine-particulate air pollution concentrations was associated with a 24% increase in the risk of a cardiovascular event and a 76% increase in the risk of death from cardiovascular disease, including myocardial infarction or stroke.[8] In addition to the adverse health effects of chronic exposure, traffic-derived air pollution is associated with the triggering of acute myocardial infarction, with people experiencing a myocardial infarction being three times more likely to have been in road traffic in the hour before the onset of symptoms.[9]

Although well-described mechanisms underlie the development of cardiovascular disease with traditional risk factors, the pathophysiological effects of air pollution are poorly understood. The exact components of air pollution responsible have yet to be specifically identified, but the epidemiological link is strongest for fine-particulate matter associated with combustion sources and particularly that associated with road traffic.[10] Recent studies have used controlled exposures to either concentrated ambient particles or dilute diesel exhaust, the latter being the predominant component of airborne fine-particulate matter in the urban environment. Diesel exhaust is of particular relevance, as in addition to all commercial vehicles approximately 40% of all new domestic vehicles are diesel powered. This proportion may well increase, given the current trend for 'green taxation' linking tax paid by the motorist to carbon dioxide emissions. Although diesel engines produce less gaseous pollution, they emit greater than 100-fold more fine-particulate matter than an equivalent-sized petrol engine with a catalytic converter.[11]

Short-term exposure to concentrated ambient particulate matter and ozone causes acute arterial vasoconstriction.[12] In healthy volunteers, we showed that a 1-h exposure to dilute diesel exhaust at a particle concentration of 300 μg/m^3 resulted in vascular endothelial dysfunction, with impaired vasomotor tone and endogenous fibrinolysis.[13] Furthermore, using a similar exposure, patients with stable coronary heart disease developed more pronounced myocardial ischaemia during exercise.[14] This is further compounded by an increase in platelet activation and thrombogenicity in the first 6 h after exposure.[15,16] In combination, these findings may explain the excess of acute and chronic cardiovascular events in the epidemiological studies of air pollution.

Regarding global and public health, controlled exposure studies have been criticized due to the relatively high concentration of fine-particulate matter used and some have questioned the relevance of these findings to everyday life. The WHO has set air quality targets for particulate air pollution (PM_{10} – particles with a mean diameter less than 10 μm) to remain below 50 μg/m^3. In many of the world's important cities (Table 1), levels of fine-particulate air pollution regularly exceed this target. In Beijing, China, this target was exceeded on 241 days out of 365 in 2006[17] and levels often exceed 300 μg/m^3,[18] as used in controlled exposure studies. In Christchurch, New Zealand, this air-quality target has been exceeded on average 29 days each year for the last 5 years, with a peak of 184 μg/m^3.[19]

Many epidemiological studies have been carried out in various countries, with differing estimates of the associated economic impact. For example, it is estimated that each year in New Zealand, air pollution leads to 1100 additional deaths, 700 additional hospital admissions for

Table 1 Average PM_{10} concentrations (particulate matter with mean diameter of 10 μm or less) in important cities worldwide (2004)

City	Urban PM_{10} (μg/m^3)
Los Angeles, USA	34
New York, USA	21
Toronto, Canada	22
Mexico City, Mexico	51
São Paulo, Brazil	40
Santiago, Chile	61
Delhi, India	150
Bangalore, India	45
Tehran, Iran	58
Moscow, Russia	21
Kiev, Ukraine	35
Nairobi, Kenya	43
Johannesburg, South Africa	32
Cairo, Egypt	169
Beijing, China	89
Bangkok, Thailand	79
Tokyo, Japan	40
Sydney, Australia	20
Auckland, New Zealand	14
London, UK	21
Barcelona, Spain	35
Rome, Italy	29
Stockholm, Sweden	11

Source: The World Bank, Development Economics Research Group Estimates (2004). (http://siteresources.worldbank.org/DATASTATISTICS/Resources/table3_13.pdf).

cardiorespiratory diseases and nearly 2 million 'restricted activity days' costing the economy around $1.1 billion each year.[20] In fact, the World Health Organization (WHO) estimates that approximately 3 million deaths worldwide (representing 5% of the total) can be attributed to air pollution exposure annually.[18]

Although average background pollution levels may not exceed the WHO target in many cities around the world, there is a large variation in the individuals' actual exposure to particulate matter. Traffic-derived air pollution is in the fine and ultrafine range and these particles remain airborne for a considerable length of time, given their small size and tiny mass. The particle concentration, when measured kerbside, can therefore actually be far higher than recorded at background monitoring stations, which tend to be located in quieter areas away from the important roads. This can be important, especially for those, such as traffic wardens, who spend a long time in kerbside environments. Their personal exposure can be as much as 5–10 times higher than that measured at the background sites.[21]

So how does the risk attributable to air pollution compare to traditionally quoted cardiovascular risk factors? The well-defined risk factors of hypertension, hypercholesterolaemia, diabetes mellitus, family history and smoking have been studied in depth in large epidemiological studies. In comparison to these variables, chronic exposure to fine particulate air pollution gives a dose-dependent and similar risk to hypertension or hypercholesterolaemia (Fig. 1). Modern medical practice is very focused on lowering patients' cardiovascular risk. We regularly monitor patients' blood pressure, cholesterol concentrations and blood sugar and often offer primary prevention medication. There are, in most places, smoking cessation services to help patients reduce tobacco consumption and many countries now have laws banning smoking in all public areas. These primary prevention strategies have a significant influence on the incidence of cardiovascular disease and the implementation of a smoking ban in Scotland recently led to a 17% reduction in the incidence of myocardial infarction.[24] What then can we do about air pollution exposure to reduce the potential health effects?

Public awareness of the problem of air pollution has led to significant pressures on countries, and local authorities, to try and reduce local emissions from both traffic and industry. Environmental policy interventions have shown that the reduction of air pollution can have substantial health

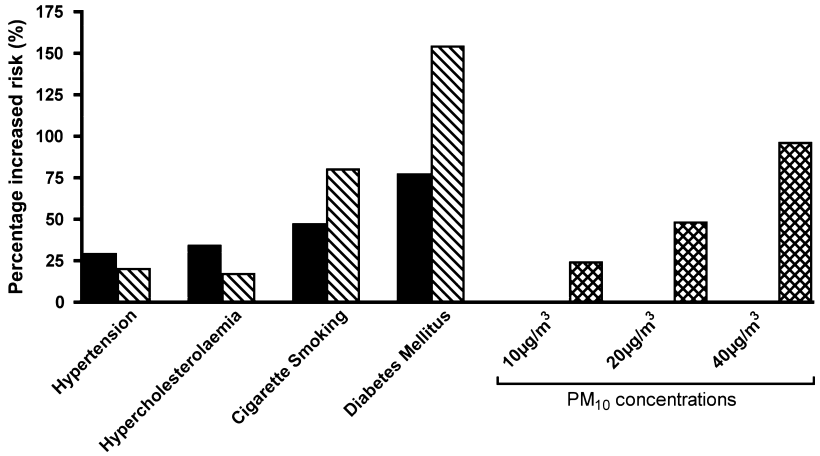

Figure 1 Percentage increase in population attributable risk with traditional cardiovascular risk factors from the Framingham[22] (solid bars) and QRESEARCH (striped bars) cohorts.[23] For comparison, risk attributed to each 10 μg/m^3 increase in $PM_{2.5}$ concentrations (hashed bars) is shown.[8] All values shown are for women only to allow comparison. Hypertension means incidence of hypertension in Framingham study and per 20 mmHg increase in systolic blood pressure in QRESEARCH. All other parameters refer only to their incidence.

benefits. For example, in Dublin, Ireland, banning the sale of bituminous coal led to a reduction in cardiovascular deaths.[25] Traffic emissions are also modifiable. Modern engines are more fuel efficient, meaning more complete combustion and fewer particle emissions. Automotive industries are now producing cars with exhaust particle traps to try and reduce the environmental impact of their products. More recently, during the Beijing Olympic Games, traffic restrictions were implemented to halve the number of vehicles on the roads based on an odd–even number plate control system. Given Beijing has more than 3.3 million registered vehicles, one would expect this to have a significant environmental and health impact, and, indeed when the Chinese government had a trial run, they reported a 40% reduction in airborne particulate matter. Personal protective equipment, such as the masks worn by the US athletes, also have a potential role in this, although their ability to negate the described health effects has yet to be shown.

Air pollution exposure, and particularly fine-particulate matter derived from combustion sources, has emerged as a new risk factor for cardiovascular morbidity and mortality and may actually lead to a similar degree of risk to the well-described risk factors, such as hypertension and hypercholesterolaemia. These effects may be mediated by changes in vascular endothelial function, leading both to the acceleration of the development of atherosclerosis and to the triggering of acute cardiovascular events. Public health measures are now needed to reduce these harmful emissions, such as the introduction of particle traps for diesel exhausts or the development of better fuels.

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Heat and haze: a forecast for myocardial infarction?

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Heat and haze: a forecast for myocardial infarction?

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Exposure to ambient air pollution is increasingly recognised as a risk factor for cardiovascular morbidity and mortality. Associations between exposure to combustion-derived fine particulate air pollution and cardiovascular mortality are consistently demonstrated in a number of large-scale epidemiological studies.[1] These reported associations have even withstood legal challenge in the USA, where industry representatives attempted to sue the US Environment Protection Agency after this evidence was used to impose strict air quality standards. An independent review of the evidence by the Health Effects Institute reaffirmed these associations and the case was lost.[2 3]

The World Health Organization estimates that indoor air pollution from the combustion of solid fuel may be responsible for up to 2.4 million deaths worldwide each year, representing the fourth leading cause of mortality in developing countries. In addition, urban air pollution may be responsible for a further 800 000 premature deaths each year.[4] Taken together, air pollution exposure is thought to be responsible for approximately 5% of deaths worldwide each year and therefore represents a significant public health concern.

Isolating the risk attributable to a single factor in epidemiological studies is challenging due to the myriad potential mediators and confounders. The effect of air pollution exposure can be modified by many factors, including temperature, geographical area and social deprivation. Similarly, investigations of the effects attributable to meteorological variables are also susceptible to confounding by exposure to ambient air pollutants, seasonal effects and infectious disease epidemics. Effects of temperature may be further modified in areas of extreme weather whereby inhabitants modify their behaviour by, for example, wearing warm clothing.

In order to tease out the effects attributable to these individual factors, a carefully performed and robust analysis of the available evidence is crucial.

AIR POLLUTION AND MYOCARDIAL INFARCTION?

In a pivotal study, Peters and colleagues[5] demonstrated in a careful case–control study employing 691 subjects with acute myocardial infarction that patients were three times more likely to have been in traffic in the hour before the onset of symptoms. The authors proposed that exposure to traffic-derived air pollution may trigger an acute myocardial infarction.

In this issue of *Heart*, Bhaskaran and colleagues[6] (***see page 1746***) present the first systematic analysis of the current evidence linking short-term exposure to air pollution and the incidence of acute myocardial infarction. The authors performed a systematic review of 26 studies that demonstrate an effect of exposure to particulate matter, ozone, carbon monoxide, nitrogen oxides or sulphur dioxide. The authors included only those studies that specifically addressed the incidence of myocardial infarction as an outcome and carefully controlled for potential confounders. They conclude that there is "fairly persuasive evidence of [a] short-term effect on myocardial infarction risk", with the risk of increasing by 5–17% for each 10 μg/m^3 increase in $PM_{2.5}$ (particulate matter with a mean diameter of $\leqslant$2.5 μm) exposure. This latter measure of exposure is most strongly associated with acute myocardial infarction and most closely representative of traffic-derived pollution. Unfortunately, $PM_{2.5}$ is not routinely measured in monitoring stations in many parts of the world including the UK.

WHAT ABOUT OTHER ENVIRONMENTAL VARIABLES?

Ambient air pollution exposure is highly variable, and depends on many different factors: the proximity of major highways, heavy industry, construction and not least local weather conditions. Meteorological conditions are often cited as a potential confounder for any studies looking at air pollution exposure. Weather is intimately linked with air pollution burden: airborne particulate is highest on cold crisp or hot humid days. In large smoggy cities, rainfall results in "clear air" as particulate matter is precipitated and washed away. This is most clearly demonstrated in the world's large polluted cities (fig 1).

Temperature itself is associated with overall (and cardiovascular) mortality. Room temperature appears to represent the "optimal" environment, with an increased incidence of events at both extremes of temperature in a "U-shaped" relationship.[7] Moreover, the effect of temperature modifies the statistical relationship between air pollution exposure and cardiovascular disease.[8]

In a separate article in this issue of *Heart*, Bhaskaran and colleagues[9] (***see page 1760***) present a second review employing the same robust and systematic approach to evaluate the evidence of an association between temperature changes and the incidence of acute myocardial infarction. The authors identify 19 studies that have looked at the incidence of myocardial infarction as a specific outcome, and controlled for potential confounders. They find that the body of evidence from these generally large and well-controlled studies suggests a similar effect on the incidence of myocardial infarction, with eight of 12 studies showing an increased short-term risk at low temperatures and seven of 13 showing increased risk at higher temperatures. While the reported effect size was highly variable, the risk of acute myocardial infarction was increased by 31–44% at the extremes of the temperature scale.

WHAT ARE THE LIKELY UNDERLYING MECHANISMS?

Epidemiological evidence requires a clear and plausible underlying mechanism. In both articles, the authors address this issue and acknowledge that these large-scale epidemiological studies can, by their very nature, demonstrate only an association and that careful controlled mechanistic studies are needed in order to tease out both the underlying mechanisms and, in the case of air pollution, the effect of

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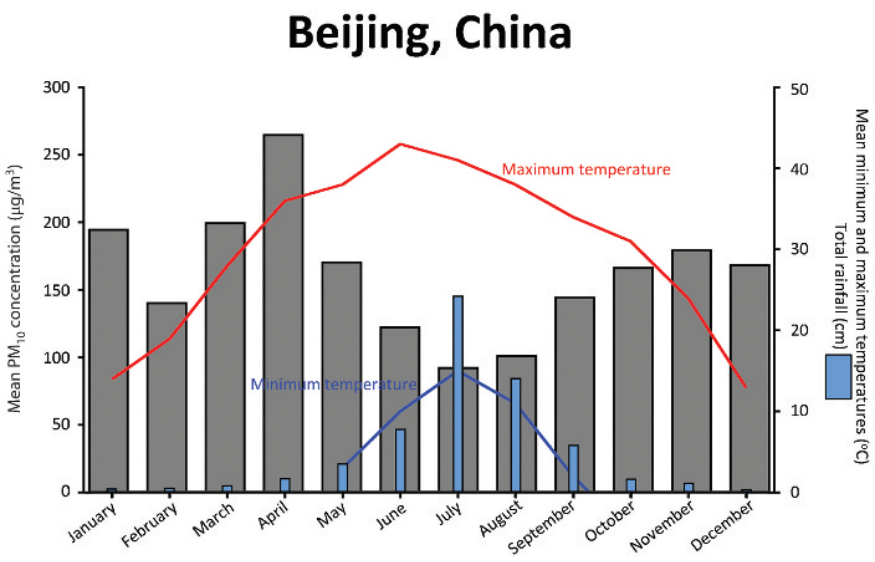

Figure 1 Recorded background urban particulate (PM_{10}; particulate matter with mean aerodynamic diameter 10 μm) air pollution from Beijing China in 2006 with average monthly minimum (blue line) and maximum (red line) temperatures. Narrow blue bars show average monthly rainfall. Data reproduced with permission from "Beijing 2008 Olympic Games: An Environmental Review" United Nations Environment Programme, 2008 (http://www.unep.org/publications/eBooks/Default.aspx) and BBC weather website (http://www.bbc.co.uk/weather/world/city_guides/results.shtml?tt=TT002100).

the different components of the air pollution mixture.

Chronic exposure to ambient air pollution increases the atherosclerotic burden in animal models and, in humans, is linked to increases in both coronary artery calcium scores and carotid intima-medial thickness. Inhaled particulate matter can also cause acute increases in blood pressure, arterial stiffness, arterial vasoconstriction and thrombogenicity.[10] Similarly, there is a consistent low-grade local inflammatory response in the lungs following exposure to fine particulate matter that appears to be driven by oxidative stress and leads to a systemic inflammatory response 6–24 h later.

The increased incidence of myocardial infarction at the extremes of temperature may share similar underlying triggers to those of air pollution. Transient and rapid increases in vasoconstriction, blood pressure and cardiac work may lead to increased arterial tissue stresses and plaque rupture. Along with increased plasma viscosity, this may also be associated with increased thrombogenicity[11] leading to acute coronary thrombosis. However, although similarities exist between the associations of acute myocardial infarction with both air pollution and temperature, there are important differences and many of the pathways will be distinct. For example, at extremes of temperature, there is a clear increase in events that are followed by a subsequent fall in the normal event rate; the so-called "harvesting effect", in which an acute trigger brings forward impending events.[12] This phenomenon is not seen in epidemiological studies of air pollution exposure, and indeed the opposite appears to occur, with the cumulative dose appearing most important.[13] Clearly, further work is needed to help identify the mechanisms and mediators of these associations if corrective or preventive strategies can be implemented.

Take home messages

Both exposure to ambient air pollution and extremes of temperature are consistently associated with an increase in cardiovascular morbidity and mortality. In two carefully performed systematic reviews published in this issue of *Heart*, Bhaskaran and colleagues[6 9] have robustly demonstrated an increased incidence of acute myocardial infarction following short-term exposure to these environmental factors. Whereas local weather conditions are outwith our control, strict attention to the reduction of ambient air pollution has the potential to reduce the burden of cardiovascular disease and result in significant public health benefits. Interestingly, environmental interventions to control ambient particulate air pollution are also likely to reduce carbon dioxide emissions that are widely accepted as being a major contributor to increasing global temperatures and may have further benefits into the future.

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Particle Traps Prevent Adverse Vascular and Prothrombotic Effects of Diesel Engine Exhaust Inhalation in Men

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Particle Traps Prevent Adverse Vascular and Prothrombotic Effects of Diesel Engine Exhaust Inhalation in Men

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Background—In controlled human exposure studies, diesel engine exhaust inhalation impairs vascular function and enhances thrombus formation. The aim of the present study was to establish whether an exhaust particle trap could prevent these adverse cardiovascular effects in men.

Methods and Results—Nineteen healthy volunteers (mean age, 25±3 years) were exposed to filtered air and diesel exhaust in the presence or absence of a particle trap for 1 hour in a randomized, double-blind, 3-way crossover trial. Bilateral forearm blood flow and plasma fibrinolytic factors were assessed with venous occlusion plethysmography and blood sampling during intra-arterial infusion of acetylcholine, bradykinin, sodium nitroprusside, and verapamil. Ex vivo thrombus formation was determined with the use of the Badimon chamber. Compared with filtered air, diesel exhaust inhalation was associated with reduced vasodilatation and increased ex vivo thrombus formation under both low- and high-shear conditions. The particle trap markedly reduced diesel exhaust particulate number (from 150 000 to 300 000/cm^3 to 30 to 300/cm^3; $P<0.001$) and mass (320±10 to 7.2±2.0 μg/m^3; $P<0.001$), and was associated with increased vasodilatation, reduced thrombus formation, and an increase in tissue-type plasminogen activator release.

Conclusions—Exhaust particle traps are a highly efficient method of reducing particle emissions from diesel engines. With a range of surrogate measures, the use of a particle trap prevents several adverse cardiovascular effects of exhaust inhalation in men. Given these beneficial effects on biomarkers of cardiovascular health, the widespread use of particle traps on diesel-powered vehicles may have substantial public health benefits and reduce the burden of cardiovascular disease.

Clinical Trial Registration—http://www.clinicaltrials.gov. Unique identifier: NCT00745446. **(*Circulation*. 2011;123:1721-1728.)**

Key Words: air pollution ■ endothelium ■ thrombosis

There is a robust and consistent association between air pollution and cardiorespiratory morbidity and mortality.[1–4] These harmful effects are most strongly associated with exposure to traffic-derived fine particles (particulate matter [PM] with a mean diameter <2.5 μm [$PM_{2.5}$]) that originate predominantly from diesel engine exhaust emissions.[5] Diesel engines are popular because of their reliability, efficiency, and relatively low running costs. However, they generate up to 100 times more fine particles than petroleum engines of a similar size and contribute substantially to the global burden of PM air pollution.

Editorial see p 1705
Clinical Perspective on p 1728

According to the World Health Organization, air pollution is responsible for at least 800 000 premature deaths worldwide each year, with an average loss of life of 1 year.[6] The long-term risk of cardiovascular death rises by 76% for each 10-μg/m^3 increase in $PM_{2.5}$.[7,8] Short-term exposure has been linked to the triggering of acute myocardial infarction,[9] with patients 3 times more likely to be exposed to traffic-derived air pollution in the hours before their acute myocardial

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infarction.[10] Despite the strength and consistency of observational studies, the underlying pathophysiological mechanisms remain unclear. Using well characterized and controlled diesel engine exhaust exposure studies in healthy volunteers, we have previously demonstrated impaired vascular vasomotor and fibrinolytic function,[11] increased arterial stiffness,[12] and enhanced ex vivo thrombus formation.[13] Furthermore, in patients with coronary heart disease, we have shown that diesel engine exhaust inhalation exacerbates exercise-induced ST-segment depression during light exercise.[14] Taken together, these adverse cardiovascular effects provide important mechanisms that help to explain the detrimental health effects of air pollution exposure.

One approach to limit traffic emissions has been the introduction of diesel engine exhaust particle traps or filters. Although the efficiency of particle traps to reduce particle emission is >90%, particles are not completely eliminated, and traps have the potential to create new and potentially more toxic particles that may outweigh the benefits of reducing the emitted particle mass.[15] We therefore sought to determine whether the introduction of a particle trap would attenuate or worsen the adverse cardiovascular effects of diesel engine exhaust inhalation.[11–14]

Methods

Twenty-one subjects were screened, and 1 subject was excluded at the initial screening. A further subject was excluded after randomization because of inability to complete all exposures, leaving 19 healthy nonsmoking men who completed the full study protocol (see Figure I in the online-only Data Supplement). The study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers.

All subjects had normal lung function and no symptoms of upper airway infection for the 4 weeks before or during the study. Exclusion criteria were regular medication, clinical evidence of atherosclerotic vascular disease, arrhythmias, diabetes mellitus, hypertension, renal or hepatic failure, asthma, significant occupational exposure to air pollution, or intercurrent illness. All subjects abstained from caffeine-containing drinks or food for at least 4 hours and from alcohol for 24 hours before each assessment.

Study Design

The primary end points were endothelial vasomotor and fibrinolytic function and ex vivo thrombus formation. Secondary end points were soluble markers of inflammation and platelet activation. Exploratory end points were markers of arterial stiffness and airway inflammation. Sample size was determined a priori and based on power calculations for the primary end points derived from our previous studies (see the online-only Data Supplement).[11,13]

In a randomized, double-blind, 3-way crossover design, subjects were exposed to filtered air, unfiltered dilute diesel engine exhaust, and dilute diesel engine exhaust that had passed through a particle trap. The order of the exposures was randomized, with an independent predetermined exposure sequence. Exposures were performed at a separate dedicated exposure facility by technical staff with no involvement in the clinical studies. Clinical studies were performed in a dedicated clinical research facility by clinical staff blinded to exposure allocation. Exposures were separated by at least 1 week and performed in a purpose-built exposure chamber, according to a previously described standard protocol.[11] During each 1-hour exposure, subjects performed moderate exercise (minute ventilation, 25 L/min per m^2 body) on a bicycle ergometer for 15 minutes alternated with 15 minutes of rest.

Diesel Exhaust

A Volvo diesel engine (Volvo TD40 GJE, 4.0 L, 4 cylinders) running on Volvo standard diesel fuel (SD-VSD-10) was used to generate the diesel exhaust. The specification of the Volvo diesel fuel is similar to the European automotive standard diesel (EN590), with a sulfur content of 5 to 7 mg/kg and polycyclic aromatic hydrocarbon content of 2% to 6% by mass. The engine worked under transient speed and load conditions in accordance with the standardized European transient cycle that mimics real-world urban driving conditions.[16] More than 90% of the exhaust was shunted away, and the residual exhaust was mixed with filtered air (Figure 1).

The concentrations of nitrogen oxides (NO, NO_2, and other nitrogen oxides) in the chamber were monitored continuously together with total gaseous hydrocarbons. During diesel exhaust exposures, we sought to generate a PM mass concentration of 300 $\mu g/m^3$. This PM mass concentration was maintained for the inlet conditions of the particle trap. Actual exposure was measured gravimetrically with standard glass fiber filter sampling together with the use of a tapered element oscillating microbalance online instrument and in accordance with a well-established protocol, as described previously.[17] In addition, a scanning mobility particle sizer system was used to determine fine (<1 μm) particle number concentration.

Particle Trap

The particle trap (diesel particulate filter–continuously regenerating trap [DPF-CRT], Johnson Matthey, Royston, UK) used is an unmodified, continuously regenerating trap filter, available commercially throughout the world as a factory-fit option or as a retrofit unit to buses and heavy goods vehicles. It is similar in design to filters produced by a number of manufacturers. It consists of a honeycomb-like complex of channels through which the exhaust is passed. A catalyst at the front of the filter oxidizes part of the NO gas in the exhaust into NO_2, which flows through the particle filter and subsequently reacts with trapped carbonaceous particles to generate CO_2 and N_2. This increases NO_2 levels in the exhaust after the particle trap, without causing significant changes in total nitrogen oxide concentrations, while achieving an efficient reduction in particle emissions.

Vascular Studies

On the basis of data from previous exposure studies,[13,14,16,18] vascular assessment was performed 6 to 8 hours after each exposure. Assessments were performed with subjects resting supine in a quiet temperature-controlled (22°C to 24°C) room. Venous cannulas (17 gauge) were inserted into large subcutaneous veins in the antecubital fossae of both arms. The brachial artery of the nondominant arm was cannulated with a 27-standard-wire-gauge steel needle. After a baseline 30-minute saline infusion, bradykinin at 100, 300, and 1000 pmol/min (endothelium-dependent vasodilator that releases tissue-type plasminogen activator [tPA]; Merck Biosciences, Nottingham, UK); acetylcholine at 5, 10, and 20 $\mu g/min$ (endothelium-dependent vasodilator that does not release tPA; Merck Biosciences); and sodium nitroprusside at 2, 4, and 8 $\mu g/min$ (endothelium-independent vasodilator that does not release tPA; David Bull Laboratories, Warwick, UK) were infused for 6 minutes at each dose. The 3 vasodilators were given in random order, separated by a 20-minute saline infusion. Verapamil was infused at 10, 30, and 100 $\mu g/min$ (endothelium-independent and NO-independent vasodilator that does not release tPA) at the end of the study protocol. The infusion of these vasodilators was undertaken to allow the assessment of distinct aspects of vascular function, including NO, endothelium, fibrinolysis, and vasomotion.

Forearm blood flow was measured in both infused and noninfused arms with venous occlusion plethysmography incorporating mercury-in-silicone elastomer strain gauges as described previously.[11] Heart rate and blood pressure were monitored in the noninfused arm throughout each study with a noninvasive, semiautomated oscillometric sphygmomanometer (Boso Medicus, Jungingen, Germany). Blood was drawn simultaneously from the venous cannulas in each arm at baseline and during infusion of each dose of bradykinin. Samples were collected into acidified buffered citrate

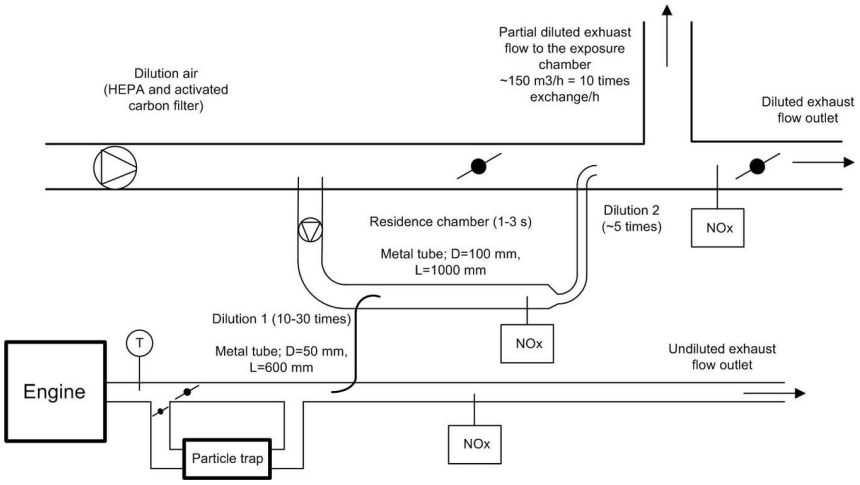

Figure 1. Schematic illustration of the diesel exhaust setup from the engine to the flow of diluted exhaust to the chamber. HEPA indicates high-efficiency particulate air; NO_x, nitrogen oxides.

(Stabilyte, Biopool International) for tPA assays and into citrate (BD Vacutainer) for plasminogen activator inhibitor type 1 assays. Samples were kept on ice before being centrifuged at 2000*g* for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at −80°C before assay. Plasma tPA and plasminogen activator inhibitor type 1 antigen concentrations were determined by enzyme-linked immunosorbent assay (TintElize tPA, Biopool EIA, Trinity Biotech, Ireland; Coaliza plasminogen activator inhibitor type 1, Chromogenix AB, Milan, Italy). Hematocrit was determined by capillary tube centrifugation of samples collected at baseline and during infusion of bradykinin at 1000 pmol/min.

Inflammatory Measures

Venous blood samples were obtained before and at 2, 6, and 8 hours after exposure. Samples were analyzed for total and differential cell count with an autoanalyzer. Plasma interleukin-6, tumor necrosis factor-α, soluble CD40 ligand, soluble P-selectin, intercellular adhesion molecule-1, and C-reactive protein were measured with commercially available enzyme-linked immunosorbent assays (R&D Systems, Abingdon, UK).

Ex Vivo Thrombosis Studies

On the basis of previous studies,[13] ex vivo thrombus formation was determined with the use of the Badimon chamber 2 hours after each exposure. In brief, a pump was used to draw blood from an antecubital vein through a series of 3 cylindrical perfusion chambers maintained at 37°C in a water bath. Carefully prepared strips of porcine aorta, from which the intima and a thin layer of media had been removed, acted as the thrombogenic substrate. The rheological conditions in the first chamber simulate those of patent coronary arteries (low-shear rate, $\approx 212\ s^{-1}$), and those in the second and third chambers simulate those of mildly stenosed coronary arteries (high-shear rate, $\approx 1690\ s^{-1}$). The model thus acts as one of deep coronary arterial injury. Each study lasted for 5 minutes, during which flow was maintained at a constant rate of 10 mL/min. All studies were performed with the same perfusion chamber, by the same operator, and according to a well-established protocol.[13]

Immediately after each study, porcine strips with thrombus attached were removed and fixed in 4% paraformaldehyde. Strips were paraffin-wax embedded, sectioned, and stained with Masson's trichrome. Images were acquired at ×20 magnification, and thrombus area was measured with a semiautomated image acquisition system (Ariol, Applied Imaging) by a blinded operator. Results from at least 6 sections were averaged to determine thrombus area for each chamber as described previously.[19,20]

Assessment of Arterial Stiffness

Please see the online-only Data Supplement.

Assessment of Airway Inflammation

Please see the online-only Data Supplement.

Data Analysis and Statistics

Plethysmographic data were analyzed as described previously.[11] Estimated net release of tPA antigen was defined as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms, as described previously.[11,14] Continuous variables are reported as mean±SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, CA). All studies, data analysis, and data exclusion were performed before the data were unblinded.

To address our primary hypothesis, the analysis plan required 2 independent assessments of the responses in the 3 randomized arms of the study. First, to confirm our previous findings, we assessed whether the inhalation of diesel engine exhaust impaired vascular function and promoted thrombogenesis. Second, we assessed whether the particle trap improved these surrogate measures of cardiovascular health. Comparisons between exposures were undertaken with a 2-sided paired *t* test and 2-way ANOVA with repeated measures, as appropriate. Factors assessed in the 2-way ANOVA were exposure and vasodilator dose. Exposure data were analyzed with a 2-sided unpaired *t* test. Statistical significance was taken at $P<0.05$.

Results

The 19 healthy male volunteers were young and normotensive, and had normal lung function (Table 1). All volunteers

Table 1. Baseline Subject Characteristics (n=19)

Age, y	25±3
Height, cm	181±5
Weight, kg	75±8
Body mass index, kg/m^2	23.4±2
Pulse, bpm	54±8
Systolic blood pressure, mm Hg	113±6
Diastolic blood pressure, mm Hg	73±8
FEV_1, L	4.6±0.6
% Predicted FEV_1	100±12
FVC, L	5.6±0.8
% Predicted FVC	103±14

Data shown are mean±SEM (n=19). FEV_1 indicates forced expiratory volume in 1 second; FVC, forced vital capacity.

Table 2. Exposure Conditions in the Chamber

	Filtered Diesel Exhaust	Diesel Exhaust	*P*
NO, ppm	2.09±0.15	5.72±0.33	<0.001
NO_2, ppm	3.44±0.33	0.69±0.02	<0.001
NO_x, ppm	5.53±0.44	6.40±0.34	0.049
Total gaseous hydrocarbons, ppm	0.84±0.06	0.91±0.05	0.387
Total PM mass concentration, $\mu g/m^3$	7.2±2.0	320±10	<0.001
Fine particle number concentration, n/cm^3	30–300	150 000–200 000	<0.001

Data shown are mean±SEM (n=19) unless indicated otherwise. Data were analyzed by 2-tailed unpaired *t* test. NO indicates nitric oxide; NO_2, nitrogen dioxide; NO_x, nitrogen oxides; and PM, particulate matter.

completed the 3 study visits. Exposures were well tolerated, with no adverse symptoms reported.

The particle trap reduced the total particle mass concentration in the chamber by ≈98% and the fine (<1 μm) particle number concentration by >99.8%. As anticipated, the particle trap, with an integrated oxidation catalyst, altered the composition of nitrogen oxides (ie, increased NO_2 and decreased NO) (Table 2). However, it had no significant effect on the concentrations of gaseous hydrocarbons.

There were no changes in blood pressure, resting heart rate, baseline forearm blood flow, or markers of arterial stiffness in between the 3 study visits (Tables II and III in the online-only Data Supplement). Hematologic variables, markers of inflammation, and soluble markers of platelet activation did not differ between exposures (Tables IV and V in the online-only Data Supplement). Markers of airway inflammation did not differ between exposures (Table VI in the online-only Data Supplement).

Vascular Studies

Vasomotor Function

There was a dose-dependent increase in forearm blood flow with both endothelium-dependent (bradykinin and acetylcholine) and endothelium-independent (sodium nitroprusside and verapamil) vasodilators after each exposure ($P<0.0001$ for all; Figure 2).

Compared with filtered air, vasodilatation was impaired after diesel engine exhaust exposure in response to bradykinin ($P=0.009$), acetylcholine ($P=0.01$), and verapamil ($P=0.03$; Figure 2). There was no significant difference in response to sodium nitroprusside ($P=0.15$; Figure 2). However, with the introduction of the particle trap, vasodilatation increased in response to all vasodilators: bradykinin ($P<0.0001$), acetylcholine ($P<0.0001$), verapamil ($P=0.001$), and sodium nitroprusside ($P=0.04$; Figure 2). Indeed, there were no differences in vasomotor responses between filtered air and filtered diesel engine exhaust except for acetylcholine, with which vasodilatation was lower with filtered air ($P=0.02$).

Fibrinolytic Function

There were no differences in baseline plasma tPA and plasminogen activator inhibitor type 1 concentrations between exposures (Table VI in the online-only Data Supplement). There was a dose-dependent increase in tPA release in response to bradykinin infusion after each exposure ($P<0.0001$ for all; Figure 3). Although numerically lower, there was no statistical difference in tPA release after exposure to diesel engine exhaust inhalation compared with filtered air ($P=0.30$; Figure 3). However, application of the particle trap was associated with an improvement in the net release of tPA compared with unfiltered diesel engine exhaust ($P=0.03$; Figure 3). There was no difference in tPA release between filtered air and filtered diesel engine exhaust ($P=0.22$).

Ex Vivo Thrombosis

Compared with filtered air, inhalation of diesel exhaust was associated with an increase of thrombus formation in the low-shear (21.8%; $P<0.001$; Figure 4) and high-shear (14.8%; $P=0.02$; Figure 4) chambers. Compared with unfiltered exhaust, the introduction of the particle trap was associated with a reduction in thrombus formation in the low-shear chamber (−15.7%; $P=0.02$; Figure 4), whereas the apparent reduction in the high-shear chamber did not reach statistical significance ($P=0.11$; Figure 4). There were no differences in thrombus formation between filtered air and filtered diesel exhaust ($P=0.78$ and $P=0.76$ for the low- and high-shear chambers, respectively).

Discussion

Short-term exposure to traffic-derived air pollution is associated with acute cardiovascular events.[9,10,21] In the present study, using complementary and relevant measures of cardiovascular health, we have reconfirmed the adverse effects of exposure to diesel engine exhaust on endothelial function and ex vivo thrombosis. In addition, for the first time, we demonstrate that reducing the particulate component of diesel exhaust with the use of a commercially available particle trap can prevent these detrimental cardiovascular effects. Our study provides support for the application of particle traps to diesel-powered vehicles to reduce urban particulate concentrations and limit a range of adverse cardiovascular effects of exposure to traffic-derived air pollution.

In a series of controlled exposure studies in human subjects, we have previously shown an impairment of vasomotor responses to endothelium-dependent and endothelium-independent vasodilators after diesel exhaust exposure.[18] These observations are consistent with other reports of brachial artery vasoconstriction shortly after exposure to dilute diesel exhaust[22] and concentrated ambient particles.[23] Such vascular impairment is not restricted to vasomotor function. We have also demonstrated increased thrombogenicity with reduced tPA release from the endothelium,[11] enhanced platelet activation,[13] and increased ex vivo thrombus formation.[13]

In the present study, we used a range of these complementary measures of cardiovascular function in a comprehensive assessment of the potential for particle traps to improve human health. We were able to confirm our earlier findings that diesel exhaust inhalation causes detrimental vascular and prothrombotic effects. On this occasion, we did not observe a statistically significant reduction in tPA release from the

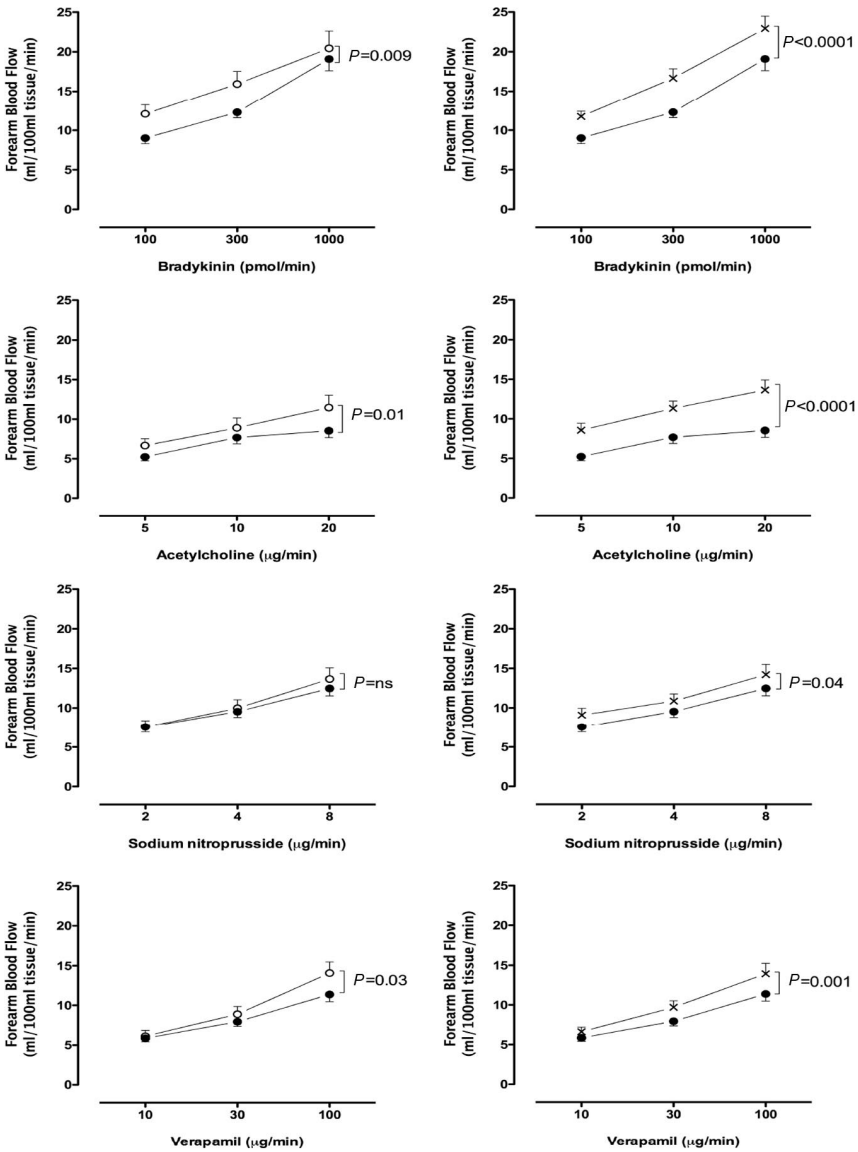

Figure 2. Infused forearm blood flow 4 to 6 hours after exposure, during intrabrachial infusion of bradykinin, acetylcholine, sodium nitroprusside, and verapamil. The left panel displays vasomotor response after exposure to air and diesel exhaust, confirming the vascular effects from previous investigations. Filtered air exposure is shown by open circles, and diesel engine exhaust exposure by filled circles. The right panel displays the main comparison of vasomotor function after exposure to unfiltered diesel exhaust (filled circles) and filtered diesel exhaust (crosses).

endothelium after diesel exhaust exposure, and this may represent a type II error or reflect the subtle differences between study protocols. However, more importantly, we were able to demonstrate that the introduction of a particle trap not only improved vasomotion, endogenous fibrinolysis, and ex vivo thrombosis but appeared to normalize them.

Although there are many potentially harmful components in ambient air pollution, traffic-derived fine and ultrafine particles are most closely and consistently linked to acute cardiovascular events. This has been the rationale for the development of, and legislation for, targeted interventions to reduce the particulate matter content of vehicle emissions.[24]

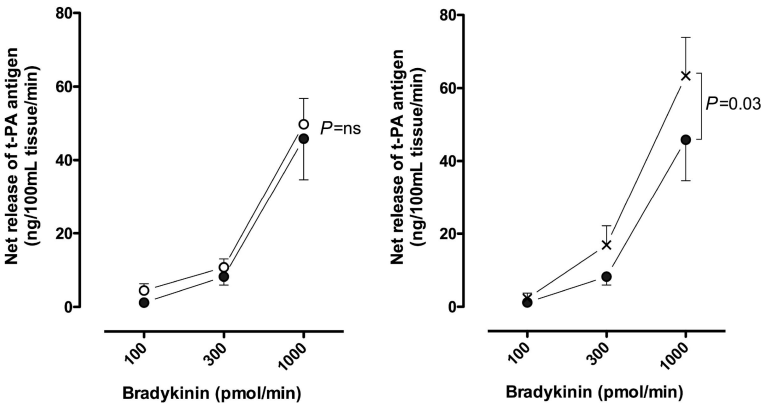

Figure 3. Tissue-type plasminogen activator (tPA) release from the forearm endothelium 4 to 6 hours after exposure, during intrabrachial infusion of bradykinin. The left panel displays fibrinolytic response after exposure to air and diesel exhaust. Filtered air exposure is shown by open circles and diesel engine exhaust exposure by filled circles. The right panel displays the main comparison of fibrinolytic function after exposure to unfiltered diesel exhaust (filled circles) and filtered diesel exhaust (crosses).

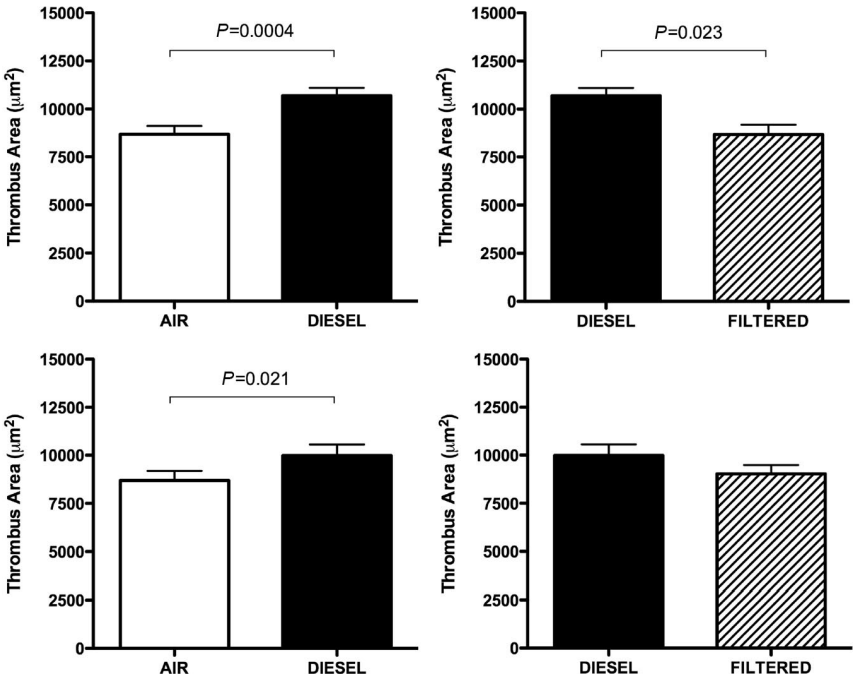

Figure 4. Ex vivo thrombus formation assessed with the Badimon chamber at 2 hours after exposure. Data from the low-shear chamber are shown in the top panels. Data from the high-shear chamber are shown in the bottom panels. Filtered air exposure is shown in white, diesel engine exhaust exposure in black, and filtered diesel exposure in hatched bars.

Although there is little doubt that particle traps are effective in reducing PM mass and number, concerns have been raised regarding the oxidation catalysts required to regenerate and maintain filter efficiency, because they may alter the toxicity of particulate and gaseous emissions. For example, soot particles generated from a low-emission diesel engine appear to have greater cytotoxic and proinflammatory effects.[15] The potential for particle traps to reduce the adverse cardiovascular effects of diesel exhaust emissions therefore needs to be assessed in humans. In the present study, we observed no adverse cardiovascular effects arising from the use of a particle trap. In fact, the only difference between filtered air and filtered diesel exhaust observed for any variable we assessed was an enhancement of vasodilatation in response to acetylcholine after exposure to filtered diesel exhaust. Although this difference was statistically significant, it was numerically small, and unlikely to be of major physiological significance. In light of these factors, we suspect that this may be a result of a type I error, although we cannot rule out an effect related to alterations in 1 or more unmeasured gaseous components.

We used a commercially available particle trap to reduce particle emissions from a heavy-duty diesel engine operating under the transient cycling conditions used as the standard for engine testing across the European Union. Particle filtration markedly reduced the mass and number of particle emissions. Taken together with our previous findings and data from observational studies, we believe that this reduction is responsible for rectifying the adverse cardiovascular effects of diesel engine exhaust inhalation. As expected, oxidation catalysts within the particle trap altered the composition of nitrogen oxides, with an increase in NO_2 and decrease in NO concentrations. However, we have recently assessed the effects of NO_2 on healthy volunteers, and did not identify any adverse effects on vascular or fibrinolytic function.[25] Although we did not observe an effect on the other gaseous components of the exposure, we acknowledge that the particle trap may have altered the composition of the exposures beyond those variables assessed during this study.

Consistent with previous studies,[11,16,18] vasodilatation was impaired after exposure to diesel engine exhaust in response to bradykinin and acetylcholine. However, there was also a reduction in vasodilatation to verapamil, implying an additional calcium flux–dependent impairment of vascular smooth muscle function. A reduction in vasodilatation in response to verapamil has been demonstrated previously after exposure to diesel exhaust generated under transient engine speed and load,[16] but not when generated under idling conditions.[11] We have speculated previously that, in exhaust generated under transient running conditions, the higher diesel-related soot content and its associated (adsorbed) organic material may cause these additional vascular smooth muscle effects, and we believe that this observation warrants further investigation.[16] Interestingly, impaired vasodilatation in response to verapamil was also normalized by the introduction of a particle trap. Although variations in responses between studies with different exposure protocols might provide insights into the pathophysiological mechanisms responsible for the adverse vasomotor effects of diesel exhaust exposure, these studies were designed and powered to detect differences within rather than between studies. Thus, although tempting, we believe that we should be cautious and circumspect in drawing conclusions from comparisons made between studies and that any such differences should be regarded as hypothesis generating and the subject of future investigations.

The exact mechanisms underlying the vascular and prothrombotic effects we observed in the current and previous studies remain only partially understood. Regarding the increase in ex vivo thrombosis seen after diesel exhaust

exposure, data from previous in vitro[26] and animal studies,[27] as well as our own previous controlled exposure studies in humans,[13] suggest that platelet activation plays a central role. Platelets are key components of arterial thrombosis, a process that underpins acute coronary syndromes, including myocardial infarction. Platelet-leukocyte aggregates, increasingly recognized as the gold standard measure of in vivo platelet activation,[28] were increased after tracheal instillation of carbon nanotubes in a murine model of vascular injury[29] and after diesel exhaust exposure in humans.[13] Debate remains regarding whether inhaled components of diesel exhaust can translocate into the systemic circulation to mediate direct effects on blood and vascular components,[30,31] or whether the induction of pulmonary inflammation and the subsequent generation of free radicals may activate platelets by reducing endothelium- and platelet-derived nitric oxide and antioxidants. Although a single observational study reported a small reduction in prothrombin time associated with ambient exposure to PM_{10},[32] the authors are not aware of any controlled exposure study demonstrating an effect of pollution exposure on plasma concentrations of coagulation factors.

The potential mechanisms underlying the adverse vasomotor effects observed in response to diesel exhaust exposure remain only partly understood,[33] and a full discussion is beyond the scope of this article. However, on the basis of data from our earlier studies in which the exposure was generated by an idling diesel engine,[11,18] we have speculated previously that oxidative stress and impaired NO-dependent signaling play a central role in the adverse vasomotor effects. Given the broader impairment of vasomotor function we observed here and in a previous study in which a transient cycling diesel engine was used,[16] we acknowledge that we cannot discount upregulation of other circulating or cellular vasoconstrictor mediators (such as Rho kinase[34]) or activation of the sympathetic nervous system as an alternative explanation for the general blunting of vasodilator responses observed.

Limitations

Our principal objective was to assess the impact of a commercially available particle trap on markers of cardiovascular health by comparing the effects of unfiltered and filtered diesel exhaust. In this regard, we clearly demonstrate that vasodilatation, endothelial tPA release, and thrombus formation are improved by particle filtration. However, we acknowledge that our approach has limitations. The use of multiple and complementary surrogates of cardiovascular health is both a strength and a weakness of this study. Replication of previous observations suggests that the findings are real and provides a clear and consistent message: Diesel exhaust impairs vascular function[11,16,18] and increases ex vivo thrombus formation.[13] The use of multiple end points with 3 exposure conditions requires multiple comparisons and increases the possibility of type I and II errors. We have not adjusted for multiple comparisons because the initial comparison between diesel exhaust and filtered air was made simply to confirm our previous findings. Both the direction and magnitude of the changes seen here are consistent with our previous findings, although the differences in net tPA release failed to achieve statistical significance. Although the study was powered prospectively on the basis of measurements of the primary end points made during previous diesel exposure studies, we acknowledge that the sample size is modest. Although we are confident that we have not missed effects on endothelial function or ex vivo thrombosis, we acknowledge that we may have insufficient power to detect changes in some of the secondary end points (Table I in the online-only Data Supplement), and thus cannot exclude the possibility of false-negative findings confounding their assessment. In addition, the study cohort consisted exclusively of young, healthy men. Although one might postulate that the benefit of particle traps may actually be greater in those with preexisting cardiovascular disease, we concede that further studies are required to assess the role of particle traps in mitigating cardiovascular effects in women and the broader population.

Conclusion

With the use of several surrogate measures, a range of adverse cardiovascular effects of diesel exhaust inhalation in men appears to be prevented by the introduction of a particle trap. Given these beneficial effects on biomarkers of cardiovascular health, the widespread use of particle traps on diesel-powered vehicles may have substantial public health benefit and reduce the burden of cardiovascular disease.

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Disclosures

None.

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CLINICAL PERSPECTIVE

There is a robust and consistent association between air pollution and cardiovascular morbidity and mortality. These harmful effects are most strongly associated with exposure to traffic-derived fine particles that predominantly originate from diesel engine exhaust emissions. Using a purpose-built exposure chamber, we have demonstrated previously the adverse vascular and prothrombotic effects of exposure to diesel exhaust in healthy men. In the present study, using complementary and relevant measures of cardiovascular health, we have reconfirmed the adverse effects of exposure to diesel engine exhaust on endothelial function and ex vivo thrombosis. In addition, for the first time, we demonstrate that reducing the particulate component of diesel exhaust with a commercially available particle trap can prevent these detrimental cardiovascular effects. Our study provides support for the application of particle traps to diesel-powered vehicles to reduce urban particulate concentrations and limit a range of adverse cardiovascular effects related to exposure to traffic-derived air pollution.

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Diesel exhaust inhalation does not affect heart rhythm or heart rate variability

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ABSTRACT

Objective Exposure to air pollution is associated with increases in cardiovascular morbidity and mortality. This study was undertaken to determine the effect of diesel exhaust inhalation on heart rhythm and heart rate variability in healthy volunteers and patients with coronary heart disease.

Design and setting Double-blind randomised crossover studies in a university teaching hospital.

Patients 32 healthy non-smoking volunteers and 20 patients with prior myocardial infarction.

Interventions All 52 subjects were exposed for 1 h to dilute diesel exhaust (particle concentration 300 $\mu g/m^3$) or filtered air.

Main outcome measures Heart rhythm and heart rate variability were monitored during and for 24 h after the exposure using continuous ambulatory electrocardiography and assessed using standard time and frequency domain analysis.

Results No significant arrhythmias occurred during or following exposures. Patients with coronary heart disease had reduced autonomic function in comparison to healthy volunteers, with reduced standard deviations of the NN interval (SDNN, $p<0.001$) and triangular index ($p<0.001$). Diesel exhaust did not affect heart rate variability compared with filtered air ($p>0.05$ for all) in healthy volunteers (SDNN 101 ± 6 vs 91 ± 6, triangular index 20 ± 1 vs 21 ± 1) or patients with coronary heart disease (SDNN 47 ± 5 vs 38 ± 4, triangular index 8 ± 1 vs 7 ± 1).

Conclusions Brief exposure to dilute diesel exhaust does not alter heart rhythm or heart rate variability in healthy volunteers or well-treated patients with stable coronary heart disease. Autonomic dysfunction does not appear to be a dominant mechanism that can explain the observed excess in cardiovascular events following exposure to combustion-derived air pollution.

INTRODUCTION

Observational and epidemiological studies consistently report that exposure to air pollutants is associated with excess cardiovascular morbidity and mortality,[1] and may be an important modifiable risk factor for cardiovascular disease.[2] If public health measures are to be implemented to reduce this risk, a better understanding of the mechanisms and components of urban air pollution responsible for these observations is urgently required.

Short-term increases in air pollution exacerbate cardiorespiratory disease leading to hospitalisation for conditions including acute myocardial infarction[3] and deaths from coronary heart disease, heart failure and arrhythmia.[2] Furthermore, in patients with implanted cardiac defibrillators there appears to be a relationship between ambient particulate matter (PM) and the incidence of ventricular tachycardia and fibrillation.[4–6] These associations are strongest for fine particulate air pollutants, with emissions from road traffic implicated both directly and indirectly in these reports.

There is an important relationship between autonomic regulation of the cardiac cycle and cardiovascular mortality.[7] Variation in the interval between consecutive heart beats, or heart rate variability, is controlled by the contrasting effects of the sympathetic and parasympathetic nervous systems. Reduction in heart rate variability reflects either an increase in sympathetic drive or a decrease in vagal parasympathetic tone. Reduced heart rate variability increases the risk of cardiovascular morbidity and mortality in both healthy individuals[8] and patients following myocardial infarction.[9]

Several panel studies have reported associations between measures of heart rate variability and high ambient PM.[10–13] There is marked heterogeneity in the nature, magnitude, direction and duration of these associations between studies with no clear and consistent effect apparent. Differences may in part be due to imprecision in the measurement of pollution exposure and the effect of potential confounding environmental and social factors. Controlled exposures of air pollutants can help to address these shortcomings by providing a precisely defined exposure in a regulated environment. Using a carefully characterised exposure system, we have previously shown in healthy volunteers that exposure to dilute diesel exhaust causes lung inflammation,[14] depletion of airway antioxidant defences[15] and impairment of vascular and fibrinolytic function.[16] Furthermore, we have described ischaemic and prothrombotic effects in patients with coronary heart disease.[17] To date, there have been no controlled exposure studies to assess the direct effects of diesel exhaust on heart rate variability in patients with coronary heart disease.

We aimed to assess the effect of dilute diesel exhaust inhalation on heart rhythm and heart rate variability in healthy volunteers and an 'at-risk' population of patients with stable coronary heart disease.

METHODS

Subjects

Fifty-two men participated in this study, which was performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Thirty-two healthy non-smoking volunteers and 20 patients with stable coronary artery disease were recruited.

Healthy non-smoking volunteers (age 20–38 years) on no regular medications and patients with coronary heart disease were recruited from the University Hospital, Umeå, Sweden. All patients (age 51–67 years) had proven coronary heart disease with a previous myocardial infarction (>6 months previously) treated by primary angioplasty and stenting, and were receiving standard secondary preventive therapy. Patients with angina pectoris (Canadian Cardiovascular Society grade ≥2), diabetes mellitus, uncontrolled hypertension, renal or hepatic failure, or those with unstable coronary disease (acute coronary syndrome or unstable symptoms within 3 months) were excluded. All volunteers were invited to a prestudy screening visit for exercise stress testing and patients unable to achieve stage 2 of the Bruce protocol were excluded. Current smokers and those with asthma, significant occupational exposure to air pollution or an intercurrent illness were excluded from the study. In patients, regular medications were continued throughout the study, with the exception of ACE inhibitor therapy that was withdrawn 7 days before each exposure.

Study design

All subjects underwent an identical randomised double-blind crossover study design, with healthy volunteers and patients attending at 08.00 h on two occasions at least 2 weeks apart for controlled exposure to dilute diesel exhaust or filtered air. Each subject was exposed for 1 h in a specially built diesel exposure chamber according to a previously described standard protocol.[14] During each exposure, subjects performed two 15 min periods of exercise on a bicycle ergometer separated by two 15 min periods of rest. The ergometer workload required to achieve a minute ventilation of 25 l/min/m^2 for each healthy volunteer and 15 l/min/m^2 for each patient with coronary heart disease was determined to ensure that subjects within each group received a similar exposure.

All subjects were fitted with Holter electrocardiographic monitors (Reynolds Medical Lifecard, Delmar Reynolds, Hertford, UK) prior to each exposure. ECG monitoring was continued for 24 h following the start of the exposure. All subjects remained indoors for the 24 h period following exposure to minimise additional exposure to ambient air pollution. Subjects were asked to abstain from alcohol for 24 h and from food, tobacco and caffeine-containing drinks for at least 4 h before each exposure.

To assess the acute effects of exposure on heart rate variability, 15 healthy volunteers and all patients were asked to rest supine in a quiet temperature-controlled room maintained at 22–24°C for 20 min immediately before and 2 and 6 h after the start of each exposure. All volunteers also underwent blood pressure measurements and a vascular assessment after the final rest period as detailed in our previous publications.[16 17]

Diesel exhaust exposure

The diesel exhaust was generated from an idling Volvo diesel engine (Volvo TD45, 4.5L, 4 cylinders, 680 rpm) from Swedish Low Sulphur Gasoil E10 (Preem, Gothenburg, Sweden) as described previously.[14 16] Over 90% of the exhaust was shunted away and the remaining part was diluted with ambient filtered air heated to 20°C (humidity ~50%) before being fed into a whole body exposure chamber at a steady state concentration. Air in the exposure chamber was continuously monitored with exposures standardised using online measurements of nitrogen oxide concentrations (NO_x) to deliver approximately 1.2×10^6 suspended particles/cm^3 at a particulate concentration of 300 μg/m^3. There was little variation in NO_x (4.45±0.02 parts per million, ppm), NO_2 (0.92±0.02 ppm), NO (3.35±0.02 ppm), CO (2.97±0.08 ppm) and total hydrocarbon (2.50±0.09 ppm) concentrations between exposures.

Data analysis

ECG recordings were analysed using the Reynolds Medical Pathfinder Digital 700 Series Analysis System (Delmar Reynolds). 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. Where less than 95% of the RR data was valid, the subject was excluded and the recording was not analysed further. RR data were analysed using the HRV Tools software package (Delmar Reynolds) to determine time and frequency components of heart rate modulation over the entire 24 h period and during the final 5 min of each rest period prior to exposure and 2 h and 6 h following the start of each exposure.

Standard time domain measures were calculated including the mean NN interval (time interval between consecutive sinus beats), SD of NN interval values (SDNN, an index that expresses overall variability), percentage 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 normalised units (LFn and HFn) to account for variation in the total power and very low frequency components, as well as the HF/LF ratio.

Statistical analysis

Continuous variables are reported as mean±SEM. Statistical analyses were performed with GraphPad Prism (Graph Pad Software, USA) using analysis of variance (ANOVA) with repeated measures and the two-tailed Student t test where appropriate. Statistical significance was taken at $p<0.05$.

RESULTS

Although one healthy volunteer and one patient were excluded from the analysis due to interference on the ECG recording, 98% and 99% of the data were valid in the remaining healthy volunteers (n=31) and patients (n=19) respectively. Patients were middle aged (60±1 years) men who were on typical cardiac medication (table 1). Patients and healthy controls did not experience any symptoms or serious arrhythmias during either exposure or during the 24 h study period.

Heart rate and heart rate variability over the 24 h study period were reduced in patients with coronary heart disease compared with young healthy controls (table 2). Inhalation of dilute diesel exhaust for 1 h did not affect time or frequency domain measures of heart rate variability over the 24 h period in either healthy volunteers or patients with coronary heart disease (table 2).

Baseline measures of heart rate and heart rate variability averaged over a 5 min interval immediately before both exposures

Table 1 Baseline characteristics of patients with coronary heart disease (n=20) and healthy non-smoking volunteers (n=32)

	Patients	Heathy volunteers
Age (years)	60±1	26±1
Cigarette smokers (non-/ex-/current)	12/8/0	32/0/0
Height (cm)	173±6	182±2
Weight (kg)	79±3	84±3
Body mass index (kg/m^2)	27±1	26±1
Hypertension	8	0
Lipid profile		
Total cholesterol (mmol/l)	4.6±0.2	–
LDL-cholesterol (mmol/l)	2.6±0.2	–
HDL-cholesterol (mmol/l)	1.3±0.1	–
Triglycerides (mmol/l)	1.5±0.3	–
Fasting glucose (mmol/l)	5.7±0.3	–
Drugs		
Aspirin	20	–
Statin	18	–
β-blocker	15	–
ACE inhibitor*/ARB	4	–

Values are presented as number or mean±SEM.
*ACE inhibitor therapy was withdrawn 7 days prior to each study. All other regular medications were continued throughout the study.
ARB, angiotensin receptor blocker; HDL, high density lipoprotein; LDL, low density lipoprotein.

were similar in each group (tables 3 and 4). Blood pressure was not affected by exposure (data not shown). There were no differences in either time or frequency domain measures of heart rate variability at 2 or 6 h in healthy volunteers. In patients with coronary artery disease there was an increase in SDNN at 2 and 6 h compared with baseline (p=0.029), but this was not affected by exposure to diesel exhaust (p=0.483).

DISCUSSION

Exposure to dilute diesel exhaust for 1 h did not affect heart rhythm or heart rate variability in healthy volunteers or patients with coronary heart disease. We suggest that the induction of autonomic dysfunction and arrhythmia is unlikely to explain the association between combustion-derived air pollution and adverse cardiovascular events. Our findings contrast with several observational studies that report associations between ambient air pollution and heart rate variability, and this apparent discrepancy requires further discussion.

Table 2 Measures of heart rate variability averaged over 24 h in patients with coronary heart disease (n=19) and healthy volunteers (n=31)

	Patients		Healthy volunteers	
	Filtered air	Diesel exhaust	Filtered air	Diesel exhaust
Heart rate (bpm)	62±2	60±2	71±2	71±2
NN interval (ms)	977±31	1008±28	848±22	843±20
SDNN (ms)	38±4	47±5	91±6	101±6
RMSSD (ms)	28±4	29±3	43±3	40±3
PNN50 (%)	10±4	9±3	20±2	18±3
Triangular index	7±1	8±1	21±1	20±1
HF power (ms^2)	269±70	314±94	607±79	540±71
LF power (ms^2)	622±182	693±245	1685±159	1533±144
HFn (ms)	33±5	33±5	23±2	23±1
LFn (ms)	65±5	65±6	75±2	75±1
HF/LF ratio	0.9±0.3	1.1±0.4	0.4±0	0.3±0

All variables are presented as mean±SEM.
Filtered air vs diesel exhaust, p>0.05 for all (paired t test).
HF, high frequency; HFn, normalised high frequency; ; LF, low frequency; LFn, normalised low frequency; PNN50, percentage of successive NN interval differences >50 ms; RMSSD, root mean square of successive NN interval differences; SDNN, SD of NN interval values.

Controlled exposure studies

Controlled exposures of air pollutants provide a precisely defined exposure in a regulated environment and can overcome many of the potential biases and confounders inherent to observational studies. Our findings are consistent with the only previous study of diesel exhaust exposure on autonomic function by Peretz *et al* who also did not identify any reproducible effects on heart rate variability in 16 young healthy volunteers.[18] Taken together with our findings, we believe this clearly demonstrates that diesel exhaust inhalation does not impact on heart rate variability or induce autonomic dysfunction.

We believe that our model of exposure is relevant both in composition and magnitude of exposure for the assessment of the acute effects of combustion-derived air pollution on autonomic function and heart rhythm in man. This is the largest controlled exposure study to date, and we have studied the effects of exposure in a cohort of patients who may be particularly susceptible to the effects of air pollution. The crossover study design employed minimises the potential for confounding by other environmental factors and within-subject comparisons increase the power to detect small changes in autonomic function. Despite this, we were unable to demonstrate an acute or persistent effect of exposure to dilute diesel exhaust on heart rhythm or heart rate variability, whether this was assessed using time or frequency domain analysis in either healthy subjects or patients with coronary heart disease.

Observational studies

While more than 30 epidemiological studies report associations between air pollution and changes in heart rate variability, there is considerable heterogeneity between these studies in the nature, magnitude, direction and duration of the associations between PM and heart rate variability (table 5). Liao *et al* were the first to report an association between fine particulate air pollution ($PM_{2.5}$) and heart rate variability in a panel of elderly subjects.[11] The authors considered their finding somewhat exploratory, but the analysis revealed an inverse correlation between same day $PM_{2.5}$ concentrations and both the HF and LF power components of heart rate variability. Reduced heart rate variability is associated with an increase in sudden cardiac death and all-cause mortality in survivors of acute myocardial infarction.[9] Liao *et al* hypothesised that an effect of PM exposure on the autonomic control of heart rate and rhythm may explain the association between PM and adverse cardiovascular outcomes. Subsequently, numerous panel studies have explored this mechanistic hypothesis by examining the associations between levels of different air pollutants and changes in heart rate variability or incidence of cardiac arrhythmia.

Most observational studies report negative associations between fine particulate air pollution and either time or frequency domain parameters of heart rate variability, but there is considerable disagreement between these studies. Differences may be due to variation in the composition of PM or the effect of confounding environmental and social factors. For example, in a multicentre study, Timonen *et al* found the effects of PM on heart rate variability in patients with coronary heart disease were dependent on local sources of PM.[19] Increases in $PM_{2.5}$ were associated with a reduction in HF power in Finland, but a similar increase in $PM_{2.5}$ was associated with an increase in HF power in Germany. In the study by Wheeler *et al*, exposure to $PM_{2.5}$ was associated with reductions in SDNN in patients with previous myocardial infarction.[20] However, the direction of this effect was reversed in patients with chronic obstructive

Table 3 Acute effect of exposure on heart rate variability in healthy volunteers during rest (n=15)

	Filtered air			Diesel exhaust			Two-way ANOVA
	Baseline	2 h	6 h	Baseline	2 h	6 h	p value
Heart rate (bpm)	69±2	65±2	65±2	66±2	67±2	64±2	0.554
NN interval (ms)	889±32	934±28	937±30	920±26	915±29	952±36	0.542
SDNN (ms)	91±9	79±6	67±5	71±4	90±7	74±7	0.836
RMSSD (ms)	54±6	58±5	48±4	50±5	54±5	52±7	0.787
PNN50 (%)	27±4	33±4	26±4	29±5	31±4	29±6	0.600
Triangular index	19±1	17±1	14±1	15±1	18±1	14±1	0.141
HF power (ms^2)	770±139	892±166	669±105	665±111	914±144	887±269	0.682
LF power (ms^2)	3058±467	2130±273	1703±244	2143±334	2474±406	1591±247	0.346
HFn (ms)	22±3	29±3	28±3	24±3	26±2	30±4	0.827
LFn (ms)	76±3	68±3	70±3	74±3	71±2	68±4	0.915
HF/LF ratio	0.3±0.1	0.5±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.5±0.1	0.862

All variables are presented as mean±SEM.
Two-way ANOVA filtered air vs diesel exhaust at each time point.
HF, high frequency; HFn, normalised high frequency; LF, low frequency; LFn, normalised low frequency; PNN50, percentage of successive NN interval differences >50 ms; RMSSD, root mean square of successive NN interval differences; SDNN, SD of NN interval values.

pulmonary disease where SDNN increased following exposure to ambient $PM_{2.5}$.[20]

Differences in statistical analyses make comparison of the effect size between observational studies difficult. In the largest study to date, Liao *et al* report weak associations between particulate air pollution and heart rate variability in a population of 6784 healthy adults, suggesting a small role for PM in modulating autonomic function.[21] An increase in 1SD of PM_{10} (11.5 $\mu g/m^3$) in the 3-day period prior to assessment was associated with an increase in heart rate of 0.32 beats/min and a decrease in SDNN of 1.03 ms. Previous studies showing that heart rate variability is an important prognostic marker have assessed the relationship between heart rate variability and long-term outcome.[9] Whether transient hourly or daily fluctuations in heart rate and heart rate variability of this magnitude impact on cardiovascular outcome is unknown.

The implication from these observational studies is that reduced heart rate variability following exposure to air pollution may predispose to serious tachyarrhythmias resulting in hospitalisation or sudden cardiac death. Direct evidence that PM may be a trigger for arrhythmia is derived from studies of high-risk patients with implantable cardioverter defibrillators (ICD). In a pilot study, fine particulate and other traffic-derived air pollutants were associated with an increase in the number of defibrillator interventions among 100 patients with ICDs.[4] However, in a larger more complete analysis with longer follow-up, there was no increase in the risk of ventricular arrhythmia unless the analysis was restricted only to those patients requiring frequent defibrillator interventions.[5] Indeed, in a much larger study, Anderson *et al* recently reported a fixed stratum case crossover analysis in 705 patients who experienced 5462 activation days over an average of 1200 days observation in London. Overall they concluded that there was little evidence of an association between air pollution and activation of ICDs.[22]

Particle composition

While controlled exposure to dilute diesel exhaust is a good model for studying the effects of combustion-derived air pollution and diesel exhaust is a major source of ambient ultrafine particles, it is important to appreciate that ambient air pollution contains a range of particulate pollutants from a variety of atmospheric sources. Notably, diesel exhaust does not contain appreciable quantities of atmospheric metals and there is some experimental evidence to suggest that metals may modify the effect of PM on the autonomic nervous system.[23] In a cohort of boiler construction workers exposed to high concentrations of PM and metals, cardiac autonomic function was associated with six common atmospheric metals including vanadium and lead.[12] Furthermore, circulating concentrations of common atmospheric metals have been found to correlate with measures of heart rate variability in healthy persons without occupational exposure.[24] Consistent with this hypothesis, exposure to pure ultrafine carbon particles does not affect heart rate variability in either healthy volunteers[25] or patients with coronary heart

Table 4 Acute effect of exposure on heart rate variability in patients with coronary heart disease (n=19)

	Filtered air			Diesel exhaust			Two-way ANOVA
	Baseline	2 h	6 h	Baseline	2 h	6 h	p value
Heart rate (bpm)	62±2	60±2	59±2	62±2	60±2	59±2	0.595
NN interval (ms)	983±28	1010±29	1040±29	1010±25	1053±31	1052±30	0.080
SDNN (ms)	32±4	44±5	40±5	34±3	48±6	41±3	0.482
RMSSD (ms)	25±3	28±3	28±3	26±2	32±3	30±3	0.190
PNN50 (%)	6±2	9±2	9±2	7±2	13±3	12±3	0.072
Triangular index	7±1	8±1	8±1	7±1	8±1	8±1	0.897
HF power (ms^2)	265±64	286±62	286±62	246±44	367±76	306±70	0.446
LF power (ms^2)	508±152	794±200	673±207	407±107	748±293	541±76	0.494
HFn (ms)	37±4	32±4	34±3	41±5	40±5	34±4	0.215
LFn (ms)	60±4	65±4	63±3	56±5	57±5	63±4	0.211
HF/LF ratio	0.8±0.2	0.7±0.1	0.6±0.1	1.0±0.2	1.0±0.3	0.9±0.3	0.094

All variables are presented as mean±SEM.
Two-way ANOVA filtered air vs diesel exhaust at each time point.
HF, high frequency; HFn, normalised high frequency; LF, low frequency; LFn, normalised low frequency; PNN50, percentage of successive NN interval differences >50 ms; RMSSD, root mean square of successive NN interval differences; SDNN, SD of NN interval values.

Table 5 Summary of observational studies assessing associations between particulate air pollution and heart rate variability

Author	Population	N	Pollutant	HR	SDNN	RMSSD	HF	LF	HF/LF
Liao *et al*. *Environ Health Perspect* 1999;107:521–5	Elderly	26	$PM_{2.5}$	–	↓	–	–	–	–
Pope *et al*. *Am Heart J* 1999;138:890–9	Healthy	7	PM	↑	↓	↑	–	–	–
Gold *et al*. *Circulation* 2000;101:1267–73	Elderly	21	PM	↓	↓	↓	–	–	–
Magari *et al*. *Circulation* 2001;104:986–91	Occupational	40	$PM_{2.5}$	↑	↓	–	–	–	–
Brauer M *et al*. *J Expo Anal Environ Epidemiol* 2001;11:490–500	COPD	16	PM_{10}	↔	↔	↔	–	–	–
Holguin *et al*. *Epidemiology* 2003;14:521–7	Elderly	34	$PM_{2.5}$	–	–	–	↓	↓	↑
Pope *et al*. *Environ Health Perspect* 2004;112:339–45	Elderly	88	$PM_{2.5}$	↔	↓	↓	–	–	–
Liao *et al*. *Am J Epidemiol* 2004;159:768–77	Community	6784	PM_{10}	↑	↓	–	↓	↔	–
Chan *et al*. *Environ Health Perspect* 2004;112:1063–7	Healthy	19	$PN_{0.02-1}$	–	↓	↓	↓	↓	–
De Paula Santos *et al*. *Eur Heart J* 2005;26:193–200	Occupational	48	PM_{10}	↔	↔	↔	–	–	–
Park *et al*. *Environ Health Perspect* 2005;113:304–9	Elderly	497	$PM_{2.5}$	–	–	–	↓	↔	↑
Schwartz *et al*. *Thorax* 2005;60:455-61	Elderly	28	$PM_{2.5}$	–	↔	↓	–	–	↔
Sullivan *et al*. *Thorax* 2005;60:462–6	Elderly	34	$PM_{2.5}$	–	–	↔	↔	–	–
Vallejo *et al*. *J Expo Sci Environ Epidemiol* 2006;16:125–30	Young	40	$PM_{2.5}$	–	↔	–	–	–	–
Timonen *et al*. *J Expo Sci Environ Epidemiol* 2006;16:332–41	CHD	84	PN	–	↔	↔	↔	–	↓
			$PM_{2.5}$	–	↔	↔	↔	–	↔
Chung *et al*. *Environ Health Perspect* 2005;113:1693–7	CHD, HTN	26	$PM_{2.5-10}$	–	↔	↔	↔	↔	↔
			$PM_{1-2.5}$	–	↔	↔	↔	↔	↔
			$PM_{0.3-1}$	–	↓	↓	↓	↓	↔
Wheeler *et al*. *Environ Health Perspect* 2006;114:560–6	COPD	18	$PM_{2.5}$	–	↑	↔	↔	↑	↔
	CHD	12	$PM_{2.5}$	–	↔	↔	↔	↔	↔
Lipsett *et al*. *Environ Health Perspect* 2006;114:1215–20	CHD	19	$PM_{2.5}$	–	↔	↔	↔	↔	–
			$PM_{2.5-10}$	–	↓	↑	↔	↔	–
Luttman-Gibson *et al*. *J Occup Environ Med* 2006;48:780–8	Healthy	32	$PM_{2.5}$	↑	↓	↓	↓	↓	–
Park *et al*. *Circulation* 2006;114:2798–805	Elderly	518	$PM_{2.5}$	–	–	–	↓	–	–
Chuang *et al*. *Am J Respir Crit Care Med* 2007;176:370–6	Young	76	PM_{10}, $PM_{2.5}$	–	↔	↔	↔	↔	–
Chahine *et al*. *Environ Health Perspect* 2007;115:1617–22	Elderly	476	$PM_{2.5}$	–	↓	–	↓	↔	–
Park *et al*. *J Expo Sci Environ Epidemiol* 2007;17:488–97	Elderly	497	$PM_{2.5}$	–	↔	–	↔	↔	↑
Chang *et al*. *Bull Environ Contam Toxicol* 2007;79:552–6	Elderly	15	PM_{1}	↔	↔	↔	–	–	↑
			$PM_{1-2.5}$	↑	↔	↔	–	↓	↑
			$PM_{2.5-10}$	↔	↓	↓	–	–	↑
Min *et al*. *Inhal Toxicol* 2008;20:435–44	Community	1349	PM_{10}	–	↓	–	↔	↓	–
Cavallari *et al*. *Inhal Toxicol* 2008;20:415–22	Occupational	36	$PM_{2.5}$	–	↓	–	–	–	–
Cardenas *et al*. *Environ Res* 2008;108:1–6	Young	52	$PM_{2.5}$	–	–	–	↓	↓	–
Baccarelli *et al*. *Circulation* 2008;117:1802–9	Elderly	549	$PM_{2.5}$	–	↔	–	↔	↔	–
Cavallari *et al*. *Environ Health* 2008;7:36	Occupational	26	$PM_{2.5\ metal}$	–	–	↓	–	–	–
Barcley *et al*. *Occup Environ Med* 2009;66:325–34	Heart failure	132	PM_{10}, $PM_{2.5}$	↔	↔	↔	–	–	–
Whitsel *et al*. *Am J Epidemiol* 2009;169:693–703	Diabetes mellitus	770	PM_{10}	↑	↔	↓	–	–	–
Wu *et al*. *Environ Health Perspect* 2010;118:87–91	Occupational	11	$PM_{2.5}$	–	↓	–	↓	↔	–

These studies were selected following a systematic search of PubMed (MEDLINE) in July 2010 using the following MeSH key words: particulate matter AND heart rate variability. Where multiple publications have arisen from a single cohort, only the primary analysis of the association between PM and heart rate variability was included.
COPD, chronic obstructive pulmonary disease; HTN, hypertension; HF, high frequency; LF, low frequency; CHD, coronary heart disease; PM, particulate matter; PN, particle number; RMMSD, root mean square of successive NN interval differences; SDNN, SD of NN interval values.

disease,[26] whereas exposure to concentrated ambient particles with a range of organic and inorganic components may influence autonomic function.[27] The effects following exposure to concentrated ambient particles are more pronounced with fine particles[28] and in elderly individuals,[29] but no study to date has attributed these differences to the metal content of PM. It appears that, even when controlling for potential environmental confounders and individual subject differences through controlled exposures, particle composition is a major determinant of the health effects of PM.

While we do not believe limitations in our study design can explain our negative findings, we acknowledge that a number of relevant factors may have influenced the outcome of our studies. Given potential safety concerns, we recruited patients who had stable and symptomatically well-controlled coronary heart disease on optimal medical therapy, which included maintenance beta-blocker therapy in the majority of patients. The beneficial effects of beta-blockade in patients with coronary artery disease and heart failure are well established and are thought to be mediated in part through enhanced cardiac vagal control,[30–32] with treatment increasing HF power by as much as 50%.[33] It is possible that an adverse effect of diesel exhaust on autonomic function could have been masked by beta-blockers or other pharmacological therapies in our patients with coronary heart disease. Indeed, a recent reanalysis of the study by Timonen[19] suggests that the use of beta-blockers in patients with coronary heart disease may have modified the association between $PM_{2.5}$ and heart rate variability, partly explaining the inconsistencies identified in the original analysis.[34] However, exposure to diesel exhaust did not affect time or frequency domain measures of heart rate variability in our young healthy volunteers, none of whom were on regular medication, suggesting that the use of cardiovascular therapies is not the primary explanation for the absence of an effect of diesel exhaust on autonomic function in our patients with coronary heart disease.

We chose to assess heart rate variability under controlled conditions 1 h following the end of the exposure to minimise any effect of exercise during the exposure on heart rate and heart rate variability. We cannot discount the possibility that exposure to diesel exhaust may have caused an immediate effect on autonomic function during the exposure itself or within the first 1 h following the exposure. While it is possible that our study was insufficiently powered to detect small effects on autonomic function, the study size was larger than any previous human exposure study addressing the effect of particle exposure on autonomic function.

Conclusions

Brief exposure to dilute diesel exhaust does not alter heart rhythm or heart rate variability in healthy volunteers or patients with coronary heart disease on optimal cardioprotective medication. While we cannot exclude an immediate effect during exposure, diesel exhaust did not impair autonomic function in the hours following exposure or over the 24 h period. Our findings from a carefully controlled exposure to an important air pollutant suggest that autonomic dysfunction may not be the central mechanism with which to explain the observed excess in cardiovascular events following exposure to road traffic or combustion-derived air pollution.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Regional Ethical Review Board, Sweden.

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Reducing Personal Exposure to Particulate Air Pollution Improves Cardiovascular Health in Patients with Coronary Heart Disease

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Background: Air pollution exposure increases cardiovascular morbidity and mortality and is a major global public health concern.

Objectives: We investigated the benefits of reducing personal exposure to urban air pollution in patients with coronary heart disease.

Methods: In an open randomized crossover trial, 98 patients with coronary heart disease walked on a predefined route in central Beijing, China, under different conditions: once while using a highly efficient face mask, and once while not using the mask. Symptoms, exercise, personal air pollution exposure, blood pressure, heart rate, and 12-lead electrocardiography were monitored throughout the 24-hr study period.

Results: Ambient air pollutants were dominated by fine and ultrafine particulate matter (PM) that was present at high levels [74 μg/m^3 for $PM_{2.5}$ (PM with aerodynamic diamater <2.5 μm)]. Consistent with traffic-derived sources, this PM contained organic carbon and polycyclic aromatic hydrocarbons and was highly oxidizing, generating large amounts of free radicals. The face mask was well tolerated, and its use was associated with decreased self-reported symptoms and reduced maximal ST segment depression (–142 vs. –156 μV, p = 0.046) over the 24-hr period. When the face mask was used during the prescribed walk, mean arterial pressure was lower (93 ± 10 vs. 96 ± 10 mmHg, p = 0.025) and heart rate variability increased (high-frequency power: 54 vs. 40 msec^2, p = 0.005; high-frequency normalized power: 23.5 vs. 20.5 msec, p = 0.001; root mean square successive differences: 16.7 vs. 14.8 msec, p = 0.007). However, mask use did not appear to influence heart rate or energy expenditure.

Conclusions: Reducing personal exposure to air pollution using a highly efficient face mask appeared to reduce symptoms and improve a range of cardiovascular health measures in patients with coronary heart disease. Such interventions to reduce personal exposure to PM air pollution have the potential to reduce the incidence of cardiovascular events in this highly susceptible population.

Key words: air pollution, blood pressure, face mask, heart rate variability, myocardial ischemia. *Environ Health Perspect* 120:367–372 (2012). http://dx.doi.org/10.1289/ehp.1103898 [Online 3 January 2012]

Air pollution exposure is an established risk factor for cardiovascular morbidity and mortality (Brook et al. 2010), especially exposure derived from traffic and industrial sources (Laden et al. 2000; Lall et al. 2011; Lipfert et al. 2006; Pope et al. 2002; Sarnat et al. 2008). Acute exposure to combustion-derived particulate matter (PM) is associated with the onset of myocardial infarction and admissions to hospital in survivors of myocardial infarction and has been proposed as a trigger for acute cardiovascular events (Peters et al. 2004; von Klot et al. 2005). Although estimates vary, chronic exposure to air pollution has been estimated to increase all-cause mortality by 2–4% per 10-μg/m^3 increase in PM (Dockery et al. 1993; Pope et al. 2002), with most deaths due to cardiovascular disease (Miller et al. 2007). The World Health Organization estimates that outdoor urban air pollution results in around 800,000 deaths worldwide each year (United Nations Environment Programme 2006).

In controlled exposure studies, inhalation of PM air pollution affects blood pressure and causes abnormalities in vascular function, coagulation, and myocardial perfusion (Mills et al. 2009). These responses provide a plausible mechanism to explain the observed increase in acute cardiovascular events and cardiovascular mortality after exposure to PM air pollution. However, although acute exposure induces these adverse effects, whether improvements in cardiovascular health can be achieved by interventions targeted to reduce exposure in those living and working in highly polluted urban environments is unclear.

Major environmental health policy interventions can have a substantial impact on the health of populations, as evidenced by major reductions in cardiovascular events after the banning of bituminous coal in Dublin, Ireland, in 1990 (Clancy et al. 2002) and, more recently, with the restriction of smoking in public places (Pell et al. 2008). However, such environmental interventions may be difficult to implement in rapidly developing countries where economic growth is dependent on road traffic and heavy industry (Smith et al. 2009). More practical solutions to reduce individual exposure and protect susceptible persons are urgently required. Therefore, we investigated the effects of a simple face mask intervention to reduce PM air pollution exposure on measures of cardiovascular health in patients with coronary heart disease.

Methods

Subjects. One hundred and two patients were recruited from the Fuwai Hospital, Beijing, China, in March 2009. All patients were nonsmokers and had a history of coronary heart disease. Exclusion criteria were a history of arrhythmia, severe coronary artery disease without revascularization, resting conduction abnormality, digoxin therapy, uncontrolled hypertension, renal or hepatic failure, or an acute coronary syndrome within the previous 3 months. Patients' medical histories were recorded from the case notes, and baseline anthropometric and biochemical measures were performed on recruitment. All subjects gave their written informed consent, and the

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study was reviewed and approved by the local research ethics committee.

Study design. Subjects attended the Fuwai Hospital or the ChaoYang Hospital in Beijing on two occasions, with at least a week between visits (median time between visits was 9 days), between March and May 2009. Each subject attended the same hospital on each visit. In a prospective randomized open blinded end point (PROBE) crossover study, subjects walked for 2 hr between 0900 hours and 1100 hours along prescribed city center routes [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ehp.1103898)] in Beijing, using a highly efficient face mask on one study visit but not the other (Dust Respirator 8812; 3M, St. Paul, MN, USA). This mask consists of a lightweight polypropylene filter, which is effective at removing airborne PM without affecting ambient gases. The mask has an expiration valve, complies with EN149:2001 FFP1 European Standard (British Standards Institute 2001), and has an assigned protection factor of 4 [i.e., it can be worn in atmospheres containing up to four times the workplace exposure limit (WEL) as defined by the U.K. Health and Safety Executive (2011). [The WEL for respirable carbon particles (carbon black), is 3.5 mg/m^3 over an 8-hr time weighted average.] Mask use was randomly assigned to the first or second visit using balanced computer-generated randomization. In order to maximize the difference in PM air pollution exposure, subjects wore the mask for 24 hr before the mask study day, in addition to wearing it during the 24 hr study day, and were given instructions to wear the mask at all times while outdoors and as much as possible when indoors. Subjects' activities after the prescribed walk were not restricted, and they were instructed to continue their normal daily routines.

Personal pollution exposure and activity monitoring. Personal air pollutant exposure was determined using monitoring equipment contained within a backpack. Fine particulate matter ($PM_{2.5}$; PM with aerodynamic diameter ≤ 2.5 μm) was determined using a DataRAM monitor (model pDR-1500; Thermo Scientific, Franklin, MA, USA), and particle number was measured using a condensation particle counter (model CPC 3007; TSI Instruments Ltd., High Wycombe, UK). Ambient temperature and relative humidity were recorded using an external sensor (Omegaette® model HH-314; Omega Engineering Ltd., Stamford, CT, USA). Gaseous pollutants were measured using a multigas analyzer with electrochemical sensors for carbon monoxide, sulfur dioxide, and nitrogen dioxide (model X-am 7000; Dräger Safety, Lübeck, Germany). During the prescribed walk, physical activity was measured using global positioning system (GPS) tracking (eTrex Summit HC GPS unit; Garmin, Olathe, KS, USA), and energy expenditure was estimated using activity data and anthropometric data as described previously (Langrish et al. 2009). The estimated PM exposure when wearing the mask was determined based on measurements of mask filter efficacy as described previously (Langrish et al. 2009).

Background pollution monitoring. Background exposure was recorded from permanent monitoring stations in the district where the patients walked on the study day (Beijing Municipal Environmental Protection Bureau 2009). Airborne PM was collected onto Teflon filters (Pall Corp., Ann Arbor, MI, USA) in three size fractions: coarse (mean aerodynamic diameter, 2.5–10 μm), fine (0.18–2.5 μm), and ultrafine (< 0.18 μm) using a MOUDI cascade impactor (MSP Corp., Shoreview, MN, USA).

PM mass was determined gravimetrically for each size fraction from the above filters after temperature and humidity conditioning and subsequently analyzed for elemental and organic carbon fractions, metals and cations, nitrate and sulfate anions, and organic matter.

Chemical and toxicological analysis of collected PM. Collected PM samples were analyzed for total carbon content, as well as elemental and organic carbon fractions, using the Sunset method (National Institute for Occupational Safety and Health 2003). Metals and cations were determined using inductively coupled plasma mass spectrometry (ICP-MS) after pretreatment with nitric acid. Nitrate and sulfate anions were determined after extraction with water using liquid chromatography paired with ICP-MS. Organic matter was extracted from filters by ultrasonification with toluene and analyzed using gas chromatography/mass spectrometry.

The oxidative potential of PM was assessed using electron paramagnetic resonance (EPR) (Miller et al. 2009). EPR was used to establish oxygen-centered free radical generation from the collected PM in the absence of tissue. Filters for all size fractions were pooled and sonicated in phosphate-buffered saline to give a final concentration of 1 mg particles/mL. Solutions were incubated with the spin-trap 1-hydroxyl-2,2,6,6,-tetramethyl-4-oxo-piperidine (Tempone-H; 1 mM) (Enzo Life Sciences, Exeter, UK), loaded into a capillary tube and assessed at 37°C in an X-band EPR spectrometer (model MS-200; Magnettech, Berlin, Germany) as described previously (Miller et al. 2009). Pyrogallol (100 μM) was used as a positive control, and samples were compared with the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) standard reference materials for urban dust (SRM-1649a; 1 mg/mL) and diesel exhaust PM (SRM-2975; 10 μg/mL).

Electrocardiography and blood pressure monitoring. Continuous 12-lead electrocardiography (ECG) was assessed with a digital Holter recorder (Lifecard 12; Spacelabs Healthcare Ltd., Hertford, UK) using the Pathfinder automated arrhythmia analysis package (version 8.701, DelMar Reynolds' Pathfinder Digital; Spacelabs Healthcare Ltd.). Identified arrhythmias were individually inspected and verified or deleted as appropriate, and heart rate variability was assessed using the DelMar Reynolds' HRV Tools software package as described previously (Langrish et al. 2009). ST segment analysis was performed at the J-point +80 msec in three representative leads (II, V2, and V5) that were analyzed separately for each subject. Maximal ST segment depression and ischemic burden (product of the change in ST segment amplitude and the duration of the recording) were determined for each lead and as a composite (Mills et al. 2007).

Ambulatory blood pressure was measured automatically (model 90217 ultralite ambulatory blood pressure monitor; Spacelabs Healthcare Ltd.) every 15 min during the 2-hr walk and then every 30 min during the day and every hour overnight (i.e., 2200 hours to 0700 hours).

Symptom questionnaire. Subjects completed a symptom questionnaire at the beginning of the study day, after the 2-hr walk, and at the end of the 24-hr visit. They were asked to report physical symptoms (e.g., headaches, dizziness, nausea), their perception of the pollution, their perceived workload, and the tolerability of the mask after the prescribed walk using a visual analog scale.

Data analysis and statistical methods. In our previous study of healthy volunteers, we demonstrated a difference (mean ± SD) in systolic blood pressure of 7 ± 5 mmHg after a 2-hr walk when a face mask was used (Langrish et al. 2009). Based on the assumption that the effect size in the present study would be considerably smaller because of the use of cardiac medications, we powered the study to detect a 2-mmHg difference in systolic blood pressure, giving a sample size of 101 at 80% power and two-sided $p < 0.05$.

Blood pressure and ECG end points were analyzed by investigators unaware of treatment allocation. All data are expressed as medians (interquartile ranges) or means ± SD unless otherwise stated. Treatment × period (order in which the mask intervention was used) interactions were assessed as described previously (Hills and Armitage 1979), before data were compared using paired Student's *t*-tests or Wilcoxon matched pairs signed rank test as appropriate. Occurrence of arrhythmias, reported symptoms, and ST segment event frequency, were compared using the chi-squared analysis. All data were analyzed using GraphPad Prism (version 4 for Macintosh; GraphPad Software, San Diego, CA, USA). Statistical significance was taken as a two-sided $p < 0.05$.

Results

Subjects and face mask intervention. Ninety-eight patients (87% male; mean age, 62 years) completed the study protocol (Table 1). Four of those originally enrolled did not complete the protocol because of withdrawn consent, cataract extraction, or withdrawal by investigators because of smoking or failure to walk the prescribed route. All subjects tolerated the mask intervention well, scoring the comfort of the mask as 0.64 ± 1.06 on a 0–10 scale (0 represents completely comfortable, and 10, intolerable). The mask intervention reduced self-reported general symptoms (Figure 1) and patients' perceived effort of work, as well their perception of the level of ambient air pollution (2.46 ± 1.67 vs. 2.73 ± 1.64 on the 0–10 visual analog scale; $p = 0.03$). There were no significant treatment × period interactions for any outcome measure.

Air quality and pollutants. Personal levels of ambient air pollutants were similar on both study days (Table 2), although we predict from previous studies of filter efficacy (97% reduction in particle number) that the PM exposure in the presence of the mask would be reduced from 89 μg/m^3 and 43,900 particles/cm^3 to approximately 2 μg/m^3 and 1,200 particles/cm^3 (Langrish et al. 2009). Temperature (17.3°C vs. 16.8°C) and ambient relative humidity (30% vs. 35%) were similar on both visits. Airborne PM was predominantly (> 99% by particle number) in the fine and ultrafine fractions (Figure 2) and contained a large amount of organic carbon and high concentrations of polycyclic aromatic hydrocarbons, nitrates, hopanes, and steranes, suggesting that much of the PM was combustion derived and related to traffic sources [see Supplemental Material, Table S1 (http://dx.doi.org/10.1289/ehp.1103898)]. These collected particles were highly oxidizing and generated large amounts of free radicals as detected by EPR, exceeding the signal seen with both the standard reference NIST urban dust material at an equivalent concentration and the intense free-radical–generating oxidant pyrogallol (Figure 2).

Effect of the face mask on cardiovascular health. Over the 24-hr period, the maximal ST segment depression recorded was lower [median (interquartile range), –142 μV (–179 to –110) vs. –156 μV (–202 to –123); $p = 0.046$] when the face mask was used. However, measures of myocardial ischemic burden were similar during the 2-hr walk and over the entire 24-hr period between visits (Table 3).

During the 2-hr prescribed city center walks, subjects walked a comparable distance (6.37 ± 1.44 km vs. 6.40 ± 1.51 km), at a similar average speed (4.25 ± 0.96 km/hr vs. 4.27 ± 1.01 km/hr), and expended the same amount of energy [2.32 ± 0.52 metabolic equivalent tasks (METs) vs. 2.33 ± 0.55 METs] between visits when the face mask was or was not used. Despite this similar workload, mean ambulatory arterial blood pressure was significantly lower (93 ± 10 mmHg

Table 1. Baseline characteristics of subjects ($n = 98$) completing the study.

Characteristic	Measure
Age (years)	62 ± 7
Male	85
Height (cm)	169 ± 6
Weight (kg)	75 ± 10
BMI (kg/m^2)	26 ± 3
Risk factors	
Hypertension	79
Diabetes mellitus	45
Stroke	15
Peripheral vascular disease	5
Previous myocardial infarction	68
Previous PCI	60
Previous CABG	38
LV ejection fraction (%; $n = 31$)	62 ± 9
Angina status	
CCS class I	67
CCS class II	31
Seattle angina score (maximum, 500)	387 ± 34
Clinical biochemistry	
Random glucose (mmol/L)	5.5 ± 1.8
Triglycerides (mmol/L)	1.5 ± 0.7
Cholesterol (mmol/L)	3.9 ± 0.9
HDL cholesterol (mmol/L)	1.2 ± 0.3
LDL cholesterol (mmol/L)	2.0 ± 0.8
Medication use	
Aspirin	92
Clopidogrel	17
Warfarin	1
ACE inhibitor or ARB	54
Beta blocker	73
Calcium channel blocker	42
Statin (fibrate, or ezetimibe)	83
Nitrate	45
Other antianginal	5
Diabetic medication	42
Traditional Chinese medicine	19

Abbreviations: ACE, angiotensin converting enzyme; ARB, angiotensin II receptor blocker; BMI, body mass index; CABG, coronary artery bypass graft; CCS, Canadian Cardiovascular Society; HDL, high density lipoprotein; LDL, low density lipoprotein; LV, left ventricle; PCI, percutaneous coronary intervention. Data are mean ± SD, or *n*.

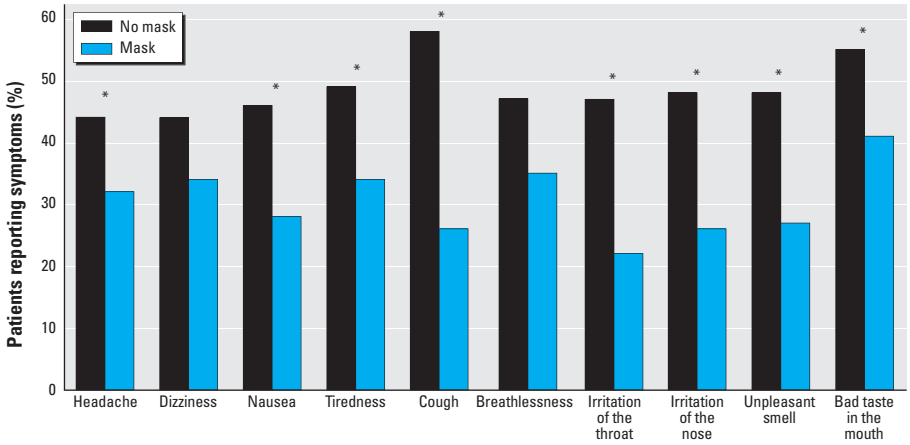

Figure 1. Self-reported symptoms of well-being in the presence or absence of the face mask.
*$p < 0.05$.

Table 2. Personal ambient pollution exposures and background pollution levels on days defined according to mask use.

Parameter	Mask	No mask
Personal $PM_{2.5}$ exposure (μg/m^3)		
Measured	61 (20–88)	89 (25–170)
Estimated	~ 2 (0.6–2.6)	89 (25–170)
Personal particle count (× 10^4 particles/cm^3)		
Measured	4.19 ± 1.29	4.39 ± 1.45
Estimated	~ 0.12 ± 0.04	4.39 ± 1.45
Personal temperature (°C)	17.3 ± 5.2	16.8 ± 5.8
Personal relative humidity (%)	30.4 ± 14.0	34.8 ± 18.2
Personal peaks > 1 ppm (number)		
NO_2	None	None
SO_2	None	None
CO	5 (2–7.5)	4 (2–8)
Background exposure		
PM_{10} (μg/m^3)	92 (70–117)	103 (83–180)
SO_2 (ppb)	38 (29–53)	54 (32–77)
NO_2 (ppb)	36 (29–42)	36 (32–47)

Abbreviations: CO, carbon monoxide; NO_2, nitrogen dioxide; SO_2, sulfur dioxide. Data are mean ± SD or median (interquartile range). Personal monitoring data were collected using portable monitoring equipment during the 2-hr walk. Background data were collected from permanent monitoring stations for the whole 24-hr period. Estimated PM exposure is calculated based on filter efficacy studies where 97% of fresh diesel exhaust PM were removed (Langrish et al. 2009).

vs. 96 ± 10 mmHg) when the face mask was used, although heart rate was similar (Table 3). During the 2-hr walk, heart rate variability [high-frequency (HF) power, high-frequency normalized power (HFn), HF:low-frequency (LF) ratio, and root mean square successive differences (RMSSD)] was higher when wearing the face mask (Table 3). There were no significant differences in overall 24-hr ambulatory blood pressure or heart rate variability. There were no significant differences in the incidence of arrhythmias between the two visits (Table 4).

Discussion

PM air pollution is a major public health concern and is associated with increases in cardiovascular morbidity and mortality. In this study, we demonstrated that reducing personal exposure to urban airborne PM by means of a simple face mask is associated with a reduction in self-reported symptoms and improvements in objective measures of myocardial ischemia, blood pressure, and heart rate variability in patients with coronary heart disease. Reducing personal exposure to PM air pollution has the potential to reduce the incidence of cardiovascular events in patients with coronary heart disease living and working in industrialized or urban environments.

Using a robust PROBE design, we conducted a randomized controlled trial to assess the impact of reducing personal air pollution exposure in patients with coronary heart disease in a polluted urban environment. Through the use of portable monitoring devices and sample collection, we completed a detailed characterization of air pollutant exposure that demonstrated the remarkably complex and toxic composition and extremely high prooxidative potential of ambient air PM in Beijing. We combined individualized pollution monitoring with a comprehensive cardiovascular assessment that incorporated hemodynamic and electrophysiological monitoring in conjunction with GPS tracking. Despite reducing exposure only for a 48-hr period in patients chronically exposed to a polluted urban environment, we observed evidence of consistent beneficial effects on a range of biomarkers of cardiovascular health after the introduction of this simple but highly efficient face mask intervention.

Myocardial ischemia. In a cohort of 20 men with stable asymptomatic coronary disease, we previously demonstrated greater exercise-induced maximum ST segment depression during exposure to diesel exhaust (Mills et al. 2007). However, although acute air pollution exposure exacerbates myocardial ischemia, many persons around the world are chronically exposed to high levels of air pollution, and it is unknown whether interventions targeted at reducing exposure will decrease myocardial ischemia.

In the present study, we showed that decreasing personal exposure to ambient air pollution reduces maximal ST segment depression over a 24-hr period in patients with coronary heart disease. The significance of silent myocardial ischemia is still debated, but it has been associated with major cardiac events in the general population (Fleg et al. 1990). Moreover, in patients with recent myocardial infarction or unstable angina, the occurrence of silent ischemia is a poor prognostic factor and is associated with a significant increase (relative risk ~ 3–4) in major cardiac events and death (Cohn et al. 2003). It seems plausible, therefore, that the modest reduction in silent myocardial ischemia seen in this study might, if sustained, result in significant reductions in major cardiac events and cardiovascular mortality.

Blood pressure. Chronic exposure to air pollution is associated with increases in blood pressure in large epidemiological studies (Auchincloss et al. 2008). Similarly controlled exposure to concentrated ambient PM and ozone in healthy volunteers results in an acute increase in diastolic blood pressure (Urch et al. 2005). Hypertension is a major risk factor for atherosclerosis, and acute increases in blood pressure may trigger plaque rupture leading to an acute cardiovascular event. Consistent with this, exercise-related increases in blood pressure are predictive of the incidence of myocardial infarction (Mundal et al. 1996), stroke (Kurl et al. 2001), and cardiovascular mortality (Kikuya et al. 2000).

We recently reported that use of a face mask that decreased personal PM air pollution exposure reduced systolic blood pressure in healthy volunteers during a 2-hr walk by 7 mmHg (Langrish et al. 2009). The more modest 3-mmHg difference in mean arterial blood pressure after a 2-hr walk observed in the present study may be explained at least in part by the lower workload during walking in this older population with heart disease (estimated energy expenditures of 2.32 METs vs. 3.61 METs in the previous study population), coupled with the modifying effects of antihypertensive medications (Barclay et al. 2009), which were used by most of the present study population. However, interventional trials of blood pressure reduction

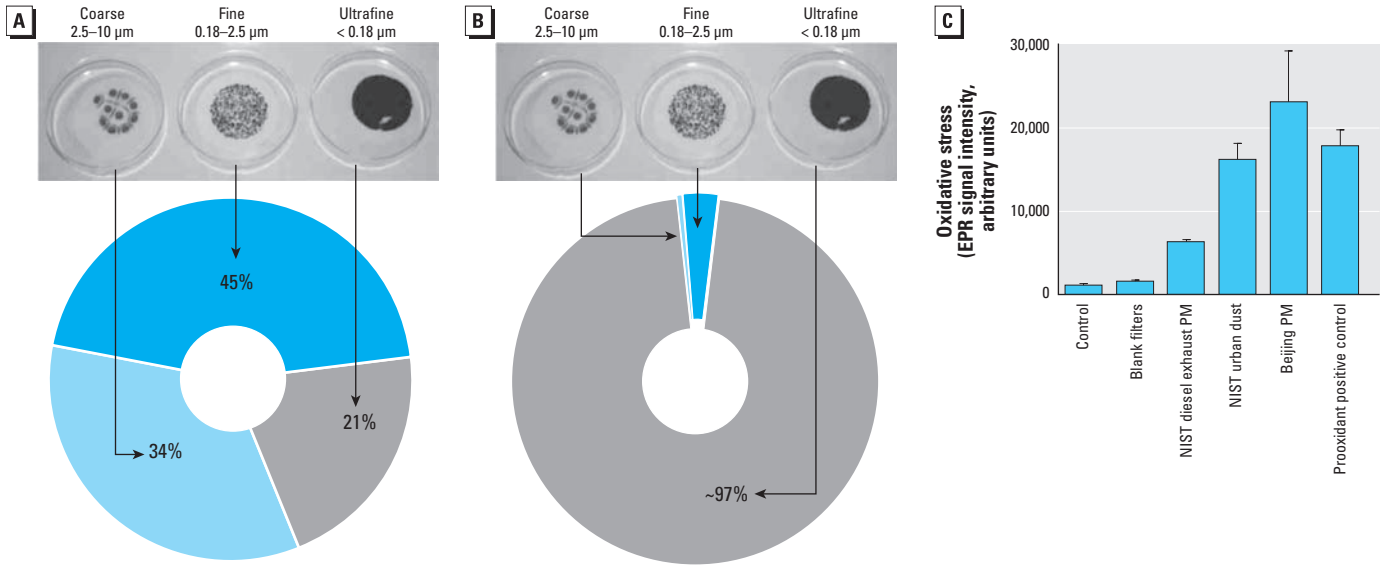

Figure 2. (*A*) Representative filter samples collected showing contribution by mass of the three size fractions averaged over 3 days. (*B*) Estimated contribution of each size fraction collected on filters by particle number. (*C*) Oxidative potential of the collected Beijing PM (1 mg/mL) using EPR to assess oxygen-centered free radical generation, compared with NIST diesel exhaust PM (10 µg/mL) and urban dust (1 mg/mL) and the prooxidant positive control (pyrogallol 100 µM) as described previously (Miller et al. 2009). Data are mean ± SD (n = 2–4).

suggest that even modest changes in blood pressure would reduce the incidence of major cardiovascular events at the population level (Williams 2005).

Heart rate variability. Heart rate variability is a reflection of the autonomic control (a balance of the sympathetic and parasympathetic nervous systems) of the heart and is a measure of the variation in the RR intervals on a continuous electrocardiogram. A reduction in heart rate variability has been demonstrated in patients with a variety of pathophysiological conditions, including hypertension, heart failure, and diabetes mellitus (Task Force 1996). Indeed, reduced heart rate variability has been linked to increased cardiovascular mortality (Nolan et al. 1998), and a large number of studies link exposure to air pollutants with a reduction in heart rate variability (Brook et al. 2010).

In the present study, we have shown that reducing personal exposure to PM air pollution in patients with coronary heart disease is associated with an improvement in heart rate variability during exercise, based on general measures of variability and variability in specific frequency bands. In this study, the changes demonstrated were predominantly in the HF-power band, which is associated with changes in parasympathetic tone, and an improvement may suggest an increased contribution of parasympathetic (vagal) tone to heart rate control. In our previous healthy volunteer study (Langrish et al. 2009), heart rate variability also increased after the face mask intervention, but changes were seen predominantly in the LF-power band, suggesting effects on sympathetic nervous system control. We suggest that this difference (HF-power vs. LF-power changes) may be related to the high use of beta-blocker therapy (74% of patients) in the present study population, which is likely to blunt any effects of exposure on sympathetic tone. The clinical relevance of acute changes in heart rate variability is not clear, although it has been demonstrated that the higher the variability, the lower the cardiovascular mortality (Kikuya et al. 2000). We suggest that a sustained improvement in heart rate variability has the potential to improve patients' prognosis and reduce the impact of air pollution on cardiovascular morbidity and mortality.

Symptoms. Patients perceived fewer self-reported symptoms, a reduction in effort of work, and lower background pollution levels when they wore the face mask. Although we observed no change in the occurrence of self-reported anginal symptoms, this is perhaps not surprising given that we recruited a highly selected population with stable coronary disease, without significant clinical angina, and who were maintained on optimal medical therapy.

Limitations. We chose a PROBE study design because we wanted to determine the acceptability of wearing a face mask, as well as its potential beneficial effects on both symptoms and objective measures of cardiovascular health. We recognize that a double-blind approach incorporating a sham mask would reduce the potential for subjective bias and would therefore be considered more scientifically robust (Smith et al. 2007). In addition, we acknowledge that such an intervention may be more readily accepted in Chinese and Asian societies, where use of face masks is commonplace because of concerns over airborne diseases, pollution, and even fashion, and furthermore, that this may have affected patients' reporting of symptom improvement. However, even a sham mask will filter air pollutants to some degree (Langrish et al. 2009),

Table 4. Cardiac arrhythmias during 24-hr electrocardiographic monitoring periods among 98 coronary heart disease patients according to face mask use.

	No. patients		Median no. of events per patient (interquartile range)	
Arrhythmia	Mask	No mask	Mask	No mask
Dropped beat	2	2	1 (1–1)	1 (1–1)
VT	1	2	2 (2–2)	1 (1–1)
Salvo	1	2	4 (4–4)	2 (1–3)
Bigeminy	15	18	4 (1–33)	6 (2–36)
Triplet	1	0	11 (11–11)	0 (0–0)
Couplet	9	4	2 (1–5)	2 (1–10)
Bradycardia	20	19	52 (3–275)	89 (9–347)
SVT	2	5	1 (1–1)	1 (1–1)
Trigeminy	3	4	12 (6–134)	10 (6–230)
Premature aberrant	79	77	17 (3–122)	22 (3–181)
Isolated aberrant	52	54	3 (1–16)	5 (1–25)
Premature normal	81	84	12 (3–56)	12 (3–42)

Abbreviations: SVT, supraventricular tachycardia; VT, ventricular tachycardia. Bradycardia defined as heart rate < 50 bpm. $p > 0.05$ for all using chi-squared (number of patients) and Mann–Whitney *U*-tests (number of events).

Table 3. Ambulatory blood pressure, heart rate variability during the 2-hr city center walk and the 24-hr study period, and myocardial ischemia measured as ischemic burden, in each individual territory and as a composite according to face mask use.

	Walk		24 hr	
Parameter	Mask	No mask	Mask	No mask
Systolic blood pressure (mmHg)	126.9 ± 15.9	128.1 ± 16.5	121.2 ± 11.9	120.8 ± 12.4
Diastolic blood pressure (mmHg)	78.0 ± 9.3	79.5 ± 8.6	73.8 ± 7.2	74.0 ± 7.3
Mean arterial pressure (mmHg)	93.3 ± 9.7*	95.7 ± 10.0	89.8 ± 7.5	90.0 ± 7.9
Heart rate (bpm)	81.5 ± 8.7	81.5 ± 10.1	77.6 ± 11.3	76.7 ± 11.1
LF power ($msec^2$)	133 (68–97)	136 (52–227)	81 (40–172)	93 (46–208)
HF power ($msec^2$)	54 (27–108)*	40 (20–69)	27 (11–77)	31 (11–68)
LFn (msec)	58.4 (45.6–69.1)*	62.9 (51.1–75.5)	67.2 (55.5–78.0)	71.1 (59.4–81.1)
HFn (msec)	23.5 (18.0–32.4)*	20.5 (13.5–27.9)	21.4 (15.0–31.6)	20.9 (12.7–30.1)
HF:LF ratio	0.418 (0.258–0.712)	0.328 (0.207–0.573)	0.301 (0.190–0.554)	0.306 (0.161–0.492)
pNN50 (%)	1.2 (0.2–2.8)	0.7 (0.0–2.3)	0.5 (0.0–3.1)	0.6 (0.0–2.6)
RMSSD (msec)	16.7 (13.2–22.5)*	14.8 (10.9–19.6)	15.5 (11.0–22.6)	14.4 (10.3–20.3)
SDNN (msec)	59.8 (46.4–79.1)	60.1 (41.0–79.3)	45.6 (30.8–70.4)	48.2 (30.0–66.3)
Ischemic burden (mV-sec)				
Inferior (II) territory	–66 (–118 to –26)	–52 (–149 to –21)	–641 (–767 to –504)	–615 (–820 to –473)
Anterior (V2) territory	–66 (–142 to –16)	–50 (–124 to –13)	–597 (–859 to –435)	–632 (–905 to –489)
Lateral (V5) territory	–37 (–104 to –8)	–43 (–85 to –18)	–604 (–811 to –429)	–586 (–790 to –412)
Sum (II + V2 + V5)	–189 (–382 to –90)	–188 (–340 to –112)	–1,930 (–2,306 to –1,541)	–1,934 (–2,391 to –1,575)

Abbreviations: LFn, low frequency–normalized; pNN50, percentage of successive RR intervals that differ by > 50 msec; SDNN, standard deviation of RR intervals. Data are mean ± SD, or median (interquartile range). LFn and HFn are normalized units to account for variation in the total power and very low-frequency components. LF and SDNN reflect mainly sympathetic nervous stimulation; HF, pNN50, and RMSSD reflect parasympathetic tone.
*$p < 0.05$ from Wilcoxon matched pairs signed rank test or Student's paired *t*-tests as appropriate, mask versus no mask.

and true blinding is difficult to achieve given that large differences in mask efficiency would be readily apparent to trial participants, and differences in mask design would be obvious to investigators. It would also be anticipated that the greater effort of breathing through a mask during exercise would lead to an increase in blood pressure rather than the reverse.

We have assessed an acute intervention, and it remains to be seen whether wearing a face mask for more prolonged periods would have sustained benefits that could affect clinical outcomes.

Conclusions

In this randomized controlled crossover intervention trial, we observed that reducing personal exposure to PM air pollution was associated with small but consistent improvements in objective measures of myocardial ischemia, exercise-related increases in blood pressure, and heart rate variability in patients with coronary heart disease. Although efforts to reduce emissions are critical to reducing exposures to the population as a whole, use of a face mask may be an effective individual-level intervention for high-risk populations. The use of a face mask has the potential to reduce the incidence of acute cardiovascular events, as well as improving patients' general well-being, particularly in developing countries where pollutant exposures are high and resources to reduce emissions are limited.

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