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**Activation of the Endothelin System in Healthy and Inflamed Lungs: A Mechanism for
Cardiovascular Effects of Air Pollution**

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Activation of the Endothelin System in Healthy and Inflamed Lungs: A Mechanism for Cardiovascular Effects of Air Pollution

Errol Thomson

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the PhD degree in Biochemistry, with a specialization in Human & Molecular Genetics

Department of Biochemistry, Microbiology & Immunology
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Abstract

Circulating levels of the potent vasoconstrictor endothelin (ET) are increased after inhalation of urban pollutants. This effect could explain the association of air pollution and cardiovascular morbidity and mortality. However, the timing, source, and specific molecular basis of the increased endothelin release are not well defined. We hypothesized that inhalation of urban pollutants rapidly activates pulmonary endothelin system genes, resulting in increased production and spillover of the peptide into circulation. A time-course study confirmed rapid and transient increases of pulmonary preproET-1 and endothelin-converting enzyme-1 mRNA in rats exposed to particulate matter and ozone. Dose-response studies revealed that each pollutant individually activated endothelin system genes, consistent with the concomitant increase of the 21 amino acid peptide ET-1[1-21] and its precursor, bigET-1, in plasma. In contrast, pulmonary preproET-3 mRNA did not correlate with plasma ET-3 levels. Analyses in other organs revealed ozone-induced increases of endothelin gene expression in the brain, pituitary, and heart, substantiating the notion of extrapulmonary effects of pollutants. Surprisingly, co-exposure to particles and ozone increased pulmonary preproET-1 mRNA but not plasma ET-1[1-21] immediately after exposure. This coincided with an increase of matrix metalloproteinase-2, an enzyme that cleaves bigET-1 to ET-1[1-32], suggesting that factors released during acute lung injury can modify circulating endothelin levels. To examine the effect of particle inhalation on existing lung pathology, we undertook microarray studies using SP-C/TNF- α mice with chronic lung inflammation and their wildtype littermates. Real-time PCR confirmed that inhalation of particles increased pulmonary preproET-1 and cytochrome p450 polypeptide 1a1 mRNA, validating

delivery of a biologically effective dose. Remarkably, microarray analyses failed to detect effects of particle exposure on pulmonary gene expression. Our data reinforce the notion that adverse health effects of acute exposure to urban particles may be dominated by physiological response cascades, with some transcriptional regulation such as activation of the endothelin pathway in target cells, rather than widespread changes in genes escaping homeostatic control. The rapid effects on a key vasoregulatory pathway in the lungs and brain provide a biological basis to explain the acute cardiovascular and cerebrovascular events that occur within hours of increased levels of air pollution.

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List of Abbreviations

ACE	angiotensin-converting enzyme
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
AP	activator protein
ApoE	apolipoprotein E
BALF	bronchoalveolar lavage fluid
CARD	caspase recruitment domain
CASP	caspase
cDNA	complementary deoxyribonucleic acid
COPD	chronic obstructive pulmonary disease
Cyp1a1	cytochrome P450 polypeptide family member 1a1
D _{AE}	aerodynamic diameter
DNA	deoxyribonucleic acid
ECE	endothelin converting enzyme
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EHC	Environmental Health Centre
eNOS	endothelial nitric oxide synthase
ET	endothelin
ET _A	endothelin A receptor
ET _B	endothelin B receptor
FDR	false discovery rate
HPLC	high performance liquid chromatography
IL	interleukin
iNOS	inducible nitric oxide synthase
LOEL	lowest observed effect level
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT1-MMP	membrane type-1 matrix metalloproteinase
NF-κB	nuclear factor- κB

NO	nitric oxide
O ₃	ozone
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
PM	particulate matter
PM2.5	particulate matter with aerodynamic cut-off diameter of 2.5 µm
PM10	particulate matter with aerodynamic cut-off diameter of 10 µm
RECK	reversion-inducing-cysteine-rich protein with kazal motifs
ROFA	residual oil fly ash
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SOD	superoxide dismutase
SP-C	surfactant protein C
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor-α
TNF mice	transgenic SP-C/ TNF-α mice
WT	wildtype

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1. Introduction

1.1. Overview

Air pollution is associated with adverse health effects. These health effects have been linked to both particulate and gaseous components of air pollution, and to both acute and chronic exposure. In terms of cardiovascular effects, the strongest and most consistent associations have been with levels of ambient particulate matter. The present study builds on previous observations that exposure of rats to particulate matter concomitantly elevates plasma levels of the potent vasoconstrictor ET-1 and blood pressure, suggesting a role for ET-1 in the acute cardiovascular effects of inhaled pollutants. The work in this thesis focussed on effects of pollutant exposure on endothelin system genes to further substantiate these findings and study the underlying molecular mechanism for the observed effects. Because the lungs are a primary target of inhaled pollutants, and are the main site of endothelin production and removal from circulation, we hypothesized that acute inhalation of urban pollutants rapidly activates pulmonary endothelin system genes, resulting in increased *de novo* synthesis and higher circulating levels of endothelin. We proposed further that the pattern of gene expression could serve as a critical endpoint of pollutant exposure for dose-response assessment, pollutant interaction studies, and investigations of independent factors of susceptibility. The following manuscripts address questions regarding the timing of endothelin activation, the specific changes in endothelin system gene expression in the lungs and other organs, and the role of inflammation in modifying the response to inhaled particles. In order to place the collected manuscripts in context, I will provide some background on key areas related to this research. First, I will review the epidemiologic evidence supporting an association between air pollution and adverse cardiovascular effects. In particular, I will

focus on the specific cardiovascular events detected, the subpopulations that appear to be most affected, and the timing of these effects, as any viable hypothesis for a biological mechanism must address these characteristics. Secondly, I will explore the characteristics that mediators responsible for these effects might possess, given the information provided by the epidemiologic studies, and review the major hypotheses that have been proposed. Specifically, I will describe work examining the role of inflammation and endothelial dysfunction, as these issues are directly relevant to the studies that I have undertaken. Third, I will discuss the regulation and biological actions of endothelin, a peptide that plays a role in the normal control of vessel tone, but at high levels is implicated in cardiovascular disease. Lastly, I will explain the rationale for this study, present the hypothesis, and discuss the choice of experimental design.

1.2. Epidemiologic evidence of acute cardiovascular effects of air pollutants

1.2.1. Air pollution is associated with increased cardiovascular morbidity and mortality

Air pollution is a complex mixture of particles and gases that can vary in composition depending on geographic location, season, and time of day. In urban settings it consists primarily of particulate matter derived from motor vehicle and industrial emissions, primary gaseous pollutants such as sulphur dioxide, carbon monoxide, and the secondary pollutants nitrogen dioxide and ozone. Respirable particles are generally classified by aerodynamic diameter and fall into three main modes: a nucleation mode (smaller than 0.1 μm); an accumulation mode (between 0.1 μm and 1 μm); and a coarse mode (larger than 1 μm). Current regulations of particulate matter set limits for the allowable mass of particles per cubic metre below a certain aerodynamic diameter. Hence, a limit for PM₁₀ refers to particulate matter with aerodynamic diameters less than 10 μm . The larger particles account

for the greatest proportion of ambient particle mass; however, fine and ultrafine particles are present in much higher numbers and present a greater total surface area per unit of mass to carry reactive co-pollutants and interact with cellular targets. Particle size will determine the probability of deposition in different regions of the airways and may impact on clearance dynamics (Ferin et al., 1992) and physiologic responses (Becker et al., 2003). Compared to larger particles, fine and ultrafine particles are more likely to deposit in the gas-exchange regions of the lungs, and may not be as readily phagocytosed as larger particles (Oberdorster, 2001). In addition to size, the inherent toxicity of particles may relate to their composition, with metals in particular being associated with toxicity (Adamson et al., 2000).

Initial epidemiologic studies focused on health effects in the days following periods of severe air pollution. Episodes of extremely high air pollution such as the Meuse Valley Fog of 1930 and the London Fog of 1952 were associated with substantial increases in cardiopulmonary morbidity and mortality, and subsequent regulatory controls were implemented primarily to reduce or eliminate the occurrence of such episodes. It was not until the early 1990s that studies began to emerge showing apparent health effects at substantially lower levels commonly experienced in urban settings. In a cohort study that followed over 8000 adults in six American cities for a period of 14-16 years (referred to as the Harvard Six Cities study), researchers found a 26% difference in the mortality rate between the most and least polluted cities, of which cardiovascular disease accounted for the largest category of excess deaths (Dockery et al., 1993). Another study that used data collected on over 500 000 adults (as part of the American Cancer Society (ACS) Cancer Prevention II project) found that a $10 \mu\text{g}/\text{m}^3$ increase in annual $\text{PM}_{2.5}$ was associated with a 4 % increase in cardiopulmonary mortality (Pope, III et al., 2002). These studies, among

others (reviewed in (Pope, III and Dockery, 2006), indicate that long-term exposure to air pollution at ambient levels increases the risk of mortality.

Short-term effects of air pollution exposure have also been detected. Analysis of pollutant levels and mortality in eight Canadian cities revealed an association between gaseous and particulate pollutants and fluctuations in daily mortality rates (Burnett et al., 2000). In a study involving 50 million people in the 20 largest cities in the United States from 1987 to 1994, daily variations of PM₁₀, and to a lesser extent ozone, were associated with increased mortality due to cardiovascular and respiratory disease (Samet et al., 2000). Ozone levels on the current or previous day correlated with increased incidence of myocardial infarction in a recent study in France (Ruidavets et al., 2005). Numerous other studies have reported similar effects of acute and chronic exposure (for example, (reviewed by (Brook et al., 2004; Pope, III and Dockery, 2006; Pope, III et al., 1995))), substantiating the notion that air pollution causes increased morbidity and mortality.

Although the relative risk for respiratory effects is often higher, cardiovascular morbidity and mortality associated with air pollution is greater due to the larger population at risk. Indeed, a recent epidemiologic study found that air pollution is associated with twice as many deaths from cardiovascular disease compared to cancer and respiratory disease (Pope, III et al., 2004). Particulate matter has been the pollutant most commonly associated with cardiovascular effects (Schwartz, 1994; Brook et al., 2004; Dockery et al., 1993). Analysis of time series data by the United Kingdom Department of Health produced an estimate that a 10 µg/m³ reduction in PM₁₀ would result in a 0.8 % reduction in cardiovascular admissions (Anderson et al., 2001). This argument has been substantiated by population studies in

which levels of a major pollutant have been reduced. For example, a ban on coal sales that reduced the concentration of black smoke by $35.6 \mu\text{g}/\text{m}^3$ was associated with a 10.3% decrease in cardiovascular mortality in Dublin, Ireland (Clancy et al., 2002). A recent evaluation of the Harvard Six Cities cohort that extended the period of analysis for an additional eight years found that reductions of particulate matter levels over this period were associated with reduced risk of mortality (Laden et al., 2006).

1.2.2. Cause-specific cardiovascular morbidity and mortality

Although there is now substantial epidemiologic evidence linking particulate matter and cardiovascular morbidity and mortality, the broad classifications typically used to identify causes of death do not provide any information with respect to associations with specific diseases. Such information would be useful in guiding toxicologic studies examining potential mechanisms of effects. Pooling of results from 12 time-series studies published prior to August 2000 revealed that a $10 \mu\text{g}/\text{m}^3$ increase in PM₁₀ was associated with increased hospital admission rates of 0.8% for congestive heart failure, 0.7% for ischemic heart disease, and 0.2% for cerebrovascular disease (Morris, 2001). However, it was noted that differences among studies such as the time lag used in the analysis, the specific disease category considered, and adjustment for co-pollutants complicated the interpretation. A recent study that examined cause-specific mortality associated with chronic exposure to air pollution found that the greatest increased risk was for ischemic heart disease, as well as increased risk of arrhythmia, heart failure, and cardiac arrest (Pope, III et al., 2004). This study did not find evidence of an association with excess mortality due to other reasons (aortic aneurysms, stroke, diabetes, hypertensive disease, or any respiratory disease). Other studies have found an association of ozone and particulate matter with incidence of

ischemic stroke (Hong et al., 2002), indicating that air pollution may be a risk factor for cerebrovascular disease. The differing results emphasize the inherent challenges in identifying specific causes of death associated with air pollution. Differences could be due to methodologic issues such as misclassification, differences in the spatial distribution and composition of pollutants in the studies, and differences in the populations being considered.

1.2.3. Populations at risk

While chronic exposure to air pollution is associated with disease progression and higher mortality rates, increased cardiovascular morbidity and mortality detected after acute exposure are generally attributed to people with existing disease. A study in Montréal found that people with chronic lung disease, coronary artery disease, and heart failure were at increased risk of acute cardiovascular mortality associated with air pollution (Goldberg et al., 2001b). Independent studies indicate that individuals with diabetes may have a higher risk of adverse cardiovascular events than nondiabetics (Goldberg et al., 2001a; Zanobetti and Schwartz, 2001). While cigarette smoking alone is associated with increased risk of cardiovascular disease, Pope et al. found that smokers were at increased the risk of arrhythmias, heart failure, and cardiac arrest due to air pollution (Pope, III et al., 2004), suggesting a possible interaction between cigarette smoking and pollution. Diseases identified in these studies are characterized by inflammation and endothelial dysfunction, suggesting that interaction of pollutant effects with such conditions may be relevant to acute health effects of air pollution.

1.2.4. Timing of acute events

Recent epidemiologic approaches that allow improved resolution of time of exposure and response have shown that adverse cardiovascular events may occur within a few hours of exposure. Such studies employ personal exposure monitors to record pollutant levels and/or electrocardiographic (ECG) monitors or hospital interviews to establish the timing of first onset of symptoms. The advantage of these approaches is illustrated by a recent study in which a stronger association between pollution levels and exacerbation of congestive heart failure symptoms was found when examining the data using an 8 h symptom onset period rather than a 24 h period (Symons et al., 2006). In addition, these studies provide insight into specific effects of inhaled pollutants that may be relevant to the cardiovascular morbidity and mortality associated with air pollutants. In an early study, elevated particle levels were associated with increased mean heart rate and decreased heart rate variability on the day of exposure (Pope, III et al., 1999). Gold et al. found that particulate matter and ozone were independently associated with reductions in heart rate variability (Gold et al., 2000). This association was strongest when averaging PM_{2.5} levels over the hour of ECG measurement and the previous three hours (4 h PM_{2.5} average), and averaging ozone levels over the previous hour (1 h O₃ average). Interestingly, a larger reduction in heart rate with PM_{2.5} exposure was found in smokers than non-smokers, indicating that smokers may have increased sensitivity to PM_{2.5}. Coarse particulate matter, carbon monoxide, sulfur dioxide and nitrogen dioxide were not associated with perturbations of heart rate variability in this study (Gold et al., 2000). Magari and colleagues undertook studies in which subjects wore both ECG monitors and personal PM_{2.5} monitors, enabling the authors to relate variations in ECG parameters to real-time PM_{2.5} levels. The authors found a significant association of PM_{2.5} levels averaged over the previous hour with reduced heart rate variability in a

relatively young working cohort of boilermaker construction workers (mean age of 32), with the magnitude of this effect steadily increasing when averaging PM_{2.5} levels over periods of up to 6 h earlier (Magari et al., 2001). The authors followed this with a study that found an association of specific metals (lead and vanadium) with altered cardiac autonomic function (Magari et al., 2002). In this case there appeared to be a 2-4 h lag in effects. Peters *et al.* (Peters et al., 2001a) found an increased risk of myocardial infarction within 2 h after acute exposure to pollution, and a second period of increased risk 1-2 days after exposure.

In brief, these studies indicate that effects of pollutant inhalation may be rapid, occurring within hours of exposure, and sustained, with increased risk of adverse events over several days. Thus, proposed mediators of acute effects should respond rapidly to pollutant exposure. However, it is important to note that the timing of health effects likely depends on a number of factors, including pollutant composition. For example, there is some evidence of an early response to fine particulates and a delayed response to ultrafine particles (Wichmann et al., 2000). Additionally, the inherent susceptibility of the host may impact these effects, potentially resulting in altered responses and response times.

1.3. Evidence supporting the biological plausibility of adverse effects of air pollution

1.3.1. Which characteristics must a mediator of cardiovascular effects possess?

The consistency of epidemiologic findings strongly suggests a causal role for air pollution in provoking adverse cardiovascular health effects. Over the past decade a number of plausible pathways have been established, and there is now a scientific consensus that the association of pollution levels with adverse health effects measured by epidemiologic studies is real (Chow et al., 2006; Pope, III and Dockery, 2006). However, there is considerable

debate over the specific pollutants and underlying mechanisms responsible for the adverse health effects. The epidemiologic data should provide clues regarding the nature of biologically plausible mediators. In terms of acute effects, the data indicate that potential mediators must be rapidly activated, since adverse cardiovascular outcomes occur within a few hours after exposure. Secondly, the mediator should be an effector of cardiovascular change, and plausibly link the route of exposure (i.e. particle deposition in the lungs) to increased likelihood of acute cardiovascular events (i.e. myocardial ischemia, altered heart rate variability). Third, effects induced by the mediator should help explain why some people (including smokers and patients with diabetes, congestive heart failure, or atherosclerosis) are most affected by increased air pollution levels. In other words, the mediator should explain how the effects of air pollution interact with host factors of susceptibility to increase the risk of acute cardiovascular events.

1.3.2. The blood coagulation hypothesis

Early epidemiologic associations of increased cardiovascular morbidity and mortality at relatively low levels of air pollution were met with some scepticism, due at least in part to a lack of biologically plausible explanations. Seaton and colleagues addressed this concern by proposing that fine combustion-derived particles may provoke inflammation in the lungs, leading to an acute phase response and the release of coagulation factors, thus initiating hemodynamic changes that put an added strain on the heart of individuals with existing cardiopulmonary disease (Seaton et al., 1995). Numerous epidemiologic and controlled exposure studies have since provided evidence supporting the contention that particle exposure can cause increased inflammation and activation of coagulation factors. Fine particles that reach the alveoli may cause pulmonary injury and inflammation through

oxidative stress pathways (Roberts et al., 2003). The inflammatory response is characterized by activation of macrophages and epithelial cells that release inflammatory cytokines (Jimenez et al., 2002), leading to the recruitment and activation of phagocytic cells such as neutrophils, eosinophils, monocytes, and lymphocytes (Driscoll et al., 1997). These inflammatory cells in turn produce additional reactive oxygen species in response to particulate matter (Voelkel et al., 2003). Several inflammatory pathways are stimulated by reactive oxygen species, including the activation of nuclear factor (NF)- κ B, a global regulator that has been shown to transcriptionally activate a wide range of inflammatory mediators (Baeza-Squiban et al., 1999). Local inflammation and oxidative stress as a result of penetration of particles into the alveolar epithelium may result in the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α and interleukins (IL)-6 and IL-8 (Jimenez et al., 2002) that can enter the circulation (Van Eeden et al., 2001). The liver may respond to circulating cytokines by releasing factors that affect blood coagulation.

Peters and colleagues found a significant increase in the probability of extreme values of blood viscosity after a period of increased pollution (Peters et al., 1997). Elevated levels of C-reactive protein (Peters et al., 2001b), a marker of inflammation (Backes et al., 2004), and increased circulating fibrinogen (Schwartz, 2001), which contributes to blood viscosity, have also been associated with air pollution levels. Controlled inhalation exposures of healthy humans to diesel exhaust resulted in modest pulmonary inflammation and increases of circulating neutrophils and platelets 6 h after a 1 h exposure (Salvi et al., 1999). In a rat model, acute lung injury following inhalation of residual oil fly ash (ROFA) is temporally associated with increases in plasma fibrinogen (Kodavanti et al., 2002), supporting the hypothesis that particle-induced inflammation can provoke an acute phase response that

could conceivably lead to increased plasma viscosity. In addition, inhaled ultrafine carbon particles have been shown to pass from the lungs into the peripheral circulation (Nemmar et al., 2002) where they may provoke direct responses in extrapulmonary organs including the heart (Kreyling et al., 2002; Oberdorster et al., 2002). However, these findings are controversial, and a recent attempt to reproduce the study failed to find significant extrapulmonary transit of inhaled particles in humans (Mills et al., 2006).

1.3.3. The endothelin hypothesis

Although there is convincing evidence that exposure to airborne pollutants can increase pulmonary inflammation and circulating markers of an inflammatory response, it is not known to what degree inflammation or acute lung injury contribute to acute or chronic cardiovascular events. In experimental animals, inhalation of urban particulate matter increased blood pressure and circulating levels of endothelin (ET)-1, a potent vasoconstrictor implicated in the pathophysiology of a number of cardiovascular diseases, in the absence of acute injury to the lungs (Bouthillier et al., 1998; Vincent et al., 2001). Based on this finding, the authors argued that the toxicity of particulate matter should not be assessed solely on the basis of its ability to cause acute injury and inflammation. In support of this notion, increased plasma levels of fibrinogen have been detected in humans 24 h after short-term exposure to concentrated ambient particles in the absence of other inflammatory mediators (Ghio et al., 2003). In an animal study, use of a histamine receptor antagonist that prevented the influx of neutrophils after intratracheal instillation of diesel exhaust particles inhibited thrombosis and platelet activation at 6 and 24 h timepoints, but not at 1 h, indicating that lung inflammation was not required for this acute effect (Nemmar et al., 2003). Nurkiewicz and colleagues found that although exposure of rats to higher levels of

ROFA (1 or 2 mg/rat) by intratracheal instillation resulted in pulmonary inflammation, they observed impairment of endothelium-dependent arteriolar dilation at a level (0.25 mg) that did not cause inflammation or acute injury (Nurkiewicz et al., 2004). These results indicate that exposure to urban particulate matter at levels that do not cause overt inflammation may affect vascular function and provoke increased production and release of factors relevant to cardiovascular function into the systemic circulation. Particulate matter has been shown to generate hydroxyl radicals in cell free media (Gilmour et al., 1996), indicating the inherent capacity of PM10 to cause oxidative stress independent of inflammatory cell activation. In addition to their role in activating proinflammatory genes, the oxidative stress-responding transcription factors NF- κ B and activator protein-1 regulate the expression of a number of other factors, including ET-1. The production of reactive oxygen species may therefore result in changes in vascular function that are parallel to, independent of, or exacerbated by inflammatory responses and existing inflammation. Thus, an examination of effects on factors implicated in the regulation of vascular function may provide clues regarding potential mechanisms related to the adverse cardiovascular effects of air pollution.

1.3.4. Potential role of the vasculature

The endothelium may be an important target of inhaled pollutants. In contrast to earlier views that the endothelium was an inactive lining of blood vessels, it is now recognized as carrying out a number of important functions critical to the maintenance of vascular homeostasis. The endothelium is involved in the production, secretion, and clearance of factors such as endothelin, prostaglandins, and nitric oxide that act through autocrine, paracrine, and endocrine pathways to regulate vascular tone and signal smooth muscle cell proliferation, platelet aggregation, fibrinolysis, and monocyte adhesion (Raupach

et al., 2006; Deanfield et al., 2007). Dysfunctional regulation of these processes can lead to a number of events relevant to cardiovascular disease, including vasoconstriction, reduced agonist-stimulated vasodilation, and increased blood coagulation (Corti et al., 2003). There is considerable evidence that passive exposure to cigarette smoke can cause endothelial dysfunction, leading to atherothrombosis and other cardiovascular events (Raupach et al., 2006). Smoking is associated with a rapid increase in circulating ET-1 (Haak et al., 1994) and impaired coronary artery dilation (Sumida et al., 1998) in humans, and increased pulmonary expression of ET-1 in animal models (Adachi et al., 2000; Wright et al., 2006; Wright et al., 2004). Furthermore, in rats exposed to cigarette smoke, constrictor responses were significantly reduced using an endothelin receptor antagonist (Rahman et al., 2007). Because air pollution and cigarette smoke share many of the same characteristics with respect to composition, route of entry, and type and rapidity of effects, it is plausible that similar mechanisms may be activated.

Rats exposed by inhalation to resuspended urban particles exhibit a concurrent increase in blood pressure and plasma ET-1 levels 24-32 h post-exposure (Bouthillier et al., 1998; Vincent et al., 2001). Levels of the endothelin isoform ET-3 were increased 2 h after exposure (Vincent et al., 2001), indicating that impacts on the endothelin system were rapid. Subsequent work in humans showed that a 2 h exposure to concentrated ambient particles and ozone resulted in elevated circulating levels of the ET-1 precursor big endothelin (Vincent et al., unpublished), arterial vasoconstriction (Brook et al., 2002) and increased blood pressure (Urch et al., 2005) immediately after exposure. These results reveal that inhaled pollutants can rapidly affect vascular homeostasis, in line with the epidemiologic evidence of acute effects within a few hours of exposure. Moreover, the data indicate that

perturbation of endothelin system regulation may be an important mediator of cardiovascular effects of air pollutants.

1.4. The endothelin system

1.4.1. Plasma endothelin: A potent vasoconstrictor

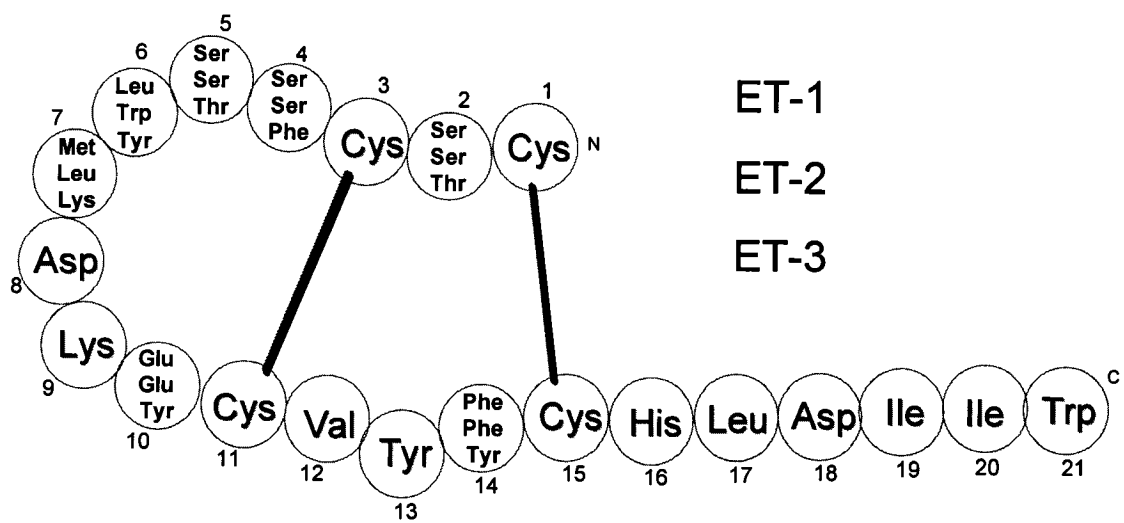
Since its isolation and characterization in 1988 (Yanagisawa et al., 1988), endothelin-1 (ET-1) has been the focus of intense study, particularly in relation to its role in the pathophysiology of cardiovascular disease. ET-1 is the most potent vasoconstrictor known (Yanagisawa et al., 1988), and is implicated both in normal vascular homeostasis and in the pathophysiology of cardiovascular disease. ET-1 is present in plasma at only picomolar levels, and there is overlap of levels in healthy individuals and in cardiovascular patients. However, elevation of ET-1 in plasma is predictive of cardiac death in congestive heart failure patients (Galatius-Jensen et al., 1996) or after myocardial infarction (Ommand et al., 1994). In addition, a 20 % decrease in ET-1 levels in congestive heart failure patients was associated with improvement of symptoms (Tsutamoto et al., 1995). Effects of plasma ET-1 on the heart appear to depend on the relative plasma level. At normal levels, ET-1 exerts a positive inotropic effect on the heart (increasing the strength of muscle contraction), whereas higher levels result in systemic and coronary vasoconstriction that increase afterload and reduce myocardial perfusion, and thus decrease cardiac output (Khan, 2005). Infusion of ET-1 at concentrations similar to those observed in heart failure caused systemic vasoconstriction in patients with left ventricular dysfunction, indicating that ET-1 can act as a circulating hormone (Cowburn et al., 1998). However, even at levels lower than those thought to induce vasoconstriction, ET-1 can potentiate contractions due to norepinephrine and serotonin (Yang et al., 1990). The half-life of endothelin peptides in plasma is on the

order of a few minutes (Anggard et al., 1989); a sustained increase in plasma therefore presumably requires sustained production and subsequent spillover into circulation, or a sustained reduction in clearance. While clearance of ET-1 from plasma is rapid (Sirvio et al., 1990), pressor effects are maintained for over an hour in rats (Mortensen and Fink, 1990) and healthy humans (Clarke et al., 1989), revealing that even transient increases in endothelin levels can have long-lasting vasoconstrictor effects.

1.4.2. Tissue production of endothelin

In addition to ET-1, there are two other endothelin isopeptides, ET-2 and ET-3, encoded by distinct genes (Figure 1), and their tissue distribution differs depending on the specific isotype (Firth and Ratcliffe, 1992). ET-1 is produced at high levels in the lungs, where it is expressed in endothelial cells, epithelial cells, macrophages, smooth muscle cells, and fibroblasts. It is also expressed at lower levels in most other organs, including the heart, liver, spleen, kidney, stomach, intestines, and brain. ET-2 is predominantly expressed in the intestines, while ET-3, like ET-1, is expressed in most organs (Firth and Ratcliffe, 1992). PreproET-3 is not expressed by endothelial cells, but rather may be expressed by neuroepithelial cells (Seldeslagh and Lauweryns, 1993). Endothelin secretion appears to occur both through a constitutive endosomal pathway regulated primarily at the level of transcription (Yanagisawa et al., 1988), and possibly by regulated release from intracellular storage bodies (Russell et al., 1998). Steady-state plasma levels are generally thought to be the result of spillover from abluminal secretion or the release of small quantities via secretory channels (Russell and Davenport, 1999), and reflect the net result of production and clearance in all organs. Although plasma levels are useful as a clinical endpoint, they may not accurately reflect local levels at the site of production. Indeed, despite the evidence

Figure 1. The three endothelin isoforms. ET-1, ET-2, and ET-3 each have 21 amino acid residues and two disulfide bonds. ET-1 and ET-2 differ by two residues, while ET-3 differs from ET-1 and ET-2 by 6 residues. Adapted from (Schiffrin, 2005).

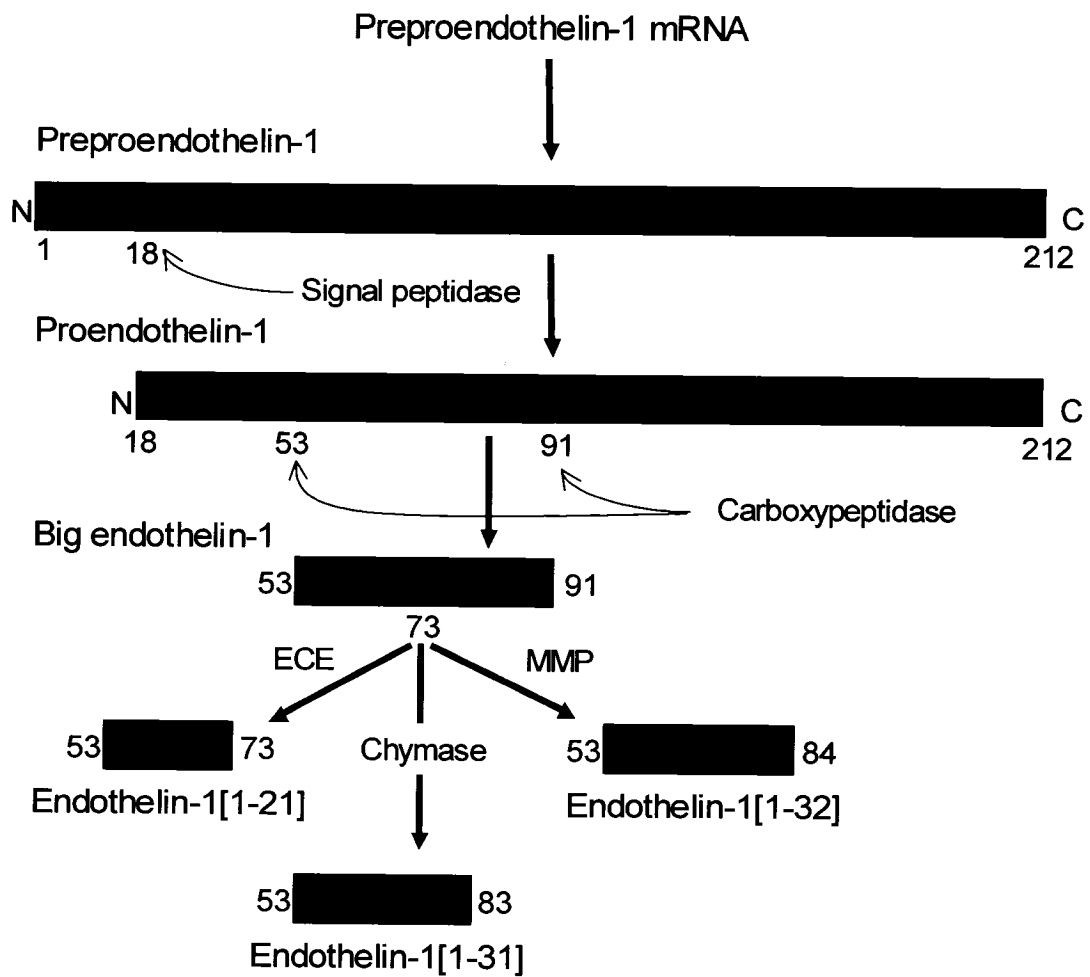


that circulating endothelin has vasomotor effects, endothelins are thought to act primarily in an autocrine/paracrine manner. Localized effects on the endothelin system therefore require organ, tissue, or even cell-specific analysis.

1.4.3. Regulation of endothelin expression

The biological activity of endothelin is regulated at several levels. ET-1 expression is thought to be regulated primarily at the level of transcription (Yanagisawa et al., 1988), and expression can be rapidly induced in response to a variety of stimuli, including inflammation (Finsnes et al., 1998) and oxidative stress (Kaehler et al., 2002; Michael et al., 1997). ET-1 can also increase its own mRNA expression via the ET_B receptor (Iwasaki et al., 1995). ET-1 production is inhibited by vasodilators such as nitric oxide, prostacyclin, adrenomedullin, and natriuretic peptides (Keith, 2000). The 3'-untranslated region appears to be important for transcript stability (Mawji et al., 2004), and the preproET-1 transcript has an intracellular half-life of only about 15 minutes (Inoue et al., 1989b). All three endothelins are successively processed from preproET forms by furin convertases to the relatively inactive precursor bigETs (Kido et al., 1998), and then to the vasoactive 21 amino acid ET forms (Figure 2). This last step is regulated by the action of specific zinc-dependent metalloproteases, the endothelin-converting enzymes (ECEs). ECE-1 mRNA expression can be suppressed by ET-1, indicating a possible negative feedback loop in the production of the mature vasoactive peptide (Naomi et al., 1998). While ECE-dependent processing of bigET-1 is thought to be the dominant pathway in the endothelium, bigET-1 can be cleaved via alternate pathways, including by chymase to yield ET-1[1-31] (Nakano et al., 1997), which is itself a substrate for ECEs (D'Orleans-Juste et al., 2003), and by matrix-metalloproteinase-2 (MMP-2) to form ET-1[1-32] (Fernandez-Patron et al., 1999). This

Figure 2. Processing of endothelin (ET) peptides. PreproET-1 mRNA is translated, and the preproET-1 peptide is successively cleaved by endopeptidases to yield the precursor peptide bigET-1. Processing of bigET-1 to the mature 21 amino acid ET-1[1-21] is mediated primarily by endothelin-converting enzymes (ECEs). However, alternative processing pathways include cleavage by chymase to yield the 31 amino acid ET-1[1-31] peptide, or by matrix metalloproteinases to yield the 32 amino acid ET-1[1-32]. Adapted from (Kido et al., 1998).

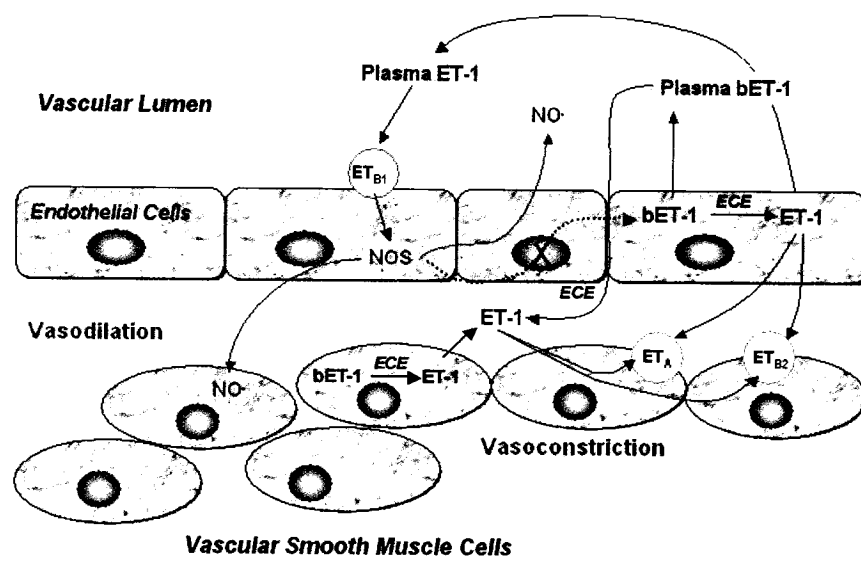


latter processing pathway may have relevance in states of tissue injury and inflammation in which MMP levels are often increased (Fernandez-Patron et al., 2001)

1.4.4. Endothelin receptors

Endothelins act through specific G-protein coupled receptors, the ET_A and ET_B receptors. The ET_A receptor has a roughly 100-fold greater affinity for ET-1 and ET-2 than for ET-3, whereas the ET_B receptor has similar affinity for all three endothelins (Inoue et al., 1989a). ET_A receptors are primarily located on vascular smooth muscle cells, where they mediate the vasoconstrictive actions of the endothelins. In contrast, ET_B receptors are more highly expressed in endothelial cells, where they mediate uptake of their ligands from circulation (Fukuroda et al., 1994) and induce relaxation through release of nitric oxide (Hirata et al., 1993) and prostacyclin (de Nucci et al., 1988). However, ET_B receptors can also be found on smooth muscle cells (and are sometimes referred to as ET_{B2} receptors) where they play a role in vasoconstriction (Haynes et al., 1995). The overall effect of ET-1 on vascular tone reflects the relative contribution of the vasoconstrictor effects mediated by smooth muscle cell ET_A and ET_B receptors, and the vasodilator effects mediated by endothelial ET_B receptors (Figure 3). Increased ET-1 levels do not strictly result in increased vasoconstriction, since this is mitigated by ET_B receptor-associated release of vasodilator factors. However, if the ET-1-dependent nitric oxide release is inhibited, as a result of disease processes (Taddei et al., 1999), ET_B receptor blockade (Verhaar et al., 1998) or nitric oxide inhibition (Cardillo et al., 2000), the strong vasoconstrictive effect of ET-1 is revealed. This may be an important distinction between the effects of ET-1 in healthy individuals as opposed to those with diseases involving endothelial dysfunction. Nevertheless, the overall physiological effect of ET-1 appears to be an increase in blood pressure, as dual ET_A/ET_B

Figure 3. Vascular effects of endothelin (ET)-1. BigET-1 synthesized by endothelial cells is cleaved by endothelin-converting enzymes (ECE) to yield the mature ET-1 peptide. Most of the synthesized ET-1 is released basolaterally towards the smooth muscle cells, where it can act on ET_A or ET_{B2} receptors to cause vasoconstriction. Smooth muscle cells can also produce the mature ET-1 peptide, and such production appears to be increased in atherosclerosis. A proportion of newly synthesized ET-1 is released or spills into the circulation. Plasma ET-1 is removed from circulation via endothelial ET_{B1} receptors, which are then internalized directed to the lysosome for degradation. Binding of ET-1 to endothelial ET_B receptors also provokes the production of nitric oxide (NO), which acts on smooth muscle cells to cause vasodilation, and can down-regulate ET-1 synthesis. The relative levels of endothelial and smooth muscle cell ET_A and ET_B receptors will determine the net physiological effect of ET-1.



receptor antagonists have a hypotensive effect (Haynes et al., 1996). ET-2 appears to exert vasoactive effects similar to ET-1 (Inoue et al., 1989a), while ET-3 acts as both a vasodilator at low doses and as a vasoconstrictor at higher doses (Crawley et al., 1992).

1.4.5. Involvement of endothelin in cardiovascular diseases

The endothelin system may be modified by disease, notably in patients with atherosclerosis, congestive heart failure, hypertension, and diabetes (Luscher and Barton, 2000; Giannessi et al., 2001), which are all groups identified as being at increased risk of adverse cardiovascular effects of air pollution. Circulating and tissue ET-1 levels correlate with the severity of atherosclerosis (Lerman et al., 1991). Atherosclerotic arteries are characterized by increased ET-1 and ECE-1 mRNA (Ihling et al., 2001), and a corresponding increased rate of bigET-1 cleavage to ET-1 compared to healthy arteries (Bohm et al., 2002; Maguire and Davenport, 1998). ET-1 may promote the growth of atherosclerotic lesions by stimulating neutrophil adhesion (Lopez et al., 1993) and enhancing platelet aggregation (Knofler et al., 1995). Administration of an ET_A receptor antagonist revealed that most of the resting tone in atherosclerotic coronary arteries was due to ET-1, implicating ET-1 in the abnormal constriction of atherosclerotic coronary arteries and the pathophysiology of the associated myocardial ischemia (Kinlay et al., 2001). Coronary vasoconstriction induced by circulating cytokines TNF and IL-2 is also mediated by ET-1 (Klemm et al., 1995). As in atherosclerosis, congestive heart failure is characterized by increased circulating ET-1, likely due to reduced ET_B receptor-mediated clearance of ET-1 in the lungs (Lepailleur-Enouf et al., 2001; Dupuis et al., 1998; Kobayashi et al., 1998) or increased pulmonary production of ET-1 (Kjekshus et al., 2000). Administration of the dual ET_A/ET_B receptor antagonist bosentan to patients with heart failure reduced vascular tone and blood pressure (Kiowski et

al., 1995). Cardiac ET-1 production appears to increase in the failing heart, and there is a corresponding increase in ET_A receptor expression and decrease in ET_B receptor expression (Asano et al., 2002). Patients with essential hypertension exhibit increased vasoconstriction to endogenous ET-1, possibly through impairment of nitric oxide synthesis (Taddei et al., 1999). Administration of bosentan significantly lowers blood pressure in hypertensive patients (Krum et al., 1998), indicating that endothelin contributes to the increased blood pressure (Schiffrin, 2005). Diabetes is characterized by increased production of ET-1 and reduced levels of nitric oxide (Haak et al., 1992), and a higher potency of endogenous ET-1 via ET_A receptor binding (Cardillo et al., 2002). As a broad generalization, each of these diseases is characterized by endothelin system changes that result in increased production of ET-1 and/or abnormal sensitivity to ET-1. A pollutant-induced surge of ET-1 might therefore be more potent in these individuals than in healthy people.

1.5. Rationale

Acute alterations in vasoregulation have been proposed as a potential mechanism to explain cardiovascular effects of air pollution. Bouthillier et al. (1998) and Vincent et al. (2001) showed that plasma ET-1 levels and blood pressure increased 24-32 h after exposure of rats to inhaled particulate matter. These effects occurred despite the absence of acute lung injury or overt inflammation, indicating that these conditions may not be required for systemic impacts of air pollutants. Plasma ET-1 levels were not altered 24 h after inhalation of ozone, and ozone did not modify the effect of inhaled particles. Controlled exposure of humans to pollutants confirmed that bigET-1 and ET-3 levels are increased in humans (Vincent et al., abstract). However, the timing, source, and specific molecular events responsible for the increased endothelin levels are not well defined. Since acute

cardiovascular health effects have been reported as early as a few hours after elevation of particulate matter (Magari et al., 2001; Gold et al., 2000; Peters et al., 2001a) and ozone (Ruidavets et al., 2005) levels, a rapid endothelin system response should be expected if endothelins are implicated in these effects. The lungs are a primary target of inhaled particles and are a major site of endothelin production and clearance both in rats (Anggard et al., 1989; Firth and Ratcliffe, 1992) and humans (Dupuis et al., 1996), and therefore could be the source of increased plasma endothelins. The specific mechanisms contributing to the higher steady state concentration of endothelins in plasma may include 1) increased *de novo* synthesis of the endothelin peptides; 2) increased cleavage by endothelin-converting enzymes; or 3) decreased clearance of circulating endothelins by endothelial ET_B receptors.

1.6. Hypothesis

We hypothesize that acute inhalation of urban pollutants rapidly activates lung endothelin system genes, resulting in increased production of endothelin and spillover of the peptide into circulation. We propose further that the pattern of endothelin system gene expression could serve as a critical endpoint of pollutant exposure for dose-response assessment, pollutant interaction studies, and investigations of independent factors of susceptibility.

1.7. Objectives

To test the hypothesis, this thesis addresses the following objectives in the series of manuscripts contained in this thesis.

1. *To determine whether inhaled pollutants activate pulmonary endothelin system genes.* A time-course study of endothelin system gene expression was performed using conventional reverse transcription polymerase chain reaction (PCR) of mRNA from the lungs of Fischer-344 rats after acute exposure to particulate matter and ozone (50 mg/m³ particles, 0.8 ppm O₃). Endothelin system gene expression was monitored at 2 h, and 1, 2, 3, 7 and 14 days after exposure. Dosimetric modeling of exposure was compared with a plausible human exposure scenario to establish the relevance of the dose used.

Thomson E, Goegan P, Kumarathasan P, Vincent R. (2004). Air Pollutants Increase Gene Expression of the Vasoconstrictor Endothelin-1 in the Lungs. *Biochim Biophys Acta* 1689: 75-82.

2. *To establish and validate a real-time PCR system permitting sensitive evaluation of effects of pollutants on endothelin system gene expression.* In order to pursue dose-response assessment of endothelin system gene expression, establishment of a more sensitive method for gene expression analysis was required. A real-time PCR assay was validated for a 96-well plate format, and the issue of plate bias was addressed.

Thomson E, Vincent R. (2005). Reagent Volume and Plate Bias in Real-Time PCR. *Anal Biochem* 337(2): 347-350.

3. *To investigate dose-response effects of acute pollutant exposure and any toxicological interactions on pulmonary ET-1 system gene expression and plasma ET-1 peptide levels.* Having established that co-exposure to particles and ozone increased ET-1 and ECE-1

mRNA levels immediately after exposure, we next examined whether the pollutants individually activated the lung endothelin system, whether the dynamics of effects differed between the two pollutants, and whether there was any toxicological interaction with respect to mRNA expression and plasma endothelin peptide levels. Real-time PCR and HPLC-fluorescence were used to assess endothelin mRNA and peptide levels, respectively, in animals exposed to particles (0, 5, 50 mg/m³), ozone (0, 0.4, 0.8 ppm), or combinations of particles and ozone, immediately and 24 h post-exposure.

Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. (2005). Differential Regulation of Lung Endothelin System Genes by Urban Particulate Matter and Ozone. *Tox Sci* 88: 103-113.

4. To examine pulmonary and plasma responses of ET-2 and ET-3 to inhaled pollution.

Plasma levels of ET-2 and ET-3, two other members of the endothelin peptide family, have been shown to increase in response to air pollution in other studies. Since the lungs respond to inhaled pollutants by increasing production and release of ET-1, we hypothesized that expression of the other two endothelin peptides might be similarly affected. Primers were designed and validated to assess the expression of these genes, and the relative mRNA and plasma peptide levels of all three endothelin peptides (ET-1, -2, and -3) were compared in rats exposed to air, particles, ozone, and the combination of particles and ozone.

Thomson E, Kumarathasan P, Vincent R. (2006). Pulmonary Expression of PreproET-1 and PreproET-3 mRNAs is Altered Reciprocally in Rats after Inhalation of Air Pollutants. *Exp Biol Med* 231: 979-984.

5. *To evaluate effects of pollutant exposure on extrapulmonary ET expression.* Since our observations did not support the hypothesis that the pollutant-induced increase of plasma ET-3 was due to increased *de novo* synthesis in the lungs, an organ screen was performed to examine whether exposure might provoke extrapulmonary endothelin production. Because ET-3 is thought to be a neuropeptide, and the pituitary is a potential source, we hypothesized that pollutants may act on the pituitary and cerebrovascular endothelin systems. Real-time PCR was used to examine endothelin system gene expression in these organs, as well as in the heart, liver, kidney, and spleen of animals described in Chapters 3 and 4.

Thomson E, Kumarathasan P, Calderon-Garciduenas L, Vincent R. Air Pollution Alters Brain Endothelin-1 and Inducible Nitric Oxide Gene Expression. *Under revision for Environmental Research.*

6. *To examine how chronic lung inflammation modifies the lung response to inhaled particulate matter and endothelin expression.* Our data and work by others suggest that endothelin production and release is altered by inflammation and lung injury. Individuals with chronic lung disease have increased susceptibility to air pollution. Transgenic TNF- α overexpressing mice with chronic pulmonary inflammation and their wildtype littermates were exposed to particulate matter, and increased ET-1 mRNA levels were confirmed by real-time PCR. Due to the potential complexity of pathways involved in host-pollutant interactions, microarray technology was used to 1) characterize the context in which the increased endothelin expression occurred; 2) detect other genes and pathways that respond to inhaled particulate matter; and 3) identify potential biomarkers of exposure that are robust to

the physiological status of the lungs. In addition, we sought to examine the utility of microarrays to detect pollutant-genotype interactions, and to determine factors important in the design of future experiments in this area.

Thomson E, Williams W, Yauk C, Vincent R. Toxicogenomic Analysis of Susceptibility to Inhaled Urban Particulate Matter in Mice with Constitutive TNF- α Expression. *In preparation.*

1.8. Rationale for experimental conditions

1.8.1. Selection of pollutants

Two primary challenges facing any researcher investigating biological mechanisms for the adverse health effects of air pollutants are 1) the difficulty in reproducing environmental exposures (Mauderly, 2006); and 2) the difficulty in extrapolating results in animal models to effects in humans (Kodavanti and Costa, 2001). To address the first issue, investigators have undertaken studies employing either pure particles (such as carbon black) or mixtures (such as ambient particles), either alone or in the presence of other pollutants (such as ozone). The advantage of using pure particles is that results can be linked to a specific source or particle size. However, pure particle preparations may not reproduce the effects of the heterogeneous mixture of particles found in the ambient air. An alternative approach is to concentrate fresh ambient particles for the immediate exposure of animals or human subjects ((Sioutas et al., 1995), reviewed by (Ghio and Huang, 2004)). This latter approach may allow a more representative assessment of the health effects of these particles, since there should be fewer changes in the physiochemical characteristics (such as particle aggregation and loss of volatile compounds) that may occur during storage. However, one

disadvantage of this approach is that subsequent experiments cannot be conducted with the same material since the exposure is dependent on the composition of particles in the air at the time of sampling. Exposures employing resuspended filter-collected urban particles have the advantage of enabling the reproduction of experimental conditions provided particles are collected in sufficient quantity. Stored particles can be fully characterized by elemental analysis and cell culture assays, such that effects measured *in vivo* can be compared to particle composition and *in vitro* bioactivity. Furthermore, the physicochemical characteristics of the particles can be modified (for example, by leaching soluble components (Vincent et al., 2001)), thus permitting evaluation of the relative contribution of certain fractions. It is possible, however, that particle storage may alter their potency through aggregation of smaller particles or loss of volatile and semi-volatile components, as fresh particles may be more toxic than stored particles (Johnston et al., 2000),.

The stored EHC particles used in the present study were collected from the bag house filters of the Environmental Health Centre (EHC) at Tunney's Pasture, Ottawa. The material EHC-93 represents the collected mass over 1993, while EHC-6802 is a blend of particles collected in 1996, 1998, 2000, and 2002. EHC-93 has been characterized extensively through elemental analysis (Vincent et al., 1997a; Vincent et al., 2001; Vincent et al., 1997b), *in vitro* toxicity studies (Vincent et al., 1997b; Mukae et al., 2000; Fujii et al., 2001; Van Eeden et al., 2001; Omara et al., 2000), *in vivo* intratracheal instillation (Goto et al., 2004; Adamson et al., 1999a; Adamson et al., 2000; Bagate et al., 2004) and inhalation studies (Vincent et al., 1997a; Bouthillier et al., 1998; Adamson et al., 1999b; Vincent et al., 2001), establishing its relevance as an environmental particle standard. EHC particles have two respirable modes at 1.3 μm and 3.6 μm which account for roughly 55% of the total mass,

and the remainder (45%) consists of a coarse mode at 15 μm (Vincent et al., 2001). The analysis performed did not permit an evaluation of ultrafine modes (which have very little mass, but may be present in large numbers); however, these may also be present. Because of the strong historic data and the prior work indicating increased circulating levels of ET-1 after exposure of rats to EHC particles (Bouthillier et al., 1998; Vincent et al., 2001), these particles are an appropriate standard for use in evaluating particle effects on endothelin system gene expression.

We exposed animals to resuspended ambient particulate matter and ozone. The physicochemical characteristics and toxicodynamics of effects of these pollutants differ greatly. In terms of mass, the bulk of particles are found in the coarse model. However, in terms of surface area and particle number, fine and ultrafine particles compose the greatest proportion of total particulate matter. Particles larger than 10 μm are generally considered non-respirable, and are predominantly trapped in the nose and upper respiratory tract. Smaller particles can deposit with greater efficiency in the alveolar gas exchange regions of the lungs. These particles may persist in the lungs for months, with particle size being one of the factors governing lung retention (Ferin et al., 1992; Oberdorster et al., 1994). In contrast, ozone is a highly reactive gas that is rapidly consumed through interactions with the lung lining. Exposure of animals to ozone causes a primary lesion and increases the permeability of lung epithelial cells in the central acinus, sparing the distal lungs (Blomberg et al., 2003; Yu et al., 1994). This may simulate the compromised epithelium of susceptible individuals with existing lung disease (Vincent et al., 1997a). The relevance of the dose used for each study is included in the respective manuscripts.

1.8.2. Rationale for animal models used

No single animal model will adequately reproduce all aspects of the human exposure and response to inhaled pollutants. Nevertheless, animal models can be chosen that at least possess similarities to the human situation with respect to the key endpoints being analysed. Rats and mice are commonly used in toxicological studies, with Fischer-344 rats routinely used as a healthy rat model. Despite differences in the respiration of rodents and humans, dosimetric modelling can be used to estimate the dose of particles delivered to the pulmonary compartment, enabling interspecies comparisons. The lungs are the main site of production and clearance of endothelin in rats (Firth and Ratcliffe, 1992; Anggard et al., 1989), mice (Chan et al., 1995; Maemura et al., 1996) and humans (Dupuis et al., 1996). Analysis of effects of pollutant inhalation on the endothelin system in rats and mice may therefore have relevance to the human situation. Increased plasma ET-1 has previously been demonstrated in Fischer-344 rats after exposure to EHC-93 (Bouthillier et al., 1998), indicating that it is a suitable model to examine effects of pollutant exposure on endothelin gene expression.

Individuals with pulmonary inflammation are thought to be at increased risk of adverse health effects from exposure to air pollution (Goldberg et al., 2001b). To examine how inflammation modifies the response to inhaled particles, we required an animal model of chronic pulmonary inflammation. In SP-C/TNF- α mice, the murine TNF- α gene is under the control of the human SP-C gene promoter, resulting in constitutive expression of TNF- α by type II alveolar epithelial cells (Miyazaki et al., 1995). These mice constitutively express TNF- α in the lungs, and consequently develop pronounced inflammation and subsequent airspace enlargement and some fibrotic lesions (Fujita et al., 2001; Miyazaki et al., 1995).

Repeated exposure to particulate matter and ozone results in increased oxidative stress and enhanced protein nitration in the lungs of SP-C/TNF- α mice compared to their wildtype littermates (Kumarathasan et al., 2005), confirming the utility of SP-C/TNF- α mice as a model of susceptibility.

1.8.3. Rationale for exposure by nose-only inhalation

Evaluation of effects of ambient particles typically involves exposure by either inhalation or intratracheal instillation. Intratracheal instillation is commonly used because of the simplicity of the technique and the reduced quantity of material required, and as a safe method to deliver highly toxic materials (Driscoll et al., 2000). However, the technique is invasive, delivers a large dose of particles to the alveolar region all at once, and may displace some of the protective lining of the lungs. While intratracheal instillation can provide important information regarding the relative potency of different particles, it likely artificially enhances inflammatory responses that may not be relevant to typical health effects of inhaled pollutants, and may confound mechanistic studies.

Exposure by inhalation is a more physiologically relevant approach. Inhalation systems require specialized expertise and equipment and large quantities of test materials for resuspension. The Environmental Health Centre at Health Canada has a unique inhalation facility that permits both whole-body chamber exposures and nose-only exposures (Vincent et al., 2001; Vincent et al., 1997a; Vincent and Adamson, 1995). The nose-only exposure system ensures controlled delivery of a known quantity of particles and/or ozone via inhalation, without the possibility of increased/altered ingestion by licking fur, or reduced

inhalation due to avoidance behaviour. A disadvantage of the nose-only approach is the potential for added stress to the animals. To minimize stress, all animals are trained in nose-only tubes for progressively longer periods of time over 5 days leading up to the exposure. All animals, including the air-exposed animal group, are on the nose-only exposure system for the same length of time (4 h), so that any stress arising from the method of exposure should not confound interpretation of pollutant effects.

1.8.4. Rationale for timing

Because of the rapidity of acute cardiovascular effects associated with air pollutants (Magari et al., 2001; Gold et al., 2000; Peters et al., 2001a), we wished to examine time-points shortly after exposure. However, there is also the potential for health effects that develop over a longer time scale (Zanobetti et al., 2003). We therefore initially chose to perform a time-course experiment covering a period of two weeks, including sampling at 2 and 24 h post-exposure to measure any acute effects. Based on our findings in the time-course study, subsequent experiments focussed on immediate and 24 h post-exposure time-points.

1.8.5. Rationale for gene expression studies

Although there is some evidence of intracellular endothelin storage (Russell and Davenport, 1999), the sustained increase in plasma ET-1 levels over a period of hours implies increased *de novo* production and secretion of the endothelin peptides, or a sustained decrease in ET_B receptor-mediated clearance, since the peptides have a half-life in plasma in the order of only a few minutes (Inoue et al., 1989b). Due to the complex effects that may contribute to an increase in peptide endothelin levels (increased production and/or cleavage,

reduced clearance, both in the lungs and at extrapulmonary sites), we chose to measure mRNA levels to identify changes contributing to increased endothelin levels. Conventional RT-PCR was used as an initial screen for pollutant effects on endothelin gene expression. However, real-time PCR is a more sensitive method to monitor perturbations of endothelin system gene expression, and so a real-time PCR assay was developed, validated, and implemented for further studies. Production of the mature endothelin isoforms was assessed by HPLC-fluorescence to verify release of the peptides into circulation.

The gene expression data presented is for the most part derived from mRNA extracted from whole tissues. This approach was chosen because inhaled pollutants may affect various anatomic regions and different cell types, and we wished to assess the broad impact of air pollutants on the tissue as a whole. An important limitation of this approach is that a decrease in expression in some cells may be masked by an increase in others, and vice versa, since results represent the sum of all mRNA in all cells in the tissue. Of course, the magnitude of increase and decrease will also be affected by such changes, as well as by the fraction of cells that are non-responding. The net effect of non-responding cells to the overall signal will be a reduction in the magnitude of the measured response. Hence, a null result does not preclude the possibility of increased expression in certain regions or cells. Moreover, small but statistically significant changes in gene expression at the level of the entire tissue may have physiological relevance since they represent a greater increase in expression in the subset of affected cells.

1.9. References

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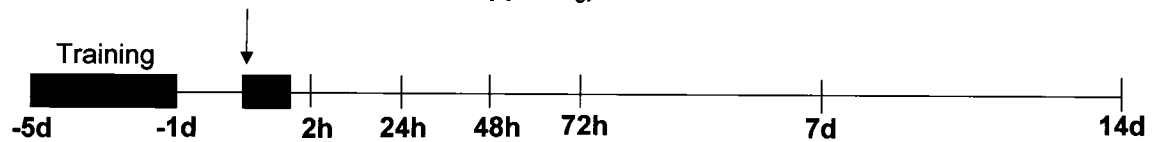
2. Thomson E, Goegan P, Kumarathasan P, Vincent R. (2004). Air Pollutants Increase Gene Expression of the Vasoconstrictor Endothelin-1 in the Lungs. *Biochim Biophys Acta* 1689: 75-82.

We conducted a time-course study to determine whether inhaled pollutants activate pulmonary endothelin system genes. Conventional reverse transcription polymerase chain reaction (RT-PCR) was used to measure mRNA levels of endothelin system genes in Fischer-344 rats at 2 h, and 1, 2, 3, 7 and 14 days after acute exposure to particulate matter and ozone (50 mg/m³ particles, 0.8 ppm O₃; Flowchart 1, p. 48). I was the lead author for the preparation of the manuscript, conducted the dosimetric modeling, analysed the data, and performed statistical analyses. Pat Goegan performed the RT-PCR work. Prem Kumarathasan and Renaud Vincent conceived and planned the experiment. Renaud Vincent was the principal investigator.

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Flowchart 1. Time-course experiment design. Fischer-344 rats were trained over a period of 5 days in nose-only tubes and then were exposed by inhalation to air or 49 mg/m³ EHC-93/0.8 ppm ozone (O₃) for 4 h. Groups of animals (n=3-9) were euthanized at 2 h, and 1, 2, 3, 7, and 14 days after exposure. RNA was extracted from lung tissue and analysed for endothelin system gene expression by conventional RT-PCR.

Exposure (Air or 49 mg/m³ EHC-93/ 0.8 ppm O₃)



Sample collection

RNA extraction

RT-PCR

**PreproET-1
PreproET-3
ECE-1
ET_A receptor
ET_B receptor**

Air Pollutants Increase Gene Expression of the Vasoconstrictor Endothelin-1 in the Lungs

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Running title

Endothelin System Genes and Air Pollutants

Keywords: air pollution, endothelin-1 (ET-1), endothelin-converting-enzyme-1 (ECE-1), ozone, particulate matter, cardiovascular disease

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Abstract

Inhalation of urban pollutants elevates the circulating levels of the vasoactive peptides endothelin (ET)-1 and ET-3 in rats. This effect could explain the association between episodic variations of urban pollutants and acute cardiopulmonary morbidity and mortality documented in epidemiological studies. Because the lungs are the primary source of circulating ET-1 and the main site of clearance from circulation, we investigated the response of endothelin system genes in the lungs of Fischer-344 rats after 4-hour nose-only inhalation of 0.8 ppm ozone plus 49 mg/m³ EHC-93 (Ottawa particles). The mRNA levels for preproET-1, preproET-3, endothelin-converting-enzyme (ECE)-1, and ET receptor subtypes A and B were determined at two hours, and 1, 2, 3, 7, and 14 days after exposure. The pollutants induced preproET-1 and ECE-1 ($p < 0.05$) after two hours, consistent with the notion of increased synthesis and conversion of the peptide ET1 in lung endothelial cells. PreproET-3 mRNA was down-regulated at 2 hours post-exposure ($p < 0.05$), and returned to control levels by 24 hours, indicating that induction of ET-3 in the lungs is not responsible for the sustained elevation of ET-3 in plasma reported after inhalation of pollutants. Our results indicate that lung endothelin system genes respond rapidly and transiently to inhalation of urban pollutants, consistent with the dynamics of urban pollutant health effects in the human population.

1. Introduction

Urban air pollution is known to cause cardiovascular and respiratory morbidity and mortality in the human population [1-5]. Individuals with chronic artery diseases and those with congestive heart failure are at particularly high risk of dying after an air pollution episode [2,3]. Acute cardiac effects have been reported less than three hours after increases of ambient fine particulate matter concentrations [6]. Although epidemiological studies have consistently indicated a significant association of air pollution with adverse health effects, it is only recently that laboratory evidence on plausible mechanisms of effects has begun to substantiate these trends. For example, we have shown that inhalation of urban particulate matter increases circulating levels of endothelin (ET)-1, ET-3 and blood pressure in experimental animals [7,8], and recent field work by others has confirmed that urban pollution is associated with elevated ET-1 in humans [9]. In addition to the hemodynamic changes documented after inhalation of urban particles in rats [8] and humans [10], instillation of urban particle suspensions in the lungs has been shown to elevate ET-1 and enhance arrhythmia in a myocardial infarction model [11] and to accelerate atherosclerosis in hyperlipidemic rabbits [12].

Endothelins are potent vasoconstrictor peptides involved in the homeostatic regulation of vascular smooth muscle tone [13]. The pulmonary endothelium is the main source of circulating endothelin, as well as the principal site for clearance of the peptide from circulation [14]. The endothelin family is composed of three 21-amino acid isomers (ET-1, ET-2, ET-3), each encoded by distinct genes (preproET-1, preproET-2, and preproET-3) [15]. The synthesized preproendothelin peptides are cleaved by specific endoproteases to

38- to 41- amino acid precursors (referred to as the big endothelins) that are themselves cleaved by endothelin-converting-enzymes (ECE) to produce the mature vasoactive peptides [16]. Endothelins act through specific G-protein coupled receptors, the ET_A receptor and ET_B receptor [17]. Binding of endothelins to ET_A and ET_B receptors on smooth muscle cells leads to vasoconstriction, whereas binding to ET_B receptors on endothelial cells stimulates the release of nitric oxide, leading to relaxation of smooth muscle cells and vasodilation (reviewed in [18]). Endothelins have a very short half-life (order of minutes) in plasma [19,20] and are cleared from circulation in part through binding to the ET_B receptor in the caveolae of lung capillary endothelial cells [17,21]. *De novo* synthesis of ET_B receptors appears to be required to maintain ET_B receptor density at the cell surface [17]. Binding of ET-1 to the ET_B receptor of endothelial cells results in internalization and targeting of the receptor-ligand complex to the lysosome [17]. Since the ET_B receptor does not appear to be recycled to the cell surface, replacement of ET_B receptors requires transcriptional activation of the cognate gene.

Increased circulating levels of ET-1 are associated with many cardiovascular diseases, including congestive heart failure, hypertension, and atherosclerosis (reviewed in Ref. [22]). Increased pulmonary spill-over of ET-1 from up-regulation of expression associated with decreased clearance through down-regulation of lung capillary ET_B receptor are thought to be fundamental changes in the progression of congestive heart failure [23,24]. Due to their important role in the regulation of cardiovascular function and their involvement in the pathogenesis of cardiovascular disease, an alteration of circulating endothelin levels offers a plausible biological mechanism to account for cardiovascular health effects of urban pollutant inhalation. We propose that sustained elevation of the steady-state plasma

concentrations of ET-1 and ET-3 levels following exposure to urban particulate matter could result from at least three possible distinct effects in the lungs, or any combination of those effects: 1) up-regulation of the cognate genes, 2) increased rate of conversion of the precursor peptide by elevated ECE expression and activity, and 3) decreased clearance of the mature endothelins from blood plasma through down-regulation of the ET_B receptor.

In a previous study, intratracheal instillation of 5 mg EHC-93 (Ottawa urban particles) in saline into the lungs of rats caused a decrease in lung ET-1 mRNA and angiotensin converting enzyme (ACE) mRNA, and an increase of inducible nitric oxide synthase (iNOS) mRNA 48 hours after exposure [25]. The particles also increased tumour necrosis factor (TNF)- α and macrophage inflammatory protein (MIP)-2 in alveoli [25]. Down-regulation of ET-1 and ACE are indicative of endothelial injury [26,27], and it is possible that the down-regulation followed an earlier induction of the genes. To our knowledge, there are at present no data on lung ET-1 mRNA in animals following inhalation of urban pollutants. In order to gain a better understanding of the relationship between pulmonary deposition of air pollutants and regulation of the endothelin system genes, rats were exposed by nose-only inhalation to ozone and the urban particles EHC-93. The mRNA levels for ET-1, ET-3, ECE-1, ET_A receptor, and ET_B receptor were examined over the course of 14 days. Our results indicate that the lung endothelin system responds to inhalation of urban pollutants with a rapid and transient increase of mRNA for preproET-1 and ECE-1, consistent with the notion of increased rates of *de novo* synthesis and conversion of the potent vasoactive peptide ET-1. The data substantiate the biological basis for the epidemiologic association between air pollutants and cardiovascular morbidity and mortality.

2. Materials and methods

2.1. *Animals*

Fischer-344 adult male rats (200-250 g) were obtained from Charles River in filter boxes at least two weeks prior to the experiments (St. Constant, Québec, Canada). The animals were housed in individual plexiglass cages on wood-chip bedding under HEPA-filtered air and a 12-hour dark/light cycle. Food and water were provided ad libitum. All experimental protocols were reviewed by the Animal Care Committee of Health Canada.

2.2. *Respirable urban particulate matter*

The urban ambient particles EHC-93 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system of the Environmental Health Centre at Tunney's Pasture in Ottawa, Canada, and cleaned through a 36 μm mesh to remove spores and debris. The particles have a median physical diameter on the order of 0.5 μm . The chemical composition, biological reactivity of the particles in cell culture models, and applications in inhalation studies have been described elsewhere [7,8,28,29].

2.3. *Inhalation exposures*

Animals were exposed to ozone and EHC-93 urban particles in a nose-only inhalation exposure system as described previously [29]. The rats were trained in nose-only exposure tubes over five consecutive days, and then exposed once for four hours to clean air or 0.8 ppm ozone plus 49 mg/m^3 EHC-93. The particle size distribution of resuspended EHC-93 in our flow-past nose-only exposure system is multimodal, with two respirable modes at 1.3 μm (aerodynamic diameter, D_{AE}) and 3.6 μm D_{AE} that together contained 55% of the mass of the

aerosol, and a non-respirable mode at 15 μm D_{AE} that contained 45% of the mass [8]. Rats were euthanized by administration of sodium pentobarbital (60 mg/kg i.p.) at 2 hours, and 1, 2, 3, 7, and 14 days post-exposure. The lungs were rapidly excised, diced, flash frozen in liquid nitrogen, and stored at -80°C .

2.4. RNA extraction

Total RNA was isolated from frozen lung samples using the TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) according to the manufacturer's instructions at a ratio of 0.2 g tissue per 1.5 mL TRIzol. The resulting RNA pellet was air-dried, resuspended in RNase-free Tris-EDTA buffer, and frozen at -80°C . Total RNA concentration was determined at 260 nm using a Spectronic photodiode array spectrophotometer (Milton Roy, Rockford, IL, USA).

2.5. Reverse transcription/polymerase chain reaction (RT/PCR)

The following primer sequences for preproET-1, preproET-3, ECE-1, ET_A receptor, ET_B receptor [30] and β -actin [31] were used:

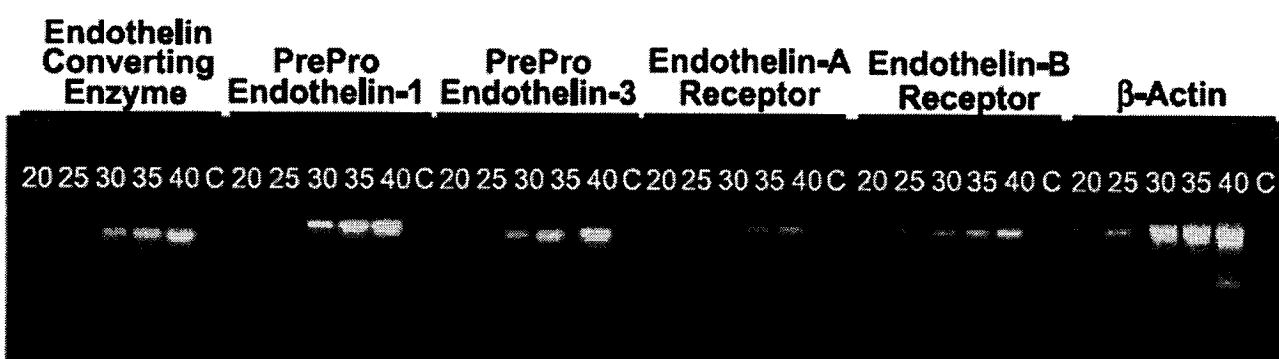
ET-1	forward 5'-GCTCCTGCTCCTCCTTGATG-3'	reverse 5'-CTCGCTCTATGTAAGTCATGG-3'
ET-3	forward 5'-GCACTTGCTTCACTTATAAGG-3'	reverse 5'-CAGAAGCAAGAAGCATCAGTTG-3'
ECE-1	forward 5'-CGTAGCGATAGTCTTAGCAC-3'	reverse 5'-GTGCCACACCAAACTACAG-3'
ET_A	forward 5'-TTCGTCATGGTACCCTTCGA-3'	reverse 5'-GATACTCGTTCCATTCATGG-3'
ET_B	forward 5'-TTCACCTCAGCAGGATTCTG-3'	reverse 5'-AGGTGTGGAAAGTTAGAACG-3'
β -actin	forward 5'-CTGATCCACATCTGCTGGAAGGTGG-3'	reverse 5'-ACCTTCAACACCCCAGCCATGTACG-3'

RT-PCR was carried out on a Thermolyne Amplitron II Thermal Cycler (Barnstead Thermolyne, Dubuque, IA, USA) using GeneAmp reagents (Applied Biosystems, Mississauga, Ontario, Canada). The reverse transcription reaction mix consisted of 5 mM MgCl₂, 1X PCR buffer II, 1 mM each dNTP, 1 unit/μL RNase inhibitor, 2.5 units/μL MuLV reverse transcriptase and 2.5 μM random hexamers. Six hundred (600) ng of total RNA were used in each reaction. Reverse transcription was carried out at 42°C for 45 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. The PCR reaction mix consisted of 2 mM MgCl₂, 1X PCR buffer II, 1.25 units/reaction AmpliTaq gold polymerase and 0.5 μM of each primer. PCR cycling involved a hot start at 95°C for 10 minutes, followed by 25 cycles of the following: 95°C for 1 minute (denaturation); 50°C for 1 minute (annealing); and 72°C for 1 minute (extension). PCR reactions were stopped in early-mid log phase, well before the plateau phase of the reaction (Fig. 1).

2.6. Visualization of the PCR products

PCR products were separated by electrophoresis on 1% agarose gels containing 1:10000 dilution of SYBR Green I (Molecular Probes, Eugene, OR) for 1 hour at an applied voltage of 80 volts. After electrophoresis, gels were scanned with a Storm 840 (Amersham BioSciences, Baie D'urfé, Québec, Canada) and the band intensities were quantified using the ImageQuant software package (Molecular Devices, Sunnyvale, CA, USA). The expression of each gene of interest was normalized to β-actin expression.

Figure 1. Composite fluorescence image (SYBR Green I) of RT-PCR products for the endothelin system genes and β -actin in the lungs of naïve Fischer-344 rats as a function of number of cycles. One major product was observed for each primer set, and all subsequent analyses were conducted at 25 cycles, ensuring quantification at early to mid-exponential phase of the amplification.



2.7. Dose modeling

Deposition of particles in our experimental animals and for a plausible human-exposure scenario were calculated using the Multiple Path Particle Deposition software (MPPDep v1.11, RIVM Publications, Bilthoven, The Netherlands) essentially as described before [8]. Model assumptions for rats were a tidal volume of 2.1 mL, a breathing frequency of 102 min⁻¹, strict nasal breathing, and an alveolar surface area of 0.34 m². Modelled deposition rates using MPPDep were estimated at 0.081 for the 1.3 µm D_{AE} mode (20% of aerosol mass), 0.047 for the 3.6 µm D_{AE} mode (35% of aerosol mass), and 0.000 for the 15 µm D_{AE} mode (45% of aerosol mass). Using these parameters, the pulmonary compartment dose in the rats was estimated at 83 µg, or 24 ng/cm² alveolar surface area. Model assumptions for a plausible human environmental exposure scenario were an average tidal volume of 875 mL and an average breathing frequency of 16 min⁻¹ over the entire day, oronasal breathing, and an alveolar surface area of 54 m². Deposition rates for the 0.05-10 µm D_{AE} size range of urban particulate matter with size cut-off of 10 µm D_{AE} (PM₁₀) containing a nucleation mode at 0.05 µm D_{AE} (5% of mass), a condensation mode at 0.2 µm D_{AE} (25% of mass) and coarse mode at 5 µm D_{AE} (70% mass) were taken as 0.20 for all modes [32]. Using these parameters, and assuming a 24-hour exposure to an average PM₁₀ concentration of 175 µg/m³ [33], a reference pulmonary compartment dose in humans was estimated as 706 µg, or 1.3 ng/cm² alveolar surface area.

Similarly, the peak centriacinar dose of ozone in the lungs of rats (68 x 10⁻⁶ µg of O₃/cm²/hour per µg of ambient O₃/m³) and humans (30 x 10⁻⁶ µg of O₃/cm²/hour per µg of

ambient O_3/m^3) can be estimated by reference to biomathematical models [34]. Exposure of our rats to 0.8 ppm ozone ($1570 \mu\text{g}$ of O_3/m^3) over 4 hours should have translated into a total centriacinar peak dose of 427 ng of O_3/cm^2 . Exposure of a human subject to 0.12 ppm ozone ($236 \mu\text{g}$ of O_3/m^3) for 12 hours, followed by 0.06 ppm ozone ($118 \mu\text{g}$ of O_3/m^3) for 12 hours would lead to a total daily centriacinar peak dose estimated at 127 ng of O_3/cm^2 [29].

2.8. Statistical Analyses

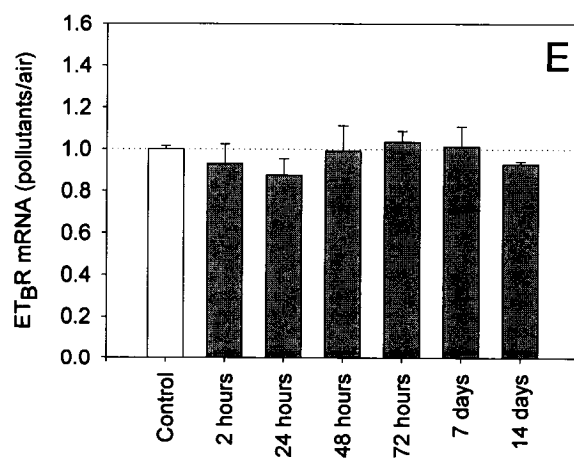
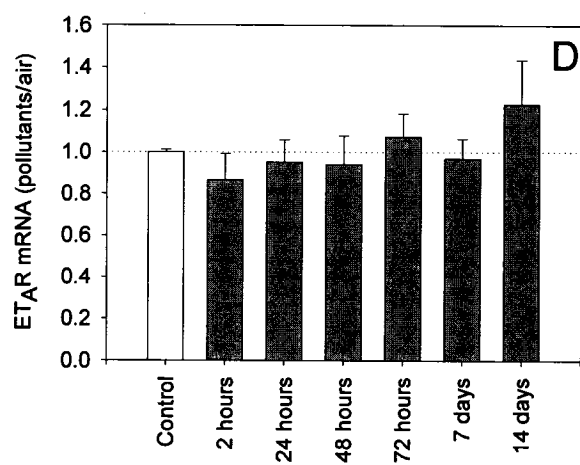
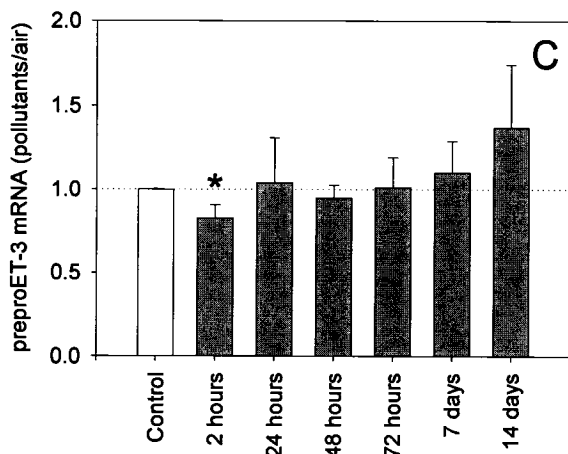
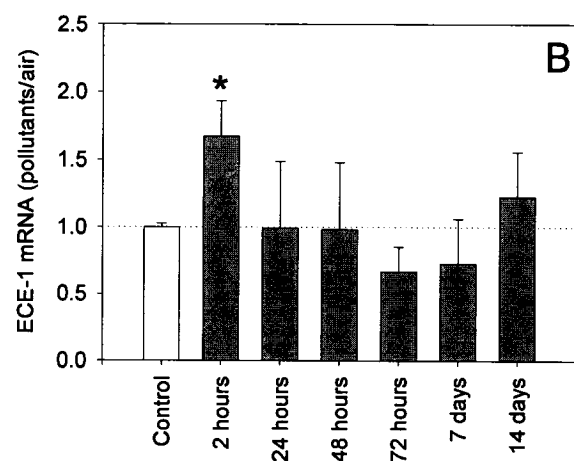
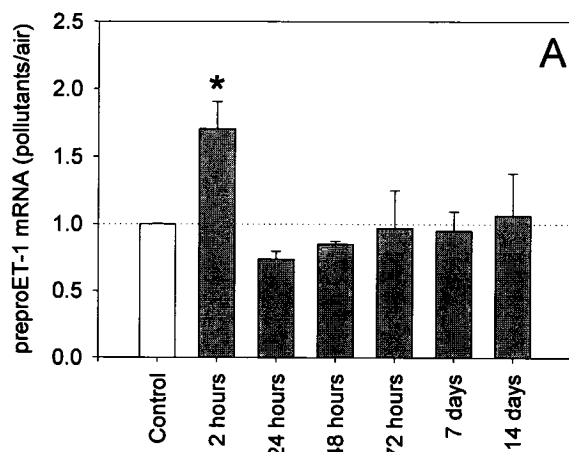
Expression data in pollutant-exposed animals at each of the recovery time-points were expressed as the ratio of the mean expression of treated animals $\pm$ S.E. over the time-matched air-control mean. The control value for statistical comparison was the aggregated data for all air control animals at all time points ($n = 30$). The effect of treatment on the expression of each gene of interest was then tested by one-way ANOVA followed by Dunnett's method to elucidate the pattern of significant effects, with $\alpha = 0.05$ (SigmaStat 2.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

PreproET-1 mRNA was elevated approximately 1.7-fold ($p<0.05$) two hours after exposure to the pollutants (Fig. 2A). Expression appeared to be reduced after recovery in clean air for 24-48 hours ($p>0.05$), consistent with the previous observations by Ulrich et al. [25], but returned to control levels by 72 hours. Examination of gene expression over a period of two weeks did not reveal sustained or delayed modulation of ET-1 mRNA expression in the animals exposed to the pollutants. Endothelin-1 is produced from the cleavage of the precursor peptide big ET-1 by endothelin-converting-enzyme (ECE)-1. Inhalation of pollutants here also rapidly induced ECE-1 mRNA expression ($p<0.05$) two hours after exposure (Fig. 2B). The upstream regions of the promoter sites of ECE-1 [35] and ET-1 [36,37,38] share several regulatory factor motifs, including AP-1/*JUN*-binding elements, acute phase responsive elements, and NF- κ B responsive motifs, and may explain co-regulation of these two genes in our model. The changes in regulation of ET-1 and ECE-1 are in line with our previous observation of increased steady-state plasma ET-1 following inhalation of urban particles [7,8], and with the lungs being the principal source of circulating ET-1 [14,39].

In contrast, preproET-3 mRNA was decreased ($p<0.05$) two hours after exposure, returning to control values after 24 hours recovery of the animals in clean air (Fig. 2C). Interestingly, this down-regulation of lung preproET-3 mRNA in the present report does not correlate with the elevated circulating ET-3 levels which have previously been documented in the plasma of rats between two and 48 hours after inhalation of urban particles [8]. Less is known about the regulation of ET-3 and the source of the peptide in the blood, but our

Figure 2. Ratio of expression of the endothelin system genes in the lungs of pollutant-exposed rats over air control rats as reflected in the abundance of mRNA and quantified by fluorimetric quantification of the RT-PCR products. Data are mean $\pm$ S.E for n = 30 (air controls, all time-points aggregated), n = 9 (two hours post-exposure), and n = 3 (all other time-points). Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison procedure ($\alpha = 0.05$). A) Preproendothelin-1. Two hours post-exposure vs air control, $p < 0.05$. B) Endothelin converting enzyme-1. Two hours post-exposure vs air control, $p < 0.05$. C) Preproendothelin-3. Two hours post-exposure vs air control, $p < 0.05$. D) Endothelin A receptor. E) Endothelin B receptor.



present data indicate that elevation of plasma ET-3 after inhalation of pollutants may not be due to increased *de novo* synthesis in the lungs. In addition to the lungs, ET-3 mRNA has been detected in the central nervous system, kidney, small intestine, stomach, and spleen [40]. The lungs, kidneys and liver are sites of ET-3 clearance [19], and diseases involving these organs (e.g. liver cirrhosis) may be associated with increased plasma ET-3 [41].

Binding of ET-1 and ET-3 to the ET_B receptors of endothelial cells is a major pathway of clearance of the circulating peptides [17,23], and ET_B receptor density appears to be controlled at the transcript level [17]. Impact of the pollutants on endothelial ET_B receptor expression could therefore affect plasma levels of the endothelin peptides. Binding of ET-1 to the ET_A receptor of smooth muscle cells leads to vasoconstriction and bronchoconstriction, and up-regulation of the ET_A receptor in target cells of the lungs could amplify the physiological impacts of the activation of endothelin system genes after inhalation of pollutants. We did not detect statistically significant changes of ET_A receptor (Fig. 2D) and ET_B receptor (Fig. 2E) expression over the period of 14 days after exposure to the pollutants. However, because of the potential large impact of slower clearance on the steady-state circulating peptide levels, the small decrease of ET_B receptor expression ($p>0.05$) seen at 24 hours post-exposure warrants further investigation. Site-specific analyses of micro-dissected samples using quantitative real-time RT-PCR, in situ PCR, or in situ hybridization may be more sensitive to focal changes in gene expression [42].

As with any biochemical changes, interpretation of molecular data in the lungs during acute lung injury can be confounded by the extensive cellular changes due to epithelial and interstitial repair proliferation and inflammatory infiltration in the septum and alveolar

lumen. The kinetics of repair and inflammation in rat lungs after an acute injury is well established and is maximal between 24 and 48 hours (for example, [43,44]). We report here clear inductions of ET-1 and ECE-1 mRNA levels as early as 2 hours after termination of exposure, prior to the maximum inflammatory cell influx in the alveoli and interstitium, and prior to epithelial and interstitial cell proliferation that are known to occur 24-48 hours after acute lung injury. Therefore, elevation of preproET-1 and ECE-1 mRNA at 2 hours post-exposure was likely due to up-regulation in pulmonary cells rather than an import of the excess transcript by infiltrating inflammatory cells. Similarly, the decrease of preproET-3 at 2 hours post-exposure is unlikely to have resulted from dilution of transcript levels after a large influx of cells that do not express the peptide. The relative contribution of inflammatory cells to the mRNA changes at the 24-48 hours time-points is not clear, but macrophages are known to express and secrete ET-1 [7].

In short, our findings verify that inhaled urban pollutants up-regulate ET-1 and ECE-1 mRNA expression in the lungs, and indicate that lung ET-1 and ET-3 are differentially regulated at the transcript level by inhaled pollutants. It remains possible that changes in circulating levels of ET-3 after inhalation of urban pollutants depend mainly on reduced clearance rather than increased production by the lungs, but direct data are lacking. To the best of our knowledge, this is the first report of an acute impact of air pollutant inhalation on gene expression of the potent broncho-active, vasoactive, and mitogenic peptide ET-1 in the lungs. The modulation of the pulmonary endothelinergic system by inhaled pollutants may have profound human health impacts. We have verified that human subjects exposed to ozone and urban particulate matter exhibit a constriction of the brachial artery [10], consistent with a pressor effect of circulating ET-1 [8]. A recent study has found that

children in south west metropolitan Mexico city have 25% higher plasma ET-1 levels as compared to children in low polluted areas [9]. The magnitude of this change is known to be associated with an unfavourable prognosis in congestive heart failure patients [45] or after myocardial infarction [46]. Transcriptional activation of preproET-1 and ECE-1 has been found to be linked with chronic inflammation and may be important in atherosclerotic plaque formation [47]. Repeated exposure to urban pollutants and the resulting chronic inflammation and endothelin system activation may therefore increase the likelihood of plaque formation and enhance progression of atherosclerosis in susceptible individuals [12]. It is noteworthy that the response of the lung endothelinergic system within a few hours of exposure of animals to the pollutants in our study is in line with epidemiological evidence of adverse cardiac effects in humans within three hours after exposure to occupational and ambient air pollutants [6].

Dosimetric relevance of the present experiment to an environmental exposure should be evaluated after scaling doses of pollutants within the lungs of rats and humans (see materials and methods, 2.7, for calculations). The ratio of experimental dose within the respiratory compartment of the rats (estimated at 24 ng particles/cm² lung alveolar surface area) to the dose calculated for a plausible human exposure scenario (estimated at 1.3 ng particles/cm²) was only 18 fold (Table 1). Similarly, the ratio of the centriacinar ozone dose in our animals (427 ng O₃/cm² alveolar duct surface area) to the estimated internal dose in a human subject under a plausible exposure scenario (127 ng O₃/cm² alveolar duct surface area) was only 3-fold (Table 2). For ethical reasons, nose-only exposures should be kept to a minimum duration, and therefore the dose-rate in our study was higher than for an

Table 1. Particle Deposition Model

Model Parameters ^A		Rat	Human
Tidal volume (mL)		2.1	875
Breathing rate (min ⁻¹)		102	16
Inhalation		Nasal	Oronasal
Alveolar surface area (m ²)		0.34	54
Exposure (hours)		4	24
Particle concentration (mg/m ³)		49 ^B	0.175 ^C
Particle distribution assumption		multimodal	PM ₁₀
Mode 1	Size (μm D _{AE})	1.5	0.05
	Mass fraction	0.20	0.05
	Deposition rate	0.081	0.20 ^D
Mode 2	Size (μm D _{AE})	3.6	0.2
	Mass fraction	0.35	0.25
	Deposition rate	0.047	0.20 ^D
Mode 3	Size (μm D _{AE})	15	5
	Mass fraction	0.45	0.70
	Deposition rate	0.000	0.20 ^D
Respiratory compartment dose (μg)		83	706
Surface relative dose (ng/cm ⁻²)		24	1.3
Dose rate (ng/cm ⁻² /hour)		6	0.05

^A From [8] unless otherwise indicated

^B Particle concentration in present study

^C High-end average daily PM₁₀ concentration in Mexico City [33]

^D Alveolar deposition rates in human adapted from [32]

Table 2. Ozone Deposition Model

Model Parameters ^A	Rat	Human ^B	
Ozone concentration (ppm)	0.8	0.12	0.06
Ozone concentration ($\mu\text{g}/\text{m}^3$)	1570	236	118
Exposure (hours)	4	12	12
Deposition rate ^C ($10^{-6} \mu\text{g O}_3/\text{cm}^2/\text{hour per } \mu\text{g O}_3/\text{m}^3$)	68	30	30
Centriacinar dose ($\text{ng O}_3/\text{cm}^{-2}$)	427	85	42
		127 ^D	
Centriacinar dose rate ($\text{ng O}_3/\text{cm}^{-2}/\text{hour}$)	107	7.1	3.5
		5.3 ^E	

^A Model parameters from [29]

^B Scenario is 12 hours at 0.12 ppm O_3 and 12 hours at 0.06 ppm O_3

^C Adapted from [34]

^D Centriacinar dose ($\text{ng O}_3/\text{cm}^{-2}$) for 24 hours

^E Average centriacinar dose-rate ($\text{ng O}_3/\text{cm}^{-2}/\text{hour}$) over 24 hours

environmental exposure spread over a 24-hour period. Nevertheless, the ratios of dose-rates for our animal-to-human comparison were approximately 100-fold for particles, and 20-fold for ozone. From the standpoint of evaluation toxicology, the internal depositions as well as the dose-rates of the pollutants in the current study are relevant to human exposures, once uncertainty factors are considered. These include the possible decay of the potency of EHC-93 by comparison to fresh particles, the known interspecies differences in sensitivity to air pollutants (with humans being more responsive than rats [48,49]), and the inter-individual differences in sensitivity within the human population (such as increased sensitivity of individuals with congestive heart failure or atherosclerosis [2,3,12]).

4. Conclusion

In summary, we have shown that inhalation of common urban pollutants in rats causes the coordinated elevation of mRNA for preproET-1 and ECE-1. The observations are consistent with previous reports of plasma ET-1 increases following pollutant inhalation [7,8]. Elevation of ET-1 and ECE-1 mRNA was early and transient, consistent with the dynamics of urban pollutant health effects in the human population, and indicated that regulation of the genes preceeded the known dynamics of inflammation and repair. The combination of pollutants (ozone and urban particulate matter) did not appear to result in sustained or delayed regulation of ET-1 over two weeks post-exposure. While urban particles are known to cause rapid and sustained increases of ET-3 in rats [8], air pollutants in this report decreased lung steady state mRNA levels for preproET-3. The data suggest that the lungs may not be the source of the excess circulating ET-3 after exposure to pollutants. Together, our data further strengthen the biological evidence for the association between ambient air pollutants and increased acute cardiopulmonary morbidity and mortality in the human population.

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3. Thomson E, Vincent R. (2005). Reagent Volume and Plate Bias in Real-Time PCR.

Anal Biochem 337(2): 347-350.

In order to pursue dose-response assessment of pollutant effects on endothelin system gene expression, establishment of a more sensitive method of measurement was required. Using a primer set designed and validated for real-time PCR, the real-time PCR assay was optimized for a 96-well plate format, and the issue of plate bias was addressed. I designed and performed all of the experiments and data analysis, and was the lead author for preparation of the manuscript. Renaud Vincent was the principal investigator.

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Reagent Volume And Plate Bias In Real-Time PCR

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Running title

Reagent volume and real-time PCR

Keywords: real-time polymerase chain reaction (PCR), SYBR Green I, 96-well plate, reproducibility, mRNA expression, quantification

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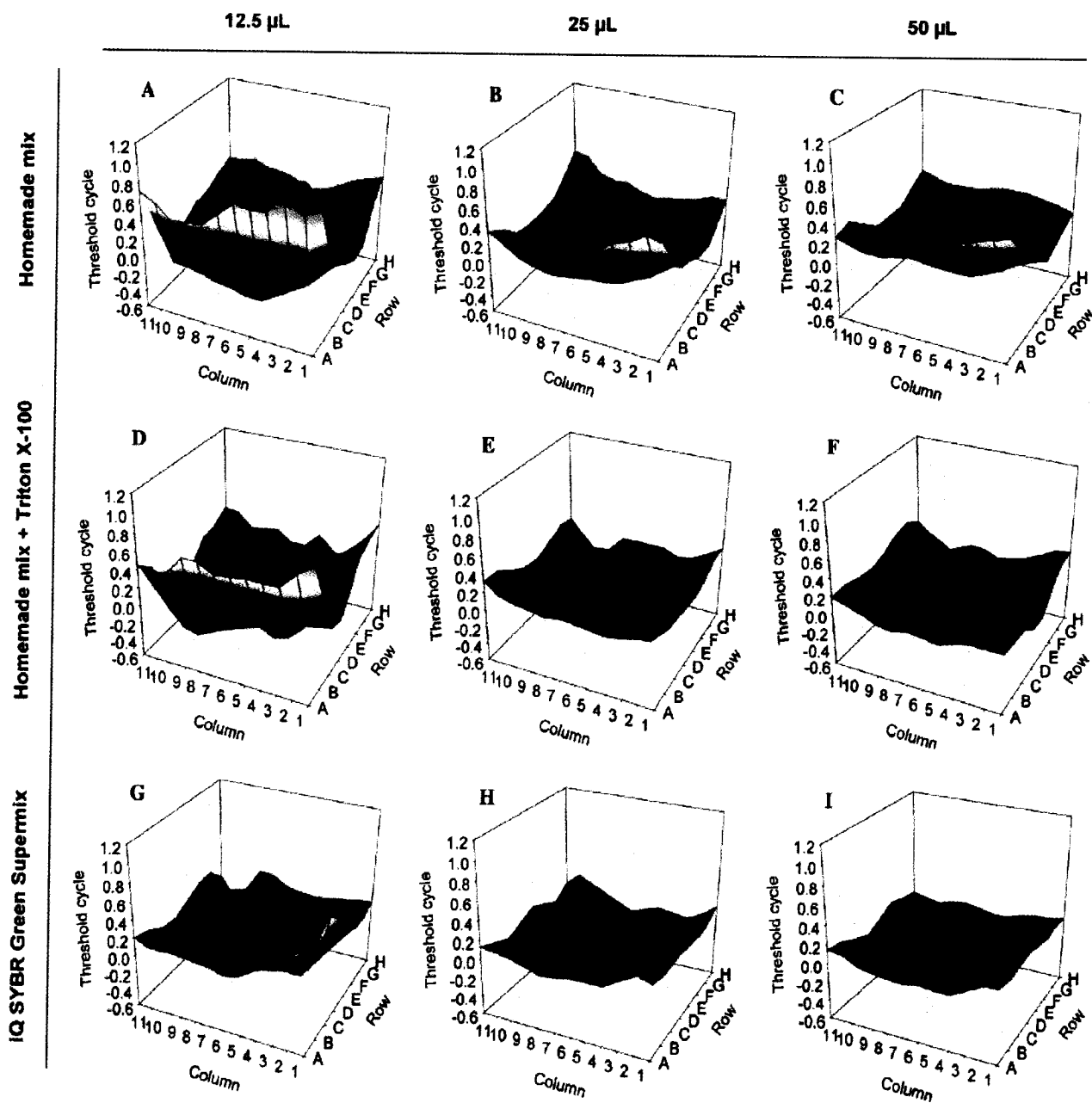
When optimal conditions are employed, real-time polymerase chain reaction (PCR) is a sensitive and accurate technique enabling the quantification of low copy number transcripts [1]. However, as with conventional PCR, small variations in initial reaction conditions are amplified exponentially and can significantly affect results [2]. Uniform reaction conditions are therefore essential to achieve accuracy and reproducibility during transcript quantification. Reagent costs are considerable, and in a high-throughput setting a reduction in the reagent volume used in each reaction would significantly reduce the cost of real-time PCR. However, the effect that reduced volume has on the accuracy of results in a plate-based system, has not, to our knowledge, been examined. Here we show that lower reagent volumes can reduce reproducibility by enhancing a bias in results across a plate.

Total RNA was isolated from confluent A549 lung epithelial type II cells (ATCC, Manassas, VA) using the TRIzol method (Invitrogen Canada Inc., Burlington, ON) and quantified using the RiboGreen Reagent and Kit (Molecular Probes Inc., Eugene, OR, USA). RNA was reverse transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, ON) according to the manufacturer's instructions. Primers for human endothelin-1 (NM_001955), the gene encoding a potent vasoconstrictor peptide relevant in a number of cardiovascular pathologies [3], were designed using Vector NTI (Informax), and double-desalted primers were ordered from Invitrogen. Annealing conditions were optimized, and high reaction efficiency (90%) was confirmed using a dilution series of A549 cDNA over a 80 ng to 0.625 ng range. We compared three reagent mixes, a homemade reagent mix, the same mix with 1.5% Triton X-100 (Fisher Scientific Ltd., Ottawa, ON) and a commercial mix (iQ SYBR Green Supermix, Bio-Rad Laboratories

Canada Ltd., Mississauga, ON) at three volumes: 50 μ L, 25 μ L, and 12.5 μ L. Master mixes of the reagents were prepared to minimize differences in reagent composition. The composition of the homemade SYBR Green reagent mix was as follows: 3 mM MgCl_2 , 200 μ M of each dNTP, 0.025 U/ μ L AmpliTaq Gold, 1X GeneAmp PCR buffer (Applied Biosystems), a 1:100 000 dilution SYBR Green I, and water. An equal concentration of cDNA (0.67 ng/ μ L) and 200 nM of each primer were added to each reagent mix. To compare the effect of volume reduction on the PCR reaction, 12.5 μ L or 25 μ L volumes were pipetted into alternating wells on the same plate as 50 μ L volumes, thus ensuring that conditions were equivalent for both volumes. This procedure was conducted on separate plates for both the homemade SYBR Green reagent mixes and the commercial iQ SYBR Green Supermix. To compare the homemade mix to the commercial mix, equal volumes of each reagent mix were pipetted into alternating wells on the same plate. All reactions were performed in the iCycler iQ spectrofluorometric thermal cycler (Bio-Rad) using the 96-well heating block module as follows: 40 cycles of 15 s at 95°C (denaturation); 15 s at 62°C (annealing); and 30 s at 72°C (elongation). Fluorescence was monitored at every cycle during the elongation step by the optical unit of the iCycler and associated software (Bio-Rad). The background, threshold value, and threshold cycles (the cycle at which the amplification curve crosses the threshold value) were determined automatically by the iCycler iQ software. SigmaPlot (SPSS Inc., Chicago, Illinois) was used for graphical display of results averaged from replicate plates for each reagent mix at the three volumes tested.

The results indicated a plate bias in which the amplification curves resulting from reactions in wells located on the periphery of the plate crossed the threshold later than those located closer to the middle (Fig. 1). The effect was most pronounced for the 12.5 μ L volume

Figure 1. Threshold cycle distribution across 96-well plates for 12.5 μ L (A,D,G), 25 μ L (B,E,H) and 50 μ L (C,F,I) of assay volume, using the homemade SYBR Green mix (A,B,C), the homemade mix plus Triton X-100 (D,E,F) or the iQ SYBR Green Supermix (G,H,I) reagents. The threshold cycle is expressed as the difference between the value for an individual well and the global mean of all threshold cycles. All plots represent the average of 2-4 individual PCR plates.



(Fig. 1A,D,G), followed by the 25 μ L volume (Fig. 1B,E,H) and the 50 μ L volume (Fig. 1C,F,I) of each reagent mix. Similarly, the standard deviation among wells and the difference between results from inner and outer wells increased at lower volumes (Table 1). Importantly, both homemade and commercial mixes exhibited similar standard deviation among inner wells, indicating that the homemade mix was of sufficient quality to permit reproducible results in the region of the plate least affected by the plate bias. However, the commercial reagent mix appeared to be less sensitive to the plate bias than the homemade mix (compare Fig. 1A-C to G-I), with the 50 μ L volumes of the commercial preparation virtually abolishing the bias (Fig. 1I). Reagent volume also appeared to affect the later progression of the reaction, as inspection of the amplification curves revealed greater spread among the replicates at the lower volumes compared to the 50 μ L volume, even when using the commercial reagent mix (see Supplemental material, Fig. 1). Plates were centrifuged briefly following the PCR run and the content of each well was weighed. Reagent volumes were uniform across the plate, indicating that the plate bias was not due to reagent losses or pipetting error (data not shown).

We observed increased droplets above the level of the reagent mix in the wells located on the edge of the plate, possibly due to increased evaporation and condensation in the outer wells due to slight differences in temperature above the heated block. Addition of Triton X-100 (1.5% v/v, [4]) to our homemade mix to wet well surfaces and help prevent beading improved performance by reducing the plate bias (Fig. 1D-F), suggesting that reagent composition can be altered to lessen to some extent the plate bias. The iCycler employs a solid block to uniformly heat all wells, and a heated roof to minimize evaporation (iCycler product literature, Bio-Rad). The 96-well PCR plate sits on the heated block such that the bottom half of each well (a 100 μ L volume) is encircled by the metal block, ensuring uniform temperature cycling conditions for the

Table 1. Comparison of the effect of reagent volume on average threshold cycle (Ct), standard deviation (SD), and coefficient of variance (CV) for replicate real-time PCR reactions across a plate using a homemade SYBR Green mix, the homemade mix plus Triton X-100, and iQ SYBR Green Supermix (Bio-Rad). All values represent the average of 2-4 independent plates.

	12.5 μ L			25 μ L			50 μ L		
	Average Ct	SD	CV (%)	Average Ct	SD	CV (%)	Average Ct	SD	CV (%)
Homemade SYBR Green mix									
All wells	23.76	0.42	1.77	24.16	0.27	1.12	23.83	0.18	0.76
Outer wells ¹	24.13	0.46	1.91	24.39	0.27	1.09	23.96	0.20	0.83
Inner wells ²	23.50	0.15	0.64	23.95	0.10	0.42	23.72	0.09	0.38
Difference Outer-Inner	0.63			0.44			0.23		
Homemade mix plus 1.5%Triton X-100									
All wells	23.42	0.39	1.67	23.38	0.17	0.73	23.40	0.14	0.60
Outer wells ¹	23.71	0.42	1.77	23.51	0.16	0.68	23.46	0.16	0.68
Inner wells ²	23.18	0.24	1.04	23.28	0.13	0.56	23.32	0.09	0.39
Difference Outer-Inner	0.53			0.23			0.15		
iQ SYBR Green Supermix									
All wells	22.85	0.25	1.09	23.02	0.13	0.56	22.92	0.09	0.39
Outer wells ¹	23.03	0.28	1.22	23.09	0.11	0.48	22.96	0.11	0.48
Inner wells ²	22.70	0.17	0.75	22.94	0.10	0.44	22.90	0.07	0.31
Difference Outer-Inner	0.33			0.15			0.06		

¹ Outer wells are defined as rows A and H, and columns 1 and 12.

² Inner wells are defined as all but the outermost two rows and columns.

PCR reagents. However, the top half of the plate rises above the solid block, and is therefore exposed to the air. There is presumably a slight loss in the temperature of the outer wells, since they are more exposed to the surrounding air than are the wells within the plate. Increased evaporation and condensation of water on the walls of outer wells will affect reagent concentration in the reaction mix in the bottom of the wells, which may well affect the kinetics of the reaction, or fluorescence readings. Lower reagent volumes would be expected to be more sensitive to fluctuations in conditions and thus more subject to interference.

We show here that using low reagent volumes as a cost saving measure may introduce variability in the data, and eventually reduce power within statistical analyses, which can only be compensated by increasing sample size. Furthermore, differences in reagent composition may affect the degree of plate bias. The bias detected in this study at the lowest volume tested would correspond to an average 60% difference in gene expression if comparing samples on the outer wells to samples on inner wells. Such a bias can have relevance in gene expression studies. For example, inhalation of air pollution in experimental animals results in a 60-70% increase in mRNA expression of lung endothelin-1, associated with physiologically relevant changes in the peptide [5]. Therefore, for experiments using low reagent volumes, it may be advisable to use the centre wells only and avoid the peripheral wells. For experiments with low sample numbers, the center wells can be used rather than distributing samples on one side of the plate or evenly across the plate.

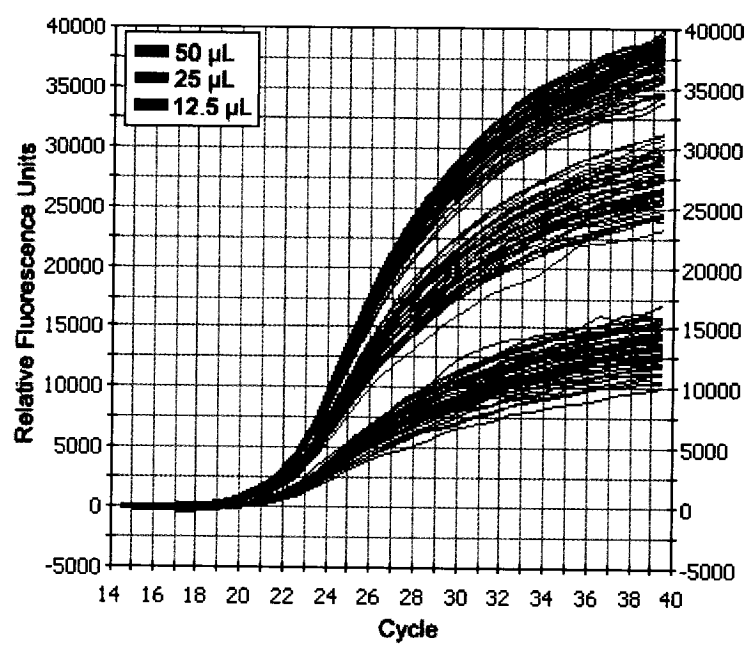
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Supplementary Material, Figure 1. Composite image of the amplification curves for 48 replicate reactions for a 12.5 μL , 25 μL or a 50 μL reaction volume of iQ SYBR Green Supermix.



4. Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. (2005). Differential Regulation of Lung Endothelin System Genes by Urban Particulate Matter and Ozone. *Tox Sci* 88: 103-113.

Having established and validated a sensitive and reproducible real-time PCR assay, I proceeded to measure pollutant effects on the pulmonary endothelin system. As we had shown that co-exposure to particles and ozone increased ET-1 and ECE-1 mRNA levels immediately after exposure, I next examined whether the pollutants individually activated lung endothelin system genes, whether the dynamics of effects differed between the two pollutants, and whether there was any toxicological interaction. Primers for real-time PCR of endothelin and nitric oxide system genes were designed and validated bioinformatically and in practice by comparative analysis of amplification efficiency (Supplementary Material, Figure 1). Real-time PCR and HPLC-fluorescence were used to assess pulmonary endothelin mRNA levels and peptide levels, respectively, in animals exposed to particles (0, 5, 50 mg/m³), ozone (0, 0.4, 0.8 ppm), or combinations of particles and ozone, immediately and 24 h post-exposure (Flowchart 2, p. 88). We also conducted subsequent work implementing techniques for the detection of ET-1[1-32] in co-exposed animals (Supplementary Material, Figure 2).

The animals used in this experiment were part of a larger exposure study conducted by Prem Kumarathasan. I was involved in the experimental design and dosimetric modelling, I performed the PCR and gelatin zymography work, statistical analyses, and data interpretation, and I was the lead author for preparation of the manuscript. Prem Kumarathasan was involved in the experimental design and supervised the HPLC analyses.

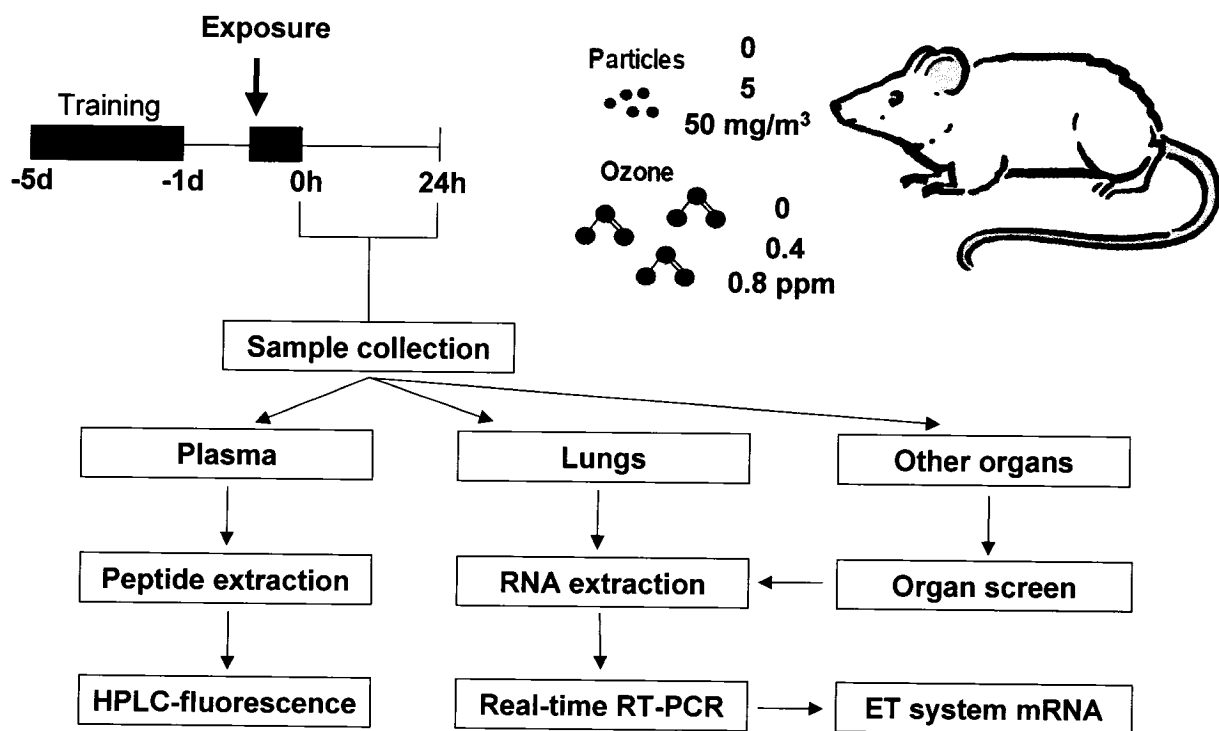
Pat Goegan was involved in organizing the experiment and performing the necropsies.

Rémy Aubin directed the sequencing of amplicons and was involved in data interpretation.

Renaud Vincent was the principal investigator.

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Flowchart 2. Dose-response and pollutant interaction study design. Fischer-344 rats were trained in nose-only tubes and then exposed to various doses and combinations of particles (EHC-93; 0, 5, 50 mg/m³) and ozone (0, 0.4, 0.8 ppm) for 4 h. Groups of animals (n=4-12) were euthanized immediately after exposure or after 24 h recovery in filtered air. Plasma, lungs, and other organs (heart, brain, pituitary, liver, spleen, kidneys) were recovered. RNA was extracted and real-time PCR was performed to examine the expression of endothelin (ET) system genes. HPLC-fluorescence was used to examine endothelin peptide levels in plasma.



Differential Regulation Of The Lung Endothelin System By Urban Particulate Matter And Ozone

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Running head

Air Pollutants Regulate Lung Endothelin System

Keywords: lung, pollution, particles, ozone, endothelin, cardiovascular

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Abstract

Periodic elevation of ambient particulate matter and ozone levels is linked to acute cardiac morbidity and mortality. Increased plasma levels of the potent vasoconstrictor endothelin (ET)-1, a prognostic indicator of cardiac mortality, have been detected in both animal models and humans after exposure to air pollutants. The lungs are the primary source of circulating ET-1, but the direct effects of individual air pollutants and their interaction in modulating the pulmonary endothelin system are unknown. Fischer-344 rats were exposed to particles (0, 5, 50 mg/m³ EHC-93), ozone (0, 0.4, 0.8 ppm), or combinations of particles and ozone for 4 h. Changes in gene expression were measured using real-time reverse transcription polymerase chain reaction immediately after exposure and following 24 h recovery in clean air. Both pollutants individually increased preproET-1, endothelin converting enzyme-1, and endothelial nitric oxide synthase mRNA levels in the lungs shortly after exposure, consistent with the concomitant increase in plasma of the 21 amino acid ET-1_[1-21] peptide measured by HPLC-fluorescence. PreproET-1 mRNA remained elevated 24 h after exposure to particles but not after ozone, in line with previously documented changes of the peptide in plasma. Both pollutants transiently increased endothelin-B receptor mRNA expression, while ozone decreased endothelin-A receptor mRNA levels. Co-exposure to particles plus ozone increased lung preproET-1 mRNA but not plasma ET-1_[1-21], suggesting alternative processing or degradation of endothelins. This coincided with an increase in the lungs of matrix metalloproteinase-2 (MMP-2), an enzyme that cleaves bigET-1 to ET-1_[1-32]. Taken together, our data indicate that ozone and particulate matter independently regulate the expression of lung endothelin system genes, but show complex toxicological interaction with respect to plasma ET-1.

Introduction

Epidemiological evidence indicates that particulate and gaseous air pollutants are linked to higher rates of cardiovascular morbidity and mortality (Burnett *et al.*, 2000; Goldberg *et al.*, 2001b; Goldberg *et al.*, 2001a; Pope, III *et al.*, 2004). Cardiac effects (e.g. decreased heart rate variability, increased myocardial infarction) have been reported within a few hours of increases of ambient ozone or respirable particulate matter (Gold *et al.*, 2000; Peters *et al.*, 2001), indicating that processes responsible for health effects are rapid. Individuals with chronic artery diseases and congestive heart failure are at higher risk of dying after an air pollution episode (Goldberg *et al.*, 2001a). Such findings have led to the suggestion that air pollutants may exert their effects through perturbations of vascular homeostasis (Bouthillier *et al.*, 1998; Vincent *et al.*, 2001). In line with this hypothesis, inhalation exposure of healthy adults to ambient fine particulate matter and ozone resulted in arterial vasoconstriction within 2 h of exposure (Brook *et al.*, 2002), consistent with the dynamics of acute health effects indicated by epidemiological studies. Experimental evidence of an elevation of circulating levels of the vasoconstrictor peptide endothelin (ET)-1 by air pollution provides a biologically plausible explanation for such effects (Bouthillier *et al.*, 1998; Kang *et al.*, 2002; Thomson *et al.*, 2004; Ulrich *et al.*, 2002; Vincent *et al.*, 2001), and field work has confirmed the association between urban pollution and elevated plasma ET-1 in humans (Calderon-Garciduenas *et al.*, 2003). However, the mechanisms governing the elevation of plasma endothelin and the respective contribution of ozone and particulate matter are not clear.

Endothelin-1 is a potent vasoconstrictor peptide involved in the homeostatic control of vascular smooth muscle tone (Haynes *et al.*, 1995). Circulating and tissue ET-1 levels are elevated in many cardiovascular diseases, including atherosclerosis, congestive heart failure, and hypertension (Luscher and Barton, 2000). The precursor preproET-1 peptide is processed by endoproteases to yield bigET-1, which is cleaved by endothelin-converting-enzymes (ECEs) to produce the mature vasoactive 21-amino acid ET-1_[1-21]. Endothelin-1 acts through specific G-protein coupled receptors, the ET_A-receptor and ET_B-receptor, and is cleared from circulation through the latter (Bremnes *et al.*, 2000) and in tissue through degradation by neutral endopeptidases (D'Orléans-Juste *et al.*, 2003). Big ET-1 and mature ET-1_[1-21] produced by endothelial cells are primarily secreted basolaterally into the interstitium towards smooth muscle cells, and circulating levels of the peptides reflect luminal spill-over from basolateral secretion. PreproET-1 mRNA has a half-life of approximately 15 min (Inoue *et al.*, 1989), and bigET-1 and ET-1 have half-lives in the blood of rats of 4 min and less than 1 min respectively (Burkhardt *et al.*, 2000). Consequently, increased steady-state levels of the peptides in plasma represent a sustained increase of *de novo* synthesis, a reduced clearance from circulation, or both. While ECE-dependent processing of bigET-1 is considered the dominant pathway in the endothelium, bigET-1 can be cleaved through a number of alternate pathways, such as by chymase to form the peptide ET-1_[1-31], which is itself a substrate for ECEs (D'Orléans-Juste *et al.*, 2003), and matrix metalloproteinase-2 (MMP-2) to form the vasoactive ET-1_[1-32] peptide (Fernandez-Patron *et al.*, 1999). This alternate processing pathway may be notably significant in tissue injury (Fernandez-Patron *et al.*, 2001).

We have reported that inhaled urban particles, while not directly injurious to normal lungs (Adamson *et al.*, 1999; Vincent *et al.*, 1997a), nevertheless increased the circulating levels of ET-1_[1-21] (Bouthillier *et al.*, 1998; Vincent *et al.*, 2001). Measurements in Wistar rats after inhalation of urban particles showed progressive increases of plasma ET-1_[1-21] and blood pressure with maximal values at 36 h post-exposure (Vincent *et al.*, 2001). In Fischer-344 rats, plasma ET-1_[1-21] was elevated 24 h after inhalation of urban particles alone and after exposure to urban particles plus ozone, but not after ozone alone (Bouthillier *et al.*, 1998). However, lung preproET-1 mRNA levels were elevated as early as 2 h after co-exposure of Fischer-344 rats to urban particulate matter and ozone (Thomson *et al.*, 2004), suggesting that the peptide might be up-regulated at an earlier time.

By factoring doses of both particulate matter and ozone, we undertook here to clarify the early effects of the individual pollutants, as well as their toxicological interaction vis-à-vis regulation of the pulmonary endothelin system genes in the lungs of Fischer-344 rats. Real-time RT-PCR was used to evaluate and quantify subtle changes in the gene expression of preproET-1, ECE-1, the endothelin receptors ET_A and ET_B, and the endothelial (eNOS) and inducible (iNOS) nitric oxide synthases immediately after inhalation exposure to the pollutants and following a 24 h recovery in clean air. The changes in gene expression were then contrasted with plasma ET-1_[1-21] and bigET-1 levels, measured by high-performance liquid chromatography (HPLC) with native fluorescence detection. We show that particulate matter and ozone independently regulate lung endothelin system genes and interact toxicologically with respect to their impact on circulating ET-1_[1-21].

Materials and Methods

Animals. Specific pathogen-free Fischer-344 male rats (200-250 g) were obtained from Charles River (St. Constant, Québec, Canada). The animals were housed in individual plexiglass cages on wood-chip bedding under HEPA-filtered air and held to a 12 h dark/light cycle. Food and water were provided *ad libitum*. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada. Distribution of the animals in experimental groups is summarized in Table 1.

Inhalation exposure to air pollutants. The ambient urban particles EHC-93 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system at the Environmental Health Centre (Tunney's Pasture, Ottawa, Canada) and mechanically sieved using a 36 μm mesh filter. The chemical composition, biological reactivity of the particles in cell culture models, and applications in inhalation studies have been described elsewhere (Bouthillier *et al.*, 1998; Vincent *et al.*, 1997a; Vincent *et al.*, 1997b; Vincent *et al.*, 2001). Animals were exposed to EHC-93 urban particles, ozone, or the combined pollutants using a nose-only inhalation system. Rats were trained in nose-only exposure tubes over 5 consecutive days, and then exposed for 4 h to clean air or to combinations of the individual pollutants EHC-93 (0, 5, 50 mg/m^3) and ozone (0, 0.4, 0.8 ppm) essentially as described previously (Thomson *et al.*, 2004; Vincent *et al.*, 1997a). The particle size distribution of resuspended EHC-93 in our flow-past nose-only exposure system was multimodal, with two respirable modes at 1.3 μm aerodynamic diameter (D_{AE}) and 3.6 μm D_{AE} that together comprised 55 % of the mass of the aerosol, and a non-respirable mode at 15 μm D_{AE} that comprised 45 % of the mass (Vincent *et al.*, 2001). Animals were

Table 1. Distribution of animals for gene expression analyses (subset number of animals for peptide analyses in parentheses)

Time post-exposure	EHC-93 (mg/m³)	Ozone (ppm)		
		0	0.4	0.8
0 h	0	12 (4)	8	8 (4)
	5	8	8	8
	50	8 (4)	8	8 (4)
24 h	0	6	--	4
	5	--	--	--
	50	4	--	8

euthanized immediately after exposure, or following 24 h recovery in filtered air.

Environmental relevance of dose regimen. Dosimetric relevance of the present experiment to an environmental exposure should be evaluated after scaling doses of pollutants within the lungs of rats and humans. To estimate deposition of particles in human lungs under an actual environmental exposure scenario, we have taken as model assumptions an average tidal volume of 875 mL and an average breathing frequency of 16 min^{-1} over the entire day (20.2 m^3 air inhaled/d), oronasal breathing, and an alveolar surface area of 54 m^2 . Deposition rates for the $0.05\text{-}10 \text{ }\mu\text{m D}_{\text{AE}}$ size range of urban particulate matter with size cut-off of $10 \text{ }\mu\text{m D}_{\text{AE}}$ (PM_{10}) containing a nucleation mode at $0.05 \text{ }\mu\text{m D}_{\text{AE}}$ (5% of mass), a condensation mode at $0.2 \text{ }\mu\text{m D}_{\text{AE}}$ (25% of mass) and coarse mode at $5 \text{ }\mu\text{m D}_{\text{AE}}$ (70% mass) were taken as 0.20 for all three modes (Schlesinger, 1989). Using these parameters, and assuming a 24 h exposure to an average PM_{10} concentration of $175 \text{ }\mu\text{g}/\text{m}^3$ (Tellez-Rojo *et al.*, 2000), a reference total dose in the pulmonary compartment of humans was estimated as $707 \text{ }\mu\text{g}$ ($175 \text{ }\mu\text{g}/\text{m}^3 \times 20.2 \text{ m}^3 \times 0.20$), or $1.3 \text{ ng}/\text{cm}^2$ alveolar surface area. Similarly, the peak centriacinar dose of ozone in the lungs of humans can be taken as $30 \times 10^{-6} \text{ }\mu\text{g O}_3/\text{cm}^2/\text{h}$ per $\mu\text{g ambient O}_3/\text{m}^3$ (Miller *et al.*, 1988). Thus, exposure of a human subject to 0.12 ppm ozone ($236 \text{ }\mu\text{g O}_3/\text{m}^3$) for 12 h ($85 \text{ ng O}_3/\text{cm}^2$), followed by 0.06 ppm ozone for 12 h ($42 \text{ ng O}_3/\text{cm}^2$) would lead to a total daily centriacinar peak dose estimated at $127 \text{ ng O}_3/\text{cm}^2$ (Vincent *et al.*, 1997a).

Model assumptions for rats were a tidal volume of 2.1 mL, a breathing frequency of 102 min^{-1} (51.4 L air inhaled/4 h exposure), strict nasal breathing, and an alveolar surface area of 0.34 m^2 . Modelled deposition rates using the Multiple Path Particle Deposition

software (MPPDep v1.11, RIVM Publications, Bilthoven, The Netherlands) were estimated at 0.081 for the 1.3 μm D_{AE} mode (20% of aerosol mass), 0.047 for the 3.6 μm D_{AE} mode (35% of aerosol mass), and 0.000 for the 15 μm D_{AE} mode (45% of aerosol mass). Using these parameters, the pulmonary compartment dose of EHC-93 particles in the rats was estimated at 8.4 μg ($5 \mu\text{g/L} \times 51.4 \text{ L} \times \{[0.20 \times 0.081] + [0.35 \times 0.047] + [0.45 \times 0.000]\}$) or 2.5 ng/cm^2 alveolar surface area, and 84 μg or 25 ng/cm^2 alveolar surface area at the 5 mg/m^3 and 50 mg/m^3 exposure concentrations respectively. Similarly, the peak centriacinar dose of ozone in the lungs of rats is taken as $68 \times 10^{-6} \mu\text{g O}_3/\text{cm}^2/\text{h}$ per $\mu\text{g ambient O}_3/\text{m}^3$ (Miller *et al.*, 1988). Exposure of our rats to 0.4 ppm (785 μg of O_3/m^3) or 0.8 ppm ozone (1570 μg of O_3/m^3) over 4 h should have translated into a total centriacinar peak dose of 214 $\text{ng O}_3/\text{cm}^2$ and 427 $\text{ng O}_3/\text{cm}^2$ respectively.

The ratio of an experimental particle EHC-93 dose within the respiratory compartment of the rats during the 5 mg/m^3 exposure (2.5 ng/cm^2) and 50 mg/m^3 exposure (25 ng/cm^2) to the particle dose calculated for a plausible human exposure scenario (1.3 ng/cm^2) is 2-fold and 20-fold respectively. The ratio of the centriacinar ozone dose in our animals at 0.4 ppm O_3 (214 $\text{ng O}_3/\text{cm}^2$) and 0.8 ppm O_3 (427 $\text{ng O}_3/\text{cm}^2$) to the estimated internal dose in a human subject under a plausible exposure scenario (127 $\text{ng O}_3/\text{cm}^2$) is only 1.7-fold and 3.4-fold respectively. For ethical reasons, nose-only exposures should be kept to a minimum duration, and therefore the dose-rate in our study was obviously higher than for an environmental exposure spread over a 24 h period. Nevertheless, from the standpoint of evaluation toxicology, the pulmonary depositions of the pollutants in the current study are directly relevant to the human experience, including the experimental dose estimated for the high particle exposure concentration once a number of reasonable uncertainty factors are

considered. These include the possible decay of the potency of EHC-93 by comparison to fresh particles, the known interspecies differences in sensitivity to air pollutants (with humans being more responsive than rats), and the heightened sensitivity within a subset of the human population, such as the known increased adverse risk of individuals with congestive heart failure or atherosclerosis (Goldberg *et al.*, 2001a).

Biological samples. Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg, ip). Blood was collected from the abdominal aorta into vacutainer tubes containing the sodium salt of ethylene diamine tetra acetic acid (EDTA) at 10 mg/mL and phenyl methyl sulfonyl fluoride (PMSF) at 1.7 mg/mL, mixed gently, and placed on ice (Kumarathasan *et al.*, 2001). Plasma was isolated by centrifugation (2000 rpm for 10 min), aliquoted, and frozen at -80°C . The lungs were washed by bronchoalveolar lavage with warm saline (37°C) at 30mL/kg body weight, then flash frozen in liquid nitrogen and stored at -80°C . The bronchoalveolar lavage fluid (BALF) was centrifuged (1500 rpm for 10 min at 4°C) to remove cells, and frozen at -80°C .

Reverse transcription of lung RNA samples. Frozen lung samples were homogenized in TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada), and total RNA was isolated according to the manufacturer's instructions. RNA was quantified using the RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon), and quality was verified by electrophoresis on a formaldehyde-agarose gel. Total RNA was reverse transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Briefly, 250 ng RNA was added to a reaction mixture of 5 mM MgCl_2 , 1X PCR

Buffer II, 1 mM each dNTP, 1 U/ μ L RNase Inhibitor, 1 μ M random hexamers, and water to produce a final volume of 50 μ L. The mixture was incubated at 42 °C for 1 h, MuLV reverse transcriptase was inactivated by heating to 99 °C for 5 min, and the reaction was cooled to 5 °C for 5 min followed by storage at -40 °C until used.

Real-time PCR primers. Primers for endothelin system genes (ET-1, ECE-1, ET_A and ET_B receptor), eNOS, and a reference gene (β -actin) were designed using Vector NTI software (InforMax, Frederick, Maryland). The primer sequences for iNOS were from Ulrich *et al.* (2002). Primers were designed to have 50 to 60 % GC content, an optimal annealing temperature of 60-62 °C, and yield PCR products 75-150 bp in length (Table 2). Primers and predicted amplicons were evaluated for any secondary structure that might inhibit primer annealing using m-fold software available online (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>). Double-desalted primers were purchased from Invitrogen. High PCR reaction efficiency was verified and compared for all primer sets using a dilution series of rat cDNA. The β -actin primer set was found to participate in high efficiency reactions at both 60 °C and 62 °C. All other primer sets were validated at either 60 °C or 62 °C. Reaction products run on 1 % agarose gels confirmed a unique band of the expected size for each amplicon. The identities of all amplicons were confirmed by TA cloning (Invitrogen) followed by automated fluorescence sequencing (3100 Genetic Analyser; Applied Biosystems Inc.) and sequence alignment against available nucleotide databases using the BLAST algorithm (<http://www.ncbi.nih.gov/BLAST/>) to verify uniqueness.

Table 2. PCR primers designed to amplify a 75-150 bp expression product

Primer (Accession #)	Sequence	Annealing temperature (C)	Size of amplicon (bp)
β -actin (J00691)	Sense (2538-): CAC TAT CGG CAA TGA GCG GTT CC; Antisense (2774-): CTG TGT TGG CAT AGA GGT CTT TAC GG	60, 62	149
PreproET-1 (NM_012548)	Sense (362-): GAC AAG GAG TGT GTC TAC TTC TGC; Antisense (446-): GGC TTC CTA GTC CAT ACG GG	62	85
ECE-1 (NM_053596)	Sense (575-): AAA AGG CGC AAG TGT ACT ACC G; Antisense (660-): CTC AAT CAG CTC CAT CAG GG	60	86
ET _A -receptor (NM_012550)	Sense (126-): CTA ATC TAA GCA GCC ACG TGG; Antisense (225-): CTA GGC AGG GCC AAA TTA GG	62	100
ET _B -receptor (NM_017333)	Sense (948-): GCT GTC CCT GAA GCC ATA GG; Antisense (1022-): AAG CAT GCA GAC CCT TAG GG	60	75
eNOS (RNO249546)	Sense (547-): CGG TAC TAC TCT GTC AGC TCA GC; Antisense (634-): CAT CCT GGG TTC TGT ATG CC	62	88
iNOS (Ulrich <i>et al.</i> , 2002)	Sense: AAT GGT TTC CCC CAG TTC CTC ACT; Antisense: CTC TCC ATT GCC CCA GTT TTT GA	62	122

Real-time PCR analysis of lung gene expression. Master mixes of the reagents were prepared to minimize differences in reagent composition and pipetting errors. Twenty ng of cDNA were incubated with 25 μ L iQ SYBR Green Supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada) and 200 nM of each primer, and the reagent mixture was brought up to 50 μ L with DNase/RNase-free water. All reactions were performed in duplicate on 96-well plates in a spectrofluorometric thermal cycler (iCycler iQ, Bio-Rad). PCR runs were initiated by incubation at 95 °C for 3 min to activate the iTAQ polymerase followed by 40 cycles of 95 °C for 15 s, the appropriate annealing temperature for 15 s (see Table 2), and 30 s at 72 °C. Fluorescence was monitored at every cycle during the elongation step. A melt curve was conducted following each run to verify product purity. Expression was calculated relative to β -actin using the delta-delta Ct method (Livak and Schmittgen, 2001), and expressed as fold change relative to air control samples.

Analysis of plasma endothelin-1. Plasma big ET-1 and ET-1_[1-21] were analysed by HPLC-fluorescence in a subset of the animals immediately after exposure as previously described (Kumarathasan *et al.*, 2001).

Gelatin zymography. BALF samples were evaluated for MMP activity by gelatin zymography in a subset of the animals immediately after exposure. Equal volumes of BALF (20 μ L) were loaded on 10% SDS-acrylamide gels containing 1 mg/mL gelatin (Sigma) and run for 1 h at 200 mV. In addition to the samples, each gel also contained pre-stained molecular weight markers (Bio-Rad) and a dilution series of a human MMP-2 standard (Calbiochem, La Jolla, California). Gels were incubated in Zymogram Renaturation Buffer (Bio-Rad) for 30 min, then incubated overnight at 37 °C in Zymogram Development Buffer

(Bio-Rad). Following incubation, gels were stained in 0.25 % Coomassie Blue R-250 staining solution (in 40 % methanol/10 % acetic acid) for 1 h, and then destained in a solution of 40 % methanol/10 % acetic acid. Clear bands were assessed by densitometric analysis using NIH shareware. To verify MMP activity, control gels were incubated under the same conditions in buffer containing 25 mM EDTA.

Statistical analyses. Data are expressed as means $\pm$ SEM. The effects of EHC-93 and ozone were tested for statistical significance by multi-way ANOVA (OZONE, EHC and TIME as factors), followed by Tukey's multiple comparison procedure to elucidate the pattern of significant effects ($\alpha = 0.05$) using Sigma-Stat (Sigma-Stat 2.0, Chicago, Illinois). The systematic description of the statistically significant effects determined from multi-way ANOVAs and post-hoc comparisons in studies involving three factors can be cumbersome. For the purpose of clarity and brevity, we have adhered to the following guidelines. Significant factor interactions are indicated in the text of the Results section. Significant main effects are described in text only if they were not part of a significant factor interaction. Statistical significance reported in the figure legends refers to the Tukey's post-hoc comparisons, as directed by significant main effects or significant factor interactions in the ANOVAs. Statistical analysis of data by 2-way and 3-way ANOVAs and post-hoc comparisons are summarized in Tables 3 and 4 respectively.

Results

The effects of ozone and particles on pulmonary endothelin system genes were investigated for the individual pollutants as well as for the combined pollutants. Factoring doses of ozone and particles provided insight into the dose-dependent changes associated with each pollutant, as well as the potential toxicological interactions at the early stages of lung response. Effects in the high exposure groups were also analysed 24 h after exposure in order to characterize the dynamics of changes in relation to the toxicokinetics of the pollutants and the inflammation and repair processes in the lungs. PreproET-1 mRNA levels were significantly increased immediately following exposure to EHC-93 (two-way ANOVA; EHC main effect, $p=0.010$; Fig. 1A) or ozone (OZONE main effect, $p<0.001$; Fig. 1A). Although both pollutants could regulate preproET-1 mRNA, there was no statistical interaction between EHC-93 and ozone with respect to the modulation of preproET-1 mRNA levels immediately after exposure (Tables 3,4). The effects of each pollutant on preproET-1 mRNA were additive. After 24 h recovery in clean air, preproET-1 mRNA expression remained elevated in the lungs of rats exposed to EHC-93 alone (three-way ANOVA; OZONE x TIME, $p<0.001$; Tukey, 0 vs 24 h within 0 ppm O₃, $p<0.05$; Fig. 1B). However, preproET-1 mRNA returned to control levels at 24 h in the lungs of rats exposed to ozone or to the combined pollutants (three-way ANOVA; OZONE x TIME, $p<0.001$; Tukey, 0 vs 24 h within 0.8 ppm O₃, $p<0.05$; Fig. 1B). The elevation of preproET-1 mRNA levels immediately after exposure coincided with a significant increase in ECE-1 mRNA expression (two-way ANOVA; EHC-93 main effect, $p<0.001$; OZONE main effect, $p<0.001$; Fig. 1C). There was no statistically significant EHC-93 and OZONE factor interaction with respect to ECE-1 mRNA expression immediately after exposure, and the

Figure 1. Particulate matter and ozone increase expression of ET-1 and ECE-1 mRNA. Rats were exposed by inhalation for 4 h to the indicated doses of particulate matter and ozone and euthanized immediately after exposure or following a 24 h recovery in filtered air. Lung ET-1 and ECE-1 mRNA expression was determined by real-time PCR. The results are expressed as mean $\pm$ SEM (n=4-12 animals/treatment). Letters over bars indicate statistical significance (Tukey, $p < 0.05$). A) PreproET-1 mRNA immediately after exposure. **a**, 5 vs. 50 mg/m³ within EHC; **b**, 0 vs. 0.4 ppm and 0 vs. 0.8 ppm within OZONE. B) PreproET-1 mRNA after 24 h recovery. **a**, 0 vs. 24 h within 0 ppm O₃; **b**, 0 vs. 0.8 ppm O₃ within 0 h; **c**, 0 vs. 0.8 ppm ozone within 24 h; **d**, 0 vs. 24 h within 0.8 ppm O₃. C) ECE-1 mRNA immediately after exposure. **a**, 0 vs. 5 mg/m³ within EHC; **b**, 0 vs. 50 mg/m³ and 5 vs. 50 mg/m³ within EHC; **c**, 0 vs. 0.8 ppm and 0.4 vs. 0.8 ppm within OZONE. D) ECE-1 mRNA after 24 h recovery. **a**, 0 vs. 50 mg/m³ EHC within 0 h; **b**, 0 vs. 24 h within 50 mg/m³ EHC; **c**, 0 vs. 0.8 ppm O₃ within 0 h; **d**, 0 vs. 24 h within 0.8 ppm O₃.

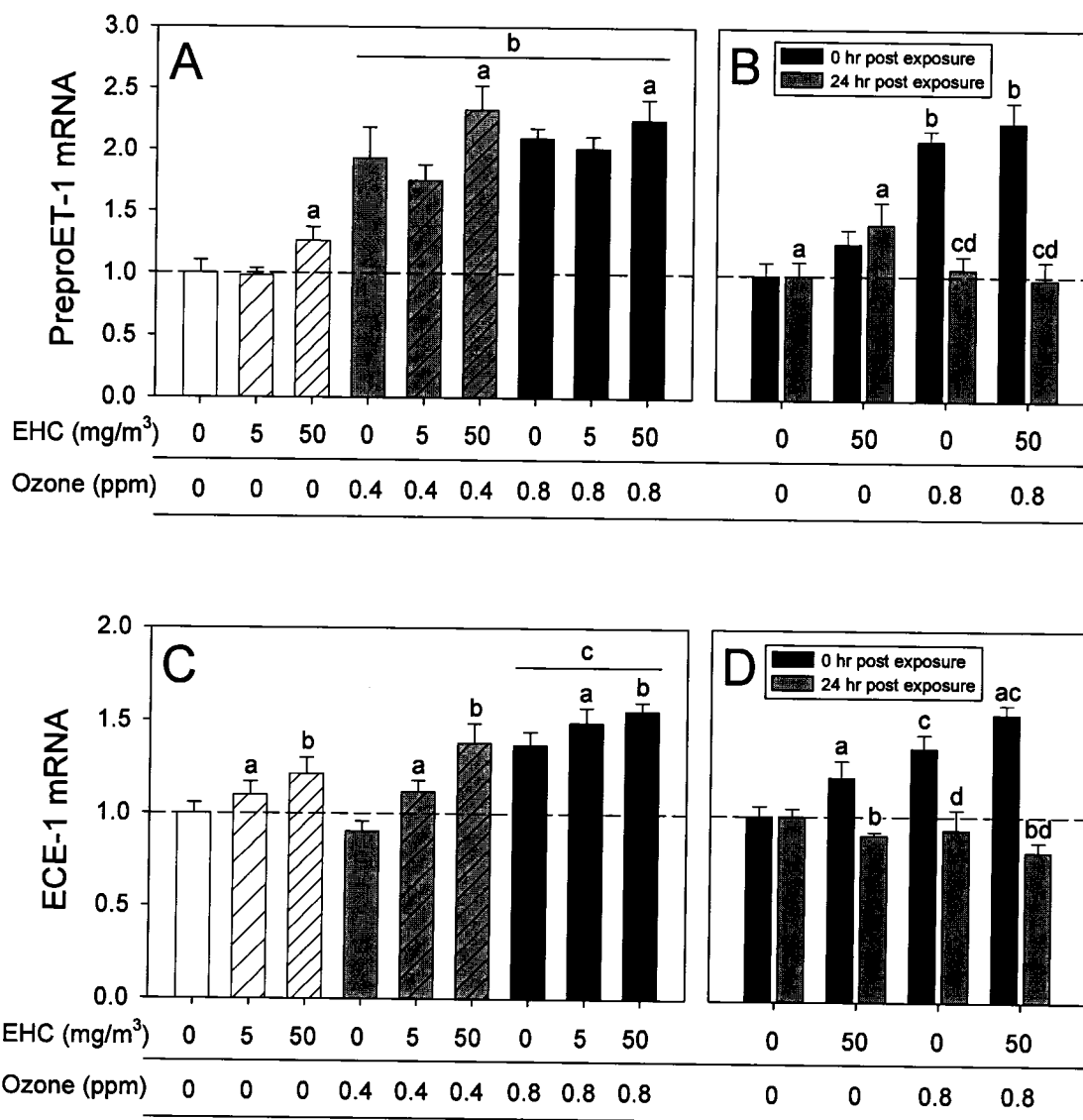


Table 3. Summary of statistical analyses of the dose-response data immediately after exposure to the pollutants (2-way ANOVA)

Endpoints	Significant Effects ^a	P	Tukey (p<0.05)
PreproET-1 mRNA	EHC	0.010	5 vs. 50 mg/m ³
	OZONE	<0.001	0 vs. 0.4 ppm 0 vs. 0.8 ppm
ECE-1 mRNA	EHC	<0.001	0 vs. 5 mg/m ³ 0 vs. 50 mg/m ³ 5 vs. 50 mg/m ³
	OZONE	<0.001	0 vs. 0.8 ppm 0.4 vs. 0.8 ppm
ET _A R mRNA	OZONE	<0.001	0 vs. 0.8 ppm
ET _B R mRNA	EHC	0.023	0 vs. 50 mg/m ³
	OZONE	0.004	0 vs. 0.8 ppm 0.4 vs. 0.8 ppm
eNOS mRNA	EHC	0.027	0 vs. 5 mg/m ³
	OZONE	0.017	0.4 vs. 0.8 ppm
Plasma ET-1	EHC x OZONE	<0.001	0 vs. 50 mg/m ³ EHC within 0 ppm O ₃ 0 vs. 50 mg/m ³ EHC within 0.8 ppm O ₃ 0 vs. 0.8 ppm O ₃ within 0 mg/m ³ EHC 0 vs. 0.8 ppm O ₃ within 50 mg/m ³ EHC
			0 vs. 0.8 ppm O ₃ within 50 mg/m ³ EHC 0 vs. 50 mg/m ³ EHC within 0.8 ppm O ₃
Plasma bigET-1	EHC x OZONE	0.003	0 vs. 0.8 ppm O ₃ within 50 mg/m ³ EHC 0 vs. 50 mg/m ³ EHC within 0.8 ppm O ₃

^a Significant main effects are identified only for factors that are not part of a significant factor interaction.

Table 4. Summary of statistical analyses of the time-course data immediately and 24 h after exposure to the pollutants (3-way ANOVA)

Endpoints	Significant Effects^a	P	Tukey (p<0.05)
PreproET-1 mRNA	OZONE x TIME	<0.001	0 vs. 0.8 ppm O ₃ within 0 h 0 vs. 0.8 ppm O ₃ within 24 h 0 vs. 24 h within 0 ppm O ₃ 0 vs. 24 h within 0.8 ppm O ₃
ECE-1 mRNA	EHC x TIME	0.004	0 vs. 50 mg/m ³ EHC within 0 h 0 vs. 24 h within 50 mg/m ³ EHC
	OZONE x TIME	<0.001	0 vs. 0.8 ppm O ₃ within 0 h 0 vs. 24 h within 0.8 ppm O ₃
ET _A R mRNA	OZONE	0.003	0 vs. 0.8 ppm
ET _B R mRNA	EHC x TIME	0.012	0 vs. 50 mg/m ³ EHC within 0 h 0 vs. 24 h within 50 mg/m ³ EHC
	OZONE x TIME	0.011	0 vs. 0.8 ppm O ₃ within 0 h 0 vs. 24 h within 0.8 ppm O ₃
eNOS mRNA	TIME	0.046	0 vs. 24 h

^a Significant main effects are identified only for factors that are not part of a significant factor interaction.

independent effects of particles and ozone were additive. After 24 h recovery of the animals in clean air, ECE-1 mRNA levels decreased in all exposure groups (three-way ANOVA; EHC x TIME, $p=0.004$; OZONE x TIME, $p<0.001$; Fig. 1D).

Endothelin-1_[1-21] was measured in plasma to assess the potential immediate systemic impacts of the lung responses to the inhaled pollutants. HPLC-fluorescence analyses of ET-1_[1-21] peptide levels revealed an interaction of particles and ozone, with both pollutants independently causing elevation of ET-1_[1-21] immediately after exposure, but not when inhaled in combination (two-way ANOVA; EHC x OZONE factor interaction, $p<0.001$; Tukey, 0 vs 50 mg/m³ EHC-93 within 0ppm O₃, $p<0.05$; 0 vs 0.8ppm O₃ within 0 mg/m³ EHC-93, $p<0.05$; Fig. 2). Plasma bigET-1 levels followed a similar pattern of response (two-way ANOVA; EHC x OZONE, $p=0.003$; Fig. 2).

The plasma levels of the mature peptide and its precursor did not strictly correlate with the mRNA levels of preproET-1 and ECE-1 in the lungs, since the combination of ozone plus particles increased lung preproET-1 and ECE-1 mRNA, but not the circulating levels of ET-1_[1-21] and bigET-1. Matrix metalloproteinase-2, known to be activated in injured lungs, can process bigET-1 to ET-1_[1-32], a peptide distinct from the ET-1_[1-21] form monitored in the HPLC assay. Analysis of bronchoalveolar lavage by gelatin zymography revealed a band in all samples that migrated with the 72 kD MMP-2 standard (Fig. 3). The intensity of this band increased immediately after exposure only in animals co-exposed to both particulate matter and ozone (one-way ANOVA; $p=0.03$), but not in animals exposed to air or the individual pollutants (Fig. 3). No gelatinolytic activity was observed in gels incubated with EDTA (data not shown). Based on its co-migration with the MMP-2

Figure 2. Immediate effects of acute exposure to particulate matter and ozone on plasma ET-1 and bigET-1 peptides. Plasma was collected from rats immediately after a 4 h inhalation exposure to the indicated doses of particulate matter and ozone, and analyzed by HPLC. The results are expressed as mean $\pm$ SEM (n=4 animals/treatment). Letters over bars indicate statistical significance (Tukey, $p < 0.05$). **a**, 0 vs. 50 mg/m³ EHC within 0 ppm O₃; **b**, 0 vs. 0.8 ppm O₃ within 0 mg/m³ EHC; **c**, 0 vs. 0.8 ppm O₃ within 50 mg/m³ EHC; **d**, 0 vs. 50 mg/m³ EHC within 0.8 ppm O₃; **e**, 0 vs. 0.8 ppm O₃ within 50 mg/m³ EHC; **f**, 0 vs. 50 mg/m³ EHC within 0.8 ppm O₃.

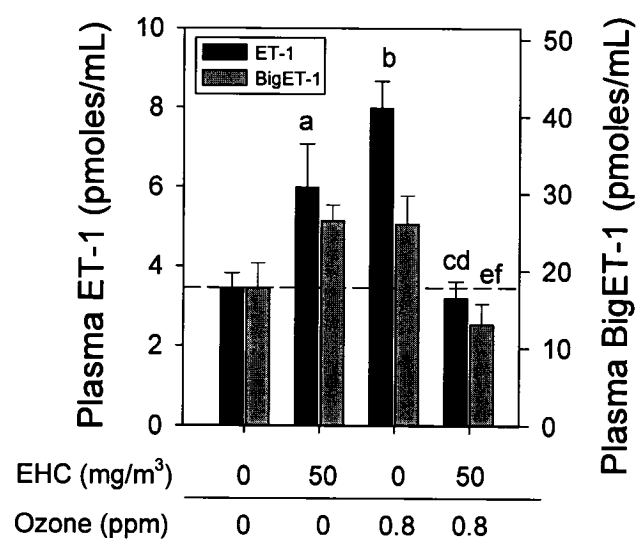
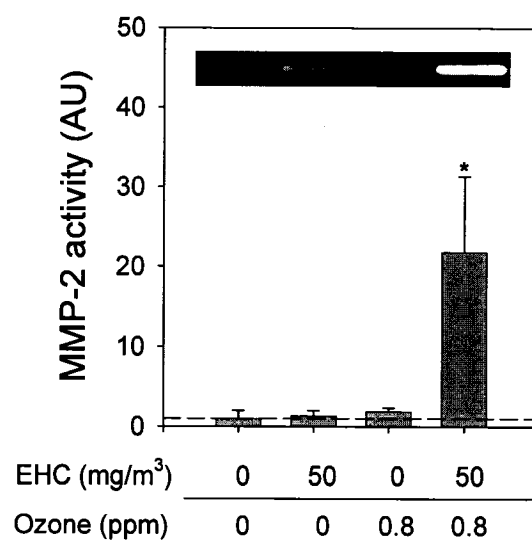


Figure 3. Gelatin zymography of bronchoalveolar lavage fluid (BALF) immediately after exposure to the pollutants. BALF was collected from rats exposed by inhalation to the indicated doses of particulate matter (EHC) and ozone and analyzed for MMP activity as described in the Materials and Methods. Cleared bands indicating gelatinase activity migrated with the 72 kDa MMP-2 standard. The results are expressed as mean $\pm$ SEM (n=3 animals/treatment). *p<0.05, (Holm-Sidak multiple comparison), 0 mg/m³ EHC/0 ppm O₃ vs. 50 mg/m³ EHC/0.8 ppm O₃. AU, arbitrary units.



standard and the inhibition of activity by EDTA, this band likely corresponds to the latent 72 kD form of MMP-2.

Changes in expression of the specific endothelin receptors have the potential to impact on the physiological significance of higher ET-1 peptide levels as well as on clearance of the peptide. Expression of ET_A receptor mRNA was reduced by ozone immediately after exposure (two-way ANOVA; OZONE main effect, $p < 0.001$; Fig. 4A) and after 24 h recovery in clean air (three-way ANOVA; OZONE main effect, $p = 0.003$; Fig. 4B), and was not affected by the urban particles. In contrast, expression of ET_B receptor mRNA increased immediately after exposure to EHC-93 or ozone. There was no evidence of pollutant interactions except for an apparent additive effect of ozone and particles (two-way ANOVA; EHC main effect, $p = 0.023$; OZONE main effect, $p = 0.004$; Fig. 4C). After 24 h recovery in clean air, ET_B receptor mRNA expression decreased about 20% below air control level in all exposure groups (three-way ANOVA; EHC x TIME, $p = 0.012$; OZONE x TIME, $p = 0.011$; Fig. 4D).

Higher expression of ET-1 in endothelial cells is usually counterbalanced by elevation of nitric oxide production. Both ozone and the urban particles independently increased eNOS mRNA expression immediately after exposure, with additive effects after exposure to both pollutants in combination (two-way ANOVA; EHC main effect, $p = 0.027$; OZONE main effect, $p = 0.017$; Fig. 5A). Overall, eNOS mRNA levels decreased to control levels after 24 h recovery (three-way ANOVA; TIME main effect, $p < 0.046$; Fig. 5B). There were no significant changes of iNOS mRNA (data not shown).

Figure 4. Particulate matter and ozone differentially modulate endothelin receptor mRNA expression. Lung ET_A and ET_B receptor mRNA levels were determined by real-time PCR after 4 h inhalation exposure to the indicated pollutant doses immediately after exposure and after 24 h recovery. The results are expressed as mean $\pm$ SEM (n=4-12 animals/treatment). Letters over bars indicate statistical significance (Tukey, p<0.05). A) ET_A receptor mRNA immediately after exposure. **a**, 0 vs. 0.8 ppm within OZONE. B) ET_A receptor mRNA after 24 h recovery. **a**, 0 vs. 0.8 ppm within OZONE. C) ET_B receptor mRNA immediately after exposure. **a**, 0 vs. 50 mg/m³ within EHC; **b**, 0 vs. 0.8 ppm and 0.4 vs. 0.8 ppm within OZONE. D) ET_B receptor mRNA after 24 h recovery. **a**, 0 vs. 50 mg/m³ EHC within 0 hr; **b**, 0 vs. 24 h within 50 mg/m³ EHC; **c**, 0 vs. 0.8 ppm O₃ within 0 h; **d**, 0 vs. 24 h within 0.8 ppm O₃.

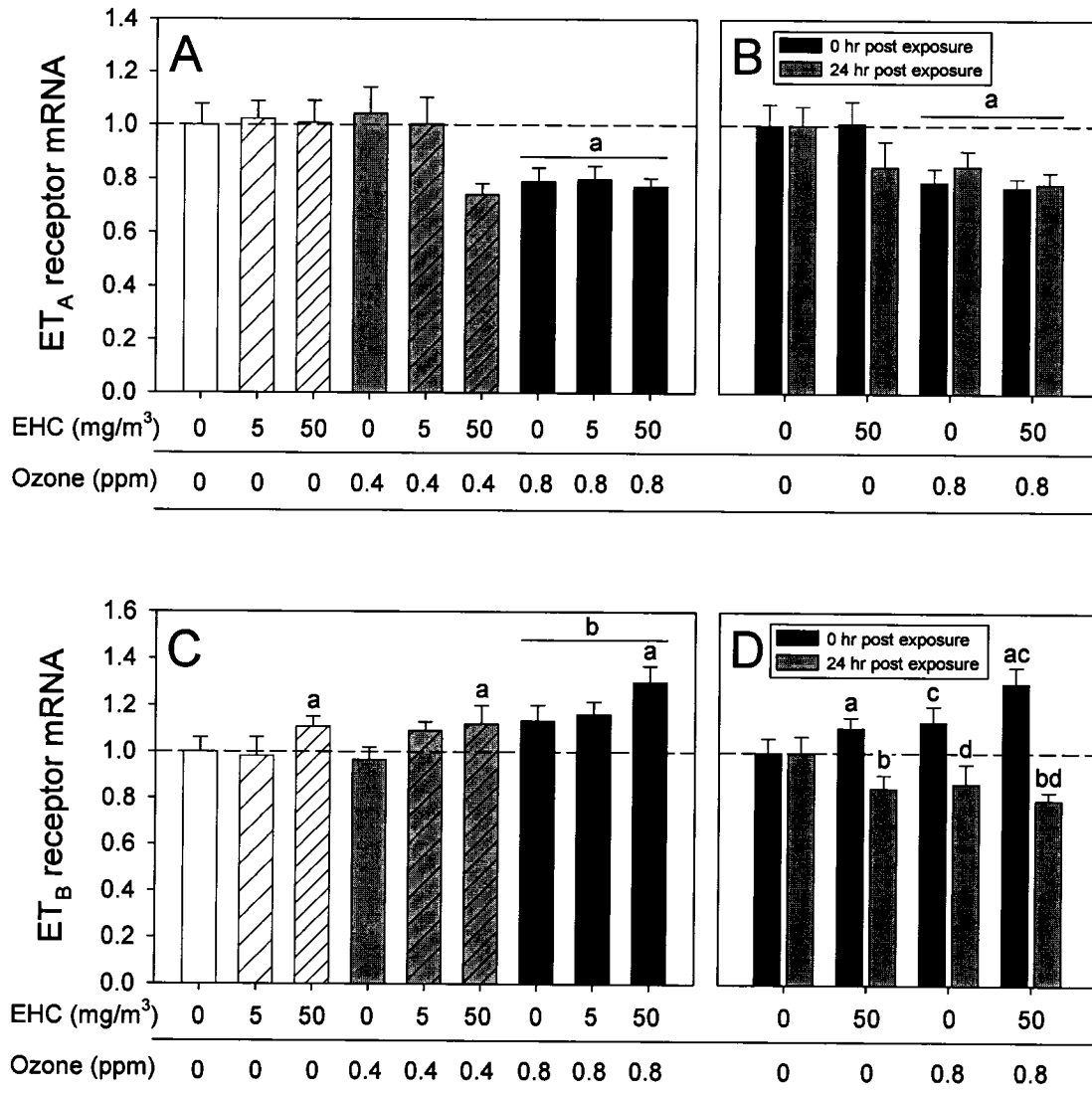
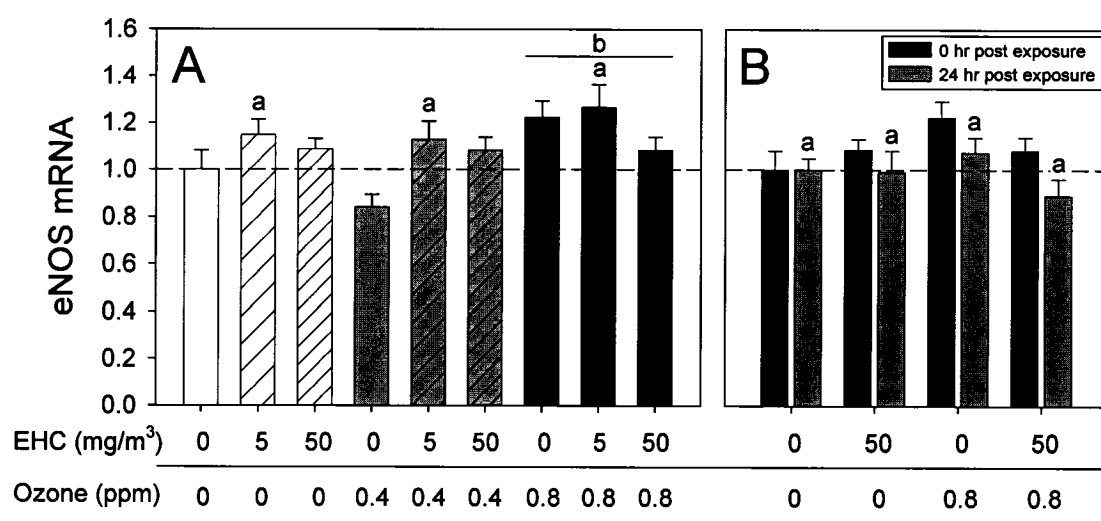


Figure 5. Endothelial nitric oxide synthase (eNOS) mRNA expression after pollutant inhalation. Lung eNOS mRNA levels were assessed by real-time PCR in rats exposed to the indicated pollutant doses immediately after exposure and after 24 h recovery. The results are expressed as mean $\pm$ SEM (n=4-12 animals/treatment). Letters over bars indicate statistical significance (Tukey, $p < 0.05$). A) eNOS mRNA immediately after exposure. **a**, 0 vs. 5 mg/m³ within EHC; **b**, 0.4 vs. 0.8 ppm within OZONE. B) eNOS mRNA after 24 h recovery. **a**, 0 vs. 24 h within TIME.



Discussion

In the present study we demonstrate for the first time that inhaled particulate matter and ozone independently regulate pulmonary endothelin system genes, and that these changes coincide with increased circulating levels of the potent vasoconstrictor ET-1_[1-21] in plasma. Unexpectedly, we found that while co-exposure to both particulate matter and ozone resulted in similar activation of lung endothelin system genes, the response immediately after exposure was not associated with increased spill-over of bigET-1 or ET-1_[1-21] peptides into circulation (Table 5).

To clarify the early impacts of the pollutants on the regulation of ET-1_[1-21], we measured gene expression in the lungs by real-time RT-PCR, and plasma bigET-1 and ET-1_[1-21] levels by HPLC-fluorescence immediately after exposure. Our data reveal a rapid increase of circulating ET-1_[1-21] after a 4 h exposure to either particulate matter or ozone. The lungs are the principal source of circulating ET-1 (Dupuis *et al.*, 1996) and concurrent increase of preproET-1 and ECE-1 mRNA in the lungs confirms that both particulate matter and ozone can regulate the pulmonary endothelin system in rats. Indeed, the impact of inhaled pollutants on pulmonary preproET-1 mRNA levels measured here is similar in magnitude to that measured in rat lungs after acute inhalation exposure to cigarette smoke (Adachi *et al.*, 2000), which is also known to rapidly increase plasma ET-1_[1-21] levels (Haak *et al.*, 1994). Changes in the precursor peptide bigET-1 correlated with levels of the mature ET-1_[1-21] peptide, confirming that elevation of circulating ET-1_[1-21] in our animal model was due, at least in part, to increased *de novo* synthesis of ET-1.

Table 5. Summary of pollutant effects on the rat lung endothelin system and plasma endothelins relative to air controls

	Time after exposure	Prepro ET-1 mRNA	ECE-1 mRNA	ET _B receptor mRNA	BigET-1 peptide	ET-1 peptide
EHC	0 h	↑	↑	↑	↑	+75 % ↑
	24 h	↑	↓	↓	— ^a	+15 % ↑ ^b
Ozone	0 h	↑	↑	↑	↑	+130 % ↑
	24 h	—	↓	↓	ND	— ^b
EHC plus	0 h	↑ ^c	↑ ^c	↑	—	—
Ozone	24 h	—	↓	↓	ND	+15 % ↑ ²

Note. ↑, increase; ↓, decrease; —, no change; ND, not determined.

^a (Vincent *et al.*, 2001).

^b (Bouthillier *et al.*, 1998).

^c This study and (Thomson *et al.*, 2004).

It is possible that the extent of oxidative stress and tissue injury produced from co-exposure to particles plus ozone, in excess of what is observed with ozone or particles alone (Vincent *et al.*, 1997a), inhibited translation of preproET-1 mRNA in the affected central acinus in these animals. Oxidative stress is known to inhibit translation of a number of proteins in the lungs (Shenberger *et al.*, 2005) and in endothelial cells (Jornot and Junod, 1989). Regulation of ET-1 is thought to be predominantly at the transcriptional level (Fagan *et al.*, 2001), but translational regulation of ET-1 has been reported in endothelial cells exposed to high density lipoprotein (Hu *et al.*, 1994). Furthermore, atrial natriuretic peptide has been shown to inhibit ET-1 synthesis while at the same time stabilizing preproET-1 mRNA (Hu *et al.*, 1992). Since translational regulation of ET-1 has not been studied to a significant extent, additional work is required to assess its relevance to ET-1 production by the lungs in normal and disease states.

On the other hand, there is ample evidence that the relative abundance of ET_A and ET_B receptors determines the effects of endothelin on target cells and impacts clearance of ET from the systemic circulation. Since the ET_A receptor is recycled back to the cell surface after binding its ligands and internalization (Bremnes *et al.*, 2000), the early decrease of ET_A receptor mRNA in the lungs after inhalation of ozone may not immediately affect ET_A receptor density. In contrast, because the ET_B receptor is not recycled (Bremnes *et al.*, 2000), the increased ET-1 peptide levels should accelerate turn-over of the ET_B receptor, which can be compensated only by increased synthesis of the ET_B protein, and hence higher mRNA levels. Binding of ET-1_[1-21] to the ET_B receptor of endothelial cells stimulates the release of the vasodilators prostacyclin and nitric oxide (Luscher and Barton, 2000). Such a response of endothelial cells to the elevated ET-1_[1-21] is substantiated here by the up-

regulation of eNOS mRNA immediately after inhalation of particles or ozone. The later 20% decrease in ET_B receptor mRNA levels in the lungs 24 h after exposure to the pollutants should result in lower receptor density and slower ET-1 clearance, which seems in agreement with the 15-20% increase in immunoreactive ET-1 reported previously (Bouthillier *et al.*, 1998). In short, our data suggest that the observed increase in circulating levels of mature ET-1_[1-21] in rats following inhalation of pollutants may be due to a combination of primary effects in the lungs, namely elevated expression of preproET-1 and ECE-1 mRNA in endothelial cells resulting in a higher rate of production, basolateral secretion and luminal spill-over of ET-1_[1-21], combined with a lower expression of ET_B mRNA in the endothelium resulting in lower receptor density and slower clearance of ET-1_[1-21].

The changes in bigET-1 in plasma tracked those of ET-1, but in contrast to clearance of ET-1 by the ET_B receptor in the pulmonary endothelium, bigET-1 is cleared from blood mainly by the liver and the kidneys through a mechanism that is not receptor-mediated (Burkhardt *et al.*, 2000). Endothelins are substrates for a variety of metallopeptidases that can be induced or activated in the injured lungs (D'Orléans-Juste *et al.*, 2003). For example, cleavage of bigET-1 by MMP-2 to produce ET-1_[1-32] may be significant in tissue injury (Fernandez-Patron *et al.*, 2001). We found that combined exposure to particulate matter and ozone, but not the individual pollutants, caused an immediate increase of MMP-2 in the alveoli. The presence of MMP-2 is in line with the enhanced septal remodeling (Vincent *et al.*, 1997a) and thickening (Bouthillier *et al.*, 1998) that results from co-exposure to EHC-93 and ozone, by comparison to the changes induced by the individual pollutants. The alveolar air-blood barrier has a thickness of less than 1 µm, and since bigET-1 is secreted basolaterally by endothelial cells, MMP-2 produced within the septum will co-locate with the

secreted peptide. Furthermore, the volume of extracellular lining fluid where alveolar macrophages distribute is small and the cells are in effect juxtaposed to type 1 epithelial cells. Consequently, any MMP-2 secreted by alveolar macrophages will immediately access the alveolar interstitium through the permeable epithelial barrier in the injured lungs of the animals co-exposed to particles and ozone.

A shift in the processing of bigET-1 in the affected areas of the lungs from the ECE-dependent production of ET-1_[1-21] to alternate pathways would explain the lack of measurable excess spill-over of ET-1_[1-21] despite increases of preproET-1 and ECE-1 mRNAs in the co-exposure group. Endothelin-1_[1-32] is a potent vasoconstrictor (Fernandez-Patron *et al.*, 1999). If our interpretation is correct that co-exposure to particulate matter plus ozone increased production of ET-1_[1-32], this alternate pathway could play a role in mediating the acute cardiovascular effects of inhaled pollutants, particularly in lungs with existing inflammation. We did not monitor alternate endothelin peptides such as ET-1_[1-31] and ET-1_[1-32] in our study, and we are not aware of studies that have actually documented ET-1_[1-32] in blood or tissues of animals, aside from simpler systems such as perfused arterial segments or *in vitro*. Confirmation of the extent and relevance of the various alternate endothelin processing pathways will require detection of those species in the plasma, lungs or BAL.

In summary, we propose that regulation of the pulmonary endothelin system by air pollutants may have profound human health impacts. Based on the responses of ECE-1 and eNOS mRNAs, the lowest-observed-effect level (LOEL) for inhaled urban particles EHC-93 with respect to changes in the endothelin system in the lungs of rats in our study corresponds

to an internal effective pulmonary dose of 2.5 ng/cm^2 . Based on the response of preproET-1 mRNA, the LOEL for ozone here corresponds to an internal dose of 214 ng/cm^2 . These values are only two-fold higher than the reference values for a plausible human exposure scenario (fine particles, 1.3 ng/cm^2 ; ozone, 127 ng/cm^2). Elevation of plasma ET-1_[1-21] and ET-3 in rats after inhalation of EHC-93 is accompanied by increased systemic blood pressure (Vincent *et al.*, 2001). In agreement with this observation, human subjects exposed to ozone and urban particulate matter exhibit a constriction of the brachial artery (Brook *et al.*, 2002). Higher plasma ET-1 levels (+25%) have been detected in children from south west metropolitan Mexico City by comparison to children from low-pollution areas (Calderon-Garciduenas *et al.*, 2003). Such an increase of ET-1 is associated with an unfavourable prognosis in congestive heart failure patients (Galatius-Jensen *et al.*, 1996) or after myocardial infarction (Omeland *et al.*, 1994). Furthermore, heart rate variability is reduced in humans within an hour of a peak ozone episode (Gold *et al.*, 2000), and high circulating ET-1 levels have been shown to correlate with decreased heart rate variability (Aronson *et al.*, 2001; Pekdemir *et al.*, 2004). Reduced ET_B receptor expression in the lungs, resulting in slower clearance of ET-1 and hence elevated steady-state levels of circulating ET-1, has been proposed as a fundamental change in congestive heart failure (Kobayashi *et al.*, 1998; Lepailleur-Enouf *et al.*, 2001), and has been shown to predispose to pulmonary edema (Carpenter *et al.*, 2003). Transcriptional activation of preproET-1 and ECE-1 is implicated in atherosclerosis progression (Rossi *et al.*, 1999), and repeated exposure of hyperlipidemic rabbits to EHC-93 has indeed been shown to accelerate plaque formation (Suwa *et al.*, 2002). Finally, acute cardiac effects in humans have now been documented within one to three hours after exposure to occupational and ambient air pollutants (Gold *et al.*, 2000; Peters *et*

al., 2001), and the rapid response of the pulmonary endothelin system in animals exposed to ozone and urban particles is consistent with these observations.

Perspectives. Our animal data suggest several verifiable theoretical implications for human health. For one, the extent of the changes to the pulmonary endothelin system induced by ambient pollutants may well depend on the pollutant mix, since ozone and particulate matter in our study appeared to display some basic differences in their toxicodynamics, as well as some level of toxicological interaction. In turn, the pathophysiological impacts and health significance of the activation of the pulmonary endothelin system should depend on host factors, such as health status or genetic predisposition. Individuals with a compromised cardiovascular system and ineffective compensation for the vasopressor effect of ET-1 may respond adversely to an acute surge of circulating ET-1. Some of the documented effects associated with higher circulating ET-1 are hypertension, decreased heart rate variability, myocardial ischemia, and arrhythmia. In individuals with underlying pulmonary inflammation, such as a lung infection, chronic obstructive pulmonary disorder, or asthma, elevation of endothelin production may enhance the pulmonary inflammation cascade and tissue hyperplasia and hypertrophy. In individuals with no apparent health conditions but nevertheless with some ET-1 and ET_A receptor polymorphisms that are associated with higher risk for asthma (Immervoll *et al.*, 2001), hypertension (Jin *et al.*, 2003), and idiopathic dilated cardiomyopathy (Charron *et al.*, 1999), it remains possible that recurring activation of the pulmonary endothelin system by air pollutants will interact with these genetic determinants of susceptibility and precipitate disease development. Molecular epidemiology tools are available to investigate such outcomes.

Acknowledgements

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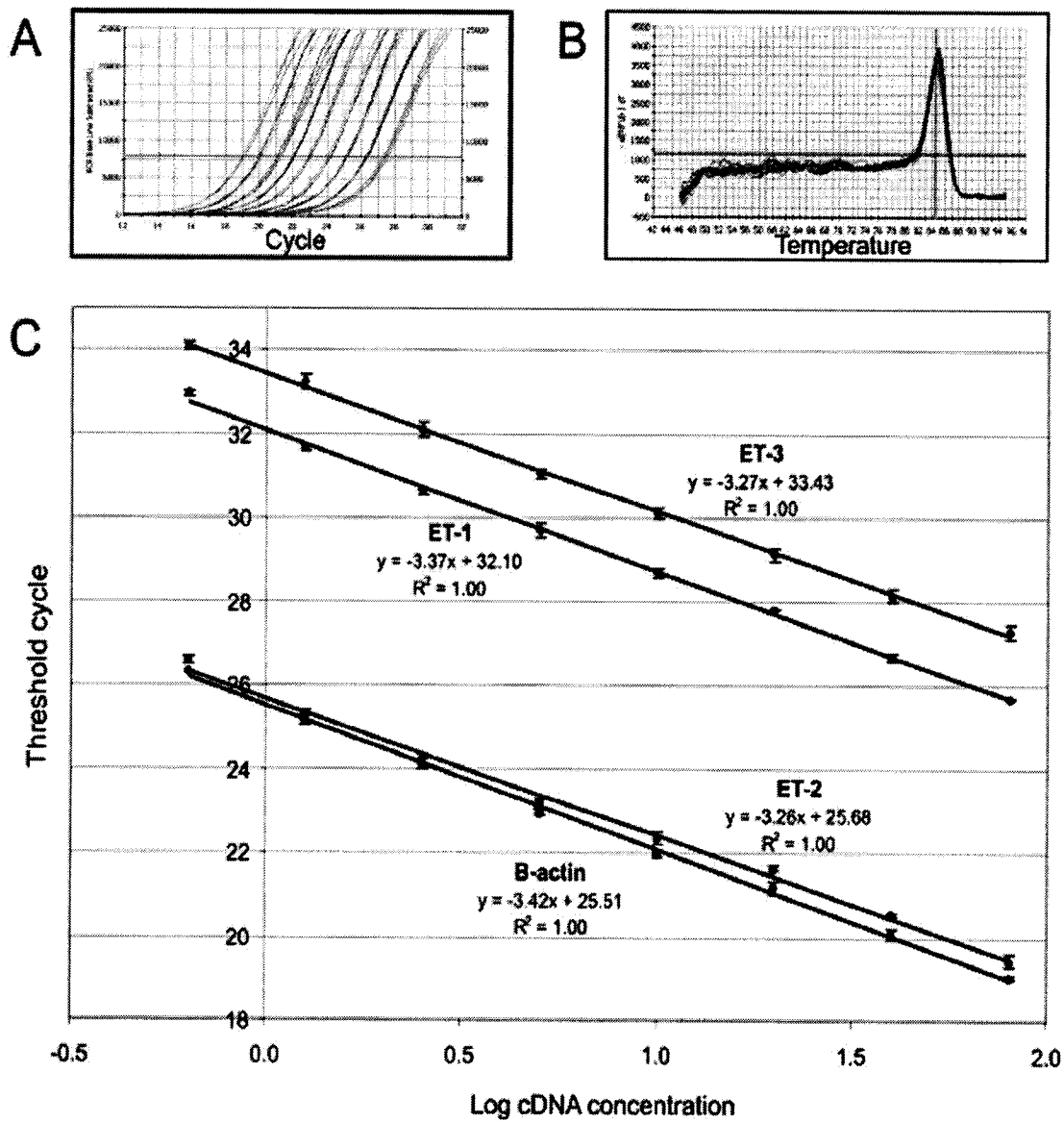
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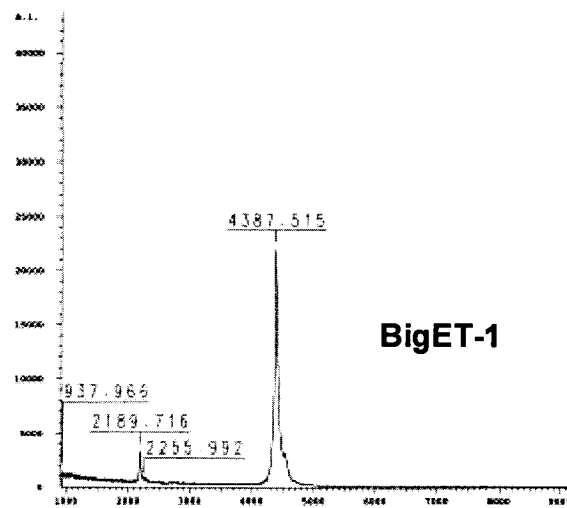
Vincent, R., Kumarathasan, P., Goegan, P., Bjarnason, S. G., Guenette, J., Berube, D., Adamson, I. Y., Desjardins, S., Burnett, R. T., Miller, F. J. and Battistini, B. (2001). Inhalation toxicology of urban ambient particulate matter: acute cardiovascular effects in rats. *Res. Rep. Health Eff. Inst.* 5-54.

Supplementary Material, Figure 1. Real-time PCR primer validation. Primers for all endothelin system genes were designed by Vector NTI (InforMax, Frederick, MD), and evaluated for any secondary structure that might inhibit primer annealing using m-fold software (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>). PCR efficiency was confirmed for all primer sets using serial dilutions of rat cDNA (an example is shown in A). The cycle at which the amplification curve crossed the threshold line was used to determine the threshold cycle for a given cDNA concentration. Melt curve analysis revealed a unique product for each primer set (B), and this was confirmed by a single band at the expected size on a 1% agarose gel (not shown). Threshold cycles plotted against log cDNA concentration revealed a linear relationship for all primer sets (an example is shown in C), indicating no change in efficiency across the dilution series. All reactions were performed in triplicate.

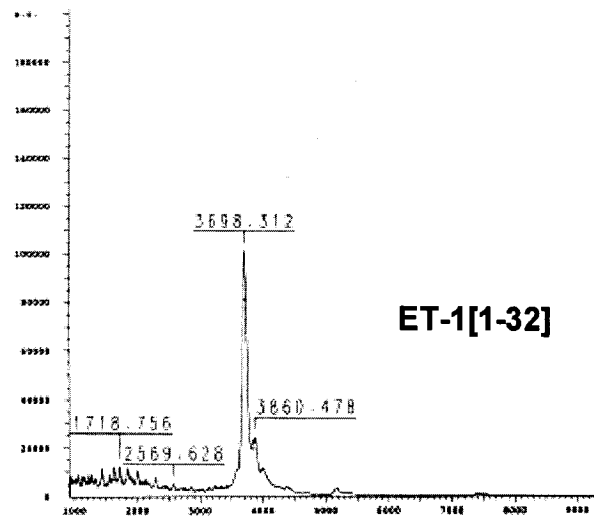


Supplementary Material, Figure 2. MALDI-TOF mass spectrometry and HPLC-fluorescence analysis of bigET-1 cleavage by matrix metalloproteinase (MMP)-2. BigET-1 was incubated overnight at 37°C in the presence of active MMP-2. Cleavage products were purified by acid-acetone extraction and molecular weight cut-off separation. (A) Big ET-1 standard (MALDI-TOF analysis). (B) ET-1[1-32] (MALDI-TOF analysis). (C) Comparison of HPLC elution of bigET-1, ET-1[1-32] and ET-1[1-21].

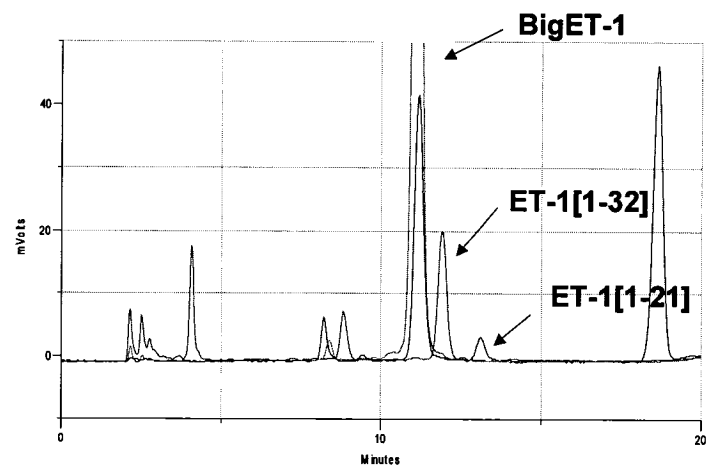
A



B



C



5. Thomson E, Kumarathasan P, Vincent R. (2006). Pulmonary Expression of PreproET-1 and PreproET-3 mRNAs is Altered Reciprocally in Rats after Inhalation of Air Pollutants. *Exp Biol Med* 231: 979-984.

Plasma levels of ET-2 and ET-3, two other members of the endothelin peptide family, have been shown to increase in response to air pollution in other studies. Since the lungs respond to inhaled pollutants by increasing production and release of ET-1, we hypothesized that expression of the other two endothelin peptides might be similarly affected. Primers were designed and validated to assess the expression of these genes, and the relative mRNA and plasma peptide levels of all three endothelin peptides (ET-1, -2, and -3) were compared in rats exposed to air, particles, ozone, and the combination of particles and ozone. The animals used in this experiment were part of a larger exposure study conducted by Prem Kumarathasan (See Chapter 4, Flowchart 2, p. 88). I was involved in the experimental design, performed the PCR and gelatin zymography work, statistical analyses, and data interpretation, and was the lead author for preparation of the manuscript. Prem Kumarathasan supervised the HPLC analyses. Renaud Vincent was the principal investigator.

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Pulmonary Expression Of PreproET-1 And PreproET-3 mRNA Is Altered Reciprocally In Rats After Inhalation Of Air Pollutants

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Running title

Reciprocal regulation of lung ET-1 and ET-3

Keywords: endothelin (ET)-1, ET-2, ET-3, lung, gene expression, real-time polymerase chain reaction (PCR), particulate matter, ozone, air pollution.

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Abstract

Perturbation of vascular homeostasis is an important mechanism in the acute health effects of inhaled pollutants. Inhalation of urban particulate matter and ozone by rats has been shown to result in increased synthesis of the potent vasoactive peptide endothelin (ET)-1 in the lungs with spillover in the circulation. In the present work, we have analyzed the inter-relationships between responses of the three major endothelin isoforms, ET-1_[1-21], ET-2_[1-21] and ET-3_[1-21] to inhaled pollutants, at the peptide and gene expression levels. Fisher-344 rats were exposed for 4 h by nose-only route to clean air, urban particles EHC-93 (0, 50 mg/m³), ozone (0, 0.8 ppm), or ozone and particles together. Circulating levels of both the ET-1_[1-21] and ET-3_[1-21] peptides were increased immediately after exposure to particulate matter or ozone. While expression of preproET-1 mRNA in the lungs increased, expression of preproET-3 mRNA decreased immediately after exposure. PreproET-2 mRNA was not detected in the lungs, and exposure to either pollutant did not affect plasma ET-2 levels. Co-exposure to ozone and particles, while altering lung preproET-1 and preproET-3 mRNA levels in a fashion similar to ozone alone, did not cause changes in the circulating levels of the two corresponding peptides. Thus, *de novo* synthesis of ET-3 in the lungs is not responsible for the increase of circulating plasma ET-3 after inhalation of pollutants, which implies regulation of preproET-3 at a remote site, and hence systemic impacts of the pollutants. Up-regulation of preproET-1 coupled with down-regulation of preproET-3 in the lungs of animals exposed to air pollutants implies a mismatch of local ET-1/ET_A receptor-mediated vasoconstriction and ET-3/ET_B receptor-mediated vasodilation.

Introduction

Air pollution levels correlate with respiratory and cardiovascular morbidity and mortality (1-3). Several recent epidemiological studies have reported increased myocardial infarction and decreased heart rate variability within a few hours of increased ambient ozone or respirable particulate matter concentrations (4, 5). Controlled exposure studies involving animal models and humans have generally supported the epidemiological data by validating potential biologically plausible mechanisms for these effects. For example, inhalation of an ambient particulate matter preparation by rats rapidly activates lung endothelin system genes (6, 7) and causes a shift in circulating levels of the vasoactive peptide endothelin (ET)-1 (8). The increase in plasma ET-1 after exposure to particles is associated with an increase in systemic blood pressure in healthy rats (9) and cardiotoxicity in a rat model of myocardial infarction (10). In line with these animal studies, healthy humans exposed to ambient fine particulate matter and ozone exhibit vasoconstriction (11). Children living in south-west Mexico city have higher ET-1 plasma levels than children living in a less polluted city (12).

Although preproET-1 is the predominant endothelin mRNA in the lungs, preproET-3 mRNA has also been detected, while preproET-2 mRNA appears to be absent or poorly expressed (13, 14). Endothelin-2 differs from ET-1 by only two amino acids (Trp⁶, Leu⁷), and exerts effects similar to ET-1 through the endothelin-A (ET_A) and ET_B receptors (15). Endothelin-3 differs by six amino acids (Thr², Phe⁴, Thr⁵, Tyr⁶, Lys⁷, Tyr¹⁴) and has a lesser affinity for the ET_A receptor, but has been shown to induce a biphasic response in the pulmonary circulation, exerting potent nitric oxide-dependent vasodilatory effects at lower levels and vasoconstriction at higher levels (16). Little is known about the regulation of

these peptides in the lungs. Recent studies indicate that exposure to air pollution, in addition to increasing spillover of ET-1 into circulation, also increases circulating levels of ET-2 and ET-3. Plasma ET-2 was elevated in aged rats after exposure to highway air pollution (17), and plasma ET-3 levels rapidly increased after inhalation exposure to particulate matter (9). The sources of the excess circulating ET-2 and ET-3 in response to inhaled pollutants are not known.

Real-time PCR is the current benchmark for sensitive and accurate quantification of mRNA levels. In the present work we set out to validate a real-time PCR system for the endothelin genes. We then investigated the inter-relationships between circulating levels of the three ET isoforms and expression of their corresponding mRNAs in the lungs of rats after inhalation of ozone and urban particles.

Materials and Methods

Animals. Specific pathogen-free Fischer-344 male rats (200-250 g) obtained from Charles River (St. Constant, Québec, Canada) were housed in individual plexiglass cages on wood-chip bedding under HEPA-filtered air and held to a 12 h dark/light cycle. Food and water were provided *ad libitum*. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

Inhalation exposure to air pollutants. The ambient urban particles EHC-93 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system at the Environmental Health Centre (Tunney's Pasture, Ottawa, Canada) and mechanically sieved using a 36 µm mesh filter. The chemical composition, biological reactivity of the particles in cell culture models, and applications in inhalation studies have been described elsewhere (8, 9, 18, 19). Rats (n=4-12) were trained in nose-only exposure tubes over 5 consecutive days, and then exposed for 4 h to clean air or to combinations of the individual pollutants EHC-93 (0, 50 mg/m³) and ozone (0, 0.8 ppm) using a nose-only exposure system essentially as described previously (7, 19). Animals were euthanized immediately after exposure, or following 24 h recovery in filtered air.

Biological samples. Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg, ip). Blood was collected from the abdominal aorta into vacutainer tubes containing the sodium salt of ethylenediaminetetraacetic acid (EDTA) at 10 mg/mL and phenylmethylsulfonylfluoride (PMSF) at 1.7 mg/mL, mixed gently, and placed on ice (20). Plasma was isolated by centrifugation (2000 rpm for 10 min), aliquoted, and frozen at

–80 °C. The lungs were washed by bronchoalveolar lavage with warm saline (37 °C) at 30mL/kg body weight, then flash frozen in liquid nitrogen and stored at –80 °C. Frozen lung samples were homogenized in TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada), and total RNA was isolated according to the manufacturer's instructions. RNA was quantified using the RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon), and total RNA was reverse transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, Ontario, Canada) according to the manufacturer's instructions.

Analysis of plasma endothelins. Plasma ET-1_[1-21], ET-2_[1-21] and ET-3_[1-21] were analysed by HPLC-fluorescence in a subset of the animals immediately after exposure as previously described (20).

Real-time PCR analysis of lung gene expression. Primers for preproET-1 (Sense: GAC AAG GAG TGT GTC TAC TTC TGC; Antisense: GGC TTC CTA GTC CAT ACG GG), preproET-2 (Sense: CAACTCCTGGCTTGACAAGG; Antisense: TAGGGAGCTGTCTGTCCTGC) and preproET-3 (Sense: CTG TCC AAC CAC AGA GGA AGC; Antisense: TGT CTG TGG AGA AGA CTG GG) genes, and a reference gene, β -actin (Sense: CAC TAT CGG CAA TGA GCG GTT CC; Antisense: CTG TGT TGG CAT AGA GGT CTT TAC GG) were designed to have 50 to 60 % GC content, an optimal annealing temperature of 60-62 °C, and yield PCR products 75-150 bp in length using Vector NTI software (InforMax, Frederick, Maryland). Primers and predicted amplicons were evaluated for secondary structure using online m-fold software (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>). Double-desalted primers were

purchased from Invitrogen. Optimal annealing temperatures were determined to be 60 °C (ET-2) and 62 °C (ET-1 and ET-3), with either temperature resulting in high efficiency amplification of β -actin. Evaluation of product purity by melt curve analysis revealed no additional products. Since primer sets amplified rat cDNA with similar high efficiency over a range of cDNA dilutions, preproET-1, preproET-2, and preproET-3 could be compared relative to β -actin to allow the determination of relative gene expression. Master mixes of the reagents were prepared to minimize differences in reagent composition and pipetting errors. Twenty ng of sample cDNA were incubated with 25 μ L iQ SYBR Green Supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada) and 200 nM of each primer, and the reagent mixture was brought up to 50 μ L with DNase/RNase-free water. All reactions were performed in duplicate on 96-well plates in a spectrofluorometric thermal cycler (iCycler iQ, Bio-Rad). PCR runs were initiated by incubation at 95 °C for 3 min to activate the iTAQ polymerase followed by 40 cycles of 95 °C for 15 s, the appropriate annealing temperature for 15 s, and 72 °C for 30 s. Fluorescence was monitored at every cycle during the elongation step. Negative RT controls (samples for which MMLV reverse transcriptase was omitted from the reverse transcription reaction mix) were included in each run to test for genomic DNA contamination, and a melt curve was conducted following each run to verify product purity. Expression was calculated relative to β -actin using the delta-delta Ct method (21), and expressed as fold change relative to air control samples.

Statistical analyses. Data are expressed as mean $\pm$ SEM. The effects of ozone and the particles EHC-93 on mRNA levels were tested for statistical significance by three-way ANOVA with OZONE (0, 0.8 ppm O₃), EHC (0, 50 mg EHC-93/m³) and TIME (0, 24 hours

post-exposure) as factors. Plasma ET data immediately after inhalation exposures were analyzed by two-way ANOVA with OZONE (0, 0.8 ppm O₃) and EHC (0, 50 mg EHC-93/m³) as factors. Tukey's multiple comparison procedure was applied to elucidate the patterns of significant effects ($\alpha = 0.05$). Significance of linear regression correlation coefficient was determined using Student's t-test ($\alpha = 0.05$). Statistical analyses were performed using the Sigma-Stat software (Sigma-Stat 3.0, Chicago, Illinois).

Results

In contrast to the increase in preproET-1 mRNA expression in the lungs immediately after exposure to the pollutants (Fig. 1a; OZONE x TIME factor interaction, $p<0.001$), preproET-3 mRNA was transiently decreased (Fig. 1e; OZONE x TIME factor interaction, $p<0.001$). Rats exposed to particulate matter exhibited a sustained decrease in preproET-3 mRNA expression in the lungs (EHC main effect, $p=0.025$). At the experimental concentrations used, ozone was more potent than the particles in elevating expression of preproET-1 mRNA, but in contrast to the sustained 24 h increase in preproET-1 mRNA by particles, the response to ozone was transient. The circulating levels of ET-1_[1-21] (Fig. 1b; EHC x OZONE factor interaction, $p<0.001$) and ET-3_[1-21] (Fig. 1f; EHC x OZONE factor interaction, $p=0.099$) peptides increased immediately after inhalation exposure of the animals to either particles or ozone, while plasma ET-2_[1-21] levels remained unchanged (Fig. 1d). Co-exposure to ozone plus particles did not result in statistically significant changes of the three endothelin peptides in plasma; in fact, levels of all three peptides were lower in the co-exposure group than in the animals exposed to either pollutant. Overall, while plasma levels of ET-1 and ET-3 were positively correlated (Fig. 2a; $r=0.60$, $p=0.014$), lung levels of preproET-1 and preproET-3 mRNA were negatively correlated (Fig. 2b; $r=-0.66$, $p<0.0001$).

Figure 1. Effects of inhalation of particulate matter and ozone on preproendothelin mRNA levels in the lungs (A, C, E) and circulating endothelin levels in plasma (B, D, E). Results are expressed as mean $\pm$ SEM (n=4-12 animals/group). Letters over bars indicate statistical significance (Tukey, $p < 0.05$). A) PreproET-1 mRNA. Three-way ANOVA, OZONE x TIME factor interaction, $p < 0.001$. **a**, 0 vs. 24 h within 0 ppm O₃; **b**, 0 vs. 0.8 ppm O₃ within 0 h; **c**, 0 vs. 0.8 ppm ozone within 24 h; **d**, 0 vs. 24 h within 0.8 ppm O₃. B) Plasma ET-1 peptide. Two-way ANOVA, EHC x OZONE factor interaction, $p < 0.001$. **a**, 0 vs. 50 mg/m³ EHC within 0 ppm O₃; **b**, 0 vs. 0.8 ppm O₃ within 0 mg/m³ EHC; **c**, 0 vs. 0.8 ppm O₃ within 50 mg/m³ EHC; **d**, 0 vs. 50 mg/m³ EHC within 0.8 ppm O₃. C) PreproET-2 mRNA. No detectable levels in lung tissue. D) Plasma ET-2 peptide. No significant effects. E) PreproET-3 mRNA. Three-way ANOVA, OZONE x TIME factor interaction, $p < 0.001$. EHC main effect, $p = 0.025$. **a**, 0 vs. 50 mg/m³ within EHC; **b**, 0 ppm vs. 0.8 ppm O₃ within 0 hr post exposure; **c**, 0 hr vs. 24 hr post exposure within 0.8 ppm O₃. F) Plasma ET-3 peptide. Two-way ANOVA, EHC x OZONE factor interaction, $p = 0.099$. Panel A and B reproduced from ref. (7) by permission of Oxford University Press.

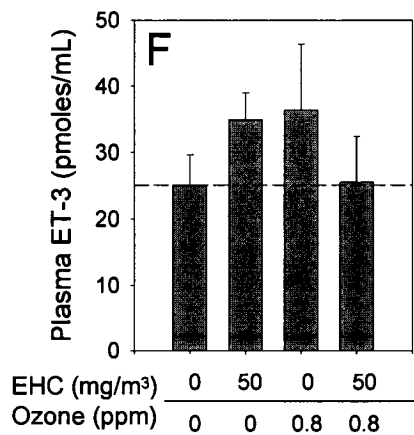
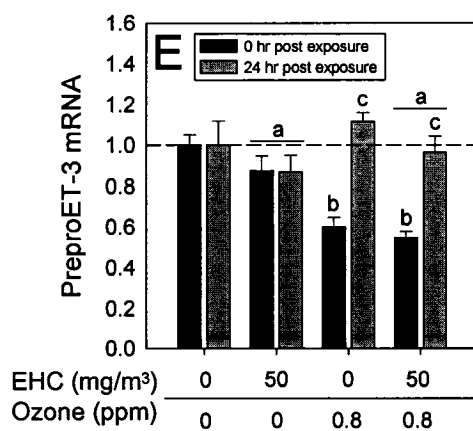
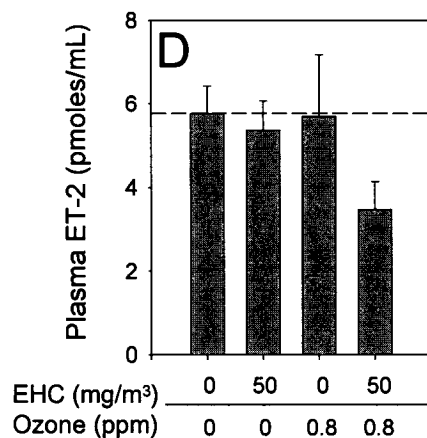
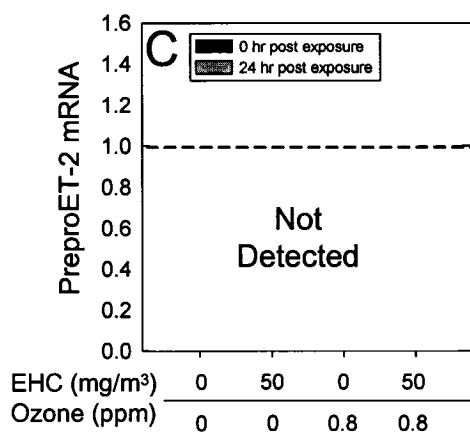
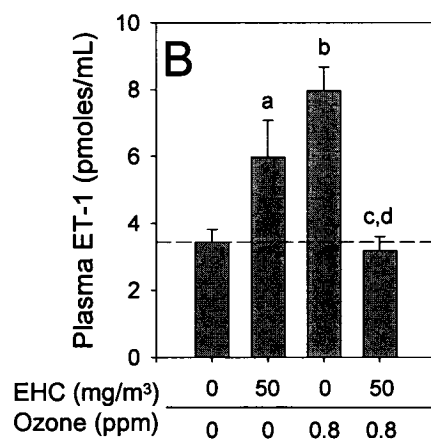
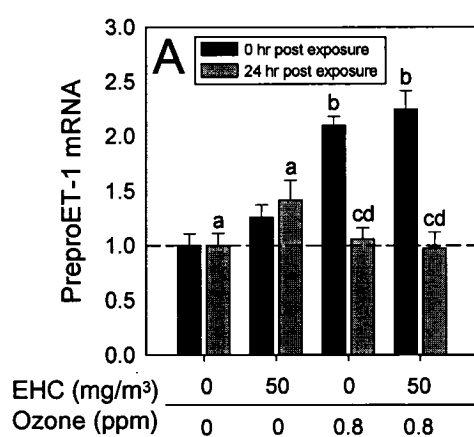
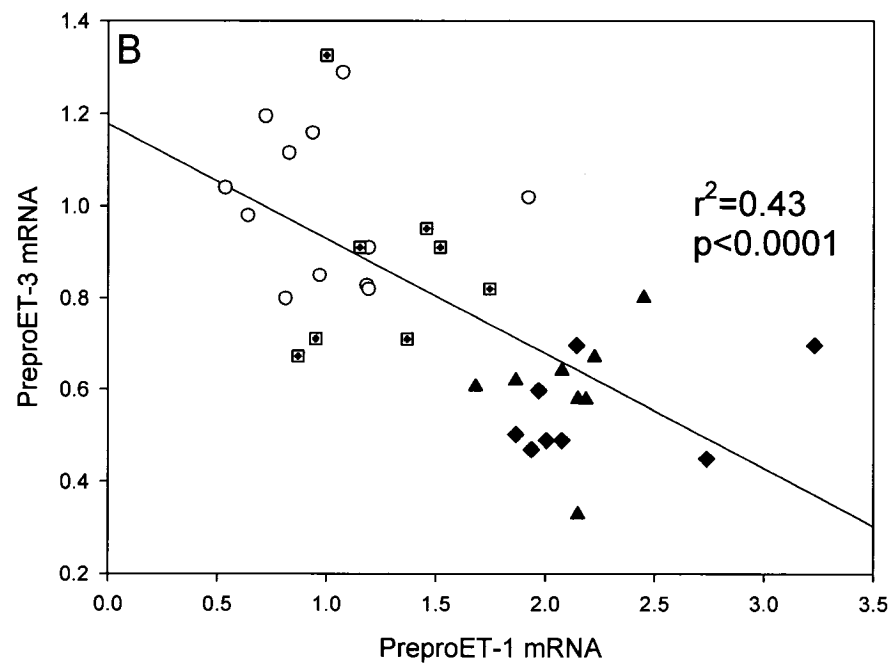
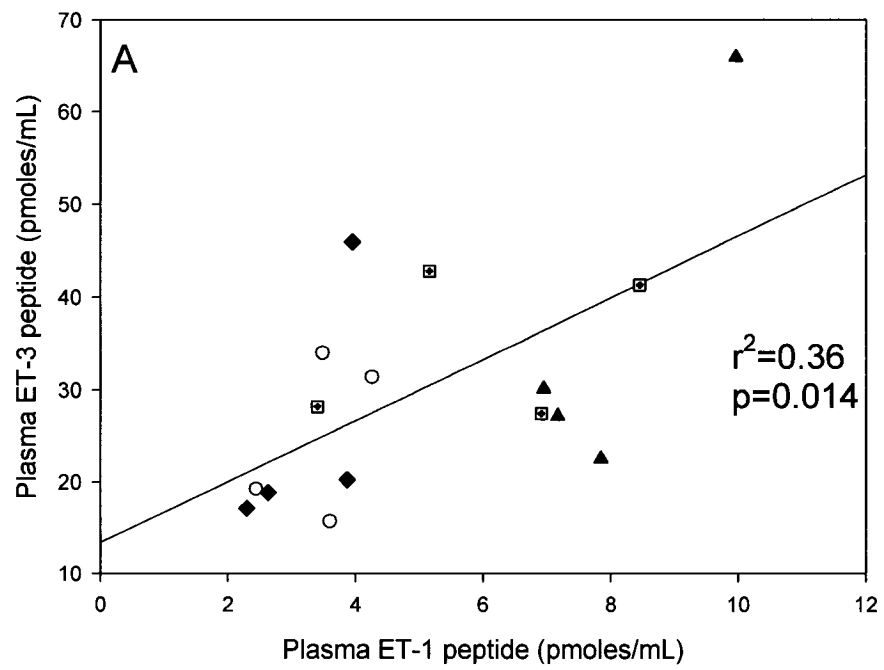


Figure 2. Linear regressions of plasma ET-1 and ET-3 peptides (A) and lung tissue preproET-1 and preproET-3 mRNA levels (B) for individual animals exposed to air (open circle), EHC-93 particles (square), ozone (triangle), or ozone plus particles (diamond).



Discussion

With appropriate controls to verify the absence of genomic DNA contamination, a SYBR Green I dye-based real-time PCR approach is a simple, relatively inexpensive, and reproducible system for gene expression analysis. We have validated a SYBR Green I dye-based real-time PCR assay for determination preproET-1, preproET-2, and preproET-3 mRNA expression in rats. In combination with primers for rat ECE-1, ET_A and ET_B receptors, and endothelial and inducible nitric oxide synthase (7), these primers allow investigation of endothelin-nitric oxide system gene expression. Using these primers we confirmed that in our animals preproET-1 was the dominant endothelin mRNA in the lungs, followed by preproET-3 mRNA, while pulmonary preproET-2 mRNA expression was negligible, consistent with previous reports (13, 22).

Increased endothelin production is a plausible mechanism to explain the association between episodic variations of ambient air pollutants and acute cardiovascular morbidity and mortality in susceptible individuals (6-9). An imbalance in the production of endothelium-derived constriction and dilation factors in the pulmonary vascular bed contributes to the alteration of vascular tone. Such endothelial dysfunction and increased vascular resistance may contribute to the progression of cardiovascular disease by increasing cardiac afterload and myocardial ischemia (23). Inhalation of either particulate matter or ozone has been shown to cause concurrent increases of preproET-1 and ECE-1 mRNA in the lungs and increased plasma steady-state concentrations of the ET-1 peptide in circulation (7). Exposure of rats to urban particles also resulted in an immediate and sustained (>48 h) two-fold increase of the steady-state levels of plasma ET-3, revealing a major impact on

regulation of the endothelin system (9). Here we report that while plasma ET-3 tends to increase immediately after inhalation of particulate matter and ozone in a pattern similar to that observed for the ET-1 peptide, preproET-3 mRNA expression in the lung actually decreases. Therefore, the data indicate that the lungs are most probably not the site of *de novo* ET-3 synthesis responsible for the sustained elevation of plasma ET-3 after inhalation of urban particles. Neuronal cells in the lungs possess secretory granules that can contain endothelin (24), and it is conceivable that EHC-93 may act as a trigger to cause the release of ET-3. However, since ET-3 has a half-life of only 1-2 minutes in plasma (25), the sustained increase in steady-state plasma ET-3 levels over 48 h after inhalation of EHC-93 (9) should require continuous *de novo* peptide production, or a sustained reduction in clearance. Since lung ET_B receptor mRNA initially increases immediately after exposure to either ozone or particles (7), decreased receptor expression and reduced receptor-mediated clearance of the peptide is not a likely mechanism to explain the rapid increase of circulating ET-3. Endothelin-3 is a neurotransmitter, and the pituitary is a site of ET-3 synthesis (reviewed in ref. (26)). Other sites of importance in the regulation of circulating ET-3 levels are the vascular beds of the spleen and kidney, which have been shown to clear ET-3 from circulation (27). The data suggest that these sites are potentially affected after inhalation of air pollutants, and organ specific expression analyses should help resolve this issue.

It is unclear what is prompting the decrease in preproET-3 mRNA in the lungs. PreproET-3 mRNA was altered reciprocally to preproET-1 mRNA in the lungs after exposure to the pollutants. Since ET-1 and ET-3 may induce similar effects when expressed at high levels, it is conceivable that our findings reflect a feedback mechanism whereby ET-1 released as a result of increased preproET-1 mRNA expression acts locally to depress

preproET-3 mRNA expression. There is some evidence to support this possibility. While initial studies of ET-1 knockout mice found no change in ET-2 or ET-3 mRNA and peptide levels in the lungs, intestines, or brains (22), preproET-3 mRNA has been shown to be over-expressed in ET-1-null astrocytes derived from these animals (28), suggesting that expression of preproET-3 is normally repressed by ET-1. In a rat model of diabetes, preproET-1 mRNA increased while preproET-3 mRNA decreased in the adrenal glands, suggesting the two genes are regulated by different mechanisms (29). Along the same lines, preproET-1 and preproET-2 are reciprocally modulated in the failing heart and during ischemia of cardiomyocytes (30). Clearly, additional work is required to identify the specific cells or areas in the lungs that are responsible for the increased preproET-1 mRNA and decreased preproET-3 mRNA, and to examine regulation of the respective genes in these cells. This mismatch between expression of preproET-1 and preproET-3 mRNAs in the lungs after inhalation of the pollutants could imply increased local ET-1 and ET_A receptor-mediated vasoconstriction, coupled with decreased ET-3 and ET_B receptor-mediated vasodilation.

When inhaled individually, particulate matter and ozone increased the circulating levels of ET-1 and ET-3. However, after co-exposure of the animals to both pollutants the plasma levels of the two peptides were not affected despite clear evidence of alteration of gene expression in the lungs. We have reported previously that co-exposure to particulate matter and ozone provokes an increase of MMP-2 activity in the lungs (7), an enzyme that has been shown to cleave bigET-1 to ET-1_[1-32] *in silico* (31), and we proposed that the excess bigET-1 produced in the injured lungs may be diverted toward ET-1_[1-32] production. Sources of circulating ET-3 peptide and the mechanistic explanation for the apparent

($p=0.099$) interaction of the two pollutants with respect to plasma levels of ET-3 remain to be determined. Nevertheless, if regulation of preproET-3 at a remote site after inhalation exposure of ozone and particles is dependent on elevated systemic ET-1_[1-21] levels from increased *de novo* synthesis and spill-over from the pulmonary capillary bed, then the pattern of response of plasma ET-3 is entirely consistent with that of ET-1.

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Since our observations did not support the hypothesis that the pollutant-induced increase of plasma ET-3 was due to increased *de novo* synthesis in the lungs, I performed an organ screen to examine whether exposure to urban pollutants might provoke extrapulmonary endothelin production. Because ET-3 is thought to be a neuropeptide, and the pituitary is a potential source, we hypothesized that pollutants may act on the pituitary and cerebrovascular endothelin systems. Real-time PCR was used to examine endothelin system gene expression in these organs, as well as the heart, liver, kidney, and spleen of rats exposed by inhalation to particulate matter and ozone. This paper describes the findings in the cerebral hemisphere and pituitary. Inhalation of particulate matter and ozone also modulated preproET-1 mRNA levels in the heart (Supplementary Material, Figure 1). Animals were part of a larger study conducted by Prem Kumarathasan (See Chapter 4, Flowchart 2, p. 88). I was involved in the experimental design, performed the PCR work, statistical analyses, and data interpretation, and was the lead author in the preparation of the manuscript. Lilian Calderón-Garcidueñas was involved in the genesis of the experiment and the interpretation of data. Renaud Vincent was the principal investigator.

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Air Pollution Alters Brain And Pituitary Endothelin-1 And Inducible Nitric Oxide Synthase Gene Expression

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Abstract

Recent work suggests that air pollution is a risk factor for cerebrovascular and neurodegenerative disease. Effects of inhaled pollutants on the production of vasoactive factors such as endothelin (ET) and nitric oxide (NO) in the brain may be relevant to disease pathogenesis. Inhaled pollutants increase circulating levels of ET-1 and ET-3, and the pituitary is a potential source of plasma ET, but the effects of pollutants on the expression of ET and NO synthase genes in the brain and pituitary are not known. In the present study, Fischer-344 rats were exposed by nose-only inhalation to particles (0, 5, 50 mg/m³ EHC-93), ozone (0, 0.4, 0.8 ppm), or combinations of particles and ozone for 4 h. Real-time reverse transcription polymerase chain reaction was used to measure mRNA levels in the cerebral hemisphere and pituitary 0 and 24 h post-exposure. Ozone inhalation significantly increased preproET-1 but decreased preproET-3 mRNAs in the cerebral hemisphere, while increasing mRNA levels of preproET-1, preproET-3, and the ET-converting enzyme (ECE)-1 in the pituitary. Inducible NO synthase (iNOS) was initially decreased in the cerebral hemisphere after ozone inhalation, but increased 24 h post-exposure. Particles decreased tumour necrosis factor (TNF)- α mRNA in the cerebral hemisphere, and both particles and ozone decreased TNF- α mRNA in the pituitary. Our results show that ozone and particulate matter rapidly modulate the expression of genes involved in key vasoregulatory pathways in the brain and pituitary, substantiating the notion that inhaled pollutants induce cerebrovascular effects.

Funding Source and Ethic Consent

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Introduction

In addition to the extensive literature documenting the association of air pollution and respiratory and cardiovascular disease, there is now evidence of a link between air pollution and increased risk of cerebrovascular and neurodegenerative disease. Epidemiologic studies have shown an association between gaseous and particulate pollutants and ischemic stroke in a number of cities (Henrotin et al., 2007; Villeneuve et al., 2006; Chan et al., 2006; Maheswaran et al., 2005; Tsai et al., 2003; Hong et al., 2002a; Hong et al., 2002b). Individuals living in a highly polluted city exhibited increased inflammation and β -amyloid accumulation in the brain (Calderon-Garciduenas et al., 2004), and dogs exposed to high levels of air pollution in Mexico City exhibit increased iNOS in cortical endothelial cells and blood brain barrier alterations (Calderon-Garciduenas et al., 2002). Direct effects of inhaled pollutants are supported by controlled animal exposures revealing translocation of particles to the brain (Oberdorster et al., 2004) and oxidative damage and increased inducible nitric oxide synthase (iNOS) expression in dopaminergic neurons after chronic exposure to ozone (Pereyra-Munoz et al., 2006).

The cerebrovascular endothelium plays a critical role in the regulation of normal vascular homeostasis, and perturbation of endothelial function is an important step in the pathogenesis of disease. For example, endothelial dysfunction and inflammation are implicated in the development of brain pathology in Alzheimer's disease (Grammas et al., 2002), and impairment of endothelium-dependent relaxation in cerebral arteries may contribute to ischemic stroke (Cosentino et al., 2001). Altered production of vasoactive factors such as endothelin (ET) and nitric oxide (NO) may be implicated in these effects.

Endothelins are potent vasoactive peptides upregulated in a number of disorders involving endothelial dysfunction, including pulmonary hypertension, congestive heart failure, atherosclerosis, cerebral vasospasm, and stroke (Volpe and Cosentino, 2000; Luscher and Barton, 2000). Activation of the cerebral ET system in pathophysiological states can cause rapid and prolonged constriction of cerebral blood vessels (Andresen et al., 2006), and administration of ET-1 to the middle cerebral artery is commonly used to induce experimental cerebral ischemia (Macrae et al., 1993). The vasoconstrictive effects of ETs are counterbalanced by vasodilators such as NO, an important regulator of cerebral blood flow (Andresen et al., 2006). We have shown that plasma levels of ET-1 and ET-3 are increased in animal models after exposure to particulate matter or ozone (Thomson et al., 2005; Vincent et al., 2001; Bouthillier et al., 1998), and higher pollution levels are associated with increased plasma ET-1 in children (Calderon-Garciduenas et al., 2003). Although activation of the pulmonary endothelin system may explain elevated plasma ET-1 after pollutant exposure (Thomson et al., 2005; Thomson et al., 2004), the source of increased ET-3 is not known, and does not appear to be due to de novo synthesis in the lungs (Thomson et al., 2006). Endothelin-1 and ET-3 are both widely expressed in the brain and pituitary gland, and ETs may be released by the pituitary into the peripheral circulation (Plonowski et al., 1997). However, the effect of air pollution on ET and NO synthase expression in the brain is not known.

Evaluation of the impact of inhaled pollutants on the development and progression of cerebrovascular and neurodegenerative diseases would require chronic exposure and an appropriate disease model. Nonetheless, it is conceivable that acute exposure of healthy animals may induce detectable changes in cerebral mRNA levels relevant to disease

pathogenesis. For example, since neurodegenerative diseases are characterized by increased inflammation, oxidative stress, rupture of the blood-brain-barrier, and early up-regulation of iNOS (Ryu and McLarnon, 2006; Grammas et al., 2006; Christov et al., 2004; Selkoe, 2001), pollutant effects on genes relevant to these conditions should be investigated. Furthermore, the increased incidence of ischemic stroke on the same day as a rise in air pollution levels (Hong et al., 2002b) implies rapid effects of acute exposure. Such acute adverse effects most likely occur in compromised individuals, such as subjects with atherosclerosis, hypertension, diabetes or other conditions associated with endothelial dysfunction. Therefore, effects of acute exposure on genes involved in key vasoregulatory pathways should be investigated. Since alteration of ET-NO homeostasis may be implicated in the pathogenesis of cerebrovascular and neurodegenerative disease, and because the pituitary is a potential source of circulating ET, in the present study we examined whether acute exposure to particulate matter and ozone modulated ET-NO system gene expression in the cerebral hemisphere and pituitary.

Materials and Methods

Animals. Specific pathogen-free Fischer-344 male rats (200-250 g) were obtained from Charles River (St. Constant, Québec, Canada). The animals were housed in individual plexiglass cages on wood-chip bedding under HEPA-filtered air and held to a 12 h dark/light cycle. Food and water were provided ad libitum. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

Inhalation exposure to air pollutants. The ambient urban particles EHC-93 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system at the Environmental Health Centre (Tunney's Pasture, Ottawa, Canada) and mechanically sieved using a 36 μm mesh filter. The chemical composition, biological reactivity of the particles in cell culture models, and applications in inhalation studies have been described elsewhere (Vincent et al., 2001; Bouthillier et al., 1998; Vincent et al., 1997a; Vincent et al., 1997b). The particle size distribution of resuspended EHC-93 particles in our flow-past nose-only exposure system is multimodal, with two respirable modes at 1.3 μm aerodynamic diameter (D_{AE}) and 3.6 μm D_{AE} that together comprise 55 % of the mass of the aerosol, and a non-respirable mode at 15 μm D_{AE} that comprises 45 % of the mass (Vincent et al., 2001). Animals were trained in nose-only exposure tubes for 5 consecutive days prior to exposure. Rats ($n=4/\text{group}$, except $n=6$ for the 0 h air group, and $n=2$ for the 24 h air group) were exposed for 4 h to combinations of 1) EHC-93 (0, 5, 50 mg/m^3) and ozone (0, 0.4, 0.8 ppm) and euthanized immediately after exposure, or 2) EHC-93 (0, 50 mg/m^3) and ozone (0, 0.8 ppm) and euthanized 24 h after exposure, essentially as described previously (Thomson et al., 2005; Vincent et al., 1997a).

Biological samples. Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg, i.p.). After exsanguination, the head was removed by decapitation at the first cervical vertebra. The brain was removed and the cerebral hemisphere was dissected, snap-frozen in liquid nitrogen, and stored at -80 °C until use. The pituitary was removed and stored in the preservative solution RNAlater (Qiagen Inc., Mississauga, Ontario, Canada) at -20 °C.

Reverse transcription of cerebral hemisphere and pituitary RNA samples. Frozen cerebral hemisphere samples were homogenized in TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada), and total RNA was isolated according to the manufacturer's instructions. Pituitary total RNA was isolated using MiniPrep kits (Qiagen). RNA was quantified using the RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon, U.S.A.). Total RNA was reverse transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, Ontario, Canada) as previously described (Thomson et al., 2005).

Real-time PCR analysis. Primers for endothelin system genes (preproET-1, preproET-3, ECE-1, ET_A and ET_B receptor), iNOS, eNOS, TNF- α , and a reference gene (β -actin) were designed using Vector NTI software (InforMax, Frederick, Maryland) and validated as previously described (Thomson et al., 2005). Master mixes of the reagents were prepared to minimize differences in reagent composition. Twenty ng of cDNA were incubated with iQ SYBR Green Supermix (Bio-Rad Laboratories (Canada) Ltd.,

Mississauga, Ontario, Canada) and 200 nmol/L of each primer, and the reagent mixture was brought up to volume with DNase/RNase-free water. All reactions were performed in duplicate on 96-well plates in a spectrofluorometric thermal cycler (iCycler iQ, Bio-Rad) using 50 μ L volumes (Thomson and Vincent, 2005). PCR runs were initiated by incubation at 95 °C for 3 min to activate the iTAQ polymerase followed by 40 cycles of 95 °C for 15 s, 62 °C for 15 s, and 30 s at 72 °C. Fluorescence was monitored at every cycle during the elongation step. A melt curve was conducted following each run to verify product purity. Expression was calculated relative to β -actin using the delta-delta Ct method (Livak and Schmittgen, 2001), and expressed as fold change relative to time-matched air control samples.

Statistical analyses. Data are expressed as means $\pm$ SEM. Where necessary, results were transformed to meet the requirements of normality and equal variance. Data were analyzed by two-way ANOVA (immediately after exposure) with OZONE (0, 0.4, 0.8 ppm) and EHC (0, 5, 50 mg/m³) as factors, or three-way ANOVA with OZONE (0, 0.8 ppm), EHC (0, 50 mg/m³) and TIME (0, 24 h post-exposure) as factors, followed by the Holm-Sidak multiple comparison procedure to elucidate the pattern of significant effects ($\alpha = 0.05$; Sigma-Stat 2.0, Chicago, Illinois).

Results

The effects of a 4 h exposure by inhalation to ozone and particulate matter on ET-NO system gene expression in the cerebral hemisphere and pituitary were assessed by real-time PCR immediately after exposure and following 24 h recovery in filtered air. Effects of exposure to ozone and particulate matter were investigated for the individual pollutants as well as for the combined pollutants to evaluate dose-dependent changes attributable to each pollutant and possible toxicological interactions. PreproET-1 mRNA levels were transiently increased immediately after exposure to ozone in both the cerebral hemisphere (two-way ANOVA, OZONE main effect, $p=0.01$; Fig. 1a) and pituitary (two-way ANOVA, OZONE main effect, $p<0.001$; Fig. 1c). There was no significant particle effect detected in the cerebral hemisphere, but there was a trend towards increased preproET-1 mRNA levels immediately after exposure in the pituitary ($p=0.059$). Unlike preproET-1, preproET-3 mRNA levels were decreased by ozone exposure in the cerebral hemisphere immediately after exposure (two-way ANOVA, OZONE main effect, $p=0.012$; Fig. 2a); this change was transient (three-way ANOVA, TIME main effect, $p=0.007$; Fig. 2b). Ozone inhalation did, however, cause a slight increase in preproET-3 mRNA expression in the pituitary (two-way ANOVA, OZONE main effect, $p=0.029$; Fig. 2c). This modest increase was sustained over 24 h (three-way ANOVA, OZONE main effect, $p=0.027$; Fig. 2d). Exposure to particulate matter tended to decrease cerebral hemisphere preproET-3 mRNA expression ($p=0.091$; Fig. 2a), but particle inhalation did not have any effect on pituitary preproET-3 mRNA expression (Fig. 2c). After 24 h recovery in filtered air, preproET-1 and preproET-3 mRNA returned to control levels in all treatment groups (Fig. 1bd, 2bd), with the exception of the sustained increase of pituitary preproET-3 mRNA in ozone-exposed animals.

Figure 1. Effects of particulate matter (EHC-93) and ozone on preproET-1 gene expression. Rats were exposed by inhalation to EHC-93 and ozone at the indicated doses and euthanized immediately or 24 h post-exposure. Cerebral hemisphere and pituitary mRNA levels were assessed by real-time RT-PCR. Results are expressed as mean $\pm$ SEM (n=4 animals/treatment). Asterisks indicate statistical significance (Holm-Sidak, $p < 0.05$). *0, 0.4 vs 0.8 ppm within OZONE, **0 vs 24 h within 0.8 ppm O₃. (A) Cerebral hemisphere preproET-1 mRNA immediately after exposure. OZONE main effect, $p = 0.01$. (B) Comparison of 0 vs. 24 h cerebral hemisphere preproET-1 mRNA. (C) Pituitary preproET-1 mRNA immediately after exposure. OZONE main effect, $p < 0.001$. EHC main effect, $p = 0.059$. (D) Comparison of 0 vs. 24 h pituitary preproET-1 mRNA. OZONE x TIME interaction, $p = 0.025$.

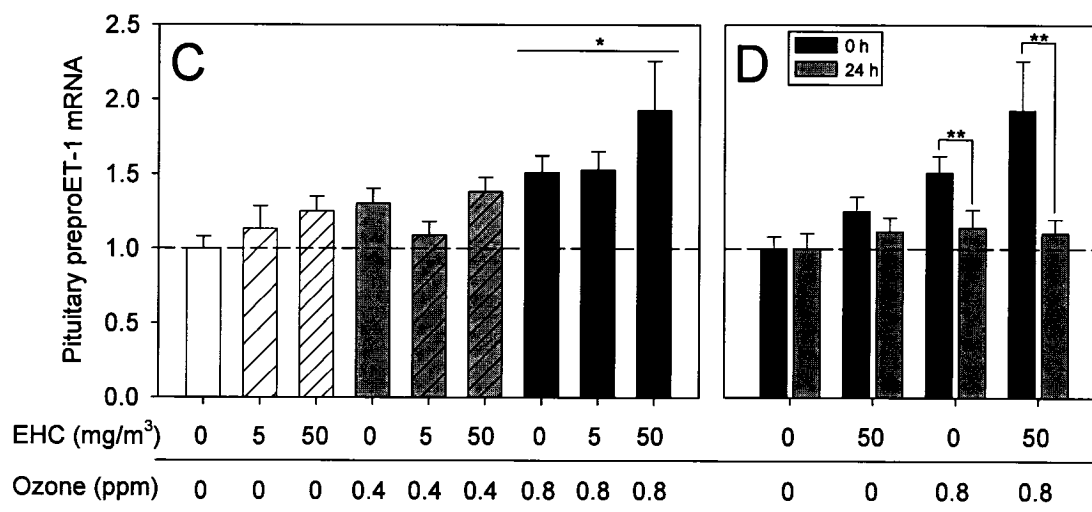
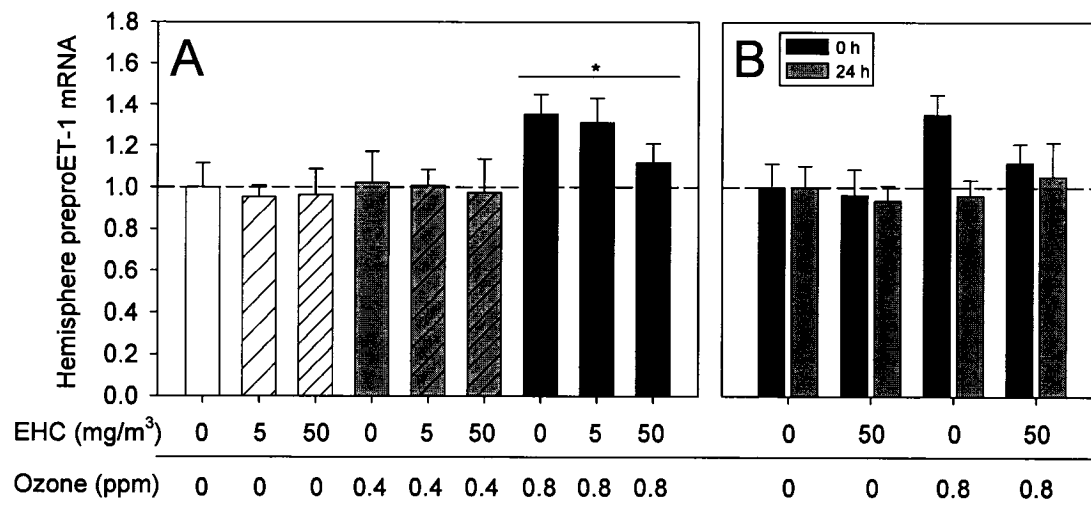
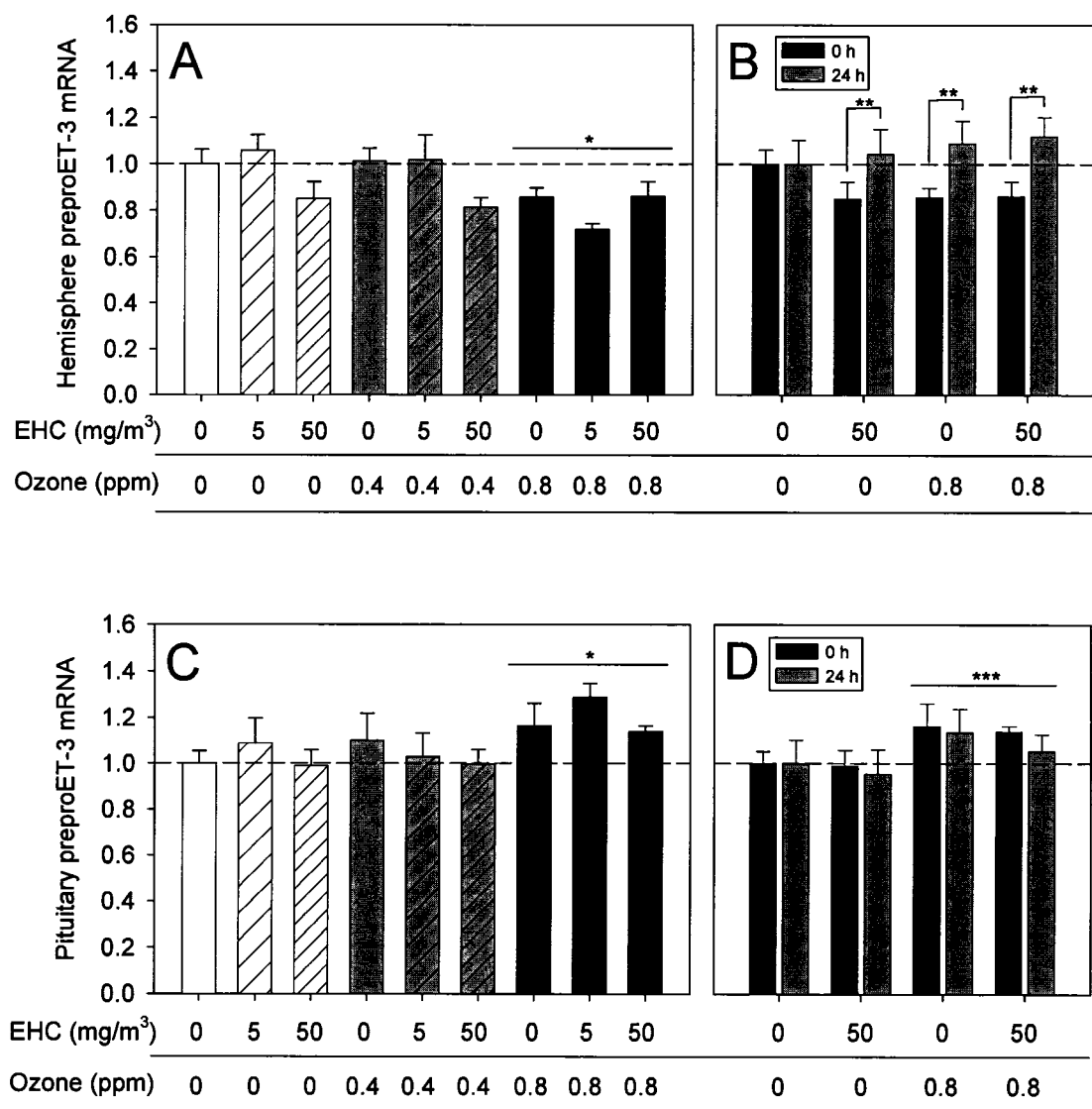


Figure 2. PreproET-3 mRNA levels in the cerebral hemisphere and pituitary of rats exposed by inhalation to particulate matter (EHC-93) and ozone. Results are expressed as mean $\pm$ SEM (n=4 animals/treatment). Asterisks indicate statistical significance (Holm-Sidak, $p < 0.05$). *0, 0.4 vs 0.8 ppm within OZONE, **0 vs 24 h within TIME, ***0 vs 0.8 ppm within OZONE. (A) Cerebral hemisphere preproET-3 mRNA immediately after exposure. OZONE main effect, $p = 0.012$. EHC main effect, $p = 0.091$. (B) Comparison of 0 vs. 24 h cerebral hemisphere preproET-3 mRNA. TIME main effect, $p = 0.007$. (C) Pituitary preproET-3 mRNA immediately after exposure. OZONE main effect, $p = 0.029$. (D) Comparison of 0 vs. 24 h pituitary preproET-3 mRNA. OZONE main effect, $p = 0.027$.



Because nitric oxide is a vasodilator that opposes the actions of ET-1, we evaluated the mRNA levels of inducible (iNOS) and endothelial (eNOS) nitric oxide synthases in the cerebral hemisphere and pituitary. Expression of iNOS mRNA in the cerebral hemisphere was significantly decreased immediately after exposure to ozone (two-way ANOVA, OZONE main effect, $p < 0.001$; Fig. 3a). After 24 h recovery, cerebral hemisphere iNOS mRNA in ozone-exposed animals was significantly elevated (three-way ANOVA, OZONE x TIME, $p < 0.001$; Fig. 3b). In contrast, there was no significant effect of pollutant inhalation on cerebral hemisphere eNOS mRNA levels (Fig. 3cd). We did not detect any significant change in iNOS or eNOS mRNA in the pituitary (data not shown).

Mature ET peptides are produced through cleavage of the precursor big-endothelin forms by ET-converting enzymes (ECEs), and act through specific receptors, the ET_A receptor and ET_B receptor, all of which are expressed in the cerebral hemisphere and pituitary. Furthermore, ET-1 can regulate NO production through binding to endothelial ET_B receptors. We therefore examined the effect of pollutant inhalation on mRNA levels for ECE-1 and the two ET receptors. There was no significant effect on ECE-1 mRNA in the cerebral hemisphere (Fig. 4ab), but ECE-1 mRNA levels were higher in the pituitary of animals exposed to 0.8 ppm ozone (two-way ANOVA, OZONE main effect, $p < 0.001$; Fig. 4c), returning to control levels after 24 h recovery (three-way ANOVA, OZONE x TIME interaction, $p = 0.01$; Fig. 4d). There was no significant effect on ET receptor expression in either the cerebral hemisphere or pituitary (data not shown).

Figure 3. Cerebral hemisphere inducible nitric oxide synthase (iNOS) and endothelial nitric oxide (eNOS) mRNA expression in rats exposed to particles (EHC-93) and ozone. Results are expressed as mean $\pm$ SEM (n=4 animals/treatment). Asterisks indicate statistical significance (Holm-Sidak, $p < 0.05$). *0 vs 0.4 ppm within OZONE, **0, 0.4 vs 0.8 ppm within OZONE, ***0 vs. 0.8 ppm O₃ within 0 h. ****0 vs 0.8 ppm O₃ within 24 h. (A) Cerebral hemisphere iNOS mRNA immediately after exposure. OZONE main effect, $p < 0.001$. (B) Comparison of 0 and 24 h cerebral hemisphere iNOS mRNA. OZONE x TIME interaction, $p < 0.001$. (C) Cerebral hemisphere eNOS mRNA immediately after exposure. (D) Comparison of 0 and 24 h cerebral hemisphere eNOS mRNA.

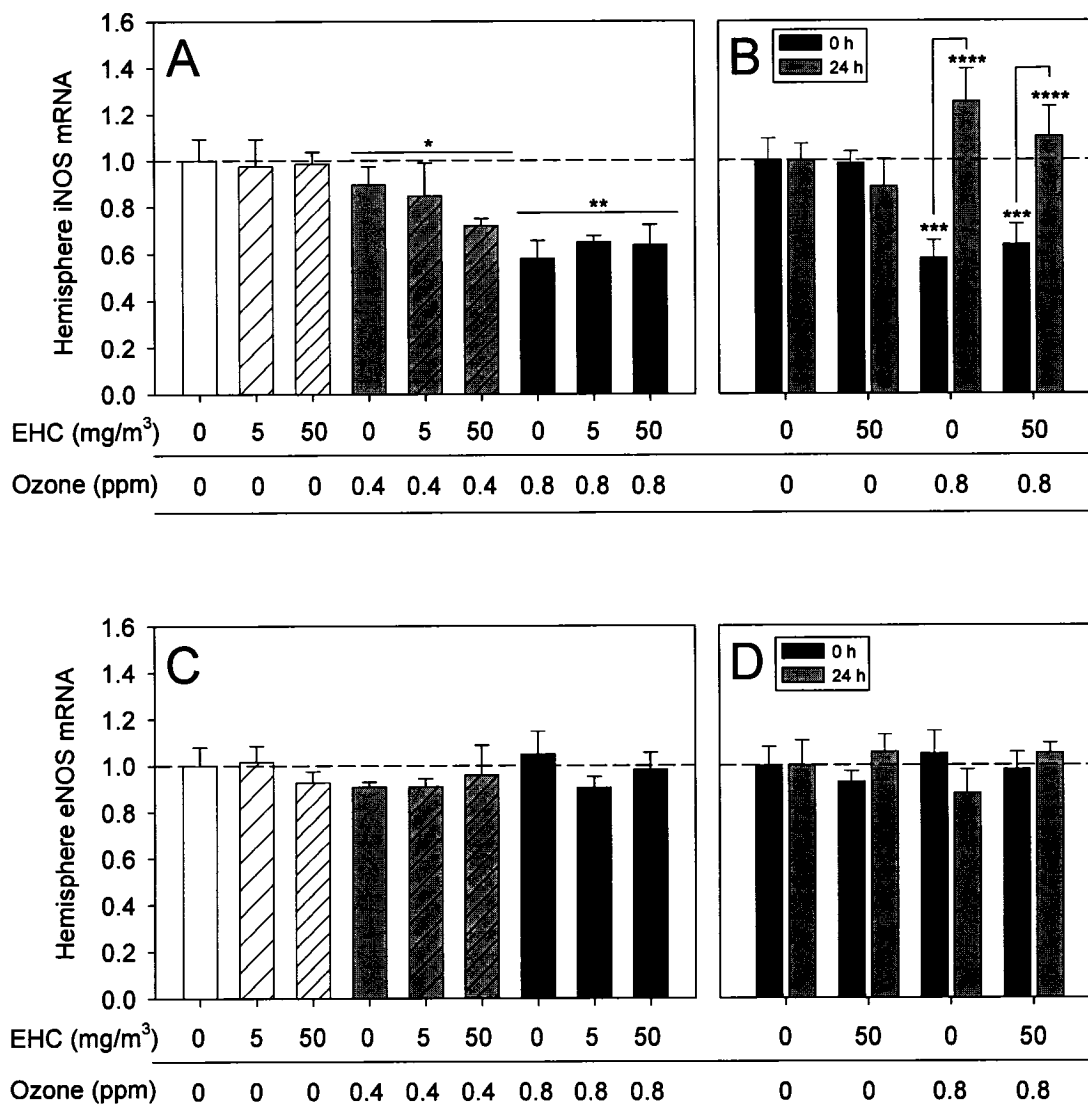
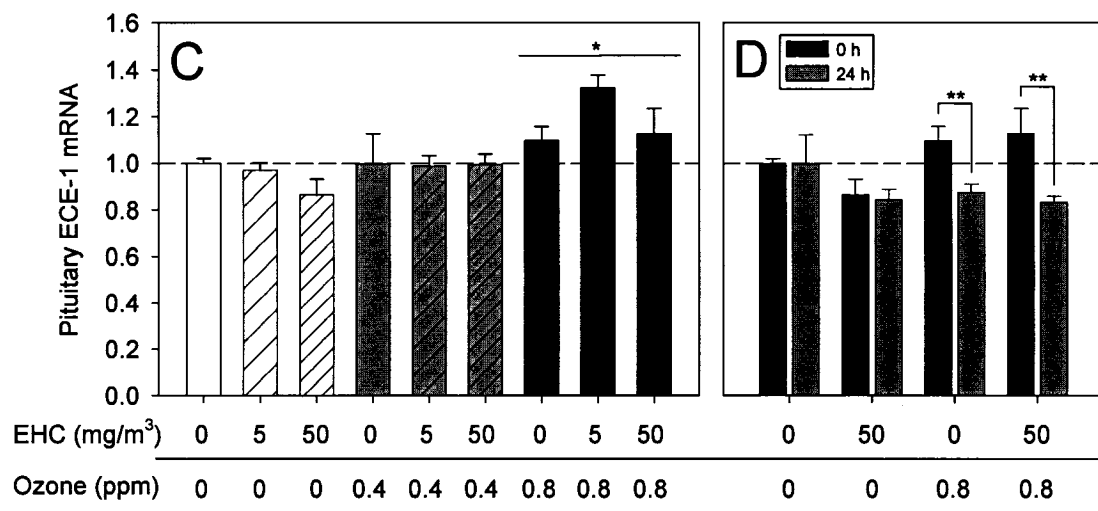
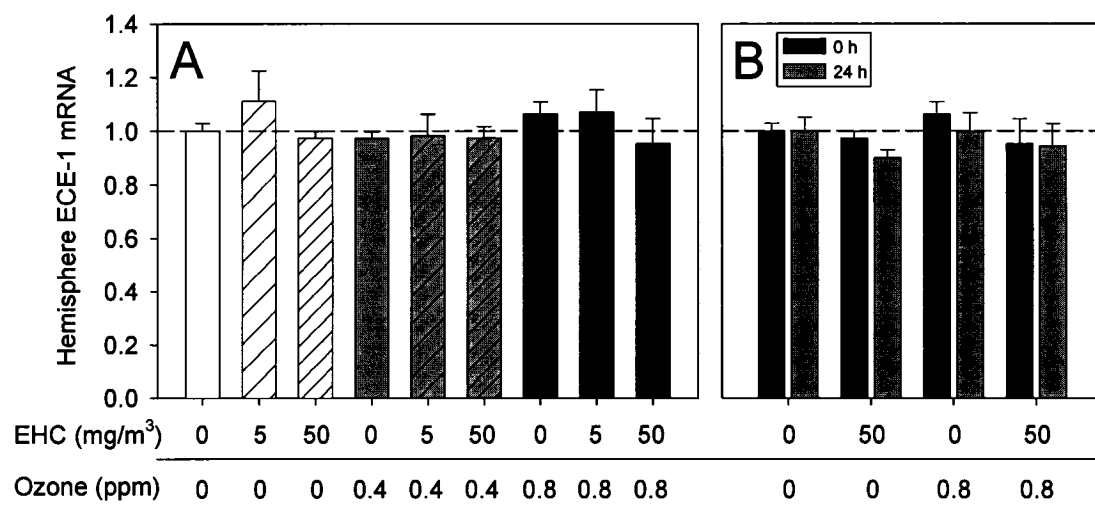
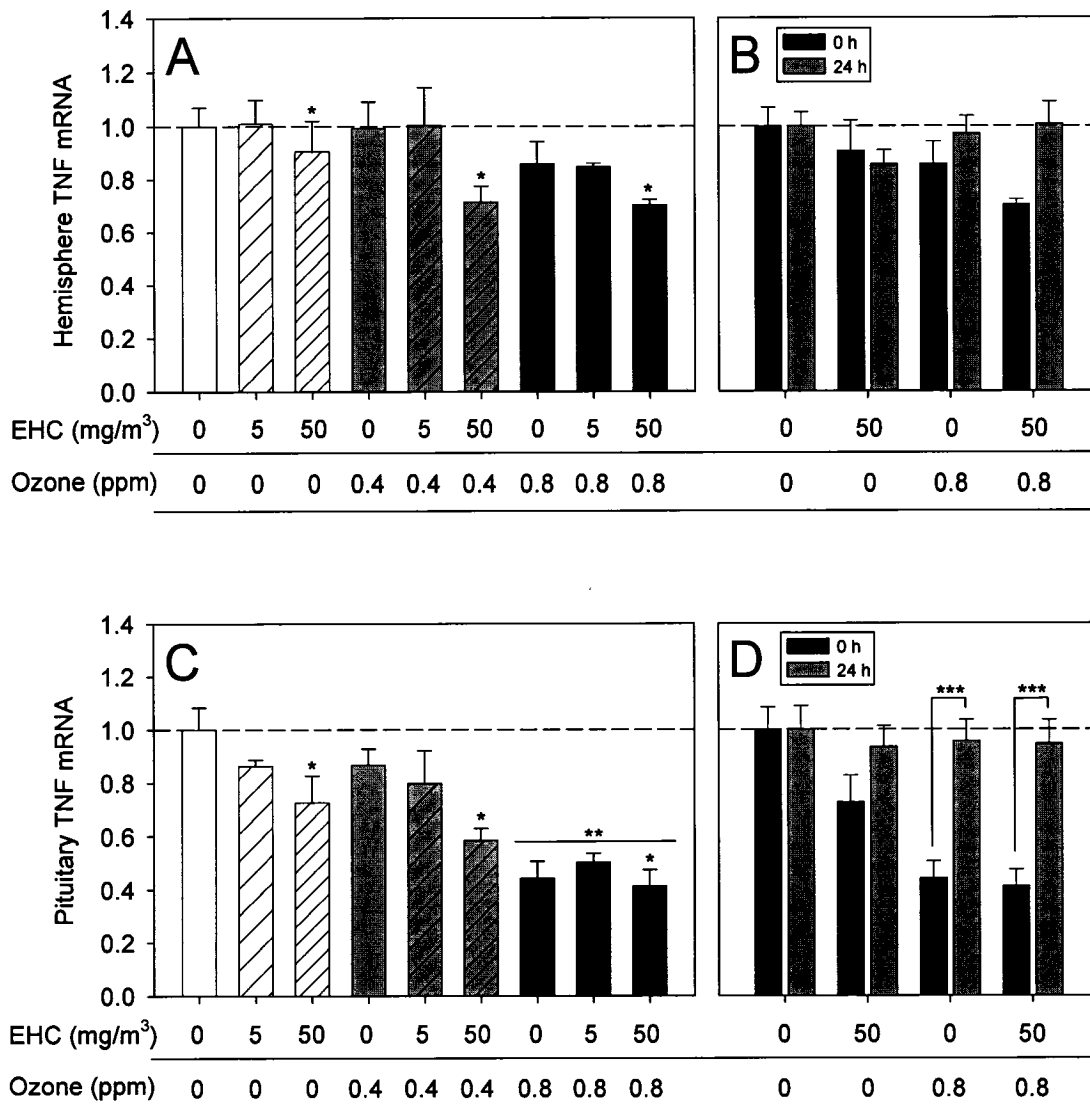


Figure 4. Pollutant effects on endothelin-converting enzyme (ECE-1) mRNA levels in the cerebral hemisphere and pituitary. Rats exposed by inhalation to particulate matter (EHC-93) and ozone at the indicated doses were euthanized immediately or 24 h after exposure and mRNA levels of were assessed by real-time RT-PCR. The results are expressed as mean $\pm$ SEM (n=4 animals/treatment). Asterisks indicate statistical significance (Holm-Sidak, $p < 0.05$). *0 vs 0.8 ppm within OZONE, **0 vs 24 h within 0.8 O₃. (A) Cerebral hemisphere ECE-1 mRNA immediately after exposure. (B) Comparison of 0 and 24 h hemisphere ECE-1 mRNA. (C) Pituitary ECE-1 mRNA immediately after exposure. OZONE main effect, $p < 0.001$. (D) Comparison of 0 and 24 h hemisphere ECE-1 mRNA. OZONE x TIME interaction, $p = 0.01$.



Since particulate matter and ozone may cause changes in brain gene expression by increasing inflammation, we examined mRNA levels of the pro-inflammatory cytokine TNF- α as an early marker of inflammatory signalling. TNF- α mRNA decreased significantly in response to particle exposure in the cerebral hemisphere (two-way ANOVA, EHC main effect, $p=0.011$; Fig. 5a). Cerebral hemisphere TNF- α mRNA levels were also transiently decreased after ozone inhalation (two-way ANOVA, OZONE main effect, $p=0.055$; Fig. 5a), returning to control levels 24 h after exposure (three-way ANOVA, OZONE x TIME interaction, $p=0.05$; Fig. 5b). In the pituitary, TNF- α mRNA levels decreased in response to inhalation of particulate matter or ozone (two-way ANOVA, OZONE main effect, $p<0.001$; EHC main effect, $p=0.009$; Fig. 5c). The effect of particles on pituitary TNF- α mRNA tended to be sustained over 24 h (three-way ANOVA, EHC main effect, $p=0.024$), but the effect of ozone was transient (three-way ANOVA, OZONE x TIME interaction, $p<0.001$; Fig. 5d).

Figure 5. Particulate matter and ozone decrease tumour necrosis factor (TNF)- α mRNA in the cerebral hemisphere and pituitary. TNF- α mRNA levels were assessed by real-time PCR in rats exposed to particles (EHC-93) and ozone for 4 h and euthanized immediately or 24 h post-exposure. The results are expressed as mean $\pm$ SEM (n=4 animals/treatment). Asterisks over bars indicate statistical significance (Holm-Sidak, $p < 0.05$). *0, 5 vs 50 mg/m³ within EHC. **0, 0.4 vs 0.8 ppm within OZONE, *** 0 vs 24 h within 0.8 ppm O₃. (A) Cerebral hemisphere TNF- α mRNA immediately after exposure. EHC main effect, $p = 0.011$. (B) Comparison of 0 and 24 h hemisphere TNF- α mRNA. (C) Pituitary TNF- α mRNA immediately after exposure. EHC main effect, $p = 0.009$. OZONE main effect, $p < 0.001$. (D) Comparison of 0 and 24 h pituitary TNF- α mRNA. OZONE x TIME interaction, $p < 0.001$. EHC main effect, $p = 0.024$ (0 vs 50 mg/m³ within EHC; not indicated).



Discussion

Brief exposure to ozone and particulate matter is associated with increased incidence of ischemic stroke (Maheswaran et al., 2005; Tsai et al., 2003; Hong et al., 2002a; Hong et al., 2002b), and chronic high levels of air pollution are associated with increased brain inflammation (Calderon-Garciduenas et al., 2004; Migliore and Coppede, 2002). This suggests that inhaled pollutants may directly target the brain, or induce systemic changes that affect the brain. In the present study we investigated whether acute exposure to ozone and particulate matter affect the expression of genes involved in key vasoregulatory pathways of the brain. We found that a single exposure by inhalation to ozone and particulate matter rapidly altered the expression of ET-NO system genes in the brain and pituitary. The effect of pollutant inhalation on the expression of most genes was transient, with the exception of the sustained increase of pituitary preproET-3 and decrease of TNF- α mRNA for 24 h. Changes of gene expression were pollutant-specific, and where both pollutants exerted an effect, the effect appeared to be additive. Furthermore, we observed a region-specific response, with the cerebral hemisphere responding to pollutant inhalation differently than the pituitary. Our findings substantiate the notion that inhaled air pollutants exert acute cerebrovascular effects.

Transcriptional activation of ET-1, eNOS and iNOS is an important step in their regulation (Kleinert et al., 2004; Fagan et al., 2001). Nitric oxide is a negative regulator of ET-1, and inhibition of nitric oxide synthesis can result in a net increase of ET-1 production by relieving the down-regulation of ET-1 synthesis (Kourembanas et al., 1993). The absence

of effects on eNOS mRNA levels (Fig. 3cd) suggests that, in our healthy animal model, the capacity of the endothelium to release NO may not be adversely affected. Nevertheless, the immediate increase of preproET-1 mRNA and decrease of iNOS mRNA in the cerebral hemisphere after inhalation of ozone (Fig. 1a, 3a) indicates that exposure to this pollutant can rapidly affect the regulation of genes implicated in the control of cerebral vascular tone. Although inducible, constitutive expression of iNOS has been detected in most regions of the normal human brain (Park et al., 2000). Furthermore, endothelin-1 has been shown to inhibit iNOS expression under some conditions (Markewitz et al., 1997), and inhibition of iNOS reduced the relaxation of middle cerebral arteries in normotensive and hypertensive rats induced by administration of the NOS substrate L-arginine (Briones et al., 1999), revealing a role for iNOS in the control of vascular tone. Our study does not provide any insight into the cellular location of the changes in preproET-1 and iNOS mRNA, and it is not known whether the observed effects occur independently or are linked. In the pituitary, increased expression of preproET-1, preproET-3 and ECE-1 mRNAs (Fig. 1c, 2c, 4c) is consistent with increased *de novo* synthesis of the preproETs and cleavage to the mature active peptides. Increased levels of the mature ET-1 peptide have recently been measured in the medulla oblongata and midbrain of normotensive and spontaneously hypertensive rats exposed chronically to cigarette smoke (Ohno et al., 2004). Our present observation of transcriptional activation of ET genes in the cerebral hemisphere and pituitary is in line with this previous study, and suggests that ambient pollutants may exert similar effects on brain ET genes. While the identity of the signal(s) responsible for the observed effects on expression of ET-1 and iNOS is unknown, both ET-1 and iNOS are rapidly activated by a number of factors, including NF- κ B and TNF- α . TNF- α , IL-1 α , and NF- κ B levels increase in the brains of

ovalbumin-sensitized mice exposed repeatedly (4 h/d, 5 d/wk for 2 wk) to concentrated particulate matter, indicating that air pollutants modify expression of these factors (Campbell et al., 2005). Our results indicate that inhaled particulate matter can rapidly decrease TNF- α mRNA expression in both the cerebral hemisphere (Fig. 3a) and the pituitary (Fig. 3b) immediately after a single exposure, suggesting that effects on ET-NO system gene expression occur in the absence of proinflammatory cytokine signalling. The physiological significance of the TNF- α mRNA decrease is unknown, but TNF- α is constitutively expressed in the normal rat brain (Ignatowski et al., 1996) and decreased TNF- α levels have been measured in the brain (Bartolomucci et al., 2003) and pituitary (O'Connor et al., 2003) of stressed animals.

Our data show that ozone, a pollutant associated with ischemic stroke (Henrotin et al., 2007; Chan et al., 2006; Tsai et al., 2003; Hong et al., 2002a; Hong et al., 2002b), alters the expression of key vasoregulatory factors in the brain of healthy rats. There have also been reports of an association between levels of particulate matter and stroke (Wellenius et al., 2005; Tsai et al., 2003; Hong et al., 2002a; Hong et al., 2002b). Living near a major road is associated with increased incidence of ischemic stroke, with risks diminishing with distance from the road (Maheswaran and Elliott, 2003). The specific agents are not known, but ultrafine particles and other traffic-derived pollutants may be involved in such effects. Nanoparticles have been shown to translocate to the brain via olfactory neurons (Oberdorster et al., 2004), and the olfactory bulb may be a route of penetration from inhaled particles of sufficiently small dimensions. Our resuspended ambient particle preparation EHC-93 does not contain a substantial ultrafine mass fraction. It may possibly contain a sufficient number

of ultrafine or nano-size particles to create a significant dose in the olfactory bulb, but no data is available to assess this. Furthermore, our EHC-93 material should have lost short-lived reactive species and some volatile components present in freshly generated particles, and may be less potent than fresh ambient fine and ultrafine particles. Despite those limitations, we report here a strong trend for increased preproET-1 mRNA in the pituitary ($p=0.059$) and decreased preproET-3 mRNA in the cerebral hemisphere ($p=0.091$) after inhalation of EHC-93 particles. These data warrant additional investigation to confirm whether the brain ET-NO system is responsive to inhalation of ambient particles, and to assess whether particle age and size are important factors in the acute brain ischemic events documented by epidemiologic studies.

The brain microvasculature is a crucial player in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and cerebrovascular diseases such as stroke, as well as in systemic diseases with a brain component such as hypertension and diabetes (Andresen et al., 2006; Birkenhager and Staessen, 2006; Arvanitakis et al., 2006; Grammas et al., 2002). It is conceivable that endothelial dysfunction in the brain microvasculature, associated with acute elevation of ET-1 and impairment of NOS-mediated vasodilation could precipitate ischemic stroke, particularly in susceptible individuals, such as those with existing hypertension, atherosclerosis, or diabetes (Cosentino et al., 2001; Volpe and Cosentino, 2000; Salom et al., 1995). In the Goto-Kakizaki rat model of type II diabetes, ET_A receptor antagonism reduced the wall-to-lumen ratio in brain vasculature, suggesting that ET-1 is involved in cerebrovascular remodelling that may contribute to the increased risk of stroke in diabetes (Harris et al., 2005). Genetic ablation of iNOS protects mice from Alzheimer's-like disease (Nathan et al., 2005), which suggests that iNOS could be a major

factor in β -amyloid deposition and disease progression. Increased iNOS expression and accumulation of β -amyloid have been observed in the brains of dogs and humans exposed to significant concentrations of urban air pollutants (Calderon-Garciduenas et al., 2004; Calderon-Garciduenas et al., 2002). Thus, the rebound elevation of iNOS in the brain of our animals 24 h after exposure to ozone could possibly constitute an adverse response.

Endothelins are also implicated in neuroendocrine homeostasis. Endothelin system genes are expressed in all regions of the pituitary, and all hormones derived from the anterior and posterior pituitary are regulated by ETs (Lange et al., 2002). Our findings of increased preproET-1 and preproET-3 mRNA in the pituitary immediately after 4 h inhalation exposure to ozone hint that acute exposure to air pollutants may induce alterations in neuroendocrine function through actions on the pituitary ET system. Moreover, complex interactions exist between the neuroendocrine and immune systems, and it is well known that endotoxins, ETs, and cytokines are capable of stimulating the hypothalamic-pituitary-adrenal axis (Morgazo et al., 2005; Turrin and Rivest, 2004; Rivest, 2001; Raab et al., 1999; Haak et al., 1997; Hirai et al., 1991). Future experiments should clarify the dynamics of circulating pituitary hormones in animals following acute exposure to ambient pollutants.

In summary, we report here that acute inhalation exposure to ozone clearly results in rapid perturbation of ET-NO system gene expression in the cerebral hemisphere and pituitary of rats. Inhalation of EHC-93 urban particles resulted in borderline ($p=0.059$) elevation of preproET-1 mRNA, and significant alteration of TNF- α mRNA levels. Our study supports the notion that common urban pollutants can exert cerebrovascular effects. The

pathophysiological significance of our observations remains to be demonstrated, for example through positive interaction in the stroke-prone spontaneously hypertensive rat model that exhibits increased ET-1 peptide and ET_A receptor levels in the brain and reduced vascular expression of NO synthase isoforms (Jesmin et al., 2004; Hirafuji et al., 2002). Chronic exposure to air pollutants in suitable animal models may provide additional insight into the physiological consequences of these alterations.

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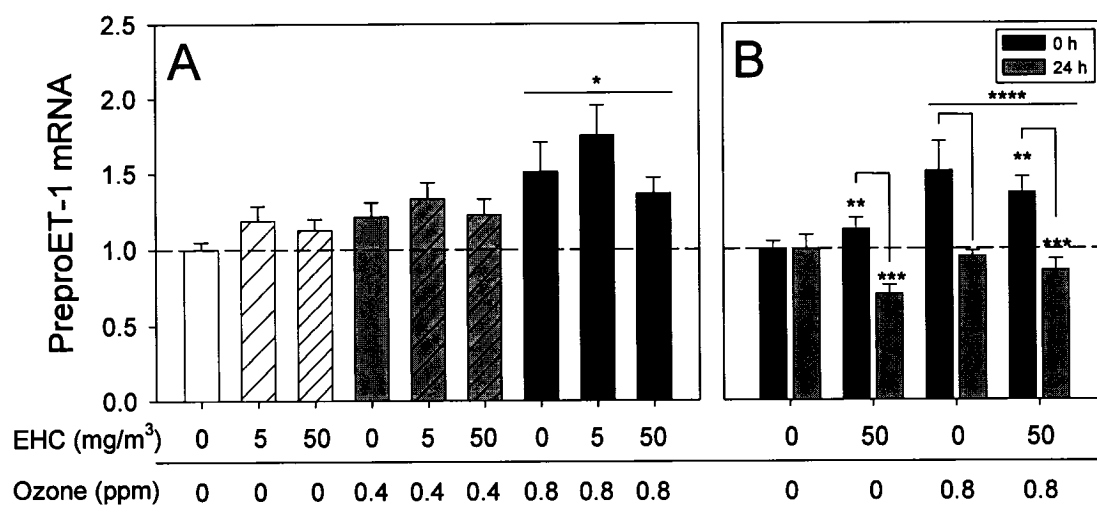
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Supplementary Material, Figure 1. PreproET-1 mRNA levels in the heart of rats exposed to air or combinations of particulate matter (EHC-93) and ozone. Results are expressed as mean $\pm$ SEM (n=4-12 animals/treatment). Asterisks indicate statistical significance (Tukey, $p < 0.05$). *0, 0.4 vs. 0.8 ppm within OZONE, **0 vs. 50 mg/m³ EHC within 0 h, ***0 vs. 50 mg/m³ EHC within 24 h, ****0 vs. 0.8 ppm within OZONE. Brackets indicate a significant effect ($p < 0.05$) of factor TIME within a given treatment group. (A) PreproET-1 mRNA immediately after exposure. OZONE main effect, $p < 0.001$. EHC main effect, $p = 0.11$. (B) Comparison of 0 and 24 h preproET-1 mRNA. OZONE main effect, $p = 0.006$. EHC x TIME interaction, $p = 0.035$.

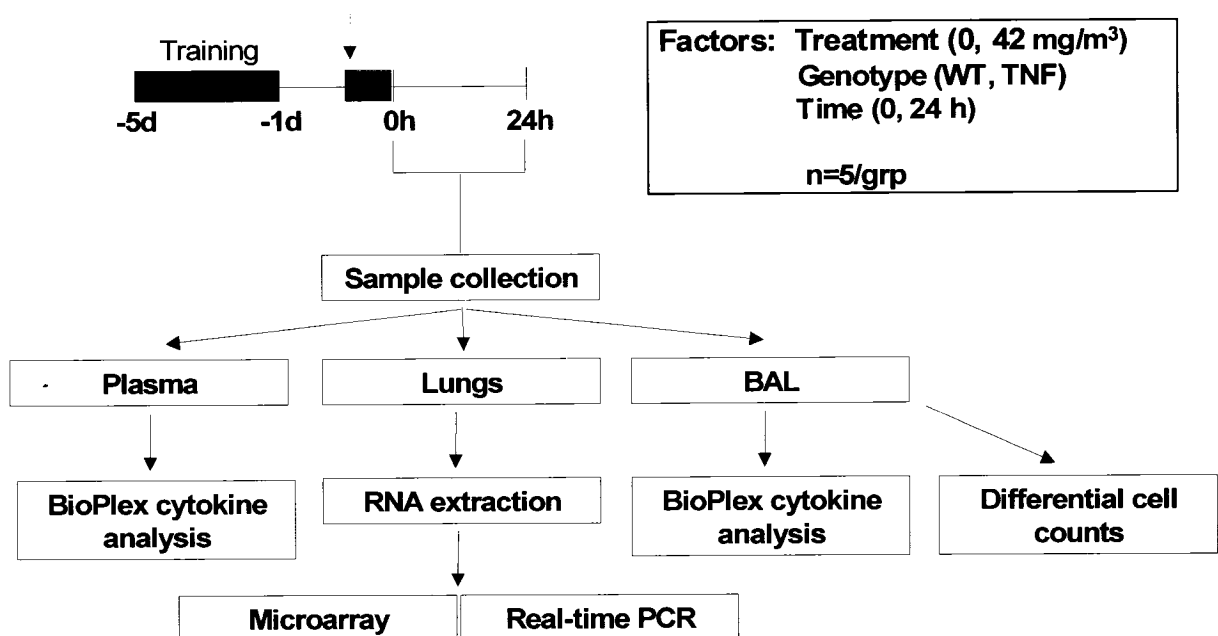


7. Thomson E, Williams A, Yauk C, Vincent R. Toxicogenomic Analysis of Susceptibility to Inhaled Urban Particulate Matter in Mice with Constitutive TNF- α Expression. *In preparation.*

Our data and work by others suggest that endothelin production and release may be modified by inflammation and lung injury. This may have relevance to the increased susceptibility of individuals with chronic inflammatory diseases to air pollution. Transgenic TNF- α mice with chronic pulmonary inflammation and their wildtype littermates were exposed to particulate matter, and euthanized 0 and 24 h post-exposure (Flowchart 3, p. 180). Due to the potential complexity of pathways involved in host-pollutant interactions, microarray technology was used to 1) characterize the context in which the increased endothelin expression occurred; 2) detect other genes and pathways that respond to inhaled particulate matter; and 3) identify potential biomarkers of exposure that are robust to the physiological status of the lungs. In addition, we sought to examine the utility of microarrays for detecting pollutant-genotype interactions, and to determine factors important to the design of future experiments in this area. Additional data validating microarray results by real-time PCR (Supplementary Material, Figure 1), and indicating matrix metalloproteinase activation in TNF- α mice (Supplementary Material, Figure 2) is provided. All authors contributed to the experimental design. I conducted the microarray analyses, data analysis and interpretation, and was the lead author for preparation of the manuscript. Andrew Williams performed the microarray data normalization and statistical analyses, and was involved in the conception of various approaches to analyse and display data. The microarray analyses were performed under the supervision of Carole Yauk. Renaud Vincent was the principal investigator.

Flowchart 3. Toxicogenomic analysis of particle effects in a mouse model of chronic lung inflammation. SP-C/TNF- α mice and their wildtype littermates were trained in nose-only tubes and then exposed by inhalation to air or 42 mg/m³ EHC-6802 for 4 h. Mice were euthanized 0 and 24 h post-exposure, bronchoalveolar lavage (BAL) was performed, and lungs and plasma were collected. Lung RNA was extracted for real-time PCR and microarray analyses of gene expression. Lung tissue, plasma and BAL were retained for potential examination of effects on cytokine production or altered expression of other proteins, as indicated by the gene expression analyses.

Exposure (Air or 42 mg/m³ EHC-6802)



Toxicogenomic Analysis Of Susceptibility To Inhaled Urban Particulate Matter In Mice With Constitutive TNF- α Expression

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Running title: Microarray analysis of particle effects in TNF- α mice

Keywords: tumour necrosis factor- α , lung inflammation, air pollution, particulate matter (PM), EHC, microarray, endothelin (ET)-1, Cyp1a1

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Abstract

Adverse health effects detected after episodic variations of air pollution are attributable to susceptible subgroups of the population, including individuals with respiratory and cardiovascular diseases. Because of the complex nature of the interaction between susceptibility and pollutant effects, comprehensive approaches such as gene expression profiling may be useful in identifying molecular networks implicated in the health effects of particulate matter. We used a toxicogenomic approach to examine how chronic pulmonary inflammation affects the transcriptional response of the lungs to inhaled urban particles. Agilent 22K oligonucleotide microarrays were used to measure pulmonary gene expression in transgenic SP-C/TNF- α mice and wildtype littermates exposed for 4h to urban particles (0, 50 mg/m³ EHC-6802, nose-only) and euthanized 0 and 24 h post-exposure (n=5 arrays/group). Bronchoalveolar lavage cell counts and composition were unchanged at either time-point after exposure, indicating that particles did not provoke an alveolar inflammatory response. Real-time RT-PCR confirmed that inhalation of particles caused a 20-40% increase in lung preproendothelin-1 mRNA and a 30-100% increase of Cyp1a1 mRNA in wildtype and TNF mice, validating delivery of a biologically effective dose. Microarray analysis identified differential expression due to genotype and time effects, but did not detect consistent differential expression attributable to particle exposure. Our data indicate that microarray analysis at the level of the whole lung may not detect physiologically relevant effects of acute particle inhalation. The present data reinforce the notion that adverse health effects of acute exposure to urban particles may be dominated by physiological response cascades, with some transcriptional regulation such as activation of the endothelin pathway

and xenobiotic-metabolizing factors in target cells, rather than widespread changes in the expression of genes escaping homeostatic controls.

Introduction

Adverse health effects associated with particulate matter are thought to be due to responses of susceptible subgroups of the population, including individuals with existing respiratory and cardiovascular diseases. For example, smokers (Pope, III et al., 2004) and people with chronic obstructive pulmonary disease (COPD) (Sunyer, 2001), coronary artery diseases (Pope, III et al., 2006), diabetes (Zanobetti and Schwartz, 2002; Goldberg et al., 2001a), and congestive heart failure (Goldberg et al., 2001b) may be at increased risk. Exacerbation of existing inflammation is one mechanism through which particles may cause adverse effects. Inhaled particles can aggravate existing epithelial lesions in the lungs (Vincent et al., 1997a), and impairment of epithelial barrier function can result in increased translocation of particles to the interstitium (Adamson and Hedgecock, 1995). Once in the interstitium, particles are less likely to be cleared via macrophage phagocytosis, and can cause interstitial inflammation, induce direct effects on local cell populations (including macrophages, fibroblasts, endothelial cells, and neutrophils) and drain to local lymph nodes (Oberdorster et al., 1994; Adamson and Prieditis, 1998). Inflammation and oxidative stress may also “prime” the lungs to respond to particles with increased production of reactive oxygen species and inflammatory mediators, exacerbating the existing disease state.

Particulate matter is a complex mixture of organic compounds, metals, salts, etc., that may provoke a number of responses in the lungs. For example, exposure of reporter cell lines to collections of urban particles revealed activation of the xenobiotic response element, Cyp1a1, metallothionein IIa, and heat shock protein 70 (Vincent et al., 1997b). We have proposed that one pathway through which particulate matter increases the risk of

cardiovascular morbidity and mortality is through perturbation of vascular homeostasis as a result of increased pulmonary production of the potent vasoconstrictor endothelin (ET)-1 and spillover into circulation (Bouthillier et al., 1998; Vincent et al., 2001; Thomson et al., 2004; Thomson et al., 2005). Activation of the pulmonary endothelin system by particulate matter appears to occur in the absence of acute injury or a pronounced inflammatory response (Bouthillier et al., 1998; Vincent et al., 1997a), as only mild focal interstitial inflammation was observed 33 h after exposure (Adamson et al., 1999). However, in the setting of acute lung injury resulting from co-exposure to particulate matter and ozone, endothelin biosynthesis and secretion appears to be modified, possibly through cleavage by matrix metalloproteinases in the lungs (Thomson et al., 2005). These observations indicate that while lung injury may not be a prerequisite for effects of inhaled particles on pathways relevant to cardiovascular health, such as increased endothelin synthesis, the physiological state of the lung may be an important modifier of effects.

The interaction between the physiological state of the lungs and the physicochemical characteristics of the inhaled particles may involve multiple pathways and genes, and such complexity is difficult to investigate by conventional means. Microarray technology enables the simultaneous monitoring of thousands of transcripts in a single assay, and has been used effectively in a number of toxicologic studies (reviewed in (Lettieri, 2006)). To date, few microarray studies have examined the impact of urban particulate matter on pulmonary gene expression. Intratracheal instillation of the urban particle preparation EHC-93 in rats produced time-dependent increases in genes involved in oxidative stress and inflammation (Kooter et al., 2005). However, cDNA from animals were pooled and hybridized to a single array per treatment, preventing statistical evaluation of biological and technical variation. A

recent sub-chronic inhalation study involving apolipoprotein E (ApoE) and low-density lipoprotein receptor (LDLr) double knockout mice exposed to concentrated ambient particles did not find any statistically significant changes in pulmonary gene expression (Gunnison and Chen, 2005). However, interpretation of the results may have been confounded by the experimental design, in which air-exposed animals were sacrificed at a different time of day from particle-exposed animals (Gunnison and Chen, 2005).

In the present study we used a toxicogenomic approach to examine how chronic pulmonary inflammation modifies the transcriptional response of the lungs to inhaled urban particulate matter. Transgenic mice with constitutive pulmonary expression of tumour necrosis factor (TNF)- α under the control of the surfactant protein (SP)-C promoter have chronically inflamed lungs that result in alveolar disruption and airspace enlargement (Miyazaki et al., 1995; Fujita et al., 2001). We have previously shown that these mice exhibit increased oxidative stress and enhanced protein nitration in the lungs after repeated exposure to particulate matter and ozone compared to their wildtype littermates (Kumarathasan et al., 2005), suggesting that they may be useful as a model of susceptibility. Using commercial microarrays we assessed the expression of roughly 21,000 transcripts in SP-C/TNF- α mice and their wildtype littermates exposed by inhalation to urban particulate matter or air. Rather than pooling biological replicates, we evaluated gene expression on a 1 sample/array basis, allowing us to perform stringent statistical analyses on the results. We had three major objectives: 1) to evaluate the suitability of using oligonucleotide microarray technology to investigate gene-pollutant interactions; 2) to identify genes and pathways that

respond to inhaled particulate matter; and 3) to identify potential biomarkers of exposure that are robust to the physiological status of the lungs.

Materials and methods

Animals. SP-C/TNF- α (TNF) mice were obtained from Dr. Mason at the National Jewish Medical and Research Centre in Denver. Male TNF mice were crossed with female C57BL/6 mice (Charles River Laboratories, St. Constant, QC, Canada). Specific pathogen-free male transgenic TNF mice and their wildtype (WT) littermates were genotyped by PCR analysis of genomic DNA isolated from ear punches as previously described (Miyazaki et al., 1995). Because of the difficulty in establishing a sufficiently large colony of mice of the same age for a single exposure, animals were exposed as three balanced cohorts, each approximately 3 months apart. Animals were 131 ± 5 days old and 28.9 ± 2.8 g (WT: 30.8 ± 2.5 g; TNF: 27.1 ± 1.7) at the time of exposure. Mice were housed in individual plexiglass cages on wood-chip bedding under HEPA-filtered air and held to a 12 h dark/light cycle. Food and water were provided *ad libitum*. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

Particulate matter preparation. The ambient urban particles EHC-6802 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system at the Environmental Health Centre (Tunney's Pasture, Ottawa, ON, Canada) in 1996, 1998, 2000, and 2002, mechanically sieved using a $36 \mu\text{m}$ mesh filter, and combined in equal proportions. Although these particles have not been characterized, they were recovered at the same site and in the same manner as the urban particle preparation EHC-93. The chemical composition, biological reactivity of the EHC-93 particles in cell culture models, and applications in inhalation studies have been described elsewhere (Vincent et al.,

1997b; Bouthillier et al., 1998; Vincent et al., 2001; Vincent et al., 1997a).

Activation of the aryl hydrocarbon (Ah) receptor by EHC-6802. The AhR-based luciferase reporter cell line H1L1.1c2 was used to assess Ah receptor-activation by the EHC-6802 particles. H1L1.1c2 cells were exposed in triplicate to EHC-6802 (1, 10, 100 $\mu\text{g}/\text{cm}^2$), 10 nM benzo(a)pyrene (positive control), and TiO_2 (negative control), and luciferase activity was measured according to the previously described method (Ziccardi et al., 2002).

Inhalation exposure to air pollutants. Mice were trained in nose-only exposure tubes over 5 consecutive days, and then exposed for 4 h to clean air or 42 mg/m^3 EHC-6802 by nose-only exposure essentially as described previously (Vincent et al., 1997a; Thomson et al., 2005). The particle concentration was monitored during each exposure at the inhalation ports by isokinetic sampling using 0.2 μm Teflon filters. Filter weight was divided by the sampling volume to provide a direct estimate of the time-weighted average particulate concentration. Particle counts and size measurements were performed at the inhalation ports (optical size range of 0.3-10 μm ; Lasair Model 301; Particle Measuring Systems, Boulder, CO, USA). Aerodynamic size characteristics were determined by gravimetric cascade impactor analyses of isokinetic samples at the inhalation ports (seven-stage Mercer cascade impactor, 1 L/min, 0.2-5.1 μm effective cut-off diameter; Intox, Albuquerque, NM, USA) or the chamber exhaust (seven-stage Mercer cascade impactor, 10 L/min, 0.2 to 9.8 μm effective cut-off diameter; Intox). Multimodal particle size distribution analysis was performed as previously described (Vincent et al., 2001).

Deposition modelling. Model assumptions for mice were strict nasal breathing, a minute ventilation of 33.58 mL/min, an alveolar surface area of 0.5 m², and a tracheobronchial surface area of 3.5 cm² (default values for the Regional Deposited Dose Ratio RDDR2 Modelling software, US EPA).

Collection of biological samples. Mice were anaesthetized by administration of sodium pentobarbital (60 mg/kg, i.p.) and euthanized by exsanguination immediately after exposure, or following 24 h recovery in filtered air (n=5 animals of each genotype/treatment/time). The lungs were washed by bronchoalveolar lavage with warm saline (37 °C) at 30 mL/kg body weight, then flash frozen in liquid nitrogen and stored at –80 °C. Bronchoalveolar lavage fluid (BALF) was centrifuged (1500 rpm for 10 min at 4 °C) to remove cells, and frozen at –80 °C. Cells were counted in a Coulter Multisizer II (Coulter Canada, Ville St. Laurent, QC, Canada) and differential cell counts were obtained from cytopspin preparations. Lavaged lungs were divided in half, with the left lung designated for RNA extraction and the right lung designated for protein analysis. The lungs were then snap-frozen in liquid nitrogen and stored at –80 °C.

RNA extraction and purification. Frozen lung samples were homogenized in TRIzol reagent (Invitrogen Canada Inc., Burlington, ON, Canada), and total RNA was isolated according to the manufacturer's instructions. The isolated RNA was further purified by spin-column using RNeasey Mini Kits (Qiagen). RNA was quantified using the RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, OR, USA), and quality was assessed using the Agilent 2100 Bioanalyzer and RNA 6000 NanoLab Chip Kit (Agilent

Technologies Canada Inc., Mississauga, ON, Canada). RNA was aliquoted into separate tubes for microarray and PCR analyses to reduce freeze-thawing of samples.

Microarray hybridization. An unbalanced block factorial design (Montgomery, 2001) was used for the factors *Treatment* (0, 50 mg/m³), *Time* (0, 24 h), and *Genotype* (WT, TNF). The design was blocked for the nuisance factors *Date of exposure* and *Date of hybridization* (Kerr, 2003). Five biological replicates per condition were used for a total of 40 microarrays. Individual 2.5 µg aliquots of RNA from each sample were amplified and labelled using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent) according to manufacturer's instructions. Agilent mouse oligo microarrays (containing approximately 22,000 probes) were hybridized with 5 µg Cy5-labelled lung RNA (1 lung/array) and 5 µg Cy3-labelled Universal Mouse Reference RNA (Stratagene, CA, USA), used as a common reference on all arrays. Arrays were incubated overnight at 60 °C in Agilent hybridization solution and washed according to manufacturer's instructions. Arrays were scanned using ScanArray Express (Perkin-Elmer Life Sciences, Woodbridge, ON, Canada), and data were acquired with ImaGene 5.5 (BioDiscovery, CA, USA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). The expression of selected genes was assessed by real-time RT-PCR. Twenty ng of cDNA was combined with Quantitect primer assays and real-time PCR supermix (Qiagen) according to the product protocol, and run at an annealing temperature of 55 °C on the iCycler (Bio-Rad). Fluorescence was monitored at every cycle during the 72 °C elongation step. Post-run melt curves were routinely inspected to verify product purity. Expression was calculated relative to β-actin using the delta-delta Ct method (Livak and Schmittgen, 2001). PCR results were

evaluated for statistical significance by one-tailed ANOVA (Sigma Stat 3.0, SPSS Inc., Chicago, IL, USA). Particle treatment effects are represented graphically as fold-change relative to the time and genotype-matched air-exposed controls.

Statistical analysis of microarray data. The background for each array was determined using the negative control (-)3xSLv1 probe. Spots with median signal intensities less than the mean plus three standard deviations of the (-)3xSLv1 probe were flagged as absent. The total number of flagged spots, the median signal intensity and standard deviation for the (-)3xSLv1 probe for each array were recorded. Other array level summary statistics including the median signal to noise ratio ($\log_2$ scale) for each channel were recorded, and this information was used to help identify microarrays with poor data quality. Lowess normalization (Yang et al., 2002) was performed using the SAS/STAT software, Version 8.2 of the SAS System for Windows (1999-2001 SAS Institute Inc., Cary, NC, USA). Ratio intensity plots for the raw and normalized data were constructed for each array using R (R Development Core Team, 2005). Other data displays produced in R included comparison boxplots, heatmaps, and volcano plots. The logarithm base 2 relative intensities were used for subsequent analyses.

Differentially expressed genes between the control and treated groups at either of the two time points for both TNF and wild type mice was determined using the MAANOVA library (Wu et al., 2003) in R. The statistical model included the main effects *Treatment*, *Time*, and *Genotype*, the three-way and all two-way interactions. The F_s statistic (Cui et al., 2005), a shrinkage estimator for the gene-specific variance components, was used and the p-values for all the statistical tests were estimated using the permutation method (1000

permutations with residual shuffling). These p-values were then adjusted for multiple comparisons by using the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995). The group means for the fold change calculation was based on the adjusted relative intensity for each gene following subtraction of the estimated *Date of exposure* and *Day of hybridization* effects from the normalized ratio. Genes were considered differentially expressed if they had a FDR-adjusted $p < 0.05$ and were present on at least 4 out of 5 arrays in the group showing a significant effect. Agilent GeneSpring software was used for additional analyses and visual representation of array data. A standard condition tree was used to examine data quality.

Based on array results, power scenarios were assessed to determine the sample size (n animals, 1 array/animal) required to detect a 1.5, 1.75, 2, 2.25, 2.5 fold change between control and treated animals. This was conducted by genotype and time point resulting in four independent assessments (WT, 0 h; WT, 24 h; TNF, 0 h; TNF, 24 h). The analysis was conducted in R using the `samr.assess.samplesize` function in the SAMR library (Tibshirani, 2006) on the residuals after controlling for the *Date of exposure* and *Day of hybridization* nuisance factors. The required sample size was identified through inspection of the plots generated from this application and corresponded to the value at which the 90th percentile for the false discovery rate was less than 0.05. The results for the four analyses were then averaged for each fold change. Lastly, the number of arrays required to detect a given fold-change were plotted relative to the minimal detectable fold change.

Results

Particle size distribution

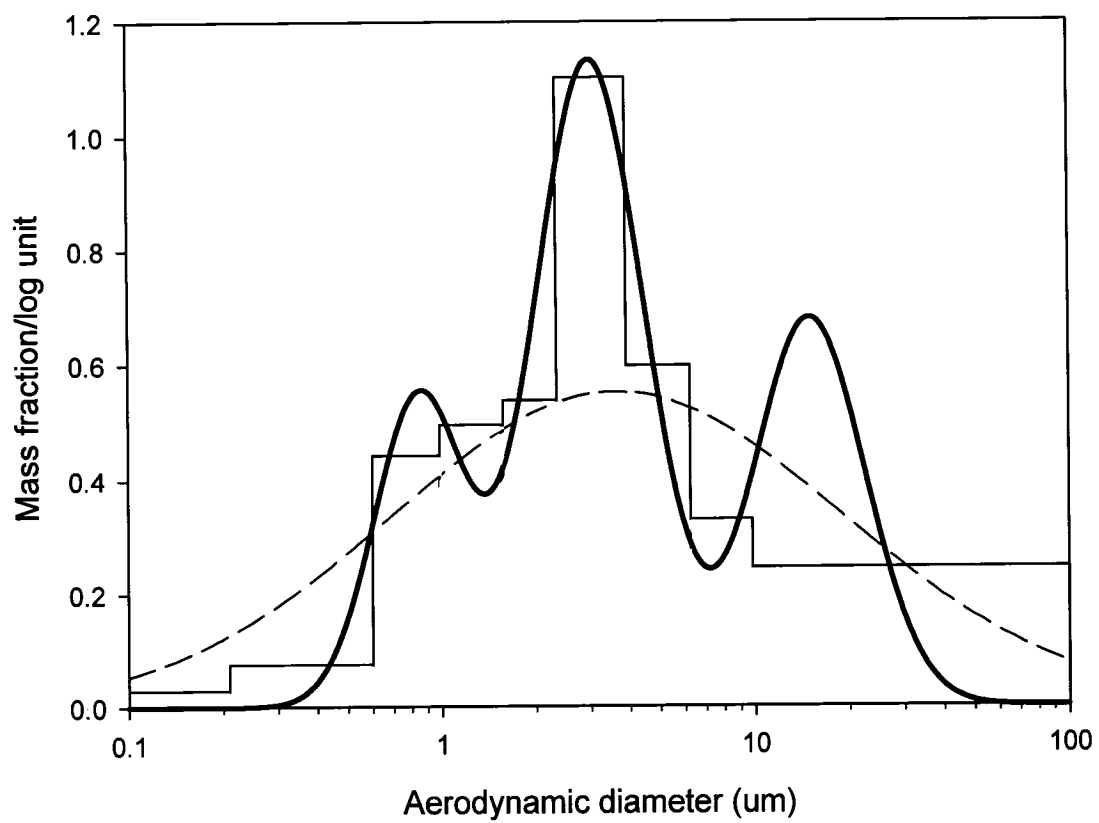
The EHC-6802 particles had a count median diameter of 0.58 μm . Analysis of cascade impactor data revealed a multimodal particle size distribution, with two respirable modes at 0.85 μm (aerodynamic diameter, D_{AE}) and 3.0 μm D_{AE} that together comprised 70 % of the mass, and a non-respirable mode at 15 μm D_{AE} that comprised 30 % of the mass (Fig. 1), similar to the particle size distribution of EHC-93 (Vincent et al., 2001).

Deposition modelling and environmental relevance of dose regimen

Dosimetric modelling was performed to allow comparison of pulmonary deposition of particles in our mouse model to a plausible human exposure scenario. Deposition for the pulmonary compartment (Regional Deposited Dose Ratio RDDR2 Modelling software, US EPA) was estimated at 11.5% for the 0.85 μm D_{AE} mode (20% of aerosol mass), 5.7% for the 3.0 μm D_{AE} mode (50% of aerosol mass), and 0.1% for the 15 μm D_{AE} mode (30% of aerosol mass). Using these values, the dose of EHC-6802 particles delivered to the pulmonary compartment was estimated at 16 μg ($42 \mu\text{g/L} \times 8.06 \text{ L} \times \{[0.20 \times 0.11] + [0.50 \times 0.051] + [0.30 \times 0.001]\}$), or 32 ng/cm^2 alveolar surface area.

The ratio of an experimental particle EHC-6802 dose within the respiratory compartment of the mice during the 42 mg/m^3 exposure (32 ng/cm^2) to the particle dose calculated for a plausible human exposure scenario over 24 h (1.3 ng/cm^2) (Thomson et al., 2004) is roughly 25-fold. For ethical reasons, nose-only exposures should be kept to a

Figure 1. Particle size distribution of EHC-6802. The histogram represents the mass of particles collected on the individual cascade impactor plates. The dotted line represents the particle size distribution assuming a single mode. The dark curving line represents the multimodal fit.



minimum duration, and therefore the dose-rate in our study was obviously higher than for an environmental exposure spread over a 24 h period. Nevertheless, from the standpoint of evaluation toxicology, the pulmonary deposition of particulate matter in the current study are directly relevant to the human experience, once a number of reasonable uncertainty factors are considered. These include the possible decay of the potency of EHC-6802 by comparison to fresh particles, the known interspecies differences in sensitivity to air pollutants (Moss et al., 2001), and the heightened sensitivity within a subset of the human population, such as the known increased adverse risk of individuals with congestive heart failure or atherosclerosis (Goldberg et al., 2001b).

Differential cell counts from bronchoalveolar lavage of mice exposed to EHC-6802

Cells were obtained by bronchoalveolar lavage from WT (Fig. 2a) and TNF (Fig. 2b) mice. Differential counts indicated greater numbers of all cell types measured in TNF mice relative to their WT littermates (*Genotype* main effect, $p < 0.001$). Particle exposure did not significantly affect cell number or composition in either the WT or TNF animals.

Confirmation of EHC-6802 particle effects on key biological pathways

To confirm delivery of a toxicologically relevant dose of particles to the lungs, we assessed the effect of EHC-6802 on pulmonary gene expression of two key transcripts. We have previously shown that urban particulate matter activates the xenobiotic-responsive Ah receptor and Cyp1a1 expression (Vincent et al., 1997b). Because EHC-6802 (but not TiO₂ particles) caused a dose-response increase in Ah receptor activity (Fig. 3a), we assessed expression of the AhR-regulated Cyp1a1 gene in the lungs of mice exposed to EHC-6802. Real-time PCR confirmed that Cyp1a1 mRNA was increased in wildtype and TNF mice after

Figure 2. Effects of EHC-6802 exposure on bronchoalveolar lavage cells. Cells were recovered by bronchoalveolar lavage of SP-C/TNF- α (TNF) mice and their wildtype (WT) littermates exposed to 0 or 50 mg/m³ EHC-6802 and euthanized 0 and 24 h post-exposure. Numbers of all cell types were significantly increased in TNF mice relative to their WT littermates (*Genotype* main effect, $p < 0.001$). There were no significant effects of particle exposure within each genotype. MNGC, multi-nucleated giant cells.

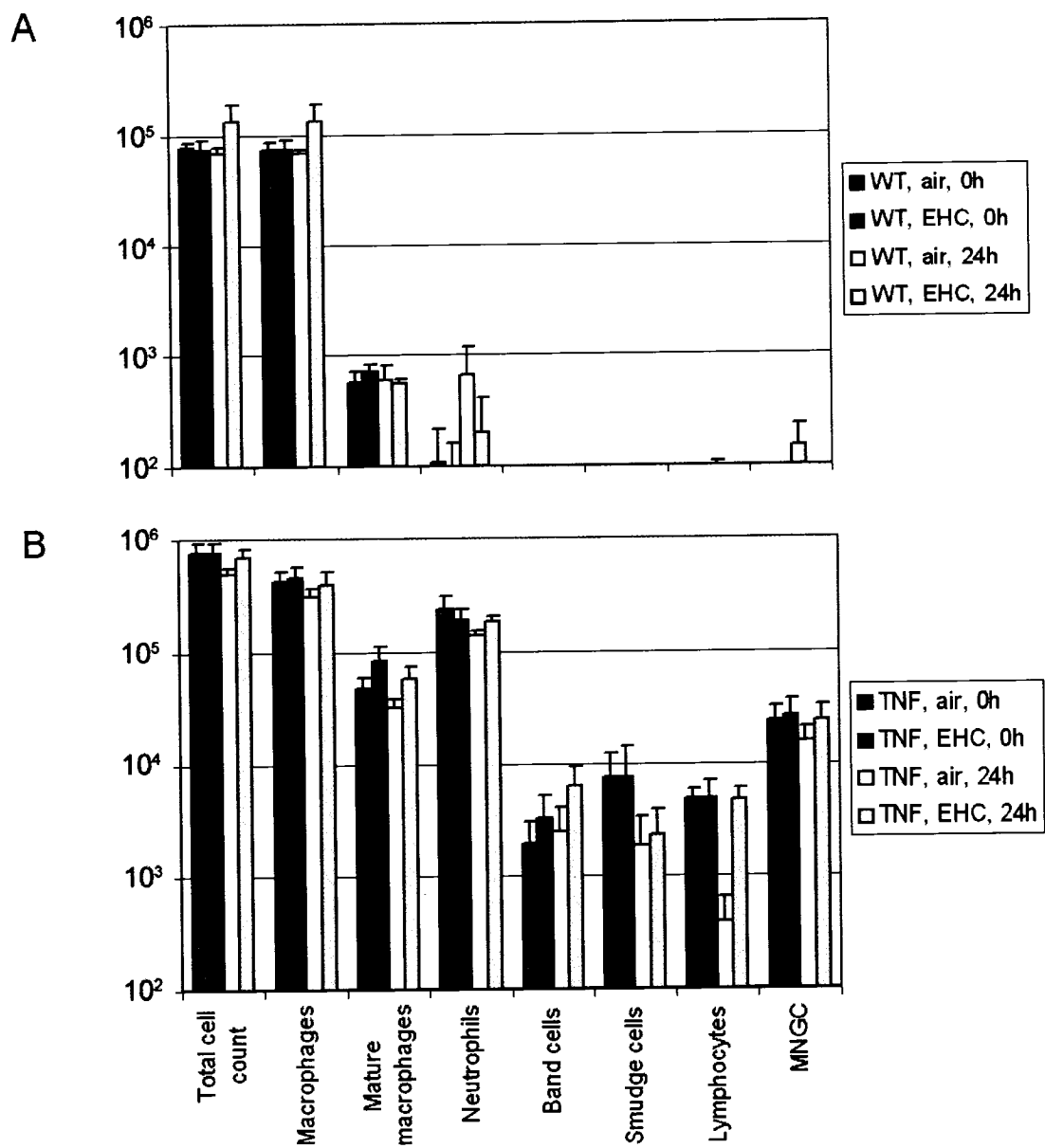
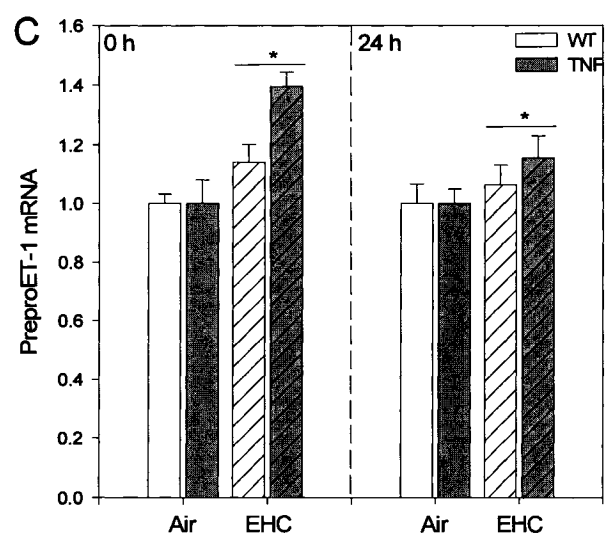
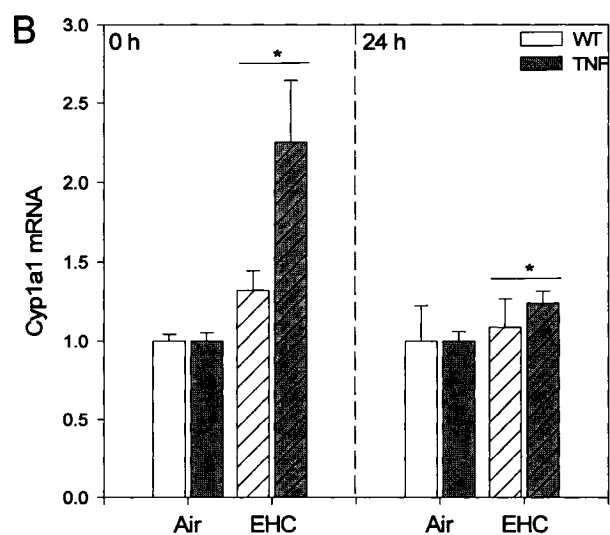
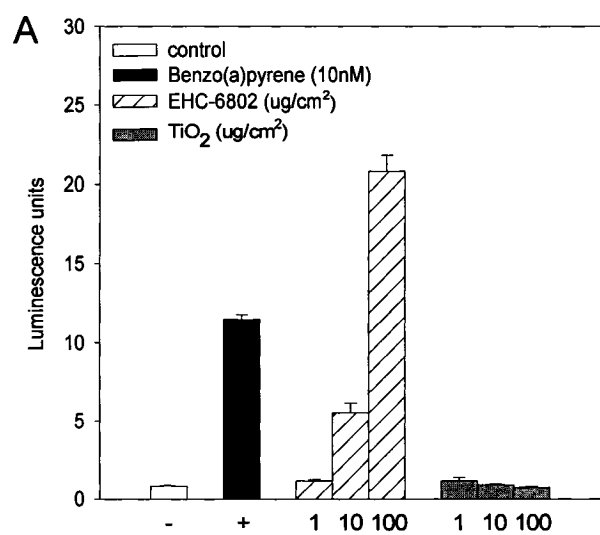


Figure 3. In vitro and in vivo assessment of induction of biologically relevant pathways by EHC-6802. (A) Aryl hydrocarbon receptor activation in vitro. H1L1.1c2 cells were exposed to vehicle, benzo(a)pyrene, EHC-6802 particles, and TiO₂, and luciferase activity was determined. Values represent the mean $\pm$ SEM of triplicate determinations. (B) Cyp1a1 mRNA levels in vivo. SP-C/TNF- α (TNF) mice and their wildtype (WT) littermates were exposed to 0 or 50 mg/m³ EHC-6802 and euthanized 0 and 24 h post-exposure. Real-time PCR was used to determine expression. Results are expressed as mean $\pm$ SEM (n=5 animals/group). **Treatment* main effect, p=0.05, one-tailed. (C) PreproET-1 mRNA levels in WT and TNF mice 0 and 24 h after exposure to EHC-6802. **Treatment* main effect, p=0.04, one-tailed.



exposure (three-way ANOVA, *Treatment* main effect, $p=0.05$; Fig. 3b). Because endothelin may be an important mediator of the health effects of inhaled pollutants (Bouthillier et al., 1998; Vincent et al., 2001), and particulate matter increases pulmonary preproET-1 mRNA levels in experimental animals (Thomson et al., 2005), we verified that preproET-1 mRNA levels were increased in the present experiment. Real-time PCR analysis confirmed that the EHC-6802 particles increased preproET-1 mRNA in WT and TNF mice (three-way ANOVA, *Treatment* main effect, $p=0.04$; Fig. 3c).

Microarray analysis of lung mRNA levels

Agilent 21K oligonucleotide arrays were used to assess lung gene expression in the experimental animals (1 lung sample/array, $n=5$ arrays/treatment). Normalization of arrays resulted in an equal median value and equivalent spread of data for all arrays (Fig. 4). The average background was 202 ± 35 for the Cy5 channel and 341 ± 101 for the Cy3 channel. Of the 20968 probes, approximately 61 % of sample channel spots were identified as having a signal above background (spots flagged: 8213 ± 838 for the sample channel; 11649 ± 706 for the reference channel; 12303 ± 673 for both channels). Hierarchical clustering of arrays was carried out on LOWESS normalized data (Fig. 5). The main branch of the tree split the arrays into two groups determined by factor *Genotype*. After clustering according to factor *Genotype*, samples clustered by factor *Time*. In contrast to the fairly tight clustering with respect to factors *Genotype* and *Time*, effects of particle exposure were subtle at the level of the entire lung. There appeared to be some clustering on factor *Treatment* immediately after exposure (7/10 WT animals, 9/10 TNF animals), with little or no clustering 24 h post-exposure (no apparent clustering in WT animals, 7/10 TNF animals). There was no apparent clustering due to nuisance factors such as date of exposure, date of hybridization, or order of

Figure 4. Box-plots of array data for all 40 arrays used in the study. The central line represents the median of the data, whereas the tails represent the upper (75th percentile) and lower (25th percentile).

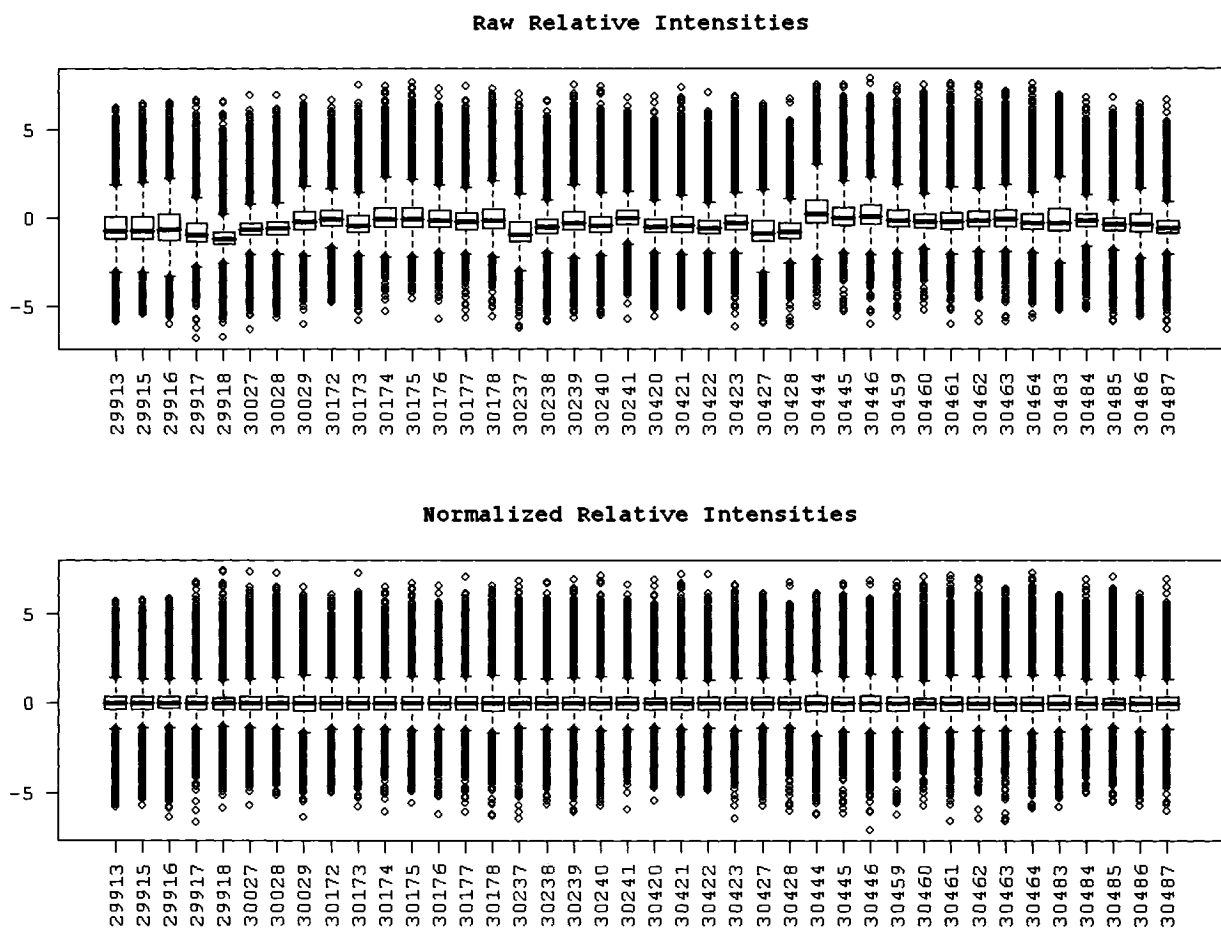
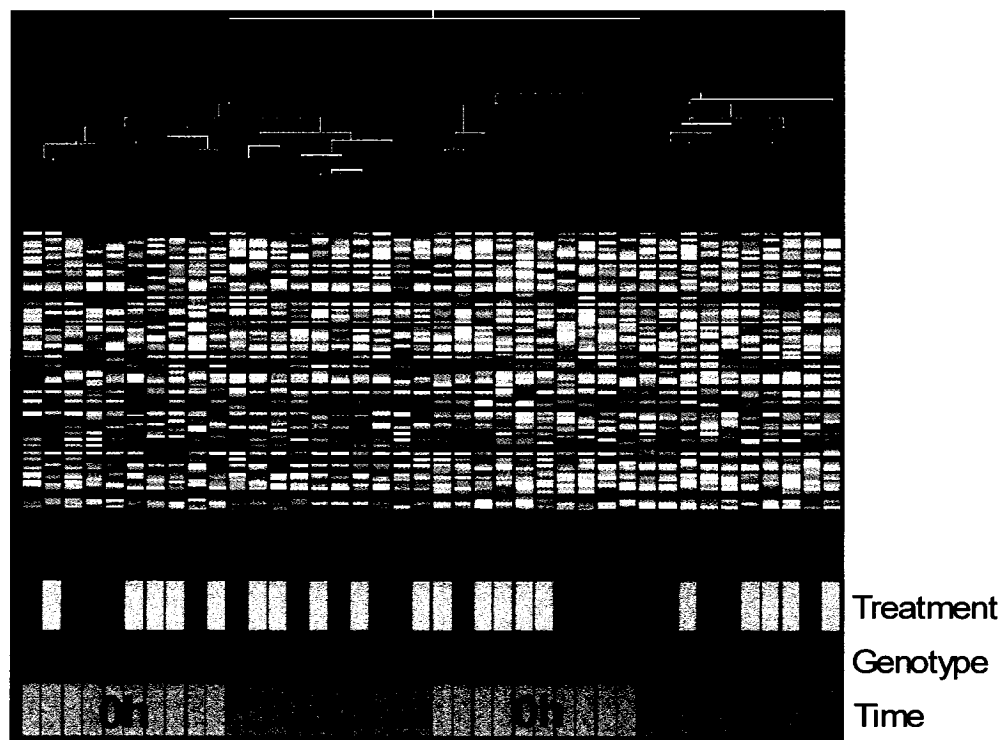


Figure 5. Hierarchical clustering of array data by standard correlation. All 40 arrays in the experiment are grouped on the x-axis, and are shown as branches of the tree. The y-axis shows all probes in the experiment, coloured by the ratio of the intensity on the sample channel to the reference channel (red and blue blocks representing high and low expression respectively, yellow indicating a roughly 1:1 ratio). Sample identity is indicated by the three coloured bars at the base of the figure, indicating the *Treatment*, *Genotype*, and *Time* factors for each array. Lines of the tree are coloured according to levels air (red) and EHC-6802 (yellow) of factor *Treatment*.



hybridization (data not shown). The hierarchical clustering confirmed that the animal genotyping was accurate, that the microarrays yielded high-quality data, and that pulmonary gene expression was influenced primarily by *Genotype*, then by *Time*, and lastly by *Treatment*.

Differential gene expression

Differential gene expression was assessed by MAANOVA (FDR-adjusted $p < 0.05$). Upper and lower bounds for the number of genes significantly affected by each factor were determined by finding the union and intersection of all probes found to be significant by pair-wise comparison involving that factor. TNF mice had significantly altered expression of 1864-3331 probes (intersection-union) compared to WT mice (Table 1), consistent with the distinct hierarchical clustering of chips by factor *Genotype*. There was a lesser effect due to factor *Time*, with 21-291 probes found to be significantly different between the two time points. Only 8 genes were differentially expressed after particle exposure, and of these, only 4 had a signal consistently above background (Table 2). None of these genes were differentially expressed in both WT and TNF animals, or at both 0 and 24 h time-points. Fold-change versus statistical significance for each of the comparisons is displayed graphically through volcano plots (Fig. 6), indicating substantial differential expression attributable to factor *Genotype*, some differential expression attributable to factor *Time*, and little differential expression attributable to factor *Treatment*.

Because of the absence of statistically significant responses to particle exposure common to both WT and TNF animals, we proceeded to look at whether there were any genes in common among the genes up- or down-regulated by at least 1.5-fold after treatment

Table 1. Comparison of numbers of differentially expressed genes for all pair-wise analyses as determined by MAANOVA.

Test		Significant probes (# above background) ^a	Probes with >1.5 FC ^b
Pairwise Comparisons	WT,0 h (Air vs. EHC)	4 (2)	147
	TNF,0h (Air vs. EHC)	2 (2)	211
	WT,24h (Air vs. EHC)	1 (0)	147
	TNF,24h (Air vs. EHC)	1 (0)	117
	WT,Air (0 vs. 24h)	279 (277)	440
	TNF,Air (0 vs. 24h)	34 (34)	269
	WT,EHC (0 vs. 24h)	177 (175)	486
	TNF,EHC (0 vs. 24h)	57 (56)	349
	0h,Air (WT vs. TNF)	3303 (3208)	2424
	24h,Air (WT vs. TNF)	2332 (2328)	2279
	0h,EHC (WT vs. TNF)	2447 (2438)	2557
	24h,EHC (WT vs. TNF)	2285 (2282)	2232
Summary ^c			
Treatment	Union	8 (4)	
	Intersection	0 (0)	
Time	Union	291 (286)	
	Intersection	21 (21)	
Genotype	Union	3331 (3238)	
	Intersection	1864 (1863)	

^a Number of statistically significant probes for each comparison after analysis of the three-way and two-way interactions, and all main effects. Numbers in parentheses indicate probes that have been declared present in at least 4 out of 5 arrays in the group showing a significant effect.

^b List of all probes with fold-change (FC) > 1.5, with no statistical filtering.

^c Summary of pair-wise comparisons. Union refers to sum of probes present in any comparison for a given factor. Intersection refers to the number of probes in common among all comparisons for a given factor.

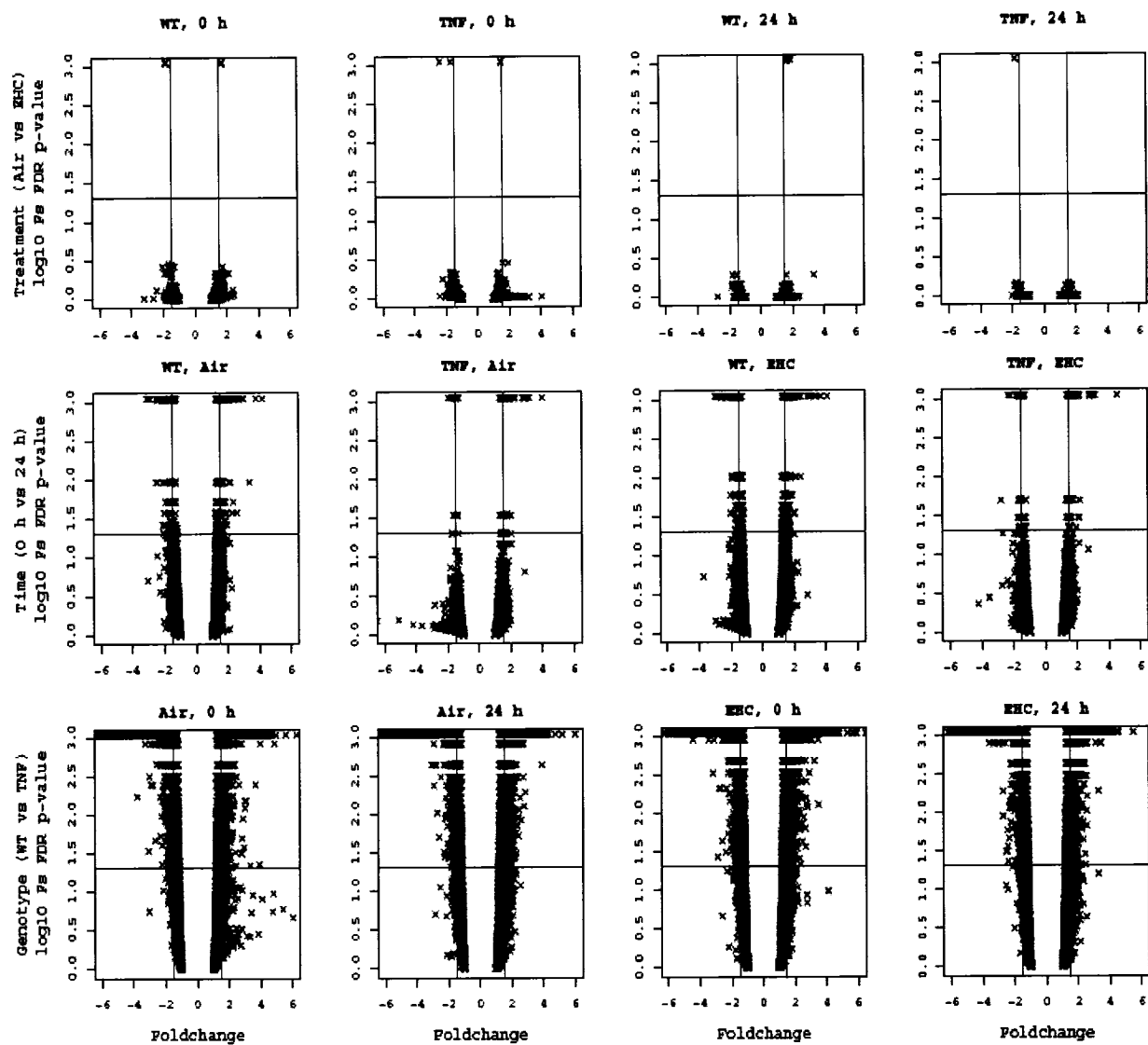
Table 2. Probes showing significant differential expression after exposure to EHC-6802 by MAANOVA analysis (FDR-adjusted $p < 0.05$).

Accession #	Fold-change ^a	Comparison ^b	Gene
AK020160	1.5	TNF, 0 h	hypothetical retroviral GAG p10 protein containing protein
NM_007657	0.6	WT, 0 h	CD9 antigen
XM_149258	0.6	TNF, 0 h	zinc finger protein 217
XM_138945	1.6	WT, 0 h	predicted protein

^a Ratio of EHC-6802-exposed mean to air-exposed mean.

^b Comparison of EHC-6802 to air-exposed animals within the indicated genotype and timepoint.

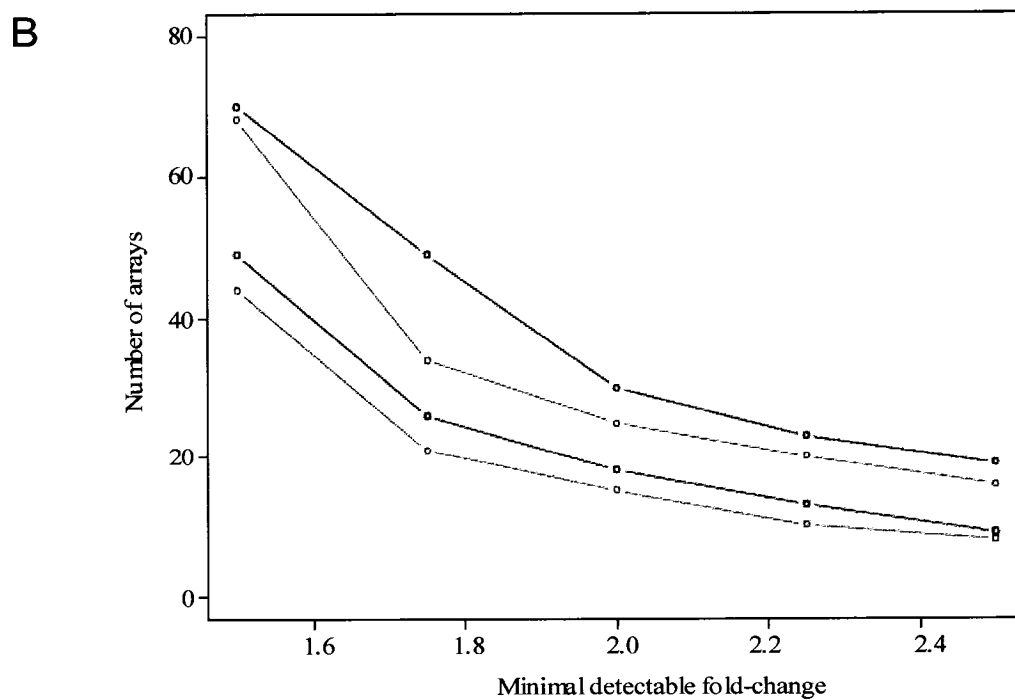
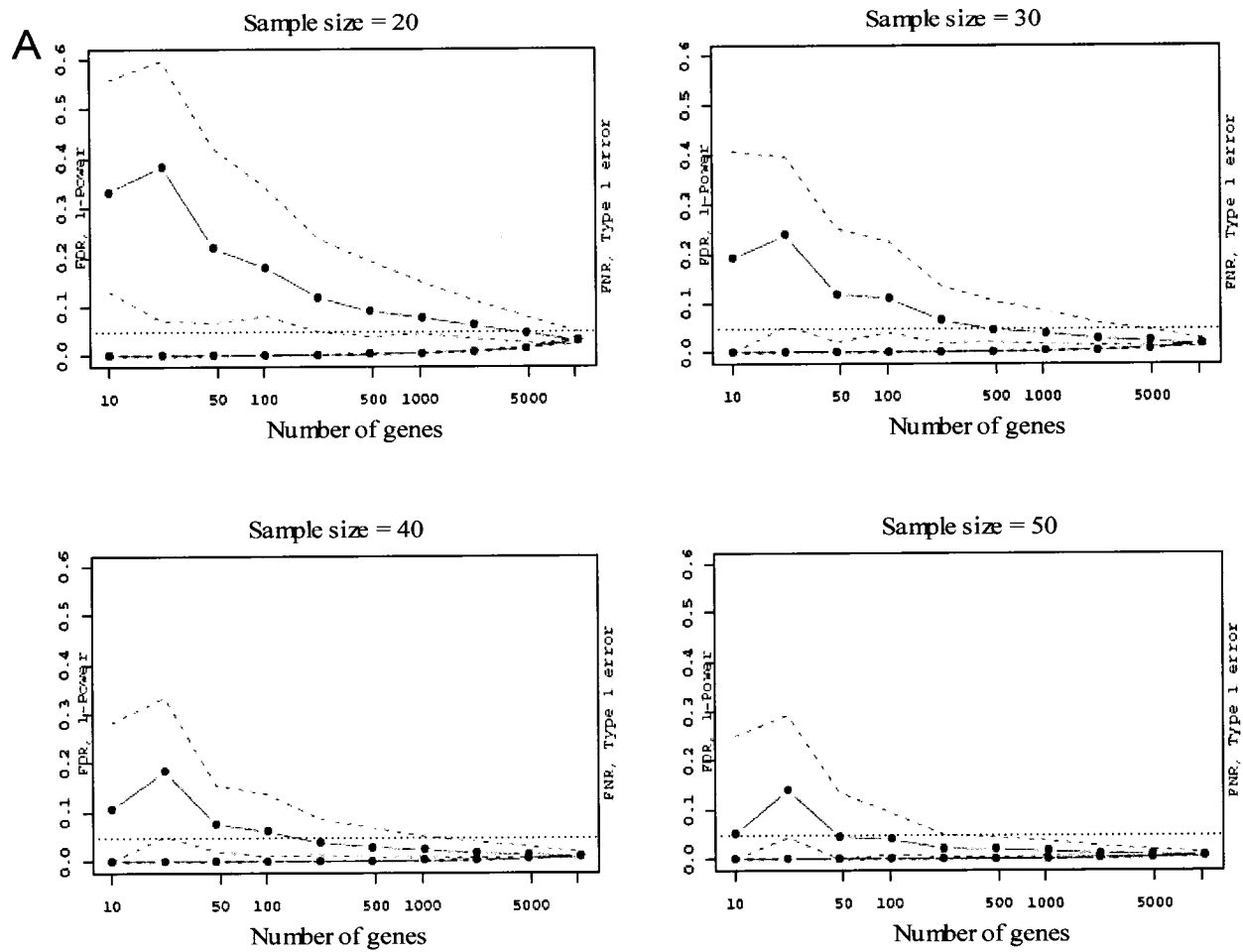
Figure 6. Volcano plots of array data for all pair-wise comparisons. Microarray data analysed in MAANOVA by Fs (y-axis) is represented relative to fold-change (x-axis) for all comparisons within factors *Treatment*, *Time*, and *Genotype*. Probes are represented by “x”. The horizontal line represents a false discovery rate (FDR)-adjusted p value of 0.05; any probe above this line was deemed significant. Vertical lines represent a fold-change of 1.5-fold.



(Table 1). This approach has previously been used to compare reproducibility across different platforms (Guo et al., 2006). Only two genes increased immediately after exposure in both WT and TNF mice: NM_009992 (Cyp1a1) and AK048310 (Mus musculus 16 days embryo head cDNA, RIKEN full-length enriched library, clone:C130048D07 product: similar to aldehyde dehydrogenase 3 family, member b1, fragment). BLAST search (NCBI) analysis of the probe sequence revealed a match with *Mus musculus* aldehyde dehydrogenase 3 family, member B2 (Aldh3b2) mRNA. Real-time PCR analysis indicated that the effect of treatment on Aldh3b2 mRNA was not significant (three-way ANOVA, $p > 0.05$, data not shown).

Power calculations performed using data generated during the present microarray study for all four analyses (WT, 0 h; WT, 24 h, TNF, 0h, TNF, 24 h) generated similar plots. A representative example is presented in Fig. 7. If expecting a small effect size, the data calculations suggest that a large number of arrays are required.

Figure 7. Power calculation using data from the present microarray study. The sample size assessment was conducted in order to determine the sample size required to detect a given fold change difference between exposed and control samples. This was examined for each genotype and time point group, resulting in four independent assessments for each fold-change analysis, all of which provided similar estimates. A representative example (detection of 1.5-fold change in WT, 0 h animals) is illustrated in (A) assuming a sample size of 20, 30, 40, or 50. The upper red bound represents the 90th percentile for the false discovery rate, whereas the red lower bound represents the 10th percentile. The solid red and green lines represent the median false discovery rate (FDR) and false negative rate (FNR). The required sample size was identified through inspection of the plots generated from this application and the sample size was determined from the point at which the 90th percentile for the FDR was less than 0.05. The results for the four analyses were then averaged, and the number of arrays required to detect a given fold-change were plotted relative to the minimal detectable fold change (B). From upper to lower, the lines represent experiments expecting 50 (blue), 100 (red), 500 (black), and 1000 (green) genes to be differentially expressed at the fold-change indicated on the x-axis.



Discussion

Chronic inflammation may increase the risk of adverse health effects from air pollution. Because of the complex nature of the interaction between susceptibility and pollutant effects, comprehensive approaches such as gene expression profiling may be useful in identifying molecular networks implicated in the health effects of particulate matter. In the present study, we used a toxicogenomic approach to examine how chronic pulmonary inflammation may modify the transcriptional response of the lungs to inhaled urban particulate matter. We had three major objectives: 1) to evaluate the suitability of using microarrays to study gene-pollutant interactions; 2) to identify genes and pathways affected by inhaled particles; 3) to identify potential biomarkers of exposure that are insensitive to the physiological status of the lungs.

Microarray technology permits the evaluation of differential gene expression at the global transcript level, and therefore may be useful in identifying unknown effects of particle exposure not examined by conventional approaches. However, there are limitations to large-scale screening approaches. The ability to detect subtle effects may be compromised when making thousands of measurements due to noise from sources such as animal-to-animal variation and the increased statistical stringency required to reduce the detection of false-positive results. Because of these issues, small but physiologically relevant changes in gene expression at the level of the whole lung may be missed. We have previously established that preproET-1 mRNA levels are increased in the lungs of experimental animals after exposure to ambient particles (Thomson et al., 2005). The elevation of preproET-1 mRNA is associated with a 25-50% increase in circulating ET-1 levels (Bouthillier et al., 1998;

Thomson et al., 2005; Vincent et al., 2001). Increases of plasma ET-1 in this range have high predictive value for chronic heart failure (Galatius-Jensen et al., 1996), indicating that the magnitude of ET-1 change need not be large to be physiologically relevant. In the present experiment we confirmed a 20-40% increase in preproET-1 mRNA expression in the wildtype and TNF animals after particle inhalation, in line with our previous work in rat models indicating endothelin system activation in the absence and presence of acute lung injury and inflammation (Bouthillier et al., 1998; Thomson et al., 2005). In addition, the urban particle preparation increased Cyp1a1 mRNA levels, consistent with the capacity of EHC-6802 to activate the aryl hydrocarbon receptor in vitro, and with earlier work showing activation of xenobiotic response element and Cyp1a1 reporter cell lines by urban particles (Vincent et al., 1997b). The response of the endothelin system and xenobiotic transformation pathways in the lungs of wildtype and TNF mice establish the delivery of internal doses of urban particles sufficient for the induction of health-relevant effects, despite the absence of visible impacts on lung lavage cytology and alteration of alveolar inflammation cascades.

Despite delivery of a biologically-effective dose, our microarray analyses detected remarkably few statistically significant (FDR-adjusted $p < 0.05$) particle-responsive genes in either genotype. Only four probes exhibited differential expression deemed to be statistically significant in any of the treatment vs. control pair-wise comparisons, and there were no consistent effects of treatment across any of the groups. The lack of treatment effects is in sharp contrast to the consistent differential expression detected for factors *Time* and *Genotype*, indicating that our negative finding is not due to large inter-animal variation, poor chip hybridization, or other technical issues. A previous inhalation study in ApoE^{-/-} and

LDLr^{-/-} double knockout mice also failed to find significant changes in gene expression in the lungs after subchronic exposure to concentrated ambient particles for 6 h/d, 5 d/wk, for 4 months (Gunnison and Chen, 2005). However, groups were small (n=3), and may have been insufficient to detect significant effects, and results were confounded by possible circadian effects due to euthanization of treated and controlled groups at different times of the day (Gunnison and Chen, 2005). Our study may have been similarly underpowered to detect subtle (less than 1.5-fold) changes, as will be discussed later. However, with 5 arrays/treatment group, this 40 array experiment is, to our knowledge, the largest microarray study to date that has examined particle effects on lung gene expression. Our results suggest that a relatively high dose of inhaled urban particles (25-fold higher than a plausible 24 h human scenario) may not cause significant changes in gene expression detectable by microarray analysis at the level of the whole lung. In addition, in the present study existing inflammation and lung injury did not increase modulation of gene expression by particulate matter as compared to healthy lungs.

Several macroarray studies using a smaller set of spotted cDNAs have indicated that exposure to diesel exhaust particles may induce some significant changes in gene expression at the level of the whole lung (Reynolds and Richards, 2001; Nadadur and Kodavanti, 2002; Wise et al., 2006). These studies suggest that smaller, more focussed approaches may facilitate detection of subtle effects. Indeed, increased pulmonary expression of a number of genes including preproET-1 mRNA was detected using an 84-gene array after instillation of residual oil fly ash or specific constituent toxic metals (Nadadur and Kodavanti, 2002). Results from a cDNA microarray study of mice exposed to diesel exhaust particles and lipopolysaccharide (LPS) suggested that LPS-induced lung injury potentiated the effect of

intratracheal instillation of diesel exhaust particles on lung gene expression (Yanagisawa et al., 2004). These studies, which like the present study used whole lung homogenates, suggest that particles can cause some changes to pulmonary gene expression detectable at the level of the whole lung, although effects were for the most part modest.

Besides the different particle and animal models used in these experiments, there are several technical issues that may account for the different results. First, our study used an inhalation approach rather than intratracheal instillation of particles suspended in saline. Although intratracheal instillation is a useful approach to compare the toxicity of different particles, it may overestimate the toxicity of particles and result in increased inflammation due to disruption of the alveolar surfactant layer by the aqueous particles delivered as a bolus. Previous studies using an intratracheal instillation approach employed particle doses ranging from 1.25-10 mg in 0.5 mL saline per animal (Nadadur and Kodavanti, 2002; Kooter et al., 2005; Reynolds and Richards, 2001; Wise et al., 2006). In comparison, the estimated pulmonary deposition in the present study was calculated to be approximately 16 μg , or 32 ng/cm^2 delivered over a 4 h period. Although this is roughly 25-fold higher than a high 24 h human exposure according to our dosimetric modelling, it is 100-1000 times lower than the dose of particles delivered in the intratracheal instillation studies.

A second important difference between our study and others was our approach towards biological replicates. In two previous studies using microarrays to examine particle effects, biological replicates were pooled and hybridized to a single array per group (Kooter et al., 2005; Andre et al., 2006). Although this is an economic approach, it does not provide any information regarding technical or biological variability, and so it is not possible to

evaluate the source of variation between arrays. Studies employing single arrays for each treatment must use fold cut-off approaches to determine a list of candidate genes. In contrast, in the present study we analyzed gene expression on a 1 animal/array basis. This approach should reduce the number of spurious results attributable to biological outliers (one animal that is very different from the others) or technical outliers (one array that is very different from the others) by allowing us to apply stringent statistical analyses to the results. If fold-change had been the sole approach available to assess effects of exposure to particles in the present study, a 1.5-fold cut-off would have resulted in 147 (0 h post, WT), 211 (0 h post, TNF), 147 (24 h post, WT), and 117 (24 h post, TNF) differentially expressed genes in the particle treatment groups relative to their respective time-matched air controls (Table 1). These lists include genes involved in oxidative stress/metabolism of toxicants (Cyp1a1, epoxide hydrolase 2, lactotransferrin, aldehyde dehydrogenase 3b2), vasoregulation (atrial natriuretic peptide), and apoptosis (cell-death inducing DNA fragmentation factor α -subunit like effector A), but also numerous genes with unclear roles in the lungs. Many of these genes have significant intragroup variation according to microarray analysis, and PCR analysis confirmed that a potential candidate biomarker identified through the array analysis, Aldh3b2, was actually not significantly affected by particle inhalation (results not shown). Had we pooled our biological replicates, we would have had no way of assessing which of these gene changes, if any, were likely to be treatment effects, and which were instead due to technical or biological variation. Our study clearly shows that inclusion of biological replicates on independent arrays is essential to permit assessment of variance and hence the significance of results. Furthermore, our results indicate that using an arbitrary fold cut-off of 2-fold or 1.5-fold may 1) result in the inclusion of false-positive results, particularly when pooling biological replicates; and 2) result in the exclusion of small (<1.5 fold) but

physiologically relevant changes, such as endothelin system activation and up-regulation of the Cyp1a1 toxicity/detoxification pathway.

Several limitations of our study should be considered. First, measurement of particle effects on gene expression at the level of the whole lung may be insensitive to effects in target cells. Using our data, we calculated that 60-70 arrays would be required to detect 1.5-fold changes, assuming 100 genes were differentially expressed with a median FDR-adjusted $p < 0.05$ (Fig. 7). Detection of 2-fold changes in gene expression would require roughly half that number (approximately 30 arrays). Studies requiring this number of arrays may not be practical for many laboratories, given the current high cost of microarrays. Appropriate pooling of animals is one approach to reduce the effect of intragroup variability, although this must be combined with the use of a sufficient number of arrays for each treatment group to permit statistical analysis (Kendzierski et al., 2005; Peng et al., 2003). Alternatively, if we assume that the responses are attributable to specialized cell populations in the lungs, an anatomically-biased or cell-type specific approach, such as through laser-capture microdissection, might uncover local impacts of particle inhalation using lower numbers of arrays. Secondly, the substantial differences in global transcript levels between WT and TNF mice may have reduced our ability to detect particle effects across genotype. Evaluation of particle effects in a more homogeneous population should increase the statistical power to detect effects. Third, due to challenges in building up a sufficiently large colony of transgenic animals of the same age, animals from different litters were exposed on three occasions. This may have introduced noise as a result of unknown factors. However, groups were balanced, exposed and euthanized at the same time of day, and microarrays were blocked for the nuisance factors *Date of exposure* and *Day of hybridization*. We

detected statistically significant changes in gene expression related to time post-exposure, suggesting that any inter-animal differences did not impede detection of differential gene expression due to environmental stimuli/stress. Time effects likely relate to stress responses from the nose-only exposure system that have subsided by 24 h post-exposure (Vincent et al., 2001); analysis of gene expression in naïve animals would be required to eliminate the possible involvement of other factors. Nevertheless, since air control animals were exposed via the nose-only inhalation system, stress effects should not confound our interpretation of results.

Summary: Our data reinforce the notion that adverse health effects of acute exposure to urban particles may be dominated by physiological response cascades rather than widespread changes in the expression of genes escaping homeostatic controls, such as in an inflammatory cascade. Nevertheless, transcriptional activation of certain key pathways, such as endothelin synthesis and xenobiotic transformation factors, may have relevance to these health effects. Microarray analysis is a useful approach for genome wide screening of effects; however, in the present study subtle effects of particle inhalation were not detectable by microarray analysis at the level of the whole lung. Increased power, site-specific gene expression analysis, or more sensitive animal models may be required to investigate effects of acute exposure on lung gene expression.

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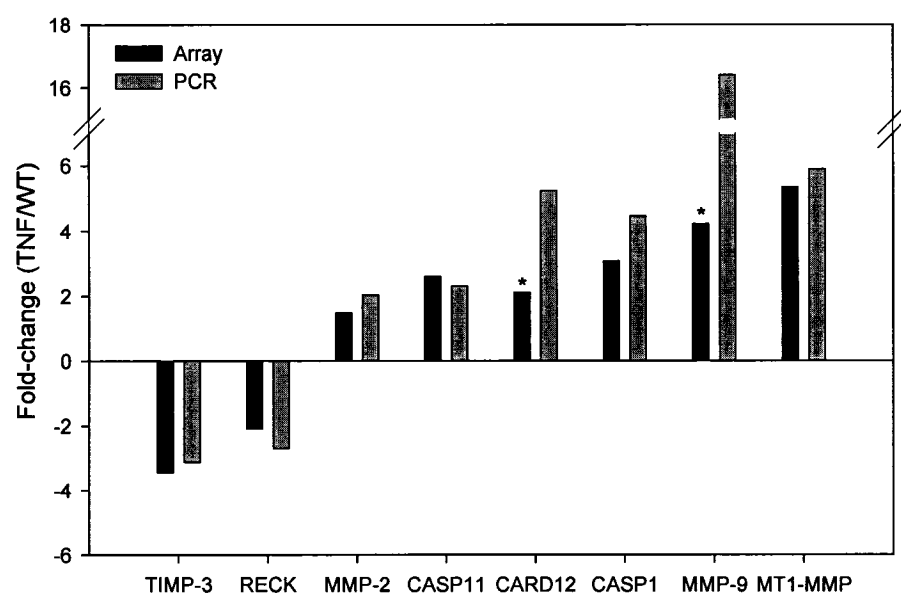
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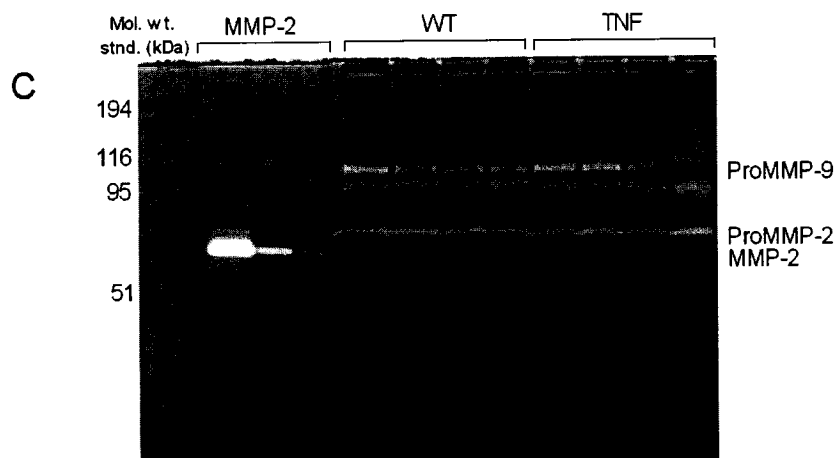
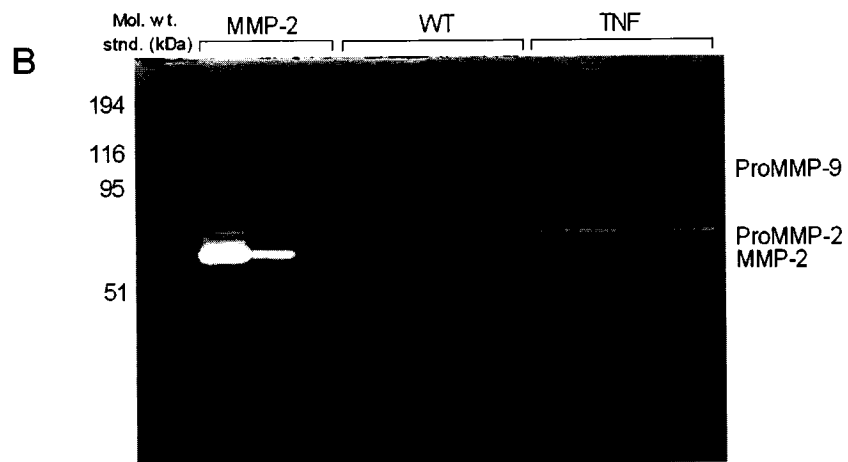
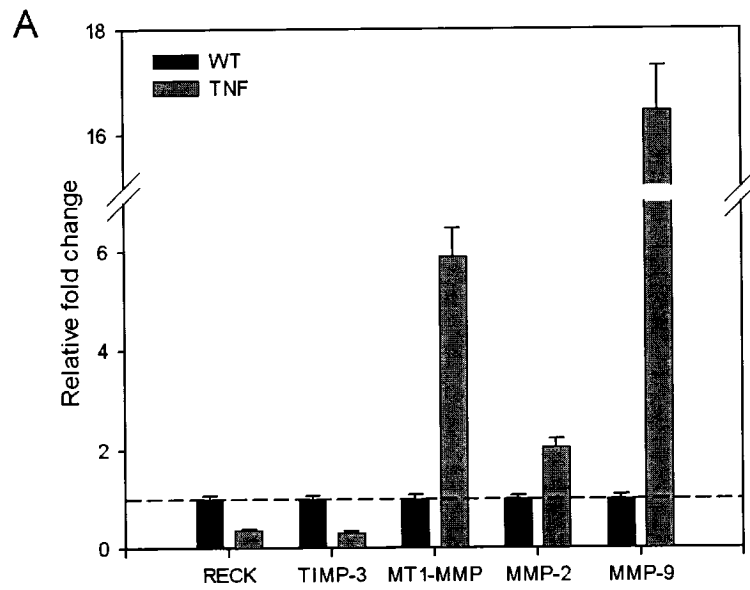
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Supplementary Material, Figure 1. Validation of microarray results. Real-time PCR was used to confirm results for a subset of genes identified by microarray analysis as being differentially expressed according to factor *Genotype*. The ratio of gene expression in unexposed SP-C/TNF- α mice to that in unexposed wildtype mice is presented. All genotype comparisons were statistically significant by PCR analysis according to t-test, $p < 0.001$. The direction of fold-change was the same by both analytical methods for all genes examined, although the magnitude of the change differed for certain genes. *A lack of microarray sensitivity (indicated by asterisks denoting probes with values close to or within background for wildtype mice) likely explains the lower fold-change determined for CARD12 and MMP-9 compared to PCR analysis. TIMP-3, tissue inhibitor of metalloproteinase-3; RECK, reversion-inducing-cysteine-rich protein with kazal motifs; MMP, matrix metalloproteinase; CASP, caspase; CARD12, caspase recruitment domain protein-12; MT1-MMP, membrane type-1 matrix metalloproteinase.



Supplementary Material, Figure 2. Comparison of pulmonary matrix metalloproteinase (MMP) system gene expression and enzyme levels in control wildtype (WT) and SP-C/TNF- α (TNF) mice. Genes were selected on the basis of differential expression according to microarray analysis, and expression was assessed by real-time RT-PCR. MMP enzyme levels were assessed by gelatin zymography. RECK is a membrane-bound inhibitor of MMPs that inhibits MMP-2, MMP-9, and MT1-MMP. TIMP-3 inhibits MMP-2 and MMP-9 activity, whereas MT1-MMP is a positive regulator of MMP-2 activation. (A) MMP system gene expression. Results are expressed as mean $\pm$ SEM (n=5 mice/genotype). All genotype comparisons were statistically significant according to t-test, $p < 0.001$. (B) MMP levels in bronchoalveolar lavage fluid recovered from WT and TNF mice. ProMMP-2 and proMMP-9 levels were increased in the lungs of TNF mice. (C) MMP levels in plasma from WT and TNF mice. Despite the MMP system activation observed in the lungs, circulating levels of gelatin-degrading enzymes appears to be similar in WT and TNF mice.



8. Discussion and Conclusions

8.1. Overview

Variation of even relatively low levels of ambient particulate matter is associated with cardiovascular morbidity and mortality. We have previously shown that impacts of particulate matter on the cardiovascular system involve perturbation of vascular homeostasis as a result of increased production of the vasoactive peptide endothelin (Bouthillier et al., 1998; Vincent et al., 2001). Since increased endothelin levels are associated with conditions relevant to the pathophysiology of cardiovascular disease, including hypertension (Schiffrin, 2005), arrhythmia (Duru et al., 2001), decreased heart rate variability (Aronson et al., 2001; Pekdemir et al., 2004), platelet aggregation (Knofler et al., 1995), and accelerated progression of atherosclerosis (Barton and Haudenschild, 2001), it is of interest to characterize the impact of air pollution on endothelin biosynthesis. Through the studies presented here, the effects of controlled exposure to urban particulate matter and ozone on expression of endothelin system genes in the lungs, the main site of endothelin synthesis and a key target of inhaled pollutants, can now be described. We hypothesized that acute inhalation of urban pollutants rapidly activates pulmonary endothelin system genes, resulting in increased production of endothelin and spillover of the peptide into circulation. We proposed further that the pattern of endothelin system gene expression could serve as a critical endpoint of pollutant exposure for dose-response assessment, pollutant interaction studies, and investigations of independent factors of susceptibility.

8.2. Urban air pollutants rapidly activate the pulmonary endothelin system

Ambient pollutants are inhaled as mixtures. We incorporated this idea in our model by studying the effects of two common urban pollutants associated with adverse

cardiovascular effects, particulate matter and ozone, both individually and in a co-exposure model. Since epidemiologic studies have indicated cardiovascular events within a few hours of increased air pollution levels (Magari et al., 2001; Gold et al., 2000; Peters et al., 2001), we initiated time-course and dose-response studies to examine the timing of endothelin system response, effects of the individual pollutants, and any toxicological interaction of the inhaled pollutants after acute exposure. The data presented here show that ET-1 is rapidly elevated at both the mRNA and peptide levels. Clearly, the timing of effects in susceptible human individuals may differ from healthy animals as a result of interspecies differences and existing pathophysiological processes. Nevertheless, in the present study, pulmonary preproET-1 and ECE-1 mRNA expression and plasma endothelin levels were increased at the earliest measurement, immediately after the 4 h exposure, in line with the epidemiologic data. Our work indicates that both particulate and gaseous components of air pollution rapidly activate the pulmonary endothelin system and provoke release of endothelin into the systemic circulation. The dynamics of response to the pollutants do, however, differ. Effects of ozone inhalation were transient, while particles tended to cause a sustained elevation of preproET-1 and a sustained decrease of preproET-3 mRNA levels. This is in line with the known toxicodynamics of these pollutants, since particles can persist in the lungs for days to months in the distal alveoli (Oberdorster et al., 1994), while the highly reactive gas ozone is rapidly consumed through interactions with the lung lining of the respiratory tract and proximal alveolar region (Hu et al., 1992).

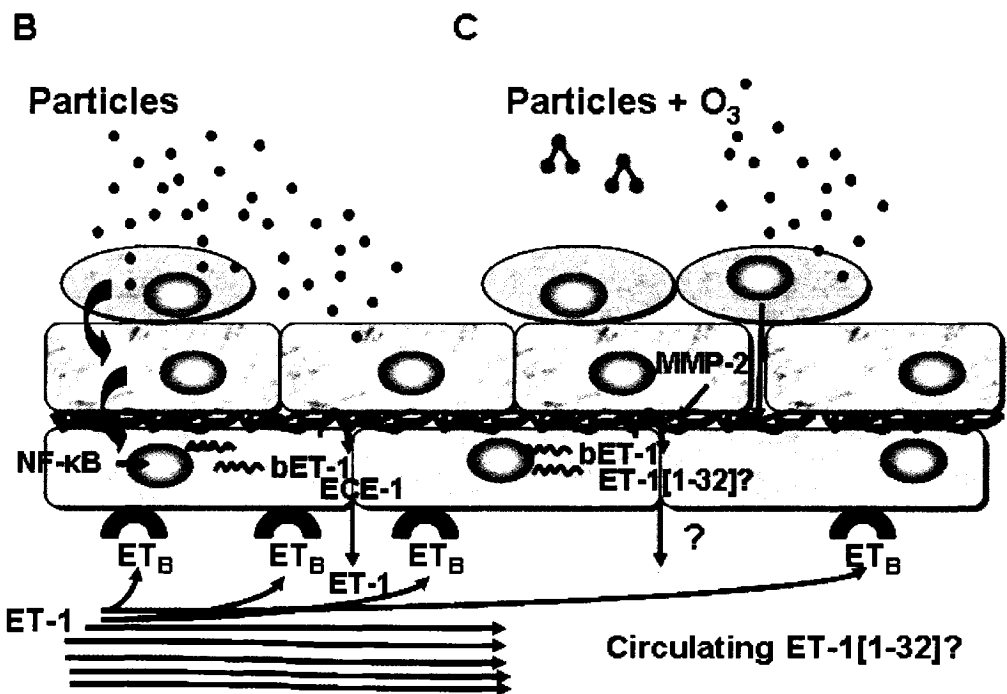
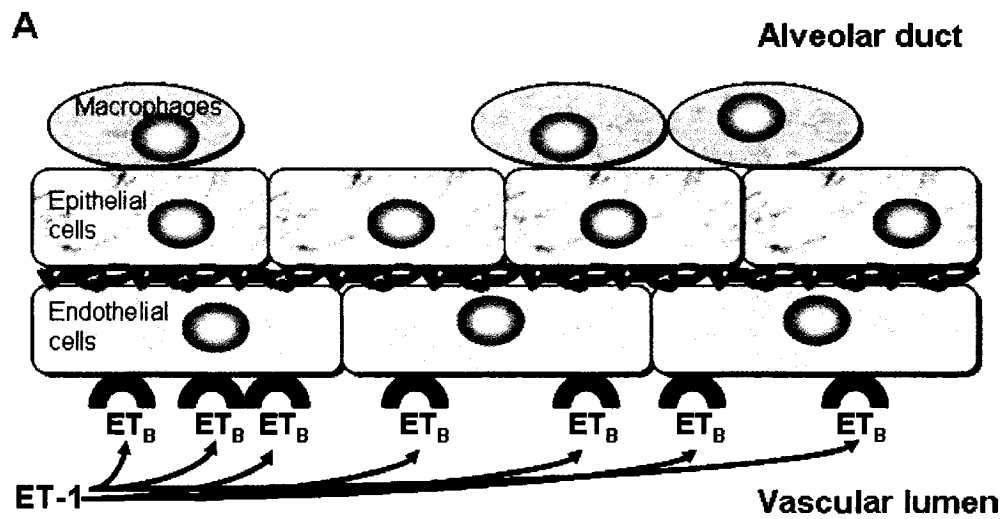
While the approach used in the present study does not allow anatomic resolution of the detected changes, the endothelium is the dominant source of ET_B receptor, ECE-1, and eNOS mRNA expression in the lung. Hence, the changes in mRNA level of these genes

immediately and 24 h post-exposure imply an effect of ozone and particles on the endothelium. Similarly, since the ET_A receptor is predominantly expressed on smooth muscle cells, the decrease in ET_A receptor mRNA 24 h post-exposure indicates an effect outside of the endothelium. Confirmation of the cellular location of these changes would require other approaches, such as in situ PCR or expression analyses in specific tissues obtained by laser-capture microdissection. The changes in gene expression of the ET system genes in the lung vascular bed after exposure to the individual pollutants are consistent with a contribution of at least three mechanisms to the higher concentration of bigET-1 and ET-1 in plasma, namely: 1) early up-regulation of preproET-1 mRNA with increased *de novo* synthesis of bigET-1; 2) early increased ECE-1 mRNA with a higher rate of conversion of bigET-1 to ET-1; and 3) later decreased ET_B receptor mRNA resulting in reduced clearance of ET-1 from circulation (Figure 1). Note that we have not measured directly the rate of bigET-1 to ET-1 conversion or the rate of clearance of circulating ET-1 (for example, see Dupuis et al., 1996). Pharmacological validation of our interpretation is complex and outside the scope of the present work. In brief, our data indicate that the pulmonary endothelin system is an early target of both particles and ozone, and that its activation soon after exposure has potential systemic effects through release of the ET-1 peptide into circulation.

8.3. The particle-induced increase in preproET-1 mRNA is insensitive to pulmonary inflammation and injury

The physiological state of the lungs is an important determinant of the effect of inhaled particles. For example, inhalation of a high dose of particulate matter alone caused only mild, focal inflammatory changes in the interstitium, with no evidence of acute lung injury (Bouthillier et al., 1998; Adamson et al., 1999; Vincent et al., 1997). However, when

Figure 1. Proposed model of pollutant effects on the pulmonary endothelin system. (A) The lungs are the main site of production and clearance of endothelin. Plasma endothelin (ET)-1 is cleared from circulation by endothelial cell ET_B receptors, and an equal quantity of ET-1 is released into circulation (for clarity, release of ET-1 is not shown on diagram). (B) Particle deposition in the alveoli activates endothelial cell production of ET-1 either through diffusion of soluble components from the particles or via signals produced by macrophages and epithelial cells. Newly synthesized bigET-1 is released basolaterally towards the interstitium, and is converted to the mature ET-1 peptide via the endothelin-converting enzyme (ECE-1). Some of the produced ET-1 then spills into circulation. Increased ET-1 in plasma will consume more ET_B receptors, since binding of ET-1 to the ET_B receptor results in internalization and lysosomal degradation. Coupled with the decreased ET_B receptor mRNA levels 24 h post-exposure, this internalization may result in the eventual decrease of receptor expression on the endothelial cell surface, and thus reduced clearance of ET-1 from plasma. (C) In the co-exposure scenario, acute lung injury results in the release of MMP-2, and possibly other factors, from macrophages and/or epithelial cells towards the interstitium. MMP-2 cleaves basement membrane proteins, facilitating cell movement and repair processes. It should co-localize with bigET-1, and may therefore process it to yield ET-1[1-32]. This would explain the lack of increased plasma ET-1 in the co-exposure scenario despite the increase in preproET-1 mRNA. It is not known whether ET-1[1-32] is increased in circulation.



inhaled with ozone, the same dose of particles caused extensive structural changes including acute epithelial injury and a pronounced interstitial inflammatory response. This response in the co-exposure model was significantly more pronounced than changes measured after exposure to ozone alone, indicating that particles exacerbated the limited lesions induced by ozone. Similarly, lungs with a compromised epithelium resulting from a chronic pulmonary condition (such as COPD, bronchitis, etc.) might be more sensitive to particle effects and have a heightened response to inhaled pollutants. Using our co-exposure model, we did not observe any difference in the primary effect of the pollutants on endothelin system gene expression compared to exposure to either pollutant individually. Changes in gene expression in the co-exposure model tended to be additive, and were entirely consistent with the response to the individual pollutants. Congruently, the preproET-1 mRNA response to particle inhalation in the chronically inflamed lungs of the SP-C/TNF- α mice was similar to that of the wildtype littermates, despite the persistent inflammation and dramatic genotypic differences in global transcript levels. These results indicate that the initial response to inhaled particles at the level of gene expression was not modified by factors released as a result of acute ozone-induced injury or chronic TNF- α -related inflammation and injury. In our animal models, the preproET-1 mRNA response was insensitive to the physiological state of the lungs, and appears to be a primary transcriptional response to pollutant exposure.

8.4. Particles and ozone interact toxicologically with respect to plasma endothelins

However, our co-exposure model did reveal a toxicological interaction of particles and ozone with respect to effects on plasma endothelin levels immediately after exposure. Whereas exposure to particles or ozone alone elicited an increase in plasma ET-1 and its precursor bigET-1, together they failed to increase plasma ET-1 or bigET-1 levels. This

observation was somewhat perplexing, as it seemed to be at odds with both the gene expression work in the present study, and with previous experiments indicating synergistic effects of particle and ozone co-exposure on endpoints related to lung injury (Bouthillier et al., 1998; Adamson et al., 1999; Vincent et al., 1997). Given the more pronounced injury and increase in proliferating cells in the lungs of co-exposed animals, it was likely that factors involved in remodelling and repair might be present. One of these factors, matrix metalloproteinase (MMP)-2, plays a key role in degrading basement membrane proteins to facilitate cell movement and repair. Intratracheal instillation of particles has been shown to increase MMP-2 in rats (Adamson et al., 2003), and MMP-2 was recently shown to cleave bigET-1 to yield ET-1[1-32], a novel vasoconstrictor (Fernandez-Patron et al., 1999).

We hypothesized that the absence of increased plasma ET-1[1-21] peptide in the co-exposure model might be explained by increased processing of bigET-1 to ET-1[1-32] as a result of higher levels of MMP-2. Gelatin zymography of metalloproteinase activity confirmed increased MMP-2 levels in the co-exposure group, but not in animals exposed to particles or ozone alone. Our findings suggest that host factors can modulate the endothelin response, potentially generating new vasoactive factors such as ET-1[1-32]. The acute lung injury and chronic inflammation in SP-C/TNF- α mice is also associated with increased transcript and enzyme levels of MMP-2 and MMP-9 (Manuscript 6, Suppl. Mat., Figure 2) and reduced plasma bigET-1 and ET-1[1-21] (Kumarathasan et al., 2005). However, direct evidence of ET-1[1-32] *in vivo* in either the co-exposure rat model or the SP-C/TNF- α mouse model is currently lacking. Conditions for detection of ET-1[1-32] by MALDI-TOF mass spectrometry and HPLC-fluorescence analyses have been optimized (Manuscript 3, Suppl. Mat., Figure 2). However, preliminary analyses of bronchoalveolar lavage fluid and plasma

by HPLC-fluorescence and mass spectrometry have failed to identify this peptide (data not shown). This may be due to insufficient sensitivity. There is currently no specific antibody for this analyte. It is also possible that ET-1[1-32] is not released into circulation or is not recoverable by bronchoalveolar lavage, but rather is bound to receptors locally, since interaction with MMP-2 implies abluminal secretion and localization to the interstitium.

Despite the similar primary response to pollutant inhalation in our rat and mouse models (i.e. increased preproET-1 mRNA levels), the net effect on circulating plasma endothelins, and hence the potential systemic effects of endothelin activation, appears to be dependent on the pathophysiological state of the lungs. This is in line with the notion that the health status of the individual is a crucial determinant of the health effects of air pollution. Since individuals may have different sensitivities to air pollution, a dose that may cause no overt immediate adverse effects in a healthy individual could cause a cascade of events leading to cardiovascular problems in a susceptible individual. Whether conditions in the lungs actually modify the response to inhaled pollutants, as occurs in our co-exposure model, or whether the inhaled pollutants instead simply exacerbate existing pathophysiological processes, the resulting increased sensitivity to inhaled particles would help to explain the apparent non-threshold association between ambient particles and health impacts indicated by epidemiologic studies.

8.5. Microarray analysis did not detect overt changes in gene expression in normal or chronically inflamed lungs after inhalation of particulate matter

Work in our laboratory has shown that a compromised epithelium resulting from exposure to ozone can potentiate the effects of deposited particles, resulting in acute lung

injury characterized by enhanced septal remodelling (Vincent et al., 1997) and thickening (Bouthillier et al., 1998), and early release of MMP-2 (present study). Because effects of low-dose particle deposition may be compensated by homeostatic mechanisms in healthy individuals but exacerbate existing pathologies and initiate a series of events leading to adverse health effects, it is important to consider the notion of biological sensitivity. Individuals with existing lung disease are thought to be at increased risk of acute cardiovascular effects of air pollution (Brook et al., 2004; Pope, III, 2000; MacNee and Donaldson, 2000; Goldberg et al., 2000). Indeed, exacerbation of existing chronic pulmonary inflammation and the subsequent induction of an acute phase response is one of the predominant hypotheses to explain the association of particulate matter levels and cardiovascular disease (Seaton et al., 1995; Donaldson et al., 2001). We therefore sought to determine whether an existing chronic lung disease state would modify the response to deposited particles. The SP-C/TNF- α mouse provided a model of chronic lung inflammation. Because of constitutive overexpression of TNF- α in type II epithelial cells, the lungs of these mice exhibit breakdown of alveolar wall structure and pronounced inflammation. Real-time PCR analysis confirmed that inhalation of particulate matter increased preproET-1 and Cyp1a1 mRNA, validating delivery of a biologically relevant dose. To gain a global perspective on transcript activity after particle inhalation, we used 22K oligonucleotide arrays enriched for toxicologically-relevant genes, including the entire endothelin system family. We hypothesized that the effects on the endothelin system may be one of many events that result from signalling cascades initiated in the lungs after particle deposition. We further hypothesized that inflammation would modify the genes and pathways activated by particles.

Remarkably, microarray analysis of gene expression failed to identify any differentially expressed genes resulting from particle exposure in either genotype. Our analyses of chip quality and the clustering of arrays by *Genotype* and *Time* post-exposure indicate that this negative result was not due to technical errors or poor chip hybridization. Indeed, we detected consistent, statistically significant differential expression of a number of genes attributed to factors *Genotype* and *Time*, and validated microarray results by real-time PCR (Manuscript 6, Suppl. Mat., Figure 1). Gene ontology enrichment analysis of the list of genes differentially expressed according to factor *Genotype* revealed enrichment of a number of pathways including inflammatory response, immune response, response to stress, cell proliferation, oxidoreductase, and protease (unpublished data), consistent with the chronic lung inflammation. It could be argued that our study lacked sufficient power to detect modest changes induced by particulate matter using a 21K microarray platform, and this indeed may be true. However, with an $n=5$ /group, and effectively 20 animals exposed to air and 20 animals exposed to particles, this is, to our knowledge, the largest array study conducted examining effects of particulate matter on the lungs. In this mouse model, at the dose used in the present study (roughly 25-fold higher than would be expected for a high human exposure over a 24 h period), there was no prominent effect of particles on lung gene expression. An important caveat is that this work examined expression at the level of the whole lung, and therefore would likely not be sensitive to small changes in a subset of lung genes in a particular anatomic region of the lungs. Laser-capture microdissection followed by array analysis would be one approach to deal with this issue. We did not observe any significant influx of inflammatory cells into the lungs, suggesting that any focal particle-induced inflammatory signalling, if present, was not sufficient to recruit significant numbers of inflammatory cells. Our findings are consistent with the view that acute effects of

particulate matter inhalation may be attributable to physiological response cascades rather than the widespread changes in gene expression that might be expected from an inflammatory cascade. Nevertheless, transcriptional activation of certain key pathways, such as endothelin synthesis and xenobiotic transformation factors, may have relevance to these health effects. An important question that remains to be investigated is whether the existing pulmonary condition affects the cardiac response to particle inhalation in these animals.

8.6. Pollutant effects on the heart: The role of endothelin

The heart is the first organ reached by oxygenated blood coming from the lungs, and is therefore particularly vulnerable to any inhaled contaminants that diffuse into circulation or to altered production of factors secreted by the lung capillary bed. It has been shown in experimental models that chronic exposure to inhaled particles with bioavailable zinc can cause myocardial injury (Costa and Kodavanti, 2003), although it is not known whether this is due to direct effects of leached materials on the heart or secondary effects through production of factors in the lungs. Endothelial dysfunction in the vasculature of the lungs, and increased release of factors such as ET-1 and ET-3, may be harmful to the heart. The resting tone of atherosclerotic coronary arteries is largely due to ET-1 (Kinlay et al., 2001), and increased plasma ET-1 is associated with both myocardial infarction (Khan, 2005) and reduced heart rate variability (Aronson et al., 2001; Pekdemir et al., 2004), both of which are associated with acute exposure to air pollution (Peters et al., 2001; Pope, III et al., 1999). In our animal model, pollutant inhalation resulted in perturbation of pulmonary endothelin system transcript levels and increases of circulating ET-1 on the order of 50% and 100% for particles and ozone respectively. A 25% increase in plasma ET-1 levels is predictive of death in congestive heart failure patients (Galatius-Jensen et al., 1996), and a 20% decrease

in plasma ET-1 was associated with improved symptoms (Tsutamoto et al., 1995), indicating that large effect sizes may not be required to precipitate adverse cardiovascular effects in susceptible individuals. Pollutant effects on the endothelin system in humans that are of similar magnitude to those measured in experimental animals in the present and previous studies (Bouthillier et al., 1998; Vincent et al., 2001; Ulrich et al., 2002) may therefore be relevant to cardiovascular effects of air pollutants.

An examination of potential physiological effects of the increased plasma endothelins on cardiac function was outside the scope of the present work. Vincent and colleagues showed that inhalation of particulate matter increased systemic blood pressure 32 h after exposure, coinciding with the rise in circulating ET-1 (Vincent et al., 2001). Others have shown that increased plasma ET-1 resulting from intratracheal instillation of particles correlated with increased arrhythmia in a animal model of myocardial infarction (Kang et al., 2002). However, direct evidence of a role for alterations in plasma endothelin levels in acute cardiovascular events will require pharmacological intervention, such as with ET_A and ET_B receptor antagonists. We did measure a local increase in preproET-1 mRNA levels in the heart in response to ozone (and a trend towards increased expression in particle-exposed animals; Manuscript 5, Suppl. Mat. Figure 1), suggesting that in addition to potential effects of plasma endothelins on the heart via ET_A and ET_B receptors, effects on the cardiac endothelin system should be considered in assessing biological effects of air pollutants.

8.7. Extrapulmonary effects of air pollution

Local effects of pollutant inhalation on pulmonary endothelin system genes appear consistent with circulating ET-1 levels, and are in line with the lungs being the main site of

ET-1 production and clearance. However, circulating levels of endothelins are the net result of production and extraction in all tissues. Pollutant effects in other organs, through direct contact by soluble factors released into circulation, or via secondary signals, may influence endothelin production and turnover and thus contribute to plasma levels. Our observations suggested that the lungs were not the sites of increased ET-3 production, raising the possibility of extrapulmonary effects. The central nervous system is a potential target for inhaled pollutants (Peters et al., 2006). The nasopharyngeal compartment receives a high dose of nasally-deposited particulate matter, and animal studies have demonstrated axonal transport of ultrafine particles to the brain via the olfactory bulb (Oberdorster et al., 2004). Since ET-3 is a neurotransmitter (Anggard et al., 1989), and the pituitary is a potential source of circulating ET-1 and ET-3 (Lange et al., 2002; Haak et al., 1997), we hypothesized that pollutants may affect pituitary endothelin production and induce changes in cerebral hemisphere endothelin-nitric oxide system genes. We found that both particles and ozone independently regulated gene expression in the pituitary and cerebral hemisphere. Our data, showing rapid effects of pollutant inhalation on key vasoregulatory pathways in the brain, support the notion of cerebrovascular effects of air pollution (Wellenius et al., 2005; Maheswaran and Elliott, 2003; Hong et al., 2002). However, the results are not consistent with the pattern of ET-3 response in plasma. Although the pituitary may contribute to circulating ET-1 and ET-3 levels, it does not appear to be responsible for the surge in ET-3 after exposure to particulate matter.

Analysis of gene expression in other organs (heart, kidney, liver, spleen, pituitary) did not provide any indication that these organs were responsible for the increased ET-3 plasma levels observed. Although the data do not allow us to conclude unequivocally that

the surge in plasma ET-3 is the result of increased extrapulmonary production and release of the peptide, it seems unlikely that the sustained (48 h) elevation (Vincent et al., 2001) could be due simply to increased release of stored ET-3 from lung cells, with no parallel increase in preproET-3 mRNA levels. It is possible that the increased ET-3 levels are the result of effects of pollutants on endothelial ET_B receptor levels, resulting in reduced clearance. However, no immediate decrease in ET_B receptor mRNA levels was observed in the lungs, the primary site of endothelin clearance. This does not preclude the possibility that a reduction of receptor-mediated clearance could also be controlled post-transcriptionally, but direct data are lacking. ET-3 is thought of as a “brain-gut” peptide (Matsumoto et al., 1989), and increased production and release of ET-3 in the gastrointestinal tract is a possibility. Clearance by coughing or via the mucociliary escalator followed by swallowing could rapidly (within minutes to hours) deposit particles in the stomach (Finch et al., 1987; Hofmann and Asgharian, 2003; Moller et al., 2004). Further work is required to investigate whether the stomach or intestines serve as sources of circulating ET-3.

8.8. Evidence in humans and animals of impaired vascular homeostasis in response to particle exposure

At least in terms of acute effects, it seems unlikely that small increases in air pollution levels would trigger an adverse cardiovascular effect in individuals without existing disease. Recent epidemiologic evidence supports this view. A study by Pope et al. showed that associations of air pollution and ischemic heart disease events were only found for individuals with at least one severely diseased coronary vessel (Pope, III et al., 2006). This is in line with the concept that short-term exposure to air pollution interacts with host factors to provoke disease, and in the absence of these host factors adverse effects may not be

detected. Nevertheless, controlled exposure of healthy human subjects may provide evidence supporting a role for increased endothelin levels and impaired vascular homeostasis in cardiovascular effects of air pollution. Indeed, bigET-1 and ET-3 levels were increased in the plasma of healthy volunteers 24 h after exposure to Toronto PM_{2.5} (Vincent et al., unpublished). The increase of bigET-1, rather than the mature ET-1 form, implies increased *de novo* production of the peptide as was observed in the present work, although reduced conversion of bigET-1 to ET-1 or reduced clearance from circulation is also a possibility. Controlled exposure of volunteers to concentrated PM_{2.5} and ozone caused a small but significant constriction of the brachial artery (Brook et al., 2002) and increased blood pressure (Urch et al., 2005), confirming vasopressor effects of this pollutant mixture. In a recent study, short-term exposure to welding fumes decreased circulating ET-1, but did not significantly affect blood levels of inflammatory markers (C-reactive protein, TNF- α , IL-6, IL-8, epithelial neutrophil activating peptide 78), coagulation factors (fibrinogen, antithrombin III, factor VIII, ristocetin cofactor), markers of endothelial dysfunction (soluble intercellular adhesion molecule-1, von Willebrand factor), or plasma cell differential counts measured 5 h post-exposure (Scharrer et al., 2007). BigET-1 was not measured in that study, and so it is not possible to assess whether the drop in ET-1 levels is due to reduced *de novo* synthesis of the precursor, reduced cleavage of bigET-1 to ET-1, increased clearance via ET_B receptors, or whether it occurs in the context of increased bigET-1. However, this study does reveal that ET-1 levels are sensitive to acute exposure to welding fumes. In line with the human exposure studies, exposure of rats to concentrated ambient particles by inhalation (Batalha et al., 2002) or Sao Paulo PM_{2.5} by intratracheal instillation (Rivero et al., 2005) has been shown to result in vasoconstriction of pulmonary arterioles.

The regulation of endothelin levels by inhaled air pollutants likely occurs in concert with other effects on the vasculature. Possible local and systemic effects of increased endothelin production should be discussed in the context of these changes. Recently, a number of studies have examined endothelial dysfunction by assessing vasomotor function in humans and animals after exposure to particles. Levels of ambient particulate matter were found to be associated with impaired endothelium-dependent and –independent dilation in the brachial artery of diabetic patients (O'Neill et al., 2005), and similar effects were observed after controlled exposure of healthy subjects to diesel exhaust (Mills et al., 2005). In line with these effects, intraperitoneal administration of diesel exhaust particles decreased endothelium-dependent vasodilation in mildly atherosclerotic apolipoprotein E knockout (ApoE^{-/-}) mice (Hansen et al., 2007). *Ex vivo* exposure of isolated coronary arteries to diesel exhaust in a tissue bath resulted in reduced nitric oxide-induced vasodilation of precontracted vessels and enhanced ET-1 mediated contraction (Campen et al., 2005). ET-1 is a more potent vasoconstrictor in nitric oxide-limited tissues, due to the lack of a vasodilator response through ET_B-receptor-mediated production of nitric oxide (Cardillo et al., 2000). Although reduced endothelium-dependent vasodilation would not in itself explain the brachial artery constriction after controlled exposure to particulate matter and ozone (Brook et al., 2002), this pollutant effect might be an important factor in explaining how increased ET-1 production causes vasoconstriction. Moreover, vascular ET-1 inhibits endothelial nitric oxide release (Barton et al., 1998), and has been shown to impair endothelium-dependent and independent vasodilation in the human forearm via the ET_A receptor (Bohm et al., 2002; Bohm et al., 2007). Although bigET-1 and ET-1 were only measured in one of these studies (and no significant effect on circulating bigET-1 or ET-1 was detected (Mills et al., 2005)), aortic ET-1 and MMP-2 mRNA levels were increased in ApoE^{-/-} mice exposed chronically

for 7 weeks to gasoline engine emissions (Lund et al., 2007). In a longer chronic exposure study, increased plaque development, vascular inflammation, and increased adrenergic-induced vasoconstriction was observed in ApoE^{-/-} mice with advanced atherosclerosis exposed to particles over a period of 6 months (Sun et al., 2005). These studies, along with ours, reveal that air pollutants may influence vascular function both through release of vasoconstrictors and through impairment of vasodilator function, which will tend to promote vasoconstriction.

8.9. Chronic effects of increased endothelin production

The present work focussed on acute effects of pollutant inhalation on the endothelin system. However, it is worth noting that acute effects of pollutant exposure may be relevant to health effects of chronic exposure. For example, while a surge of ET-1 resulting from acute exposure could destabilize plaques and provoke an acute cardiovascular event, chronic exposure and repeated activation of factors such as ET-1 could accelerate the progression of atherosclerosis. In fact, chronically elevated air pollution in Los Angeles has recently been shown to be associated with carotid-intima media thickness, a measure of subclinical atherosclerosis (Kunzli et al., 2005). In both the acute and chronic exposure scenarios, the initial response to pollutant exposure (i.e. increased ET-1) may be the same; it is the end result (triggering of an acute event vs. accelerated progression of a disease) that differs. Expanding on this point, chronic exposure to pollutants at levels that do not cause immediate adverse effects but that do cause slight perturbations of vascular homeostasis would be expected to increase the likelihood of adverse health effects in response to other triggers. For example, ET-1 levels were higher in children living in a polluted city relative to others living in a less polluted environment (Calderon-Garciduenas et al., 2003). A follow up study

by the same authors confirmed the association of chronic exposure to air pollution with increased endothelin levels, and found that individuals with the highest ET levels were more likely to have increased pulmonary arterial hypertension (Calderon-Garciduenas et al., unpublished).

There is increasing evidence that air pollution may act through several pathways simultaneously, but effects may only be apparent in pathways in which there is an existing susceptibility. Understanding why people are dying from air pollution exposure will therefore likely require analysis of the interaction of airborne contaminants with the pathophysiology of the respective existing disease or phenotype.

8.10. Future directions

There is a substantial literature supporting a role for endothelin in cardiovascular disease. Our work provides a basis for future investigations using endothelin gene expression as a critical endpoint for air pollution risk assessment. Confirmation of the role of pollution-induced increases in endothelin production in humans on endpoints such as brachial artery constriction, blood pressure, arrhythmia, reduced heart rate variability, etc., will require the use of specific endothelin receptor inhibitors. These are available – bosentan, for example, an ET_A/ET_B receptor inhibitor that has been shown to reduce blood pressure in patients with essential hypertension (Krum et al., 1998). Controlled exposure of those people hypothesized to respond the greatest to air pollution (i.e. people with existing disease) poses ethical questions. However, blood samples taken as part of an epidemiologic study could be assessed for endothelin levels. Because of substantial variations of baseline

endothelin levels within the population, it would be useful to perform repeated sampling over time. With sufficient power it should be possible to determine whether circulating endothelin levels in humans are associated with periodic variations in pollutant levels, and also whether certain pathophysiological states predispose an individual to an increased endothelin response, or to an enhanced physiological response to the rise in endothelin levels.

Animal studies should continue to be a useful approach. A particularly promising model is the ApoE^{-/-} mouse model of atherosclerosis, which has received increased attention in the field of inhalation toxicology due to the apparent effects of air pollutants on plaque destabilization, myocardial infarction, and progression of atherosclerosis. ET-1 and ECE-1 are highly expressed in atherosclerotic plaques (Ihling et al., 2001), and young ApoE^{-/-} mice with no atherosclerotic lesions exhibit a heightened vasoconstrictive response to exogenous ET-1 (Maguire et al., 2006). Atherosclerotic plaques are also sites of increased MMP-2 activity, raising the possibility of alternative processing of bigET-1. Since preproET-1 and MMP-2 mRNA are both upregulated in atherosclerotic arteries of these animals after exposure to gasoline engine exhaust (Lund et al., 2007), the ApoE^{-/-} mouse model of atherosclerosis is a useful model to study the interrelationship of ET-1, MMPs, and air pollution with respect to enhanced plaque development, plaque destabilization, vasoconstriction, and the involvement of endothelin in these processes. The stroke-prone hypertensive rat, which is characterized by increased cerebral hemisphere ET-1 production and reduced cerebral blood flow (Jesmin et al., 2004), might be a useful model to investigate the effects of inhaled pollutants on the brain, and to determine whether the apparent activation of the endothelin system in the cerebral hemisphere increases the risk of ischemic

stroke. Diabetic rats have been shown to exhibit an increased ET-1 response to intratracheal instillation of PM_{2.5} particles (Lei et al., 2005), suggesting an enhanced effect on the endothelium due to interaction of particulate matter exposure and disease pathophysiology. Of note, this study did not find any interaction between diabetes and particulate matter with respect to levels of IL-6, TNF- α , and C-reactive protein, indicating that effects on plasma ET-1 levels occurred in the absence of changes in circulating markers of inflammation.

The upstream effects leading to activation of endothelin synthesis are currently being investigated. Intravenous administration of a superoxide dismutase (SOD) mimetic abolished the rise in ET-1 after pollutant exposure (Ganesh, Blais, Goegan, Guenette, Thomson, Kumarathasan, Crapo, Vincent, unpublished), consistent with the involvement of oxidative stress in this pathway. Pulmonary preproET-1 and ECE-1 mRNA and plasma bigET-1 responses to the pollutants were not affected by treatment by the SOD. It is possible that oxidative stress is not required for transcriptional activation of preproET-1 by the pollutants, but rather for the regulated release of the peptide from intracellular storage sites in endothelial cells (Russell and Davenport, 1999). Cardiac mRNA was also increased after exposure to particles with or without treatment with the SOD mimetic, indicating that this too is independent of oxidative stress and circulating ET-1 levels. In rat endothelial cells *in vitro*, oxidative stress decreased preproET-1 mRNA and ET-1 release (Michael et al., 1997). In contrast, xanthine oxidase increased preproET-1 mRNA and ET-1 release in cultured human endothelial cells (Kaehler et al., 2002). Further research is required to investigate the upstream signalling events that lead to endothelin activation.

8.11. Summary: Activation of pulmonary endothelin system genes: A mechanism of cardiovascular effects of air pollution?

If the epidemiologic evidence provides insight into the biological effects that explain the association between periodic variations in air pollution and adverse cardiovascular effects, prospective mediators should satisfy the requirements set out in the Introduction. Namely, the mediator must 1) respond rapidly to air pollution; 2) link particle deposition in the lungs to adverse cardiovascular effects; and 3) interact with, or be modified by, existing host factors in individuals thought to be at increased risk.

1. Our data indicate that, with respect to the timing of response, ET-1 does respond rapidly to both inhaled particulate matter and ozone in our animal models. The increased gene expression and peptide levels immediately after acute exposure indicate that the ET system responds within the time range identified by epidemiologic studies, and is consistent with work in other models showing rapid *de novo* synthesis of ET-1 in response to a stimulus (Finsnes et al., 1998; Marsden et al., 1991).
2. The ET-1 response occurs as a result of direct pollutant effects in the lungs. Since there is no evidence of damage to the epithelial barrier in animals exposed to particles alone, the increased expression of genes predominantly expressed in the endothelium implies diffusion of soluble factors from particles or transduction of signals from alveolar macrophages and epithelial cells that reach the endothelium (Figure 1). If particles do indeed translocate to the systemic circulation, our data suggest that this is not required for effects on circulating ET-1 levels. Since the endothelin response precedes the influx of inflammatory cells, our results reveal that inflammation is not a prerequisite for pollutant effects on this pathway. In the

mouse model, inhalation of particulate matter at a high but toxicologically relevant dose increased preproET-1 mRNA but did not induce widespread changes in gene expression detectable by microarray analysis, and did not cause a significant influx of inflammatory cells in either normal or chronically inflamed lungs.

Although measurement of physiological correlates of the increase of ET-1 peptide was outside the scope of the present work, there is considerable evidence to support a role for increased ET-1 production in adverse cardiovascular effects, as described in the Introduction. These range from rapid effects on blood pressure, heart rate variability, and disruption of atherosclerotic plaques that can result in myocardial infarction, to longer term effects on the progression of atherosclerosis. Epidemiologic studies have indeed correlated air pollution levels with increased blood pressure (Ibald-Mulli et al., 2001), reduced heart rate variability (Magari et al., 2001), and the triggering of myocardial infarction (Peters et al., 2001) within 2-4 hrs of exposure, and progression of atherosclerosis with chronic exposure (Kunzli et al., 2005). Effects on the endothelin system are not limited to the lungs. We also observed changes in the cerebral hemisphere, pituitary, and heart, revealing that inhaled pollutants provoke extrapulmonary effects on important vasoregulatory pathways.

3. Various components of the endothelin system are affected by, and play a role in, the pathogenesis of cardiovascular disease. The physiological relevance of the increased ET-1 production observed in the present study is established by the substantial literature describing adverse cardiovascular outcomes in individuals with existing disease. Our data indicate that the endothelin response occurs in healthy lungs, in lungs with acute injury, and in chronically inflamed lungs, indicating that the primary effect of particle deposition is not modified by

these existing pathologies. However, the physiological state of the lungs appears to influence plasma endothelin levels. Activated factors in the injured/inflamed lungs may modify the processing and release of endothelins, and thus influence the systemic effects resulting from increased pulmonary endothelin production (Figure 1).

In summary, this thesis provides evidence in experimental models that the pulmonary and cerebral endothelin systems are targets of inhaled air pollutants. Given the pollutant effects on endothelin system genes measured in the present study and the substantial literature supporting a role for ET-1 in cardiovascular disease, we propose that the pattern of endothelin system expression should be considered as a critical endpoint for assessment of biological effects of urban air pollution. The impact of air pollution on human endothelin levels and subsequent interactions with host susceptibility factors warrant further investigation.

8.12. References

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May - Dec. 1999

Research Assistant

CANMET, Natural Resources Canada, Ottawa

- Research involving enzyme purification and immobilization
- Development of a biosensor

PAPERS

Thomson E, Kumarathasan P, Calderón-Garcidueñas L, Vincent R. Air Pollution Alters Brain Endothelin-1 and Inducible Nitric Oxide Gene Expression. *Environ Res. In press.*

Thomson E, Kumarathasan P, Vincent R. (2006). Pulmonary Expression of PreproET-1 and PreproET-3 mRNAs is Altered Reciprocally in Rats after Inhalation of Air Pollutants. *Exp Biol Med* 231: 979-984.

Chauhan V, Breznan D, Thomson E, Vincent R. (2005). Effects of Ambient Air Particles on the Endothelin System in Human Pulmonary Epithelial Cells (A549). *Cell Biol Tox* 2: 191-205.

Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. (2005). Differential Regulation of Lung Endothelin System Genes by Urban Particulate Matter and Ozone. *Tox Sci* 88: 103-113.

Thomson E, Vincent R. (2005). Reagent Volume and Plate Bias in Real-Time PCR. *Anal Biochem* 337(2): 347-350.

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POSTERS

Thomson E, Williams A, Yauk C, Vincent R. Toxicogenomic Analysis of Susceptibility to Inhaled Urban Particles in Mice With Constitutive Pulmonary TNF- α . 10th International Inhalation Symposium, Hannover, Germany, May 31-June 3, 2006.

Williams A, Thomson E, Dong H, Padhi BK, Berndt L, Pelletier G, Vincent R, Douglas G, Yauk C. Combining Multiple Scans to Improve the Detection of Differentially Expressed Genes. *Health Canada Research Forum*, Ottawa, Ontario, Oct. 24-25, 2005.

Williams A, Padhi BK, Thomson E, Hierlihy A, Dong H, Berndt L, Pelletier G, Wade M, Curran I, Vincent R, Douglas G, Yauk C. Identifying and Controlling Nuisance Parameters in Microarray Studies. *Health Canada Research Forum*, Ottawa, Ontario, Oct. 24-25, 2005.

Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. Urban Pollutants Interact Toxicologically to Regulate Lung Endothelin System Genes and Plasma Endothelin. *Ninth International Conference on Endothelin (ET-9)*, Park City, Utah, Sept. 11-14, 2005.

Ganesh G, Thomson E, Blais E, Goegan P, Guénette J, Kumarathasan P, Crapo J, Vincent R. Activation of the Endothelinergic System in the Heart and Lungs after Inhalation of Air Pollutants is Reversed by the Superoxide Dismutase Mimetic AEOL 10150. *Society of Toxicology of Canada*, Montréal, Québec, Dec. 6-7 2004.

Chauhan V, Breznan D, **Thomson E**, Vincent R. **Inflammatory Cytokines and Endothelin are Regulated Separately in Human Pulmonary Epithelial Cells Exposed to Urban Particles.** *Society of Toxicology of Canada*, Montréal, Québec, Dec. 6-7 2004.

Thomson E, Kumarathasan P, Vincent R. **Differential Regulation of the Lung Endothelin System by Urban Particulate Matter and Ozone.** *Health Canada Research Forum*, Ottawa, Ontario, Oct. 18-19, 2004.

Thomson E, Kumarathasan P, Calderon-Garciduenas L, Vincent R. **Inhaled Urban Pollutants Regulate Endothelin Expression in the Brain.** *Health Canada Research Forum*, Ottawa, Ontario, Oct. 21-23, 2003.

Breznan D, Chauhan V, **Thomson E**, Kumarathasan P, Vincent R. **In Vitro Models for Cardiovascular Effects of Inhaled Urban Particles.** *Health Canada Research Forum*, Ottawa, Ontario, Oct. 21-23, 2003.

Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. **Systemic Effects of Air Pollutants: Expression of Endothelin in the Heart and Brain of Rats after Inhalation of Ozone.** *Canadian Federation of Biological Societies 46th Annual Meeting*, Ottawa, Ontario, June 11-15, 2003.

Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. **Ambient Particulate Matter and Ozone Modulate Expression of Endothelin System Genes in the Lungs and Heart.** *Experimental Biology 2003*, San Diego, California, April 11-14, 2003.

Chauhan V, Breznan D, **Thomson E**, Kumarathasan P, Battistini B, Vincent R. **Effects of Urban Particles on the Vasoactive Agents Endothelin-1 and Nitric Oxide in A549 Human Lung Epithelial Cells.** *Experimental Biology 2003*, San Diego, California, April 11-14, 2003.

Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. **Real-Time RT-PCR Analysis of Endothelin Expression in Rats Exposed to Ambient Particulate Matter and Ozone.** *Health Canada Research Forum*, Ottawa, Ontario, Nov. 18-19, 2002.

SEMINARS

Errol Thomson. Activation of the Pulmonary Endothelin System: A Mechanism for Cardiovascular Effects of Air Pollutants. *Environment & Occupational Toxicology Seminar Series*. LCDC Building, Tunney's Pasture, Ottawa, March 7, 2006.

Errol Thomson. Pollutant Regulation of Endothelin: Is it Modified by Inflammation? *Graduate Student Seminar Series*. LOEB Institute, Ottawa, April 22, 2005.

Errol Thomson. Particulate Matter and Ozone Modulate Expression of Endothelin System Genes. *Graduate Student Seminar Series*. University of Ottawa Heart Institute, Ottawa, March 10, 2003.

OTHER INTERESTS

Academic	Health Canada Graduate Student Journal Club organizer (2004-2006) Co-op student supervisor (2002-2003)
Community involvement	Volunteer , Los Alerces National Park, Argentina (2001) Volunteer Squash Coach , Junior Program, Orléans Recreation Complex (1994-1999)

SCHOLARSHIPS, FELLOWSHIPS, AND OTHER AWARDS HELD

Name of the award	Value (CAN\$)	Level	Type	Location of tenure	Period held (mm/yy)
10 th International Inhalation Symposium Poster Award – 2 nd place	1,000	International	Research	Hannover, Germany	06/06
9 th International Endothelin Conference Young Investigator Travel Award	1,000	International Travel Award	Research	Park City, Utah	09/05
NSERC PGS-B	42,000	National	Academic/Research	University of Ottawa	09/04-09/06
1 st Prize PhD Biochemistry Graduate Poster Competition	120	Institutional	Research	University of Ottawa	04/04
National Excellence Scholarship	Tuition fees + 2,000	Institutional	Academic	University of Ottawa	09/02-present
NSERC PGS-A	34,600	National	Academic/Research	University of Ottawa	09/02-09/04
Strategic Areas of Development Scholarship	6,000	Institutional	Academic/Research	University of Ottawa	05/02-12/02
Excellence Scholarship	Tuition fees	Institutional	Academic	University of Ottawa	01/02-08/02
Ontario Graduate Scholarship	15,000	Provincial	Academic/Research	University of Ottawa	01/02-08/02
Senate Medal for Outstanding Academic Achievement	-----	Institutional	Academic	Carleton University	Awarded upon graduation
Dr. Harry Katznelson Memorial Scholarship	150	Institutional	Academic	Carleton University	09/00-12/00
NSERC Undergraduate Scholarship	4,500	National	Academic	Carleton University	05/00-08/00
Harry H. Southam Scholarship	1500	Institutional	Academic	Carleton University	01/00-12/00
Henry Marshall Tory Scholarship	1500	Institutional	Academic	Carleton University	09/98-04/99
James and Jane Fraser Roy Scholarship	1500	Institutional	Academic	Carleton University	09/97-04/98
Carleton University Entrance Scholarship	1500	Institutional	Academic	Carleton University	09/96-04/97