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Inhalation Toxicology of Urban Ambient Particulate Matter: Acute Cardiovascular Effects in Rats

Renaud Vincent, Premkumari Kumarathasan, Patrick Goegan, Stephen G Bjarnason, Josée Guénette, Denis Bérubé, Ian Y Adamson, Suzanne Desjardins, Richard T Burnett, Frederick J Miller, and Bruno Battistini

Includes a Commentary by the Institute's Health Review Committee

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The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe and has published over 100 research reports.

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HEI STATEMENT
Synopsis of Research Report 104

Acute Cardiovascular Effects in Rats from Exposure to Urban Ambient Particles

BACKGROUND

Particulate matter (PM) in ambient air is a complex mixture containing particles of different sizes and chemical composition. Short-term increases in ambient PM levels have been associated with short-term increases in morbidity and mortality from cardiovascular causes. However, the biologic mechanism by which PM may affect cardiovascular events and the roles of particle size and composition are not well understood. One hypothesis is that particle deposition in the lung induces the release of factors that affect blood cells and vessels throughout the body. The Health Effects Institute funded the study described in this report to address this hypothesis and to elucidate how the chemical composition of the particles could change the effects of PM on the cardiovascular system.

APPROACH

Dr Renaud Vincent, of Health Canada, Ottawa, and his colleagues hypothesized that ambient PM would cause changes in certain cardiovascular parameters; for example, heart rate and blood pressure or the concentration of compounds that regulate the constriction and dilation of blood vessels. The investigators implanted rats with radiotransmitters (to collect continuous data on heart rate, blood pressure, and body temperature) and indwelling catheters for repeated blood sampling. The animals were exposed for 4 hours to clean air or one of four types of resuspended particles: ambient particles (Ottawa dust), ambient particles that had been washed in water to remove soluble components, diesel soot, or carbon black. The investigators used stored resuspended particles rather than fresh particles to ensure that particle concentration and chemical composition were identical among experiments. The investigators measured several endpoints in the rats' blood and plasma (endothelins, nitric oxide products, and catecholamines), changes in heart rate, blood pressure, and general activity, and injury to lung tissue.

RESULTS AND IMPLICATIONS

The investigators found that exposure to urban PM, washed urban PM, and diesel soot (but not carbon black) caused an increase in endothelin-3 and to a lesser extent endothelin-1. This is important because endothelins help to regulate normal cardiovascular homeostasis between vasoconstriction and vasodilation. Elevation of endothelin-1 is an indicator of the severity of congestive heart failure in some patients and may predict cardiac death, whereas its decrease may herald an improvement in symptoms; elevation of endothelin-3 may be associated with systemic vasoconstriction in cardiac patients.

Urban PM also caused an increase in blood pressure; the effect disappeared when washed particles were used. The increase in blood pressure was not observed with diesel soot or carbon black. This indicates that one or more of the soluble components in the unwashed urban particles may be responsible for the blood pressure effects of urban PM. The investigators suggested that soluble metals may have been responsible for this adverse effect; among other candidates, zinc may be important because zinc-dependent enzymes are involved in the regulation of endothelins, which may then impact blood pressure. Whether this is indeed the case remains to be determined.

The increase in blood pressure after exposure to urban PM was small, however, and its significance is unclear because the stress caused in the animals by handling and surgery was substantial; this was reflected in the variability of the baseline physiologic parameters (ie, heart rate, blood pressure, and body temperature) and blood catecholamine measurements. The study could have benefitted from additional analyses of changes in electrocardiogram or in the daily rhythm variations in heart rate and blood pressure. Additional short-term and transient changes in these physiologic parameters at different time points after exposure to the particles, if present,

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr Renaud Vincent of the Safe Environments Programme, Health Canada, Ottawa. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

would strengthen the argument that PM may have adverse effects on the cardiovascular system.

The investigators found no evidence of damage to lung epithelial cells from exposure to any of the particles by using an assay that measured lung cell proliferation several days after exposure. These findings confirm previous studies with the same urban particles in the same laboratory. In those studies, however, urban PM did increase other indices of lung injury, ie, levels of inflammatory markers in lung lavage fluid. It is possible that exposure to washed urban particles, diesel soot, or carbon black might also have affected those markers of lung inflammation.

The use of resuspended particles rather than fresh particles was a valid choice to ensure the stability of the administered concentration and the chemical composition of the particles among experiments. The relevance of the effects of resuspended particles to those observed with fresh particles is in question, however, because they likely underwent chemical and physical changes during storage. Due to aggregation, these particles are presumably larger than fresh particles and it is unclear whether larger particles penetrate the deep lung. Nevertheless, the finding that both resuspended ambient particles and diesel soot affected the same endothelins strengthens the confidence in these data.

Main strengths of this study were that the investigators compared several classes of particles and measured diverse endpoints. The finding that urban PM changed endothelin levels might be strengthened by future research using several PM concentrations and by reconstituting the urban PM from the washed PM, which would show whether or not adding the soluble components to the washed particles would restore the biologic effects.

In conclusion, the investigators found effects on endothelins after exposure to resuspended urban PM, washed particles, and diesel soot, and effects on blood pressure after exposure to urban PM. Although extrapolating results from animal studies to humans and from the high levels used in this study to ambient levels always involves uncertainties, these findings open interesting avenues to further explore the possible mechanisms by which inhaled PM may cause adverse cardiovascular events. More research is needed to assess whether changes in endothelin levels would occur in humans exposed to ambient levels of particulate matter, and whether these changes may contribute to the adverse cardiovascular events that have been associated with air pollution episodes in epidemiology studies.

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HEALTH EFFECTS INSTITUTE

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Research Report 104

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HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

PREFACE

The Preface describes the general regulatory and scientific background for the HEI Research Program that produced this and other reports on related topics.

INVESTIGATORS' REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

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PREFACE

In 1994, HEI initiated a research program to investigate the complex issues associated with the health effects of exposure to particulate matter (PM*) in the air. This program was developed in response to growing concern about the potential public health significance of reported associations between daily fluctuations in levels of PM and changes in daily morbidity and mortality in time-series epidemiology studies. These results were questioned for a variety of reasons, including the lack of support from experimental studies and the lack of a mechanism to explain how such effects would occur. To address these issues, HEI undertook research initiatives in 1994, 1996, and 1998.

In 1994, the Particle Epidemiology Evaluation Project (Samet et al 1995, 1997) evaluated six of the time-series epidemiology studies that had reported effects of PM on mortality, and a program of epidemiology and toxicology studies were funded that aimed at understanding how PM might cause toxicity and what factors might affect susceptibility (RFA 94-2, "Particulate Air Pollution and Daily Mortality: Identification of Populations at Risk and Underlying Mechanisms"). In 1996, HEI issued RFA 96-1, "Mechanisms of Particle Toxicity: Fate and Bioreactivity of Particle-Associated Compounds", which sought studies that would improve our understanding of toxicologically relevant characteristics of ambient particles. In 1998, HEI issued RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter", which targeted a broad and ambitious set of research goals relating to both exposure assessment and health effects. In all, HEI has issued five requests for research on PM and funded 34 studies or reanalyses over the last 5 years.

This Preface provides general regulatory and scientific background information relevant to studies funded from RFA 98-1, including the study by Dr Renaud Vincent that is described in the accompanying Report and Commentary. This is one of 14 studies funded from RFA 98-1. The "HEI Program Summary: Research on Particulate Matter" (Health Effects Institute 1999) provides information on all PM studies funded since 1996.

BACKGROUND

Particulate matter is the term used to define a complex mixture of anthropogenic and naturally occurring airborne particles. In urban environments, these particles derive mainly from combustion, including mobile sources such as motor vehicles and stationary sources such as power plants. The size, chemical composition, and other physical and biologic properties of PM depend on the sources of the particles and the changes the particles undergo in the atmosphere. The most commonly used descriptor of particles is size measured as *aerodynamic diameter*. On the basis of this parameter, ambient particles tend to fall into three size classes (often defined as modes): ultrafine or nuclei mode (particles less than 0.1 μm in diameter); fine or accumulation mode (particles between 0.1 and 2.5 μm in diameter), and coarse (particles larger than 2.5 μm in diameter). Fine and ultrafine particles are found primarily in emissions from combustion processes, whereas coarse particles are mostly generated by mechanical processes from a variety of noncombustion sources. Generally, the ultrafine and fine fractions are composed of carbonaceous material, metals, sulfate, nitrate, and ammonium ions. The coarse fraction consists of insoluble minerals and biologic aerosols, with smaller contributions from primary and secondary aerosols and sea salts (US Environmental Protection Agency [EPA] 1996).

A number of early epidemiologic studies indicated that human exposure to high concentrations of PM, such as London fog, had deleterious effects (such as an increased number of deaths) particularly in children, the elderly, and those with cardiopulmonary conditions (Firket 1931; Logan 1953; Ciocco and Thompson 1961; Gore and Shaddick 1968). Because of this apparent relation to increased mortality, the EPA has regulated the levels of ambient PM since 1971, when the Clean Air Act was first promulgated. This act authorized the EPA to set National Ambient Air Quality Standards (NAAQSs) for a number of potentially harmful air pollutants (including PM) in order to protect the health of the population, particularly those people thought to be sensitive to the effects of pollution.

The first NAAQSs for PM were based on controlling total suspended PM or particles up to 40 μm in diameter. In 1978, the standards were revised to regulate inhalable particles, or particles that can deposit in the respiratory tract and therefore have greater potential for causing adverse health effects. These particles measure 10 μm or smaller in aerodynamic diameter (PM_{10}). More recent epidemiologic studies, published in the early 1990s, indicated a relatively consistent association between short-term small increases in PM levels and increases in both morbidity and mortality from respiratory and cardiovascular diseases

* A list of abbreviations and other terms appears at the end of the Investigators' Report.

Preface Table 1. Current National Ambient Air Quality Standards for Particulate Matter (Set in 1997)

Time Period	PM_{10}	$PM_{2.5}$
Daily	150 $\mu g/m^3$	65 $\mu g/m^3$
Annual	50 $\mu g/m^3$	15 $\mu g/m^3$

(reviewed by the Committee of the Environmental and Occupational Health Assembly, American Thoracic Society [Bascom et al 1996]).

Some studies also suggested that long-term exposure to low levels of PM is associated with adverse effects (Dockery et al 1993; Pope et al 1995). These latter studies also pointed to a possible role of fine particles (less than 2.5 μm in aerodynamic diameter [$PM_{2.5}$]). In 1997, the EPA decided that the evidence for the effects of fine particles was sufficient to promulgate a $PM_{2.5}$ standard while retaining the PM_{10} standard (US EPA 1997) (see Preface Table 1). The next review of the PM NAAQSs is scheduled to be completed by 2002.

HEI'S PARTICULATE MATTER RESEARCH PROGRAM

The wealth of epidemiologic data published in the early 1990s suggested an association between PM and health effects, but aspects of these findings were not well understood. Problems involved uncertainties in the exposure estimates, confounding by weather or other factors, the role of copollutants, and the mechanisms by which particles may cause effects. Moreover, although the epidemiologic findings were consistent across different communities exposed to distinct mixtures and levels of pollutants, they were not well supported by either human exposure chamber studies or animal inhalation studies aimed at delineating the pathologic changes that might result in death. Failure of the experimental studies to provide support for the epidemiologic findings was attributed to insufficient statistical power, use of particles not representative of ambient particles, or use of animals not representative of the individuals susceptible to increased mortality.

By the mid 1990s, it became apparent that the research to advance our understanding of the association between exposure to particles and daily mortality found in the epidemiologic studies needed to focus on identifying (1) susceptible populations, (2) mechanisms by which particles may lead to increased mortality, and (3) characteristics of the particles responsible for the effects. It was recognized that both epidemiologic and experimental studies would be required. The HEI program from RFA-94 was aimed at addressing these research needs. In 1994, HEI also initiated the Particle Epidemiology Evaluation Project to address the validity and replicability of key epidemiologic studies (Samet et al 1995, 1997). Out of that project evolved the National Morbidity, Mortality, and Air Pollution Study to continue the epidemiologic evaluation in a large number of cities across the US with varying levels of PM and other air pollutants (Samet et al 2000a,b). Subsequently, HEI funded studies under RFA 96-1 that would use fine and ultrafine particles to test specific hypotheses related to the role of particle constituents in PM toxicity.

With increased financial support from the EPA and industry, in January 1998 HEI requested applications targeting both exposure assessments and health effects. HEI held a workshop at the Offices of the National Research Council that brought together scientists and representatives of the EPA and the motor vehicle and oil industries to discuss research needs. Out of this discussion, RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter", was developed and issued. The exposure objectives included (1) characterizing personal exposure to particles in different indoor and outdoor microenvironments and in geographic locations that differ in the types and sources of particles, topography, and climate; and (2) improving particle characterization to increase the accuracy of exposure estimates in epidemiologic studies. The health effects objectives included (1) characterizing potential pathophysiologic effects caused by PM in sensitive subjects; (2) defining the relation between particle characteristics and dose, distribution, and persistence of particles in the respiratory tract; (3) identifying the kinds of particles or particle attributes that may cause toxicity; (4) investigating the diseases or conditions that affect sensitivity; and (5) delineating how copollutants affect or contribute to the physiologic response to particles. From this RFA, HEI funded a comprehensive set of exposure assessment and health effects studies.

Three exposure assessment studies were designed to investigate personal exposure to PM in potentially sensitive population subgroups in several US and European cities with diverse climatic and geographic features. These studies focused on (1) characterizing indoor concentrations of and personal exposure to $PM_{2.5}$ for subjects in two European cities (Bert Brunekreef of Wageningen/Utrecht University); (2) characterizing exposure to $PM_{2.5}$, ozone, nitrogen dioxide, carbon monoxide, and sulfur dioxide in children, healthy seniors, and subjects with chronic

obstructive pulmonary disease (Petros Koutrakis of Harvard School of Public Health); and (3) assessing personal exposure to $PM_{2.5}$ and characterizing PM in terms of mass, functional groups, trace metals, polynuclear aromatic hydrocarbons, and elemental and organic carbon (Barbara Turpin of Environmental and Occupational Health Sciences Institute). A fourth study aimed to validate a newly developed method for measuring the acidic component of ultrafine particles or $PM_{0.1}$ (Beverly Cohen of New York University Medical Center).

Four human experimental and epidemiologic studies focused on several potentially important endpoints that may help elucidate the mechanisms of particle toxicity. The epidemiologic studies investigated the relation between PM levels and specific cardiac events: arrythmias (Douglas Dockery of Harvard School of Public Health) and nonfatal myocardial infarctions (Annette Peters of GSF-Forschungszentrum für Umwelt und Gesundheit). The experimental studies investigated cardiovascular and pulmonary effects in healthy and asthmatic subjects exposed to ultrafine carbon particles (Mark Frampton of University of Rochester) and concentrated ambient particles (CAPs) from Los Angeles air (Henry Gong of Los Amigos Research and Education Institute).

Six animal studies addressed a number of hypotheses about susceptibility to and toxicity of particulate matter components using different health endpoints, animal models, and types of particles. These studies investigated (1) whether coexposure to CAPs from Boston air and ozone causes a synergistic amplification of asthmatic airway inflammation and hyperresponsiveness in juvenile mice with hypersensitive airways (Lester Kobzik of Harvard School of Public Health); (2) the genetic determinants of susceptibility to morbidity and mortality from nickel particles in inbred mouse strains (George Leikauf of University of Cincinnati); (3) whether exposing healthy rats to CAPs from New York City air causes changes in blood coagulation parameters that may be involved in thrombotic effects (Christine Nadziejko of New York University Medical Center); (4) the effects of particle size and composition on the lung inflammatory and histopathologic responses in old rats and rats with preexisting inflammation (Fletcher Hahn of Lovelace Respiratory Research Institute); (5) whether exposure to resuspended particles from Ottawa air samples causes changes in heart function and vascular parameters in adult rats (the study presented here; Renaud Vincent of Health Canada); and (6) the effects of CAPs from Detroit air on the airway epithelium in rats with preexisting hypersecretory airway disease (Jack Harkema of Michigan State University).

CONTINUING RESEARCH

Many of the key questions identified in the early 1990s are still relevant and many research projects continue to address them. The research strategies have evolved, however, as results from completed studies have provided insights into which animal models and which endpoints may be the most helpful to evaluate. In addition, advances in exposure assessment and statistical methods have pointed to new approaches for conducting epidemiologic studies. In the past two years HEI has published several reports from its PM research program (Checkoway et al 2000; Gerde et al 2001; Godleski et al 2000; Goldberg et al 2000; Gordon et al 2000; Krewski et al 2000a,b; Lippmann et al 2000; Oberdörster et al 2000; Samet et al 2000a,b; Wichmann et al 2000). Many additional PM studies that are currently under review will be published in 2001 and 2002.

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INVESTIGATORS' REPORT

Inhalation Toxicology of Urban Ambient Particulate Matter: Acute Cardiovascular Effects in Rats

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ABSTRACT

Wistar rats were exposed for 4 hours by nose-only inhalation to clean air, resuspended Ottawa ambient particles (EHC-93*, 48 mg/m^3), the water-leached particles (EHC-93L, 49 mg/m^3), diesel soot (5 mg/m^3), or carbon black (5 mg/m^3). Continuous data for physiologic endpoints (heart rate, blood pressure, body temperature, animal's activity) were captured by telemetry before and after exposure. Blood was sampled from jugular cannulas 1 to 3 days before exposure and at 2 and 24 hours after exposure, and by heart puncture on termination at 32 hours (histology group) or 48 hours (telemetry group) after exposure. Lung injury was assessed by ^{3}H-thymidine autoradiography after the rats were killed. We measured endothelins (plasma ET-1, big ET-1, ET-2, ET-3) to assess the vasopressor components; nitric oxide (NO)–related metabolites (blood nitrate, nitrite, nitrosyl compounds, and plasma 3-nitrotyrosine) to assess the vasodilator components; and catecholamines (epinephrine, norepinephrine, L-DOPA, dopamine) and oxidative stressors (*m*- and *o*-tyrosine) for additional insight into possible stress components.

Lung cell labeling was uniformly low in all treatment groups, which indicates an absence of acute lung injury. Inhalation of EHC-93 caused statistically significant elevations ($P < 0.05$) of blood pressure on day 2 after exposure, plasma ET-1 at 32 hours after exposure, and ET-3 at 2, 32, and 48 hours after exposure. In contrast, the modified EHC-93L particles, from which soluble components had been extracted, did not affect blood pressure. The EHC-93L particles caused early elevation ($P < 0.05$) of the plasma levels of ET-1, ET-2, and ET-3 at 2 hours after exposure, but the endothelins returned to basal levels 32 hours after exposure. Exposure to diesel soot, but not carbon black, caused an elevation ($P < 0.05$) of plasma ET-3 at 36 hours after exposure; blood pressure was not affected by diesel soot.

Our results indicate that inhalation of the urban particles EHC-93 can affect blood levels of ET-1 and ET-3 and cause a vasopressor response in Wistar rats without causing acute lung injury. Furthermore, the potency of the particles to influence hemodynamic changes appears to be modified by removing polar organic compounds and soluble elements. Because the pathophysiologic significance of elevated endothelins has been clinically established in humans, our observations suggest a novel mechanism by which inhaled particles may cause cardiovascular effects. These findings in rats contribute to the weight of evidence in favor of a biologically plausible epidemiologic association between ambient particulate matter and cardiovascular morbidity and mortality in human populations.

* A list of abbreviations and other terms appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report 104, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr Renaud Vincent, Associate Professor, Safe Environments Programme, Tunney's Pasture 0803C, Health Canada, Ottawa K1A 0L2, Ontario, Canada.

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INTRODUCTION

Human epidemiologic investigations over the past 20 years have provided strong evidence of acute health effects from low concentrations of ambient air particulate matter (eg, Bates and Sizto 1983; Burnett et al 1994, 1995, 1999, 2000; Schwartz 1994; Fairley 1999; Neas et al 1999). For example, an increase of 13 μg/m^3 in ambient sulfate, equivalent to about 30 μg/m^3 $PM_{2.5}$ (particulate matter smaller than 2.5 μm in aerodynamic diameter) or 50 μg/m^3 PM_{10}, on the day before a hospital admission was associated

with a 3.7% increase in respiratory admissions and a 2.8% increase in cardiac admissions in Ontario (Burnett et al 1995). The epidemiologic data imply an unusually high potency of ambient particulate matter. In fact, these levels of environmental exposure to ambient particles are much lower than occupational exposure limits established for a number of respirable materials (American Conference of Governmental Industrial Hygienists [ACGIH] 1995) such as amorphous silica (3 mg/m^3), quartz (100 μg/m^3), and copper fumes (200 μg/m^3) (8-hour time-weighted averages). For the most part, the exact biologic and toxicologic bases for the health impacts of ambient particles remain unclear.

It is known, however, that the physicochemical characteristics of particles are relevant to their potency. Induction of stress responses in cell cultures by suspensions of ambient particles was related to the chemical composition of the particles, notably their content of bioavailable transition metals and organic species (Goegan et al 1998; Vincent et al 1997b). In addition, hospital admissions were reduced in the Utah Valley during the closure of a local steel mill in 1986 and 1987, which correlated with a lower fraction of bioavailable transition metals in PM_{10} and a lower potency of the particles in cell culture assays (Frampton et al 1999). A number of cell culture studies have shown that oxidative stress, mediated by both the soluble and insoluble fractions of particles, is a key mechanism of cellular effects (Carter et al 1997; Dye et al 1999; Ghio et al 1999a,b; Kennedy et al 1998; Omara et al 2000; Prahalad et al 1999). These studies have demonstrated that, after cells are activated by particles, the increased release of free radicals and cytokines can affect tissue integrity both by direct toxicity and by amplifying inflammatory changes. Oxidative stress and cytokines generated by similar mechanisms may also suppress other key cellular physiologic pathways such as cellular functions of innate immunity (Nadeau et al 1996) and acquired immunity (Omara et al 2000).

Our experimental work in vivo indicates that toxic potency of respirable ambient particles should not be defined solely on the criteria of high biologic reactivity or cytotoxicity. The direct induction of structural lung injury may not be essential for ambient particles to cause adverse biologic responses. We have collected Ottawa urban dust (EHC-93 particles) from ambient air in bulk form and prepared particulate material for applications in cell culture studies and animal exposure experiments. We have demonstrated that the EHC-93 particles were as potent as $PM_{2.5}$ and PM_{10} in inducing stress genes (such as heat shock protein 70 and metallothionein) in cell cultures (Vincent et al 1997a). The material was also directly toxic to the lungs by intratracheal instillation in saline (Adamson et al 1999a), having a potency comparable to that of residual oil fly ash (Costa and Dreher 1997). The toxic potency of aqueous suspensions of EHC-93 to the lungs could be attributed to the soluble fraction (Adamson et al 1999a) and notably to the soluble zinc content (Adamson et al 2000). Intratracheal delivery of the EHC-93 particles into the lungs of rats as a dispersed dry bolus did not cause remarkable changes in markers of acute lung injury, such as protein and neutrophils, in bronchoalveolar lavage fluid (our unpublished observations). It is likely that intratracheal injection of an aqueous suspension of ambient particles displaces the protective extracellular lining of the lungs and overestimates the direct toxicity of the material if it were inhaled and deposited in healthy lungs. Nevertheless, such experiments do reveal an inherent toxicity of ambient particles and may actually mimic the impact of the particles in compromised lungs in which the extracellular lining may already be altered, thus allowing particles to have more direct access to target epithelial and inflammatory cells.

In support of this interpretation, inhalation exposure of rats to high concentrations of the EHC-93 particles alone caused only mild focal, interstitial inflammatory changes in the lungs with no evidence of epithelial injury (Adamson et al 1999b; Bouthillier et al 1998; Vincent et al 1997b). Interestingly, elevation of cytokine production (eg, macrophage inflammatory protein-2) could be documented in macrophages isolated by lung lavage, which confirmed the dose delivered and the production of some direct effects in the lungs by the inhaled particles (Bouthillier et al 1998). However, in contrast to exposure to particles alone, inhalation exposure of rats to EHC-93 particles in combination with ozone resulted in severe structural changes in the lungs. These changes were characterized by an acute epithelial injury and a strong interstitial inflammatory response in terminal bronchioles and alveolar ducts, the magnitude of the changes being severalfold higher than those measured for inhalation of ozone alone (Adamson et al 1999b; Bouthillier et al 1998; Vincent et al 1997b). The limited lung lesions induced by ozone in our animal model can be viewed as a surrogate for the compromised epithelial and other cellular changes associated with a number of pulmonary conditions such as asthma, bronchitis, and viral infection.

These toxicologic observations indicate that ambient particles are not innocuous and do contribute to the health impact of air pollution. Although intact lungs may be relatively well protected from inhaled ambient particulate matter, compromised lungs respond quite differently to a deposited dose of ambient particles. The characteristics of particles that determine their toxic potency most likely

interact with host factors to produce a cascade of events leading to adverse effects. Thus it is conceivable that low internal doses of particulate matter that may be sufficient to regulate cellular responses but insufficient to cause acute adverse changes in the lungs of healthy individuals (ie, a dose too low to initiate immediate adverse effects in normal lungs, or a "subthreshold dose") could nevertheless adversely impact the already compromised lungs of sensitive individuals (ie, a dose sufficient to exacerbate established pathophysiologic processes, or a "nonthreshold dose"). This interpretation of the toxicologic evidence would explain the apparent nonthreshold association between ambient particles and the health effects indicated by the epidemiologic data and would substantiate the biologic plausibility of the epidemiologic evidence.

Although one can conceive that even low concentrations of ambient particles could exacerbate a pulmonary condition, plausible mechanisms for cardiovascular effects from low particle exposures remain more elusive. A somewhat perplexing observation from our previous studies was that, although inhaled EHC-93 particles were not directly injurious to the lungs of rats, inhalation of the particles was associated with a significant elevation of immunoreactive endothelin-1 (ET-1) in the systemic circulation (Bouthillier et al 1998). The increase of plasma ET-1 was observed in animals exposed to particles alone and in combination with ozone, but the magnitude of the response was not affected by the presence of ozone. This indicated that the endothelin response was not associated with centriacinar injury or a strong inflammatory component, but probably with the more diffusely deposited dose of particles in the alveoli. We have speculated that soluble components from the particles, diffusing from the site of deposition in alveoli to the endothelial cells of the capillary bed, could have affected endothelin regulation either by (1) reducing ET_B receptor–mediated clearance of circulating endothelins by the lung capillary endothelial cells, (2) modulating neutral endopeptidase activity, or (3) increasing the spillover of endothelins from the lungs into the systemic circulation. More work is required to establish the mechanisms of action of the particles on systemic levels of endothelins, the exact pathophysiologic significance of the changes, and which potency determinants in the particles modulate this response.

The observation that urban particles deposited in the lungs can alter the homeostasis of the most potent vasoconstrictors (endothelins) in the systemic circulation, with no evidence of acute lung injury in healthy rats offers a critical lead for investigating the cardiovascular toxicity of ambient particulate matter (Bouthillier et al 1998). The role of endothelins in normal cardiovascular homeostasis and their significance as effectors of cardiovascular pathophysiologic processes are well documented (Haynes and Webb 1998; Kramer et al 1997; Parris and Webb 1997; Schiffrin 1999; Warner 1999). Despite overlapping ranges of plasma endothelins between healthy and compromised humans (Battistini et al 1993), elevation of plasma ET-1 by about 25–50% is known to correlate with severity of disease in patients with congestive heart failure and to predict cardiac death (Galatius-Jensen et al 1996). Increased plasma endothelin levels were also found to predict mortality one year after acute myocardial infarction, which suggests that an acute increase of ET-1 may be cardiotoxic by promoting infarct size (Omland et al 1994). Improvement of ET-1 levels (ie, a decrease on the order of 20%) in patients with congestive heart failure was associated with improvement of symptoms, leading investigators to postulate a direct role for ET-1 in pathophysiologic processes (Tsutamoto et al 1995). Evidence suggests that a surge or a sustained elevation of endothelins in cardiac patients can be cardiotoxic and arrhythmogenic (Alexiou et al 1998; Maxwell et al 1992; Omland et al 1994; Sharif et al 1998).

Much of the research on endothelins has focused on the ET-1 form produced mainly by endothelial cells, but also by many other cell types (Battistini et al 1993). Other forms of endothelins, including ET-2 and ET-3, produced by a number of cell types in tissues may also be relevant to cardiovascular homeostasis. For example, elevation of circulating ET-3 is apparently associated with systemic vasoconstriction in cardiac patients (Cowburn et al 1999). Although a strong correlation between individual plasma endothelin values and systemic blood pressure may not always be evident, elevation of endothelins has been shown in experimental models and in patients with hypertension to be associated with a vasopressor response (Martinez et al 1996; Schiffrin 1999).

We have proposed that the mechanisms by which ambient particles affect cardiovascular function involve changes in vasoregulation. Changes in the regulation of endothelins in the lungs by inhaled ambient particles could very well occur both in healthy individuals and in individuals whose cardiovascular functioning is compromised. Whereas healthy subjects may not show symptoms of such changes in vasoregulation because of rapid homeostatic adjustments, in subjects with compromised cardiovascular systems vasoregulation changes may precipitate symptoms or a cascade of adverse responses including life-threatening effects such as cardiac ischemia or arrhythmia.

The purpose of the present study was to test the preliminary components of this hypothesis by confirming and extending our original observation that the levels of plasma ET-1 in healthy rats change after exposure to

particles and by identifying physiologic correlates of this effect. The experiments presented here were conducted in healthy Wistar rats exposed via inhalation to one of four particle preparations or clean air; before and after a single 4-hour exposure, we monitored cardiovascular physiologic status (heart rate, blood pressure, body temperature, and animals' activity) and measured vasoregulators (endothelins and NO-related products) and possible stress components (oxidative stress markers and catecholamines).

Several investigative approaches are available to pursue the toxicology of inhaled particles: (1) resuspended environmental particles (Adamson et al 1999b; Bouthillier et al 1998; Kodavanti et al 1999; Vincent et al 1997a), (2) model particles created by reconstituting binary or ternary mixtures (Jakab et al 1996; Kleinman et al 1999), and (3) concentrated present-day ambient particles (Gordon et al 1998, 1999; Sioutas et al 1995). Because we wanted to reproduce our key observation of a response of plasma endothelins, we exposed rats to EHC-93 (Bouthillier et al 1998; Vincent et al 1997a). To assess what the rapidly bioavailable components of the EHC-93 particles contribute to the particles' cardiovascular potency, we washed away soluble components and lyophilised the solid fraction; we referred to these as EHC-93L (leached) particles. Finally, because ambient air particulate mass includes carbonaceous particles linked to fossil fuel combustion, we exposed animals also to diesel soot particles and to carbon black particles.

To monitor cardiovascular and physiologic status before and after exposure to the particles, the animals were implanted with radiotransmitters and data on heart rate, blood pressure, body temperature, and animals' activity were captured by telemetry, which records longitudinal data without disturbing physiologic processes or adding stress to the animals (Bazil et al 1993; Brockway et al 1991; Desjardins et al 1996; Deveney et al 1998; Gelzer and Ball 1997; Watkinson et al 1998).

Our previous observations on the response of plasma ET-1 to inhaled particles were obtained using an ELISA assay that presented significant cross reactivity from other endothelin isoforms. We have since developed a high-pressure liquid chromatography (HPLC) procedure with fluorescence detection that allows direct determination of ET-1, ET-2, and ET-3 in a single sample (Kumarathasan et al 2000). Endothelins are known both to be regulated by NO and to induce NO release via activation of endothelial ET_B receptors (Junbao et al 1999; Marsen et al 1999; Schena et al 1999). Nitric oxide is a potent vasodilator that has a critical role in controlling systemic vascular tone. There is a homeostatic relationship between these two vasoregulatory systems, and changes in circulating endothelins will automatically result in a compensatory response from the NO system (Marshall and Johns 1999; Martinez et al 1996; Miki et al 2000; Warner 1999; Zhang et al 1999). To determine the impact of particles on this balance between endothelins and NO in the animals, we estimated NO from total blood nitrite, nitrate, and nitrosyl compounds (Sonoda et al 1997), and from plasma 3-nitrotyrosine (Kooy et al 1997; Tabrizi-Fard et al 1999).

The integrity of the vascular endothelium is essential for cardiovascular homeostasis; a number of studies over the past decade have documented the impact of endothelial dysfunction in hypertension, atherosclerotic heart diseases, and congestive heart failure (Boulanger 1999; Drexler and Hornig 1999; Garcia-Palmieri 1997; Rosen et al 1998). Oxidative stress can result in endothelial cell dysfunction with potential impact on regulation of NO and endothelin systems (Harrison 1997; Love and Keenan 1998; Michael et al 1997; Romero and Reckelhoff 1999; Rosen and Tschope 1998; Saito et al 1998; Yura et al 1999). To monitor the possible oxidative stress induced in the animals by inhalation of particles, we recorded the levels of a series of protein oxidation markers in plasma, namely 3-nitrotyrosine, *o*-tyrosine, *m*-tyrosine, and L- DOPA (Fu et al 1998; Halliwell and Kaur 1997; Kelly and Lubec 1995; Kristal et al 1998; Leeuwenburgh et al 1999; van der Vliet et al 1994). Furthermore, the catecholamines norepinephrine, epinephrine, and dopamine are known to be elevated in a number of cardiovascular conditions and in response to stress or injury (Maddens and Sowers 1987). Norepinephrine is produced by the sympathetic nervous system, and epinephrine and dopamine are released in the blood stream by the adrenal gland. Norepinephrine has a vasopressor effect and could increase systemic blood pressure, especially if its release is a response to stress or is a reflex in the upper respiratory tract (Grassi 1998). Spillover of cardiac norepinephrine from heart tissue into the blood stream has also been observed in ischemic heart disease and hypertension (Esler et al 1997; Remme 1998; Rahn et al 1999). Therefore, we have measured these catecholamines in the plasma samples used for the oxidative stress endpoints.

SPECIFIC AIMS

- **Specific Aim 1.** To investigate the biologic plausibility that exposure to ambient particulate matter causes cardiovascular effects in Wistar rats by analyzing changes in circulating levels of endothelins after acute inhalation exposure to EHC-93 Ottawa urban dust and by identifying physiologic correlates of these changes.

- **Specific Aim 2.** To investigate the relationship between potency of ambient urban particles and their chemical composition by analyzing responses in rats after inhalation exposure to EHC-93L particles from which a significant fraction of soluble components had been extracted.
- **Specific Aim 3.** To investigate whether inhaled diesel soot and carbon black particles can modulate blood pressure and plasma levels of endothelins in rats.

METHODS AND STUDY DESIGN

We investigated in rats the effects of four different particle preparations: Ottawa urban particles EHC-93, water-leached EHC-93L particles, diesel soot, and carbon black particles. Throughout the study, we purchased six batches of animals and conducted 18 3-week experimental sessions. Each particle preparation was studied separately in different batches of purchased animals. Some animals from each batch were assigned to a control group and exposed to clean air in parallel during each 3-week experimental session to obtain longitudinal data on endpoints across the entire study period (Table 1).

ANIMALS

Our initial study design allocated 50 animals from which we would gather ongoing cardiovascular data via telemetry and biochemistry data via scheduled blood samples taken from jugular cannulas, and a separate set of 46 animals from which we would gather histologic and autoradiographic data at the end of exposure (a total of 96 animals). However, some animals were lost because of surgical complications or problems with cannula patency and blood clotting. To resolve these problems, we added cannulas to rats assigned to histology and autoradiography. Table 2 summarizes the numbers of animals assigned to exposure groups and data acquisition compared with the numbers of animals that actually contributed data for different cardiovascular, histologic and radiographic, and biochemistry endpoints.

All experimental protocols were reviewed by the Animal Care Committee of the Health Protection Branch, Health Canada, as set forth in the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. In our previous work and in pilot studies for the present research, F344 rats were used to examine shifts of plasma endothelin after inhalation of EHC-93 (Bouthillier et al 1998). However, F344 rats have a lower body weight (adults 250–300 g) than other strains and implantation of the 13-g radiotransmitter combined with jugular cannulation resulted in levels of stress and discomfort that we considered unacceptable for the purpose of our study. Therefore, Wistar rats were used because of their larger size (adults ~400 g). Animals were received from the commercial supplier (Charles River, St Constant Québec) in filter boxes, housed in individual plexiglass cages on wood-chip bedding, and supplied with charcoal- and HEPA-filtered air and a 12-hour dark (18:00–06:00) and 12-hour light (06:00–18:00) cycle. Food and water were provided ad libitum. Sentinel animals were monitored throughout the study and were found to be free of infections.

When the animals were received, they were weighed, randomized, and allowed to acclimate for 5 days. Each

Table 1. Distribution of Animals Among Exposure Groups

Exposure Atmosphere	Concentration (mg/m^3)	Animals from *x* of 6 Batches Purchased[a]	Number of 3-Week Experimental Sessions Conducted[a]	Animals			
				Total Number	Body Weight[b] (g)		
					Before Surgery	Day of Exposure	At Termination
Clean air	0	6 of 6	18	29	391 ± 6	389 ± 6	388 ± 5
EHC-93	48	3 of 6	10	26	393 ± 9	399 ± 9	403 ± 10
EHC-93L	49	1 of 6	2	12	401 ± 6	387 ± 9	384 ± 9
Diesel soot	4.2	1 of 6	3	16	401 ± 12	400 ± 9	396 ± 9
Carbon black	4.6	1 of 6	2	13	402 ± 8	399 ± 11	396 ± 9
Total animals				96			

[a] Some animals from every batch purchased were exposed to clean air concurrently with every particle exposure atmosphere during every 3-week experimental session.

[b] Values are means ± SE.

Table 2. Distribution of Animals at Start of Study and Animals Contributing Data at End

	Distribution of Animals at Start				Number of Animals for Data Collection at End				
Exposure Atmosphere	Cardio-vascular (Telemetry)	Histology and Auto-radiography	Total	Cannulas Added to Histology Animals[a]	Cardio-vascular (Telemetry)[b]	Auto-radiography[c]	Catechol-amines[d]	Endo-thelins[d]	NO Products and Oxidative Stress Compounds[d]
Clean air	15	14	29	12	12–15	6	4–12	4–11	4–12
EHC-93	16	10	26	6	9–16	6	2–9	4–9	2–9
EHC-93L	6	6	12	6	6	6	3–7	4–9	3–9
Diesel soot	6	10	16	4	6	6	2–6	2–6	1–6
Carbon black	7	6	13	6	5–7	6	5–9	4–10	5–11
Total animals	50	46	96	34	38–50	30	16–43	18–45	15–47

[a] For biochemistry data collection.

[b] Ranges are due to malfunctioning transmitters, signal interference, or premature interruption of data collection.

[c] Lungs were recovered from all animals for autoradiography but only six animals per group were actually examined.

[d] Not all cannulas were patent at the time of final data collection. Samples were obtained from all animals at time of termination (32 hours after exposure for histology group; 48 hours after exposure for telemetry group).

3-week experimental session generally followed this plan: The rats were trained in the nose-only tubes over 5 consecutive days. Radiotransmitters and jugular cannulas were implanted (10 days after the animals had arrived). In all but the earliest experimental sessions, animals to be used for histology (none of which had radiotransmitters) were implanted with jugular cannulas to increase sample sizes for the blood biochemistry endpoints. Recovery time from the surgery before inhalation exposure was 5 to 8 days. Telemetry data capture was initiated 3 to 4 days before exposure. Blood samples were collected through the cannula between 1 and 4 days before exposure to determine the group baseline values of blood endpoints. After exposure to clean air or to the particles by nose-only inhalation for 4 hours, the animals were returned to their individual cages. Blood samples were obtained from the jugular cannulas at 2 hours and 24 hours after exposure. The animals were killed (by sodium pentobarbital, 65 mg/kg IP) at 32 hours (histology group) or 48 hours (telemetry group) after exposure and terminal blood samples were obtained by heart puncture.

ELECTROCARDIOGRAM AND BLOOD PRESSURE RADIOTRANSMITTERS

Food was withheld from the animals for 12 hours before surgery. They were anesthetized with 2–3% isofluorane/100% oxygen. The abdomen was incised and a radiotransmitter (TL11M2-C50-PXT implant, 13 g, 20 mm × 30 mm; Data Sciences International, St Paul MN) was placed in the abdominal cavity with the leads and blood pressure probe facing posteriorly (Figure 1). The dorsal aorta was carefully isolated from the vena cava and the blood pressure probe was inserted into the aorta (the open tip of the probe facing anteriorly); it was held in place using a small piece of surgical patch and surgical glue. The electrocardiogram (ECG) leads were placed as described by Sgoifo and colleagues (1996). The positive lead was sutured to the underside of the xiphoid process. The negative lead was brought through the abdominal wall to the left of the midline and the abdominal wall was closed with suture, fixing the transmitter in place with the same sutures used to close the abdominal wall. An incision was made in the midline of the throat area exposing the sternocephalicus muscles. The negative lead was tunneled subcutaneously from the abdominal area to the thoracic opening, inserted in the

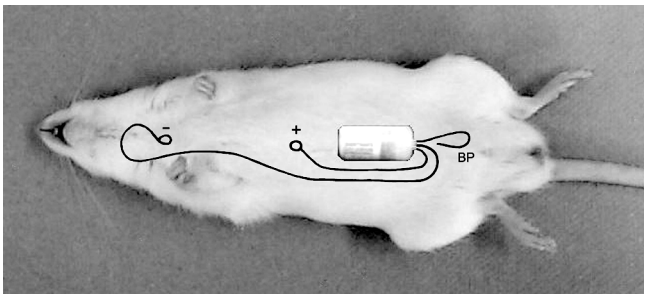

Figure 1. Location of the ECG leads and the blood pressure probe of the intraperitoneal radiotransmitter. The positive (+) lead was attached to the dorsal side of the xiphoid process and the negative (−) lead was placed in a small depression in the muscles at the entrance to the thoracic inlet. The blood pressure probe was inserted into the abdominal aorta, between the renal arteries and the bifurcation into the femoral arteries.

depression between the muscles, and sutured at the entrance of the thoracic inlet.

The jugular catheter consisted of 25 mm of silastic tubing (0.027 inches ID) connected to 155 mm of polyethylene tubing (0.023 inches ID). Working within the same incision site, the jugular vein was isolated and the silastic end of the cannula inserted in the jugular vein and tied in place. The polyethylene tubing was tunneled under the skin to the back of the animal. A loop in the free end of the cannula was made and tucked under the skin, and the cannula was fixed in place using surgical staples. The animal was given 5 mL lactated Ringers solution through the catheter to improve recovery. The catheter was then blocked with 40 μL of a 1:1 mix of glycerol and heparin (1000–2000 IU/mL heparin in saline). Tissue plasminogen activator (2000 U/mL; Sigma-Aldrich Canada, Oakville Ontario) or urokinase (2.7 U/mL; Sigma-Aldrich) was added to the blocking solution in 29 of the animals in an attempt to extend the patency of the cannulas. Each animal was given buprenorphine (30 μg/kg IM; Temgesic; Reckitt and Coleman, Hull England) immediately after surgery. Every second day, the blocking solution was drawn, the catheter was flushed with 0.5 mL saline, and fresh blocking solution was added.

DATA ACQUISITION

All telemetry components for data acquisition and analysis were from Data Sciences International (St Paul MN). Individual receivers (model RPC-1) for capturing the radiotransmitter signals were located directly under the animal cages in the holding room. The captured data were relayed through the data exchange matrices to a Pentium computer (Dataquest ART acquisition and analysis software, and CQ2010 card). Continuous data for ambient air pressure were also acquired (ambient pressure unit APR-1).

Each animal was monitored for a 10-second period every 5 minutes over a timespan of 6 to 7 days and the data were stored in the proprietary file format of the software. Data for ECG were collected at a rate of 1000 points/second and blood pressure data at 500 points/second. Using the Dataquest ART data analysis program, these data points were used to reconstruct the ECG and blood pressure waveforms and the discrete values for heart rate, mean blood pressure, systolic blood pressure, diastolic blood pressure, QA interval, body temperature, and animal's activity. Heart rate and blood pressure data were derived from the ECG (1000 points/sec) and pressure (500 points/sec) waveforms. The QA interval is defined as the time (msec) between the Q wave and the onset of the aortic blood pressure pulse, and is an index of cardiac contractility (Cambridge and Whiting 1986). Animals' activity measurements were derived by the radiotransmitters from variations in signal strength as the animals moved about in their cages.

SOURCE OF PARTICLES

We recovered the ambient urban particles EHC-93 in a dry form from the videlon filters of the bag-house of the single-pass air-purification system of the Environmental Health Centre in Ottawa (100% outdoor air). The material was mechanically sieved (300 μm, 100 μm, 80 μm, 56 μm, 36 μm) to remove debris and large biologic compounds such as spores. The sieved dust recovered after the 36-μm mesh filter, referred to as EHC-93 bulk particles, was stored at −80°C until use. The toxicity of EHC-93 was studied in both cell culture (Goegan et al 1998; Nadeau et al 1996; Omara et al 2000; Vincent et al 1997b) and in vivo (Adamson et al 1999a,b; Bouthillier et al 1998; Costa and Dreher 1997; Vincent et al 1997a) models to establish its relevance as an environmental material.

To prepare the water-leached particles, 20 g of bulk EHC-93 dust was suspended in 25 mL of filtered water (Milli-Q, Millipore, Bedford MA), sonicated for 20 minutes, and centrifuged at 500 × *g* for 20 minutes. The pellet was washed four more times with 20 mL of water and the water-soluble leachate was pooled. The water-leached solid particles and the water-soluble leachate were lyophilized separately. Mass recovery was 97%, 80% being recovered as solid leached particles and 17% as soluble material. These modified particles, from which a significant fraction of readily soluble elements had been removed, are referred to as water-leached particles, EHC-93L. Diesel soot (SRM 1650) was obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg MD). Carbon black particles were a gift of Dr Kent Pinkerton, University of California at Davis.

NOSE-ONLY INHALATION EXPOSURES

The animals were progressively trained in the nose-only restraint tubes over 5 consecutive days before surgery. The animals were introduced in the tubes and left inside their cages for 1 hour on the first day, 2 hours on the second and third days, and 4 hours on the fourth and fifth days. On the day before inhalation exposure, animals were reintroduced to the nose-only tubes for 4 hours. The animals were exposed in inhalation chambers to EHC-93 particles (48 mg/m^3), EHC-93L particles (49 mg/m^3), diesel soot (4.2 mg/m^3), or carbon black (4.6 mg/m^3). After aspiration through a 1-mm nozzle (powder disperser model 3433; TSI, St. Paul MN), the particles were dispersed in a venturi and directed to a flow-past nose-only exposure manifold

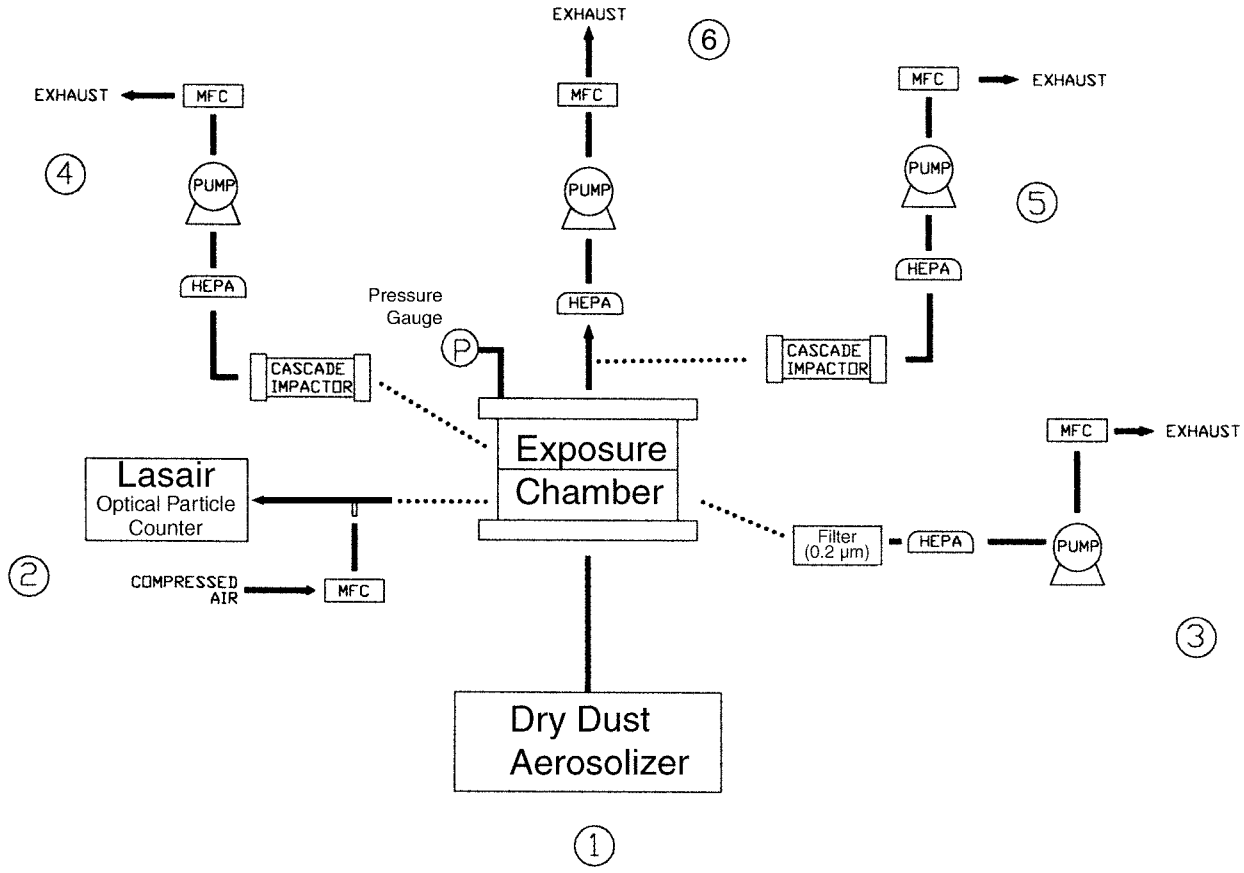

Figure 2. Nose-only exposure system. Particles were resuspended into an air stream using a dry dust aerosolizer (1) and delivered to the flow-past nose-only exposure manifold. Isokinetic sampling at inhalation ports was performed for real-time counting and sizing of particles using a laser optical particle counter (2), and for sampling on a 0.2 μm filter to determine time-weighted concentration (3). Cascade impactors were used to assess particle size distributions at the nose-only port level (4) and at the exhaust from the system (5 and 6). All flows were regulated using mass flow controllers (MFC).

(12 ports per stack × 2 stacks; CH Technologies, Westwood NJ) at a flow rate of 24 L/min (Figure 2). A known mass of particles was packed inside the groove of the metal disc, and then aerated with a fine wire. Groove size and disc rotation determined the rate of mass delivery to the venturi.

The aerosol concentration was evaluated at the inhalation ports by isokinetic sampling using 0.2-μm Teflon filters (TF-200, 47 mm; Gelman Sciences, Ann Arbor MI). Two to four filters were sampled during each 4-hour exposure. Filter weight change divided by the sampling volume provided a direct estimate of the time-weighted average particulate concentration. Real-time particle counts and size measurements at the inhalation ports (optical size range of 0.3 to 10 μm; Lasair Model 301; Particle Measuring Systems, Boulder CO) were obtained continuously throughout each 4-hour exposure and provided measurements of count median diameter to confirm concentration stability. The aerodynamic size characteristics of the particulate atmospheres were determined by gravimetric cascade impactor analyses on isokinetic samples at the inhalation ports (seven-stage Mercer cascade impactor, 1 L/min, 0.2 to 4.6 μm effective cutoff diameter [ECD]; Intox, Albuquerque NM) or the chamber exhaust (seven-stage Mercer cascade impactor, 10 L/min, 0.2 to 9.5 μm ECD). The particulate atmospheres were multimodal. Treatment of particle size distribution data is described in the dosimetry section below.

SAMPLING PARTICLES FOR CHEMICAL ANALYSIS

Size-fractionated samples were collected from the nose-only exposure system on filters during exposure runs without animals to compare chemical composition in the exposure chamber with that of the bulk material. Total atmosphere samples were collected at the inhalation ports using a filter cassette (TF-200, 47 mm; Gelman). Size-fractionated particulate mass was recovered from the inhalation ports on the terminal filter of the 1-L/min cascade impactor (0.2 μm Teflon membrane, TF-200, 25 mm;

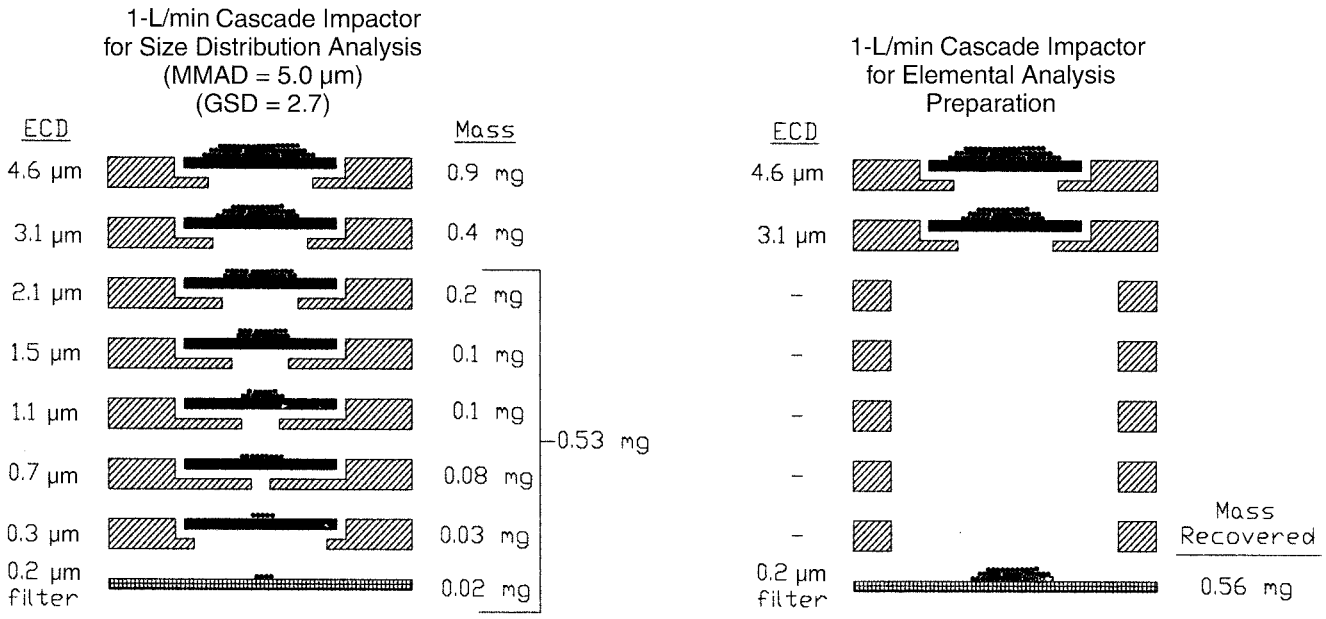

Figure 3. Cascade impactor configurations for analyzing particle size distribution (left) and preparing 3-µm ECD filter samples for elemental analysis (right). Average mass of particles recovered (at 1 L/min for 1 hour) on each stage of filter is indicated. Terminal filters are at the bottom.

Gelman) and at the chamber exhaust on the 10-L/min cascade impactor (TF-200, 47 mm; Gelman). The combination of stages loaded in the cascade impactors determined the range of particle sizes collected on the terminal filters (Figure 3). For example, the top two stages of the 1-L/min cascade impactor provided an ECD of 3.1 µm median aerodynamic diameter (MAD), whereas the top five stages provided an ECD of 1.1 µm MAD. Dummy stages, consisting of an annular ring sized to fit in the cascade impactor, were used to replace the missing stages. Impactor plate combinations and air flow (12–14 L/min) were adjusted to match the ECDs between the cascade impactors.

ELEMENTAL ANALYSIS OF THE EHC-93 PARTICLES

Sample Preparation

Bulk EHC-93 particles and material recovered on filters were digested with 3.7 mL of concentrated nitric acid and hydrofluoric acid (HNO_3:HF, 3.3:0.4) in closed Teflon vessels. The 25-mm filters were sonicated as whole filters, whereas the 47-mm filters were cut in half. For digestion, the mixture was progressively heated for 20 minutes in a microwave oven, and maintained at a pressure of 100 psi for 10 minutes. After cooling (pressure ~14 psi), the digest was diluted to 100 mL to produce a resulting solution of 3.5% HNO_3 and 0.22% HF. Urban dust standards SRM 1648 and SRM 1649 (NIST) and spiked filters were also prepared for elemental analysis. To estimate the solubility of elements, bulk EHC-93 material and Teflon filter samples of EHC-93 particles were sonicated in deionized water for 15 minutes, centrifuged for 15 minutes, and filtered through membrane filters (0.2 µm). This procedure was repeated two more times, and the three sequential leachates were combined, diluted, and acidified to 3.5% HNO_3. Variable volume-to-mass ratios were investigated.

Analytic Conditions

Most of the elemental concentrations were ascertained by multielement determinations using a Perkin-Elmer Elan 5000 inductively coupled plasma–mass spectrometer equipped with a pneumatic nebulizer and platinum cones. The nebulizer flow rate was 0.95 L/min, and the auxiliary and plasma argon flow rates were 0.8 and 16 L/min, respectively. The injection flow rate was 1.0 mL/min, the radio frequency power was 1000 W, and the dwell time was set at 100 msec or more. Terbium and rhodium were used as internal standards. The solutions SRM 1643d (NIST), TM27, and TMDA51 (NWRI) were used as reference materials. Plasma emission spectrometry was used for the determinations of sodium (589.59 nm), silicon (251.61 nm), calcium (317.93 nm), and iron (259.94 nm). A direct-current argon plasma atomic emission spectrometer (ARL Spectraspan 7 DCP-AES) was used in simultaneous multichannel mode and in single mode (for silicon). Solutions SRM 1643d (NIST), TMDA 53–54 (NWRI) and ION 95–96 (NWRI) were used as reference materials.

PLASMA ENDOTHELINS

Sample Preparation

Blood samples from jugular cannulas (0.6–1 mL) or heart puncture (5–8 mL) were recovered in vacutainer tubes containing the sodium salt of ethylenediaminetetraacetic acid (EDTA) (final, 10 mg/mL) and phenylmethylsulfonyl fluoride (final, 1.7 mg/mL) and were mixed gently. Aliquots (100 μL) were set aside for assay of nitrite (see below) and the remaining blood samples were centrifuged at 500 × *g* for 10 minutes. Aliquots of plasma (250 μL) were transferred to separate 1.5-mL centrifuge tubes for the different analyses. (Subsequent steps for tyrosines and catecholamines are described below.)

For analyses of endothelins, the samples were processed as follows. The conversion of big ET-1 (the inactive precursor that has 38 to 41 amino acids) to ET-1 (the mature peptide that has 21 amino acids) was prevented by adding 3,4-dichloroisocoumarin to the plasma samples at a final concentration of 48 mg/mL. Note that ET-2 and ET-3 originate similarly from big ET-2 and big ET-3 precursors, which are cleaved by endothelin-converting enzymes (ECEs). Protein was precipitated with 1.5 volume of ice-cold acid acetone (acetone:1N HCl:water, 40:1:5). Samples were vortexed and clarified by centrifugation at 9000 × *g* for 10 minutes and the supernatants were concentrated by evaporation under nitrogen flow. The concentrated samples were treated a second time with acid acetone and centrifuged. Molecular weight cutoff filters (30 kDa; Sigma Chemicals, St Louis MO) were washed with 50 mL of deionized water by centrifugation at 5000 × *g* for 10 minutes. The concentrated supernatants were then loaded on the molecular weight cutoff filters and centrifuged at 5000 × *g* for 30 minutes. The filters were washed with 75 mL of 50% methanol. Filtrates were dried under a flow of nitrogen. The samples were either stored dry at −40°C or immediately processed for HPLC analysis. The samples were reconstituted with 75 mL phosphate-buffered saline, vortexed, and transferred into 1.5-mL amber glass vials with inserts for HPLC analysis.

HPLC Analyses

Endothelin-related peptides were measured by HPLC as described by Kumarathasan and colleagues (2001). The HPLC unit consisted of a Gilson solvent delivery system (Mandel Scientific, Guelph Ontario), a Gilson autosampler (model 231 XL; Middleton WI), a Supelcosil LC-318 reverse-phase column (25 cm length, 4.6 mm ID, 5 μm particle size, 300 Å pore dimension; Supelco, Oakville Ontario), and a Shimadzu fluorescence detector (model RF 551; Columbia MD). Injection volume was 20 μL. Analytes were resolved using a gradient elution with two solvents: solvent A consisted of 30% acetonitrile and 0.1% trifluoroacetic acid (TFA) in water; solvent B consisted of 90% acetonitrile and 0.1% TFA in water. The mobile phase (flow at 1 mL/min) was programmed as follows: initial mobile phase 100% solvent A; 3-minute ramp to 92:8, A:B solvent ratio; held for 12 minutes; 10-minute ramp to 100% solvent B; held for 5 minutes; 5-minute ramp to 100% solvent A; held for 3 minutes. Total run time was 38 minutes. The fluorescence detector was optimized for high sensitivity detection with the excitation wavelength set at 280 nm and emission wavelength at 340 nm (Figure 4). A manual integration method was used with a drop-line method of integration applied to shoulders or peak overlaps. The limit of detection of the endothelins was 0.2–0.5 pmol on the column. Linear performance (r^2 = 0.99) of the detector established for all endothelins analyzed (big ET-1, ET-1, ET-2, and ET-3) was 1–100 pmol. Analytic precision determined with standards was higher than 96%. Spiked plasma samples were analyzed to verify any changes in the elution characteristics attributable to matrix effects. Recovery of endothelins from spiked plasma samples was: big ET-1, 75%; ET-1, 97%; ET-2, 60%; and ET-3, 70%. Analytic precision determined with spiked plasma samples was: big ET-1, 95%; ET-1, 98%; ET-2, 94%; and ET-3, 80%.

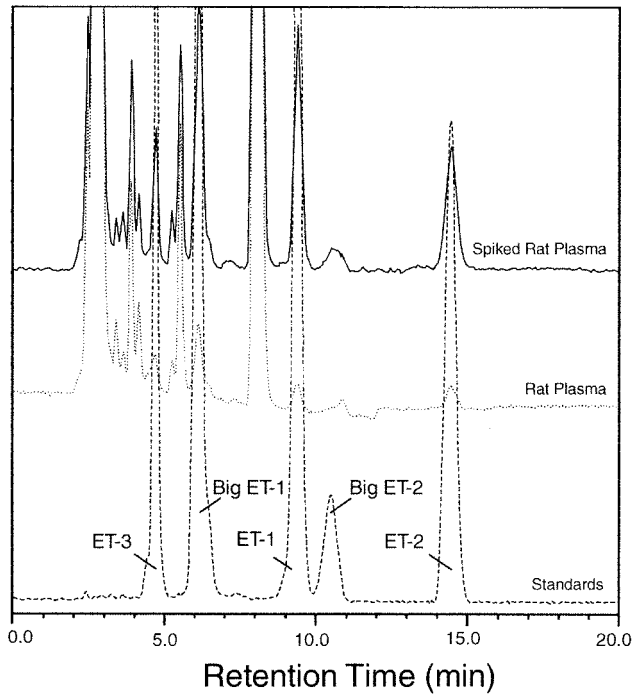

Figure 4. Analytic profiles of plasma endothelins separated by HPLC and identified by fluorescence detection.

PLASMA CATECHOLAMINES AND TYROSINES

Sample Preparation

Plasma samples (250 μL) in 1.5-mL centrifuge tubes were treated with diethylenetriaminepentaacetic acid (final, 15 mM) and butylated hydroxy toluene (final, 43 mM) and then vortexed. Protein was precipitated with 1.5 volume of ice-cold acid acetone (acetone:1N HCl:water, 40:1:5). The samples were vortexed and centrifuged at 9000 × *g* for 10 minutes and the supernatants were concentrated by evaporation under nitrogen flow. The concentrated supernatants were treated again with acid acetone, centrifuged, and concentrated under nitrogen. Molecular weight cutoff filters (30 kDa) were washed with 50 μL of deionized water by centrifugation at 5000 × *g* for 10 minutes. The concentrated samples were then loaded on the filters and centrifuged at 5000 × *g* for 30 minutes. The filters were washed with 75 μL of 50% methanol. The filtrates were then dried under a flow of nitrogen. The samples were either stored dry at −40°C or immediately processed for HPLC analysis. Samples were reconstituted with 200 μL of acidified water (1N HCl:deionized water, 1:4), vortexed gently, and diluted as required for HPLC analysis.

HPLC Analyses

The HPLC unit consisted of a BAS 400 solvent delivery system (Bioanalytical Systems, West Lafayette ID), a Gilson autosampler (model 231 XL; Middleton WI), a Supelcosil LC18 reverse-phase column (25 cm length, 4.6 mm ID, 5 μm particle size; Supelco, Oakville Ontario) and an electrochemical detector equipped with a glassy carbon working electrode and an Ag/AgCl reference electrode (Bioanalytical Systems amperometric detector, model LC-4B). The mobile phase (pH = 4.75) was composed of 97.2% sodium citrate (final concentration, 26.3 mM) in acetate buffer (final concentration, 10.9 mM) and 2.8% methanol. Isocratic elution of analytes was carried out at a flow rate of 1 mL/min and the analyses were performed at an oxidation potential of +1.2 V. The limits of detection for the catecholamines and tyrosines were better than 1 pmol on the column. The injection volume was 100 μL. Linear performance ($r^2 = 0.99$) of the detector established for catecholamines and tyrosines was in the range of 5–200 pmol. Recovery of catecholamines and tyrosines from spiked plasma samples ranged from 70–100%.

NITRIC OXIDE–RELATED COMPOUNDS IN WHOLE BLOOD

Nitric oxide generated in vivo is converted to nitrite, nitrate, nitrosyl hemoproteins, nitrosyl metal complexes, and *S*-nitroso compounds. Thermolysis of NO-related compounds in whole blood followed by reduction to nitrite provides an estimate of the steady-state levels of NO in the vascular system. Levels of NO were determined in whole blood by the method of Sonoda and colleagues (1997) with modifications. Blood samples (100 μL) were mixed with 50 μL of 1 mM diethylenetriaminepentaacetic acid (final, 125 μM), 50 μL of 1N HCl (final, 125 mM), and 200 μL of 10 mM Tris-HCl, pH 7.2 (final, 1.25 mM) in screw-capped amber-glass vials. The samples were sonicated for 5 minutes, heated to 86°C for 30 minutes, cooled on ice, and neutralized to pH 7.2 with 50 μL of 1N NaOH. The samples were sonicated for 5 minutes, transferred to eppendorf tubes, centrifuged at 16,000 × *g* for 15 minutes, and the supernatants were filtered through molecular weight cutoff filters (10 kDa) at 4000 × *g* for 1 hour.

The samples (50 μL) were loaded in triplicate into 96-well plates for assay of nitrite as follows. Nitrate was reduced to nitrite by adding 50 μL Tris-HCl (stock, 10 mM), 20 μL NADPH (stock, 0.5 mM), 20 μL nitrate reductase (stock, 0.83 U/mL), and incubating the mixture at room temperature for 1 hour. Nitrite was reacted with 12 μL 2,3-diaminonaphthalene (stock, 0.05 mg/mL in 0.62 M HCl) and incubated at room temperature for 10 minutes. Samples were basified by adding 6 μL NaOH (stock, 2.8 N) and the plates were read on a Cytofluor 2350 multiplate fluorescence detector (Millipore, Bedford MA) at an excitation wavelength of 360 nm (slit width 40 nm) and an emission wavelength of 460 nm (slit width 40 nm). A linear calibration curve was established for nitrite concentrations in the range of 0 to 1000 pmol.

LUNG HISTOLOGY AND AUTORADIOGRAPHY

After exposure to clean air or to the particles for 4 hours, the rats were returned to clean air and maintained under normal housing conditions for 32 hours. Animals selected for autoradiography received an intraperitoneal injection of ^{3}H-thymidine (0.1 mCi/100 g body weight). After 90 minutes, the animals were anesthetized with sodium pentobarbital (65 mg/kg, IP), and a terminal blood sample was obtained by heart puncture for plasma preparation. The animals were then killed by sectioning the abdominal aorta. The trachea was cannulated, and the lungs were collapsed and inflated with 2% glutaraldehyde in 0.085 M sodium cacodylate and 0.05% (w/v) calcium chloride, pH 7.4, at a pressure of 25 cm H_2O above the chest for 10–15 minutes. The lungs and trachea were excised, immersed in fixative, and stored at 4°C. Three lung tissue blocks (5 × 5 × 7 mm) sampled below the hilum of the left lobe were post-fixed in formalin for 24 hours, dehydrated in ethanol, and embedded in glycol methacrylate.

Autoradiographic analyses on whole sections were performed by standardized methods. In brief, 0.75-μm sections on glass slides were coated with Kodak NTB2 photographic emulsion, exposed for 2 weeks at 4°C, developed, and stained with basic fuchsin. Sections were scanned at 100× magnification for labeled cells (at least three silver grains over the nucleus). For three lung tissue blocks per animal, 1500 cells per section were examined for each of the parenchymal and bronchiolar compartments, and the number of labeled cells was recorded. Labeled and total nuclei for each compartment were added to generate a single estimate of percentage of labeling per animal. This procedure is particularly sensitive to an increase in the frequency of labeled epithelial cells in the lungs, either the nonciliated bronchiolar epithelial cells or the alveolar type 2 epithelial cells, which are the progenitor cells of the bronchiolar and alveolar epithelium, respectively. In normal lungs, these two cell populations turn over at a very low rate. An acute primary epithelial injury induces the cells to replicate, with a peak mitotic activity measured at 36 hours in adult and in senescent rats (Vincent and Adamson 1995).

PARTICLE DOSIMETRIC MODELING

Cascade impactor data were analyzed for distribution of multiple size modes according to Hewett and McCawley (1991). This procedure assumes that a multimodal particle size distribution can be described by a linear combination of weighted, lognormal distributions. In brief, histograms of the frequency distribution of mass as a function of aerodynamic diameter (mass per cascade impactor stage) were prepared. Visual inspection of the plots allowed us to identify the number of underlying size distributions, the geometric aerodynamic diameter and standard deviation (GSD) of each distribution, and the relative contribution of each distribution to the overall distribution (ie, percent of the total mass).

These approximated values were used as initial input parameters in the model. A fit was then calculated from these input parameters according to the following equation, which describes the frequency for each distribution at any particle aerodynamic diameter (AD):

$$f_N(\log AD) = \frac{1}{\log_{10} GSD\sqrt{2\pi}} \exp\left[\frac{-(\ln AD - \ln GM_N)^2}{2(\ln GSD_N)^2}\right]$$

where GM_N is the geometric mean and GSD_N is the geometric standard deviation of the Nth underlying distribution.

The smooth, weighted multimodal fit was then superimposed graphically over the histogram of mass frequency distribution. The sum of the squared difference per interval between the area under the curve and the corresponding area under the histogram provided an estimate of the strength of the fit, e^2. Model parameters were then modified by iteration to optimize visually the quality of the fit, aiming at $e^2 \leq 10$ if possible. The polydisperse distributions of all four particulate atmospheres could be described in this way as being composed of three distinct modes.

The mass median aerodynamic diameter (MMAD), GSD, and mass fraction of each mode of the different experimental particulate atmospheres used in our study were determined by the procedure outlined above. The deposition efficiencies (fraction of the inhaled mass that is deposited) for each mode of the particle size distributions were determined for the tracheobronchial compartment and the pulmonary compartment of the Wistar rats using the MPPDep software (RIVM Publications, Bilthoven, The Netherlands). This software uses the biomathematic model of multiple path deposition developed by Anjilvel and Asgharian (1995). In brief, calculations of the depositions through diffusion and sedimentation mechanisms are done for an asymmetric branching airway structure that was defined from airway morphometric measurements for 2,404 conducting airway paths in Long Evans rats (Raabe 1975; Mènache et al 1991) and a symmetric eight-generation acinus model attached to the terminal bronchiole (Yeh et al 1979).

We have used as model input parameters for the Wistar rats a tidal volume of 2.1 mL, a breathing frequency of 102/min, and strict nasal breathing. Density of the EHC-93 and EHC-93L particles was taken as 3.3 g/cm^3, and the density of the diesel soot and carbon black particles was taken as 1.8 g/cm^3 (Lide 1999; Kroschwitz and Howe-Grant 1992). Inhalability corrections were introduced (Ménache et al 1995). Dose estimates were then calculated for the Wistar rats assuming an exposure time of 240 minutes to a steady-state concentration of the particles, a pulmonary compartment surface area of 0.34 m^2, a tracheobronchial surface area of 22.5 cm^2, and a nasopharyngeal surface area of 15 cm^2 (Vincent et al 1997a). The chemical compositions of the particles in the size modes were factored into this dosimetric modeling in order to define lung deposition of soluble and insoluble elements.

For estimating internal doses of fine particles in a human subject, we have assumed a pulmonary deposition rate of 25%, a tracheobronchial deposition rate of 5%, a minute ventilation of 13.8 L/min for an exposure duration of 24 hours, a pulmonary surface area of 54 m^2, and a tracheobronchial surface area of 3200 cm^2. In the first scenario, we have used as pollutant levels the 95th percentile

of the measured elemental concentrations associated with $PM_{2.5}$ in eight major Canadian cities between 1986 and 1996 (Burnett et al 2000). In the second scenario, we have used a particle mass concentration of 250 μg/m³, for which the following values for soluble elemental contents were adapted from data on the chemical composition of $PM_{2.5}$ samples (Vincent et al 1997b): aluminum 2,000 μg/g; barium 500 μg/g; boron 4,000 μg/g; cadmium 15 μg/g; calcium 10,000 μg/g; chromium 20 μg/g; cobalt 2 μg/g; copper 500 μg/g; iron 2,000 μg/g; lead 250 μg/g; magnesium 1,000 μg/g; manganese 200 μg/g; molybdenum 10 μg/g; nickel 20 μg/g; silicon, 800 μg/g; sodium 10,000 μg/g; strontium 50 μg/g; tin 10 μg/g; titanium 500 μg/g; vanadium 30 μg/g; zinc 1,000 μg/g.

STATISTICAL METHODS AND DATA ANALYSIS

Primary Endpoints of Effects

The primary cardiovascular physiology variables in our study were blood pressure and heart rate. The primary biochemical variables were the plasma levels of ET-1 and ET-3 and total blood levels of nitrite. All other variables were supporting endpoints considered in the overall interpretation of effects. All data were compiled on Quattro Pro (Corel, Ottawa Ont). All statistical analyses were performed using SAS v6.12 (SAS Institute, Cary NC) or SigmaStat v2.03 (SPSS, Chicago IL). We did not impute missing data values. Rather, the general linear model analyses used all available data to provide least-square regression estimates of the means.

Cardiovascular Telemetry Data

The many data generated by telemetry were summarized for timely manipulation and presentation. The ECG waveforms were not analyzed. To facilitate analyses, we defined day 0 as the 24 hours that ended with the 4-hour exposure, day 1 as the 24 hours immediately after exposure, and day 2 as the next 24 hours. (The inhalation exposures were conducted for 4 hours between 08:00 and 12:00, therefore 1 day is defined as noon to noon; data acquired 1 hour before and 1 hour after the exposure were excluded from the analyses to avoid introducing artifacts related to animal handling.) "Before exposure" refers to day 0 data collected before exposure began and "during exposure" refers to data collected at the end of day 0 during the 4-hour exposure period. For initial statistical analyses and to limit the number of comparisons, we summarized the data on days 0, 1, and 2 as 18-hour daily averages (13:05–07:00). The cardiovascular (heart rate, mean blood pressure, systolic pressure, diastolic pressure, QA interval), body temperature, and activity data were verified for normality.

Normal data were then analyzed separately within each exposure atmosphere group (air, EHC-93, EHC-93L, diesel soot, carbon black) by one-way repeated-measures analysis of variance (RM ANOVA), followed by the Tukey multiple comparison procedure to elucidate the patterns of significant effects ($\alpha = 0.05$). The nonnormal data were analyzed separately within each exposure atmosphere group (air, EHC-93, EHC-93L, diesel soot, carbon black) by rank using a Friedman RM ANOVA followed by the Tukey multiple comparison procedure ($\alpha = 0.05$). The specific physiologic endpoints that were ranked are identified in the tables.

After we had established positive treatment effects through this initial robust approach, we summarized the circadian patterns for the cardiovascular data (heart rate, mean blood pressure), body temperature, and activity data as 4-hour averages for 20 hours before inhalation exposure and during the 4-hour exposure (day 0), and on the 2 days after inhalation exposure (days 1 and 2). None of these 4-hour datasets could be normalized. Therefore, the data within each exposure atmosphere group (air, EHC-93, EHC-93L, diesel soot, carbon black) were ranked, and then analyzed separately using a two-way RM ANOVA with a two-factor repetition technique using day (day 0, day 1, day 2) and time interval within day (4 [12:00–16:00], 8 [16:00–20:00], 12 [20:00–24:00], 16 [00:00–04:00], 20 [04:00–08:00], 24 [08:00–12:00]) as factors. If we found a significant day × time interval interaction ($P < 0.05$), the pattern of significant effect of day within time interval was elucidated using the Tukey multiple comparison procedure ($\alpha = 0.05$).

Biochemistry Data

The blood concentration of nitrite, and the plasma concentrations of big ET-1, ET-1, ET-2, ET-3, 3-nitrotyrosine, *m*-tyrosine, *o*-tyrosine, epinephrine, norepinephrine, L-DOPA, and dopamine were calculated from each sample. Data were examined for normality and then analyzed by two-way ANOVA to verify a treatment (air, EHC-93, EHC-93L, diesel soot, carbon black) × time interval (before, and 2, 24, 32, and 48 hours after exposure) interaction ($\alpha = 0.05$). If we found significant two-way factor interactions, one-way ANOVA was then applied to determine the time interval effect within each treatment group, followed by the Dunnett test using the preexposure value as the control value, or the Tukey procedure to compare all time intervals ($\alpha = 0.05$).

In our study, cannula patency was not optimal, which led to incomplete time-series data; RM ANOVA techniques

could not be applied to the biochemical data. To increase power, animals for both the cardiovascular and the lung histology assessments were cannulated. Thus, the animals used for the biochemical analyses may or may not have had a radiotransmitter, and we have verified whether the different intensities of these two surgeries (cannula alone or cannula plus radiotransmitter) could have impacted differently on the patterns of effects. Similarly, because of poor cannula patency, we added either tissue plasminogen activator or urokinase to the blocking solution in a subset of the animals to improve the useful life of the cannulas. To detect any bias introduced by these experimental variables, for all endpoints with a significant treatment × time interval interaction, the data were verified for normality and analyzed by three-way ANOVA with treatment (air, EHC-93, EHC-93L, diesel soot, carbon black), surgery (cannula alone, cannula plus radiotransmitter), and thrombolytic agent (none, tissue plasminogen activator or urokinase) as the factors, followed by the Tukey multiple comparison procedure ($\alpha = 0.05$).

RESULTS

MONITORING ANIMAL DATA

Receivers for capturing the radiotransmitted physiologic data were located on the nose-only inhalation exposure systems for real-time monitoring during exposure and in the animal holding room for continuous monitoring before and after exposures. We initially verified that the stress of immobilizing the animals during training in the nose-only tubes and during the four-hour inhalation exposure would not alter the cardiovascular status of the animals and confound our measurements of physiologic endpoints. Electrocardiogram and blood pressure waveforms captured in different conditions of animal housing are illustrated in Figures 5 and 6. We observed no differences in the quality of the signals between freely moving animals (Figures 5A and 6A) and animals placed inside nose-only restraint tubes but left in their cages (Figures 5B and 6B).

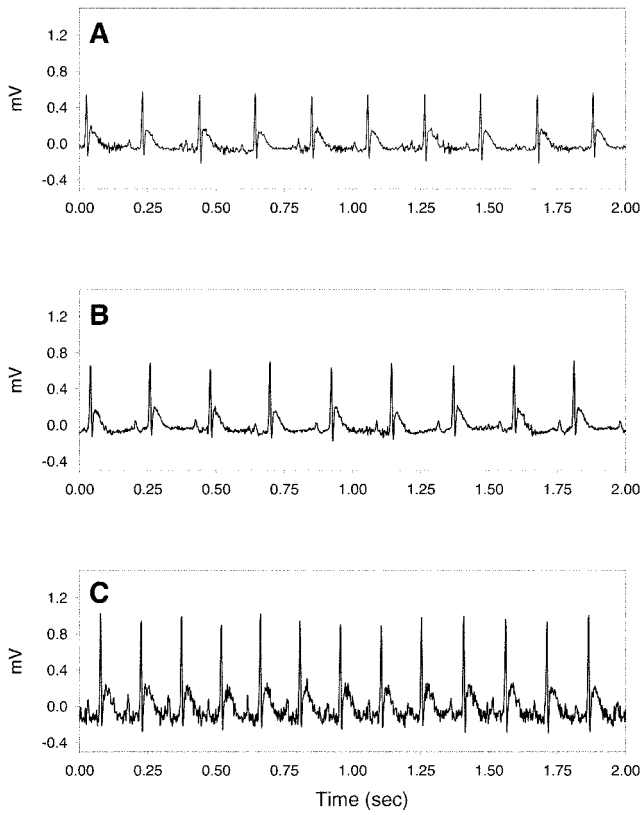

Figure 5. Representative sample of two-second electrocardiogram waveforms. Rats (A) freely moving; (B) in the nose-only exposure tube during training; (C) and in the nose-only exposure system during exposure.

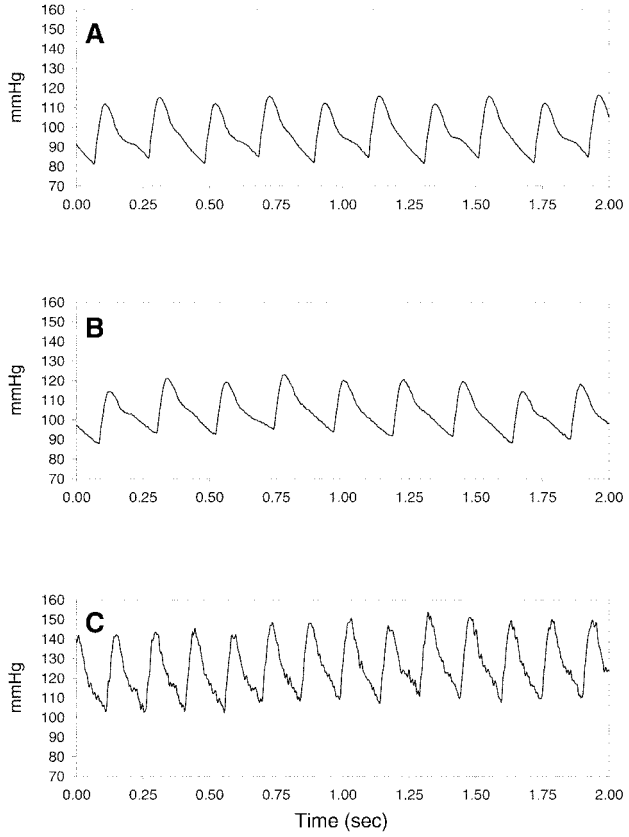

Figure 6. Representative sample of two-second blood pressure waveforms. Rats (A) freely moving; (B) in the nose-only exposure tube during training; (C) in the nose-only exposure system during exposure.

Disturbing the animals, for example by introducing them into the nose-only tubes or immediately after removing them from the tubes, caused transient increases in heart rate and blood pressure, which returned rapidly to normal when the animals were held in the nose-only tubes or returned to their cages where they could move about freely. Furthermore, continuous measurements of heart rate and blood pressure during the 5-day training period in the nose-only tubes did not reveal any progressive changes in heart rate and blood pressure profiles with time (data not shown). Heart rate (Figure 5C) and blood pressure (Figure 6C) did increase while animals were in the inhalation chamber regardless of exposure atmosphere, presumably due to stress from vibrations and noise in the laboratory. For this reason, all our experiments included control animals exposed to clean air in the nose-only exposure system to control for possible confounding by this response to the laboratory environment.

The surgical procedure for the telemetry animals involved inserting the radiotransmitter into the abdominal cavity, placing two ECG electrodes, inserting the blood pressure probe into the dorsal aorta with transient interruption of blood flow, and finally inserting the cannula into the jugular vein. Body weight loss (20 g) over the first 4 days after surgery was approximately 5%, but weight gain began to resume on day 5 (Figure 7A). Blood pressure was elevated for the first 3 days after surgery, and then declined progressively to stabilize on day 5 (Figure 7B). The amplitude of the circadian pattern for heart rate was reduced during the first 4 days after surgery (Figure 7C). This was attributable to a decreased activity of the animals during this early phase of postoperative recovery (Figure 7D). ("Activity" was movement recorded by the radiotransmitter and reported as an arbitrary unit to compare relative activity levels among groups of animals.) The data summarized in Figure 7 indicate that the activity pattern, blood pressure, and heart rate were all affected by the surgical procedure, but nevertheless had returned to normal and had stabilized 5 days after the surgery.

Our ideal study protocol would have included a 2-week period of recovery after surgery and before inhalation exposure. For example, the circadian patterns for heart

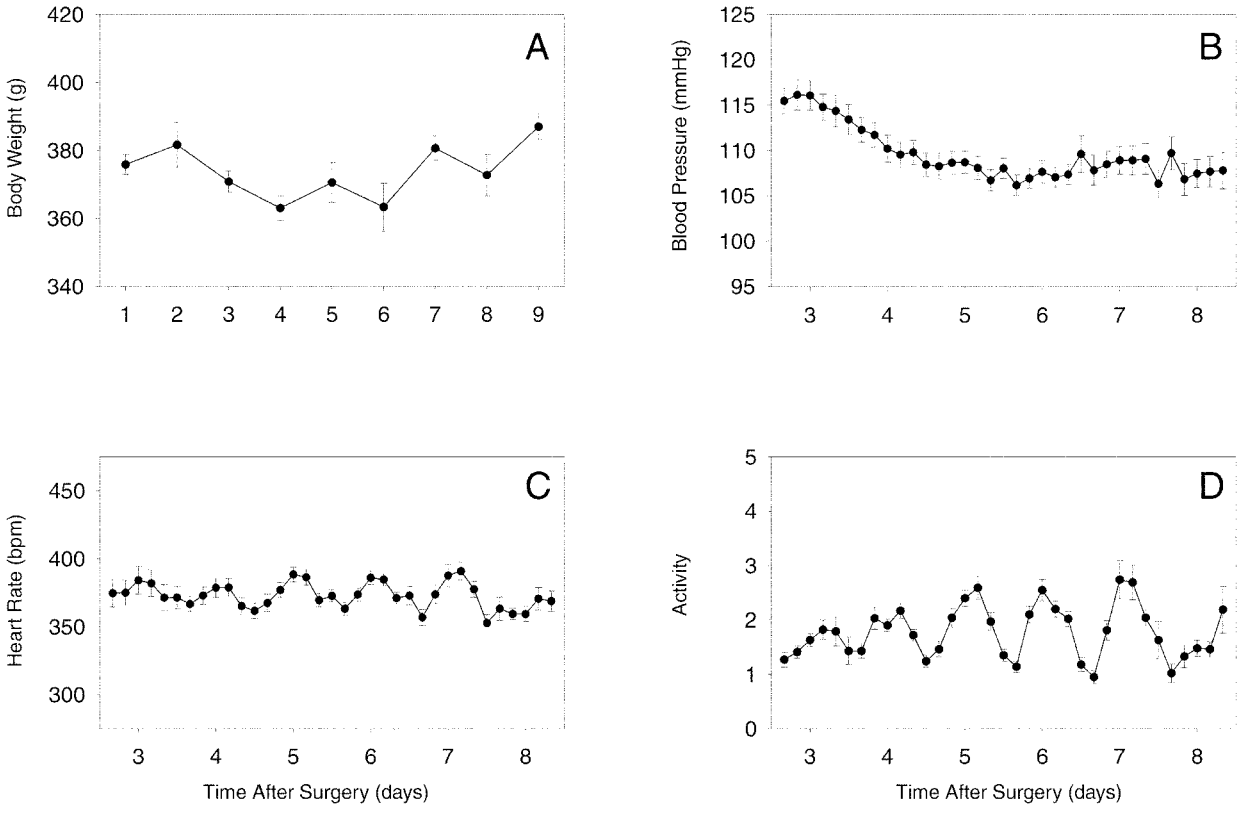

Figure 7. Recovery of the animals after surgery. Body weight (A) is corrected for the weight of the radiotransmitter. Animals lost weight after surgery but began to regain weight 4 days later. Mean blood pressure (B) had stabilized by 5 days after surgery. Heart rate (C) fluctuations closely followed the pattern of activity (D) of the animals. All available data from the study were pooled for illustration, and each point is the mean ± SE for n = 8–45 animals.

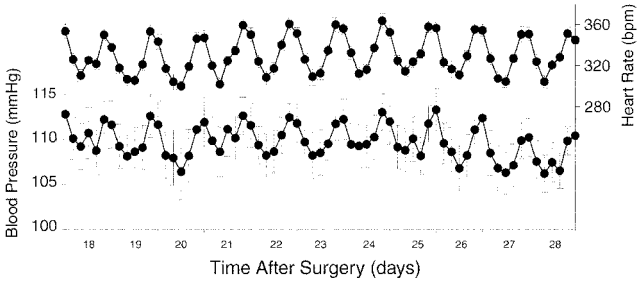

Figure 8. Mean blood pressure (lower line) and heart rate (upper line) profiles in Wistar rats during a period of 11 days after recovery from surgery for 18 days. Data are means ± SE for 7 animals. The diurnal variation in blood pressure parallels the changes in heart rate. Data are from sentinel animals, which underwent surgery but were not otherwise included in the experimental sessions.

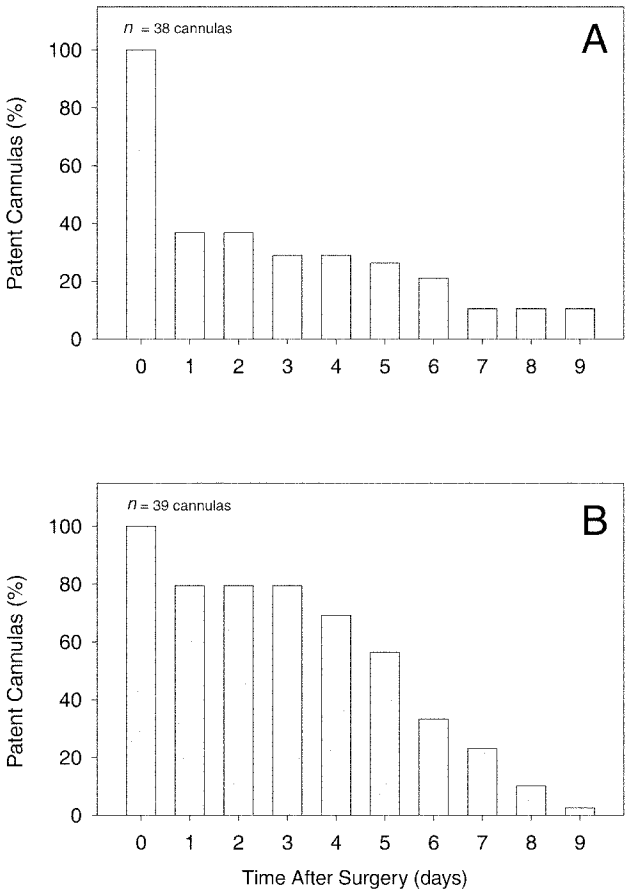

Figure 9. Patency of the jugular cannulas. (A) Heparinized glycerol in saline as the blocking solution. (B) Addition of a thrombolytic agent (tissue plasminogen activator or urokinase) to the blocking solution.

rate and blood pressure during an 11-day period starting on day 18 after surgery are illustrated in Figure 8. In the future, the quality of such telemetry data should allow more comprehensive analyses of time-series data, in particular data that document circadian patterns. In the current study, however, one objective was to match the kinetics of changes in hemodynamic endpoints with changes in plasma endothelins; acquiring such data required long-term jugular cannula patency to obtain multiple blood samples from the animals before and after exposure to the particles. We had also planned to conduct a dose-response assessment on the total EHC-93 particles, in addition to comparing animal responses to a number of particle types, which would have required rapidly reusing the limited number of radiotransmitters. Unfortunately, the performance of the jugular cannulas was highly variable among animals and among experiments in the early part of the project. Most cannulas were no longer patent 3 to 4 days after surgery (Figure 9A). The tubing material used to prepare the cannulas for our study might be adequate for repeated intravenous injections, but we found that it was less than optimal for repeated blood sampling. A small clot formed at the tip of the cannula, sealing it when we drew fluid; this may not be a problem when the cannulas are used for injecting fluid. New materials that are far less thrombogenic are now available commercially and these should have been used for constructing our jugular cannulas.

This poor cannula performance resulted in incomplete data sets of biochemical endpoints for many of the rats, precluding the use of repeated-measures statistical techniques for analyzing the biochemical endpoints, and eliminating the possibility of meaningful direct animal-by-animal correlations between the biochemical and physiologic endpoints in the time-series data. The time-series data for the biochemical endpoints were nevertheless essential, in particular to provide additional insight into the dynamics of the endothelin response. To improve cannula patency, we added a small amount of tissue plasminogen activator or urokinase to the cannula blocking solution to reduce the likelihood of clot formation at the tip of the cannula. This resulted in about 60% of the jugular cannulas remaining patent for blood sampling after 5 days (Figure 9B) compared with only 25% without using any thrombolytic agent (Figure 9A). Extending the life of the cannulas increased our chances of obtaining blood samples before inhalation exposure and early after exposure. The preexposure samples to determine the basal values of the biochemical endpoints were recovered after a maximum of 3 to 4 days after surgery, such that basal values could be determined for most animals. The animals were exposed by inhalation to particles between 5 and 8 days after surgery, such that hemodynamic changes attributable to the surgery did not prevent detecting effects attributable to the inhaled particles (Figures 7B and 7C). In addition to

the animals that received a cardiovascular telemetry implant, we systematically cannulated the animals for the histologic assessment to improve our sample sizes and the statistical power of our biochemical endpoints.

Consequently, although blood samples could not be obtained from every animal at each time point, a blood sample was available from most animals before the inhalation exposures, from many animals at 2 hours and at 24 hours after exposure, and for all animals at termination, which was at 36 hours (histology animals) or 48 hours (telemetry animals) after exposure. These biochemical data from the telemetry and the histology animals were combined for statistical analyses and data presentation.

Note that two portions of our original proposal could not be completed within the compressed time frame specified in the RFA: First, we had planned to conduct a dose-response assessment of the total EHC-93 particles; this was prevented by delays created in our attempts to resolve the issue of cannula patency. Second, we proposed to monitor angiotensin II in the animals; however, to gain a better appreciation of the impact of stress on the animals after surgery and the nose-only inhalation exposure, we used the limited volumes of plasma at the different time points (0.3–0.5 mL) to monitor only the catecholamine levels.

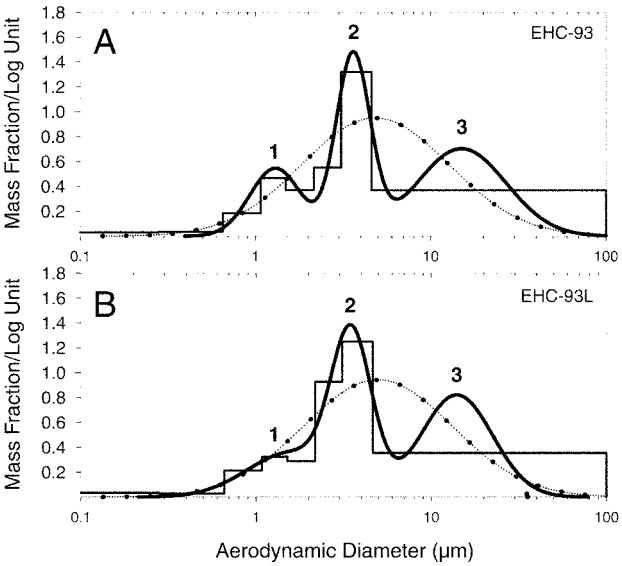

Figure 10. Particle size distributions for the EHC-93 and EHC-93L exposure atmospheres. The histogram (blocked line) is the mass of particles recovered on the individual cascade impactor plates. The dotted line is the forced log-probit fit of the particle size distribution assuming a single mode. The dark curving line is the multimodal fit assuming a linear combination of weighted lognormal distributions. See Table 3 for summary data on MMAD, GSD, and mass fraction of the different modes, and Table 4 for estimates of lung depositions.

DEVELOPMENT OF THE EXPOSURE ATMOSPHERES

The particle size distributions of the exposure atmospheres for EHC-93 urban dust (Figure 10A), modified EHC-93L particles (Figure 10B), diesel soot (Figure 11A), and carbon black particles (Figure 11B) were multimodal. Plotting the mass distribution of either of these materials as a function of aerodynamic diameter produced GSDs that were generally greater than 3, which is a good indicator that a size distribution is multimodal. The procedure of Hewett and McCawley (1991) allowed a better description of the various size modes, particularly in the resuspended urban dust preparations.

In the early portion of our study, we attempted to reduce the overall MMAD of the EHC-93 and EHC-93L atmospheres by removing the coarse fraction of the particles directly through a cyclone, on an online impactor plate, and eventually through a virtual impactor with a theoretical aerodynamic diameter cutoff of 3 to 4 μm, but mass recovery was highly variable (usually less than 5%). With hindsight, it appears that an impactor system or a cyclone with a sharp cutoff at 5 to 7 μm should be sufficient to remove the coarse mode 3 (Figure 10). However, because of the stringent time lines of the contract, we opted to continue our experiments using total particles regardless of size of the resuspended EHC-93 and EHC-93L materials. The presence of nonrespirable coarse mode particles did

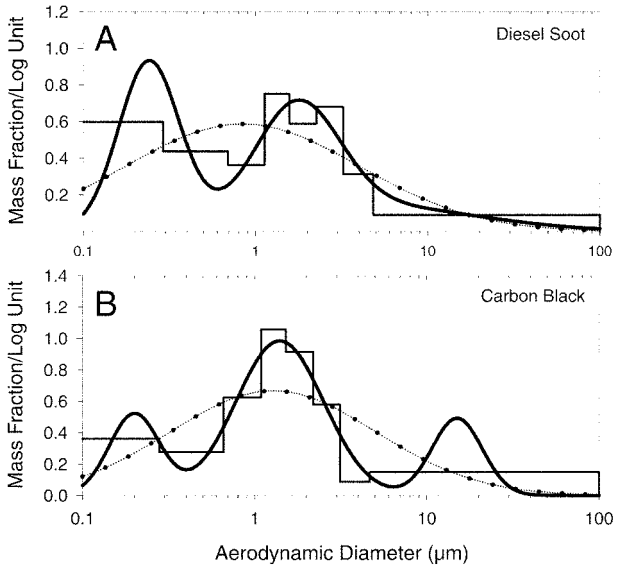

Figure 11. Particle size distributions for the diesel soot (A) and carbon black (B) exposure atmospheres. The histogram (blocked line) is the mass of particles recovered on the individual cascade impactor plates. The dotted line is the forced log-probit fit of the particle size distribution assuming a single mode. The dark curving line is the multimodal fit assuming a linear combination of weighted lognormal distributions. See Table 3 for summary data on MMAD, GSD, and mass fraction of the different modes, and Table 4 for estimates of lung depositions.

not constitute a serious confounder in these studies because such particles would simply not contribute to a pulmonary dose. We recognized that the chemical compositions of the particles measured in the bulk particle preparations or in the filter samples could differ strongly from the experimental exposure atmospheres and from the actual chemical composition of the respirable particle size modes that contributed to the internal particulate doses. Thus, we conducted a thorough comparative analysis of the chemical compositions of the different size modes and we have modeled biomathematically the deposition of elements in the lungs of the animals (see section below on Dosimetric Modeling).

The resuspended EHC-93 particles contained a respirable mode at 1.3 μm MAD (mode 1) and a respirable mode at 3.6 μm MAD (mode 2). These two respirable modes accounted for about 55% of the total aerosol mass (Table 3). An additional coarse mode at 15 μm MAD (mode 3) accounted for the remaining 45% of the aerosol mass. The modified EHC-93L particles also contained two respirable modes, at 1.6 μm MAD and at 3.5 μm MAD, that accounted together for about 58% of the mass, as well as a coarse mode at 14 μm MAD that accounted for the remaining 42% of the mass of the aerosol. Extracting the EHC-93 particles in water, which removed about 17% of the chemical constituents of the particles, obviously did not modify very significantly the overall mass distribution as a function of aerodynamic size. These particle size analyses, based on inertial impaction, are not sensitive to the presence of an ultrafine mode in the preparations. The mass of particles recovered on the terminal filter of the cascade impactors in our experiments was often higher than the mass on the preceding impactor plate, which suggests the presence of a mode of ultrafine particles in the EHC-93 and EHC-93L preparations; however, we lacked direct evidence and quantitative data on particle counts for this ultrafine mode.

The deposition efficiencies of mode 1 for both EHC-93 (1.3 μm MAD) and EHC-93L (1.6 μm MAD) particles were estimated at 8% for the pulmonary compartment and 4% to 5% for the tracheobronchial compartment (Table 3). The deposition efficiency for mode 2 (3.5–3.6 μm MAD) of both particle types was estimated at 4% to 5% in both the pulmonary and the tracheobronchial compartments. Note that biomathematic modeling predicted that deposition of the coarse mode 3 (14–15 μm MAD) from both of these particulate preparations in the pulmonary and tracheobronchial compartments should be insignificant. The values of deposited mass were then computed, taking into account mass concentration, exposure duration, respiratory rate, and deposition efficiency (Table 4). Therefore, the doses of deposited particles in the pulmonary compartment of the rats were estimated at 24 ng/cm^2 for the EHC-93 particles (exposure concentration of 48 mg/m^3) and 26 ng/cm^2 for the leached EHC-93L particles (exposure concentration of 49 mg/m^3). The dose of deposited particles in the tracheobronchial compartment was estimated at about 3 μg/cm^2 for both the EHC-93 (2,626 ng/cm^2) and EHC-93L (2,994 ng/cm^2) particles. The dose of deposited particles in the nasopharyngeal compartment was estimated at about 55 μg/cm^2 for both the EHC-93 and EHC-93L particles. From a toxicologic standpoint, we concluded that the

Table 3. Experimental Exposure Atmospheres

Exposure Atmosphere	Exposure Concentration (mg/m^3)	Yield[a]	Mode	MMAD (μm)	GSD	Mass (%)	Deposition Efficiency[b]		
							Pulmonary	Tracheobronchial	Nasopharyngeal
EHC-93	48	25%	1	1.3	1.4	20	0.081	0.044	0.078
			2	3.6	1.3	35	0.047	0.043	0.456
			3	15.0	1.8	45	0.000	0.000	0.323
EHC-93L	49	25%	1	1.6	1.8	23	0.078	0.046	0.162
			2	3.5	1.3	35	0.050	0.045	0.443
			3	14.0	1.6	42	0.000	0.001	0.341
Diesel soot	4.2	86%	1	0.2	1.5	40	0.146	0.036	0.053
			2	1.8	1.8	36	0.070	0.046	0.190
			3	4.0	4.5	24	0.042	0.022	0.360
Carbon black	4.6	36%	1	0.2	1.4	19	0.143	0.035	0.052
			2	1.4	1.8	63	0.074	0.043	0.124
			3	15.0	1.4	18	0.000	0.000	0.329

[a] The measured concentration divided by the amount of aerosolized material.

[b] The deposition efficiency is the fraction of the inhaled mass that is deposited in the pulmonary, tracheobronchial, and nasopharyngeal compartments. The deposition efficiencies were determined with the MPPDep software (V1.11, RIVM, The Netherlands) with inhalability correction.

Table 4. Modeling of Particle Deposition

Exposure Atmosphere	Exposure Concentration (mg/m³)	Mode	MMAD (μm)	Pulmonary		Tracheobronchial		Nasopharyngeal	
				μg	ng/cm²	μg	ng/cm²	μg	μg/cm²
EHC-93	48	1	1.3	40	12	22	965	38	2.6
		2	3.6	41	12	37	1,651	394	26
		3	15	0	0	0	0	359	24
Total				81	24	59	2,616	791	53
EHC-93L	49	1	1.6	45	13	27	1,184	94	6.3
		2	3.5	44	13	40	1,763	391	26
		3	14	0	0	1.1	47	361	24
Total				89	26	68	2,994	846	56
Diesel soot	4.23	1	0.24	13	3.7	3.1	138	4.6	0.3
		2	1.8	5.4	1.6	3.6	159	15	1.0
		3	4	2.2	0.6	1.1	51	19	1.2
Total				21	6	8	348	39	3
Carbon black	4.6	1	0.2	6.8	2.0	1.7	74	2.5	0.2
		2	1.4	11	3.2	6.4	285	18	1.2
		3	15	0	0	0	0	14	0.9
Total				18	5	8	359	35	2

experimental internal particulate mass doses created by inhalation exposure of the animals to the urban particles were essentially identical between the EHC-93 particles and the modified EHC-93L particles.

Thus, if biologic responses to inhaling these two materials are found to differ, those results should be related to differences in the physicochemical characteristics of the particles and should imply some role in the biologic effects for the water-soluble (and therefore bioavailable) fraction of the particles. In contrast, identical patterns of effects between the two materials could suggest either that deposition of the particulate mass is a main determinant of potency or that surface characteristics of the particles may be more important than a rapidly bioavailable fraction.

In principle, total elimination of the mode 3 particles from the experimental EHC-93 and EHC-93L atmospheres would have resulted in reducing the nasopharyngeal dose of only 45% from the estimated value of 55 μg/cm². Thus, although the coarse mode 3 of urban particles constituted almost half of the particulate mass in the experimental exposure atmospheres, the respirable particles in mode 2 contributed more significantly than mode 3 particles to the nasopharyngeal dose. Consequently, the overall internal dosimetry and the interpretation of biologic effects was probably not very sensitive to mode 3 particles.

The diesel soot and carbon black aerosols both consisted mostly of respirable mass. A respirable mode 1 at 0.2 μm MAD and a respirable mode 2 at 1.4–1.8 μm MAD together accounted for about 80% of the total aerosol mass (Table 3). Mode 3 at 4 μm MAD in the diesel soot aerosol and a coarse mode 3 at 15 μm MAD in the carbon black accounted for about 20% of the aerosol mass. The depositions of particles in the animals exposed to diesel soot or carbon black were estimated at 5 to 6 ng/cm² in the pulmonary compartment, about 350 ng/cm² in the tracheobronchial compartment, and about 2 to 3 μg/cm² in the nasopharyngeal compartment (Table 4). Thus, the deposited doses were essentially identical between the diesel soot and the carbon black aerosols. Again, differences between the biologic responses to the diesel soot and to the carbon black aerosols should indicate that chemical constituents, and not mass deposition, are determinants of potency. The doses of diesel soot and carbon black aerosols were about one fifth the doses of the urban dust EHC-93 and the modified EHC-93L particles in the pulmonary compartment and about one tenth in the tracheobronchial compartment. Because we had limited amounts of diesel particles, we did not conduct inhalation exposures at higher concentrations.

LUNG HISTOLOGY AND AUTORADIOGRAPHY

When the lungs of six animals exposed to each particulate atmosphere or clean air were examined histologically and for autoradiographic labeling with ^{3}H-thymidine (see Table 2), we found no exposure-related changes. Representative histologic sections are shown for animals exposed to

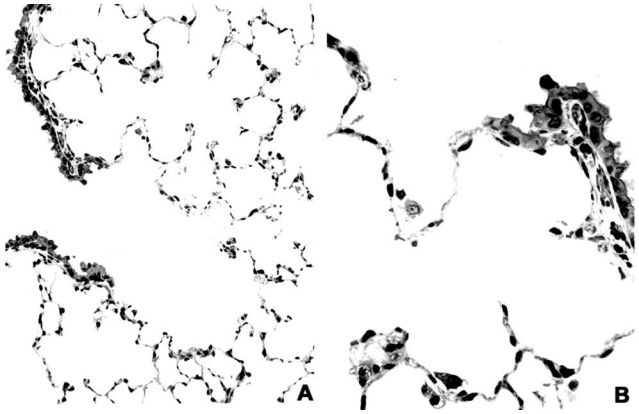

Figure 12. Lung section showing a bronchiole-alveolar duct junction after inhalation exposure to EHC-93 particles (48 mg/m³) for 4 hours followed by 32 hours in clean air. Magnification is ×320 (A) and ×900 (B).

EHC-93 particles (Figure 12) and to diesel soot (Figure 13). For all exposure groups, autoradiographic labeling was on the order of 0.25% in the lung parenchyma (including intravascular labeling) and 0.03% in terminal bronchioles (Table 5). Occasional foamy macrophages and peribronchiolar lymphocytes were observed in some animals, but these were incidental findings that were not associated with exposure atmosphere. Macrophages with phagocytized particles could be seen in the bronchiolar-alveolar duct regions of animals exposed to each particulate atmosphere, which confirmed delivery of particles to the pulmonary compartment; the dark pigmentation of the diesel soot (Figure 13B) and carbon black particles was particularly noticeable. Overall, none of the particulate materials used in our inhalation study resulted in histopathologic changes in the lungs; no neutrophils were seen in the alveolar spaces or the pulmonary interstitium, which indicates a lack of inflammatory response to any of the particles. Therefore, differences among the cardiovascular responses to the inhaled materials in the present experiments should not be confounded by differences in acute pulmonary toxicity.

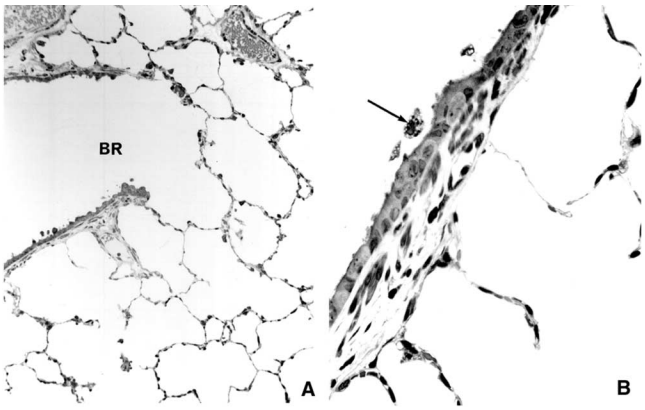

Figure 13. Lung section showing a bronchiole-alveolar duct junction after inhalation exposure to diesel soot (4.2 mg/m³) for 4 hours followed by 32 hours in clean air. Magnification is ×320 (A) and ×900 (B). In panel A, BR = bronchiole. In panel B, an arrow indicates a macrophage containing diesel particles.

Table 5. Percentage of ^{3}H-Thymidine–Labeled Lung Cells[a]

Treatment	Alveolar Cells	Bronchiolar Cells
Clean air	0.25 ± 0.08	0.02 ± 0.01
EHC-93	0.19 ± 0.04	0.03 ± 0.01
EHC-93L	0.17 ± 0.06	0.03 ± 0.01
Diesel soot	0.25 ± 0.02	0.03 ± 0.01
Carbon black	0.33 ± 0.07	0.04 ± 0.02

[a] Data are means ± SE; $n = 6$ animals.

PHYSIOLOGIC ENDPOINTS

Blood pressure and heart rate were elevated during the 4-hour nose-only inhalation exposure period. Thus, before analyzing the complex telemetry database for statistically significant changes, we calculated 18-hour averages from the data (13:05–07:00), removing the data collected during the 4-hour inhalation period and the 1-hour time periods before and after exposure, and tested the 18-hour averages by one-way repeated-measures ANOVA. The rationale for this procedure was to simplify the model and reduce the number of comparisons. The results from this robust analysis confirmed particle exposure effects (as detailed in Tables 6 through 12). The physiologic data were then summarized as 4-hour averages over 3 consecutive days for each exposure atmosphere. Figures 14 through 18 show the four physiologic endpoints in Wistar rats before, during, and after a 4-hour nose-only exposure to each of the five atmospheres. In each figure, the reference line is the mean blood pressure, heart rate, body temperature, or activity of the animals for the first 20 hours of day 0 (ie, excluding the 4-hour exposure period). Data bars show 4-hour averages of continuous telemetry for 20 hours before and 4 hours during exposure (day 0) and for 1 and 2 days after exposure. The empty bar at the 24-hour time point (08:00–12:00) on day 0 is the period of exposure. Bars are means ± SE for n = 12–15 animals. Letters indicate that a statistical difference was detected by two-way RM ANOVA with two-factor repetition (day × time interval; $P < 0.001$). Bars with different letters are statistically different by the Tukey test ($P < 0.05$; comparison of days within time intervals).

Examining the daily profiles for each endpoint across the atmosphere exposure groups indicated that the exposures did not disrupt the physiologic endpoints in dra-

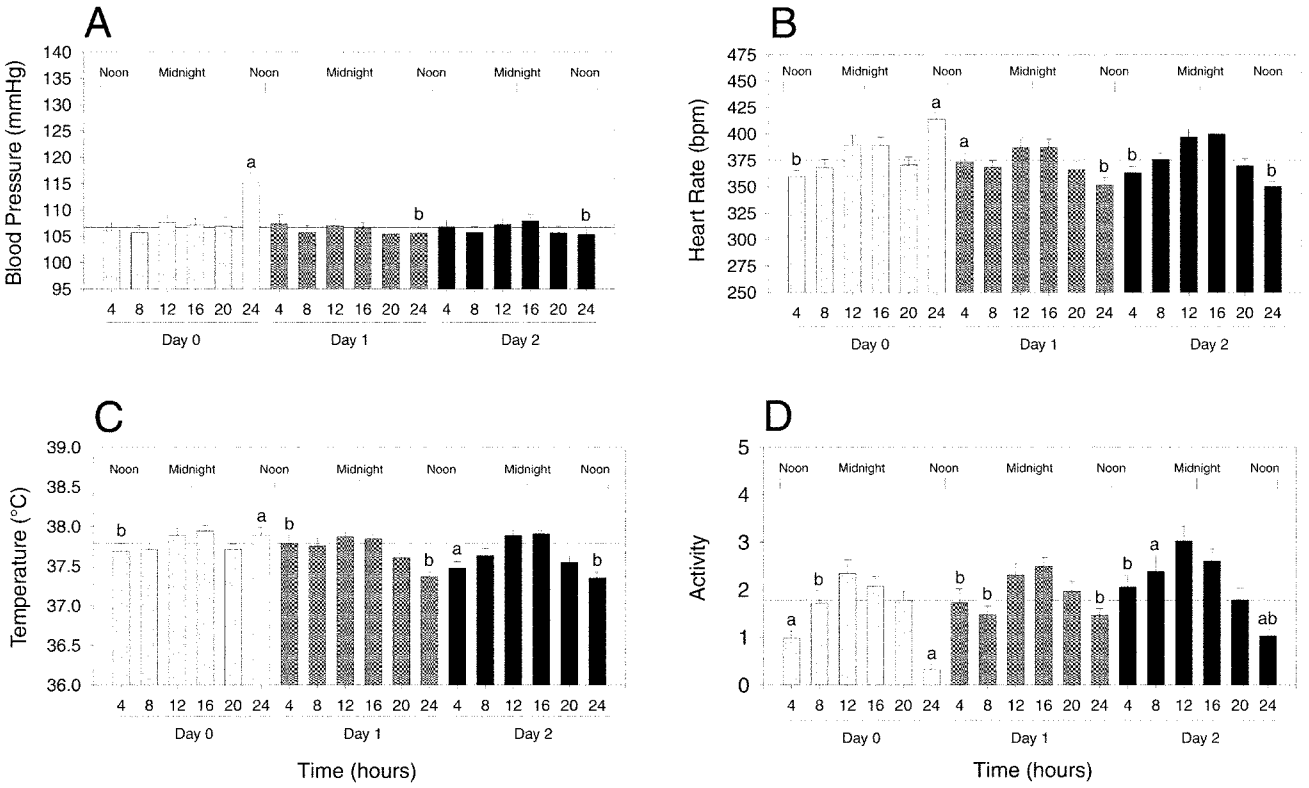

Figure 14. Clean air. (A) Mean blood pressure was elevated during exposure and returned to normal immediately after exposure. (B) Heart rate was elevated during and immediately after exposure. (C) Body temperature was elevated during exposure. (D) The activity pattern of the animals was modified immediately after exposure (during both the 4 and 8 time intervals) and again during the three time intervals at the end of day 1 and beginning of day 2 (24, 4, and 8). For further explanation, see text on page 24.

matic ways. In the air-exposed control animals, heart rate (Figure 14B and Table 6) and activity (Figure 14D and Table 7) on day 2 were significantly elevated by comparison with day 0 before exposure. Similarly, the activity patterns of the animals exposed to EHC-93 (Figure 15D) and the animals exposed to diesel soot (Figure 16D) on day 2 were also statistically different by comparison with day 1 (Table 7). Overall, body temperature was not affected by the exposure when considering the 18-hour averages (Table 8), but the two-way repeated-measures ANOVA conducted on the 4-hour averages revealed elevated body temperature during the exposures and sometimes immediately after exposure by comparison with the same time intervals on subsequent days. The transient changes in heart rate and body temperature were statistically significant, but could be attributed to confinement in the nose-only tubes and to mild stress from noise and vibrations in the inhalation laboratory. It is not clear whether the statistically significant changes in the animals' activity revealed some nonspecific disturbances of the activity patterns caused by the experimental protocols, or whether they represent a carryover of the stress of surgery (Figure 7D). Furthermore, the animals groomed immediately after the inhalation exposures, a period of the day during which they are normally resting. Overall, exposure to the different particulate atmospheres did not appear to modify directly the activity levels of the animals, which could constitute a major confounding factor in interpreting the impact of the particles on the hemodynamic endpoints. We have no explanation for the nonsignificant dip in nocturnal activity just before midnight (time period 12 [20:00–24:00]) on day 1 after exposure in the animals exposed to EHC-93 (Figure 15D) and to EHC-93L (Figure 17D).

The only statistically significant hemodynamic change attributable to the particle exposures was the elevation of blood pressure after animals were exposed to EHC-93 (Figure 15A). By comparison with the value before exposure (day 0, 18-hour average), the change was on the order of +2.5 mm Hg on day 1 ($P > 0.05$), and +4 mm Hg on day 2 ($P < 0.05$) for mean blood pressure (Table 9), systolic pressure (Table 10), and diastolic pressure (Table 11). Analyzing the data by two-way repeated-measures ANOVA, with day and time interval as factors, followed by the Tukey multiple comparison procedure indicated that the

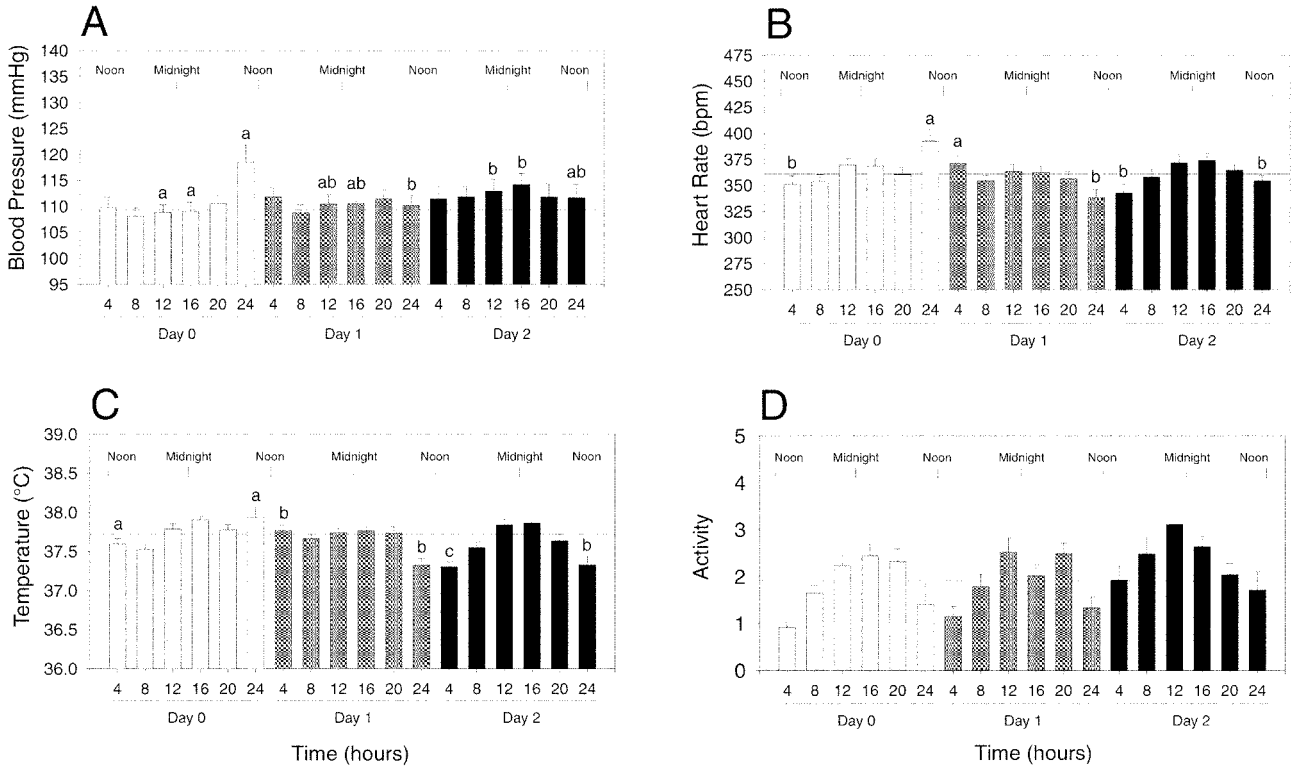

Figure 15. EHC-93 particles (48 mg/m³). (A) Mean blood pressure was elevated during exposure and on day 2 after exposure at time intervals 12 and 16 (20:00–04:00), which corresponds to the time of maximal activity for the animals. (B) Heart rate was elevated during and immediately after exposure. (C) Body temperature was elevated during and after exposure. (D) Activity of the animals was not significantly modified by the exposure session. For further explanation, see text on page 24.

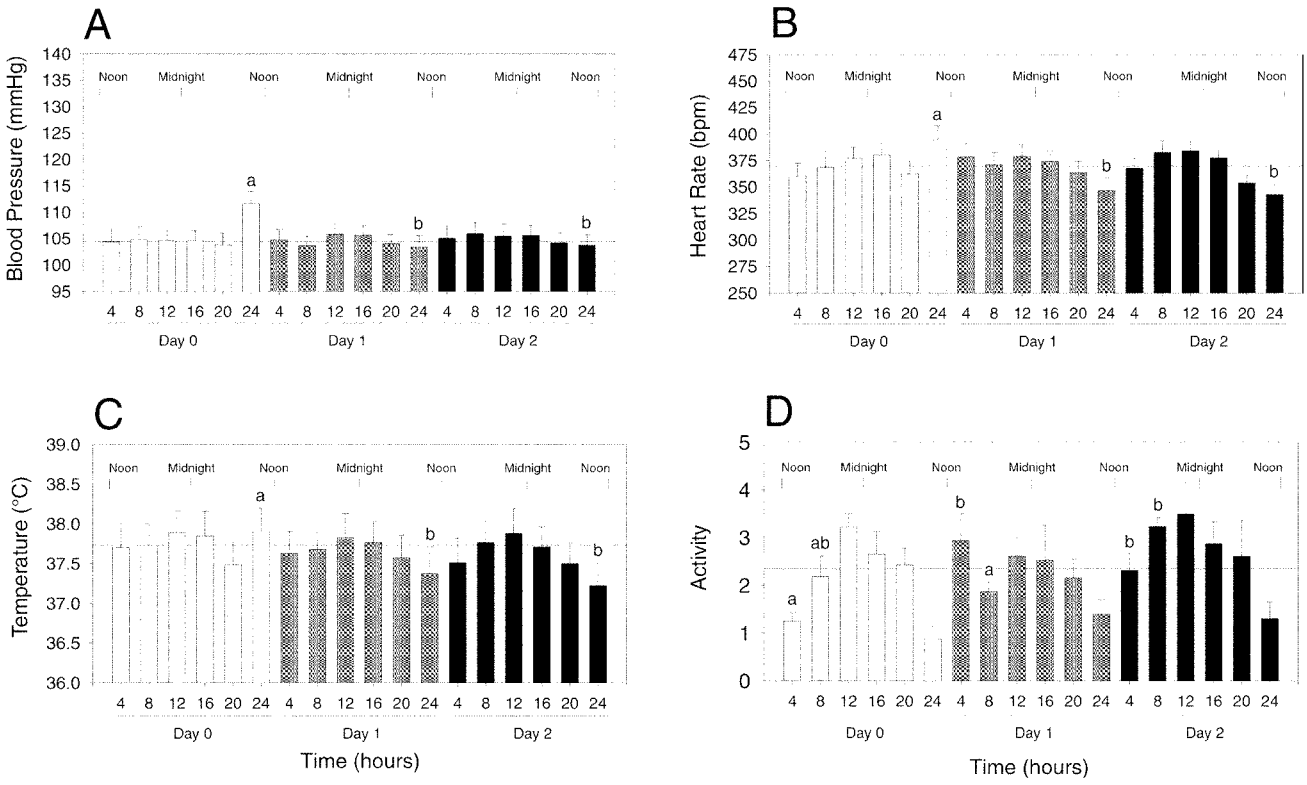

Figure 16. Diesel soot (5 mg/m³). (A) Mean blood pressure was elevated during exposure. (B) Heart rate was elevated during exposure. (C) Body temperature was elevated during exposure. (D) Comparing days 0, 1, and 2, activity of the animals was perturbed mainly during time intervals 4 and 8 (12:00–20:00). For further explanation, see text on page 24.

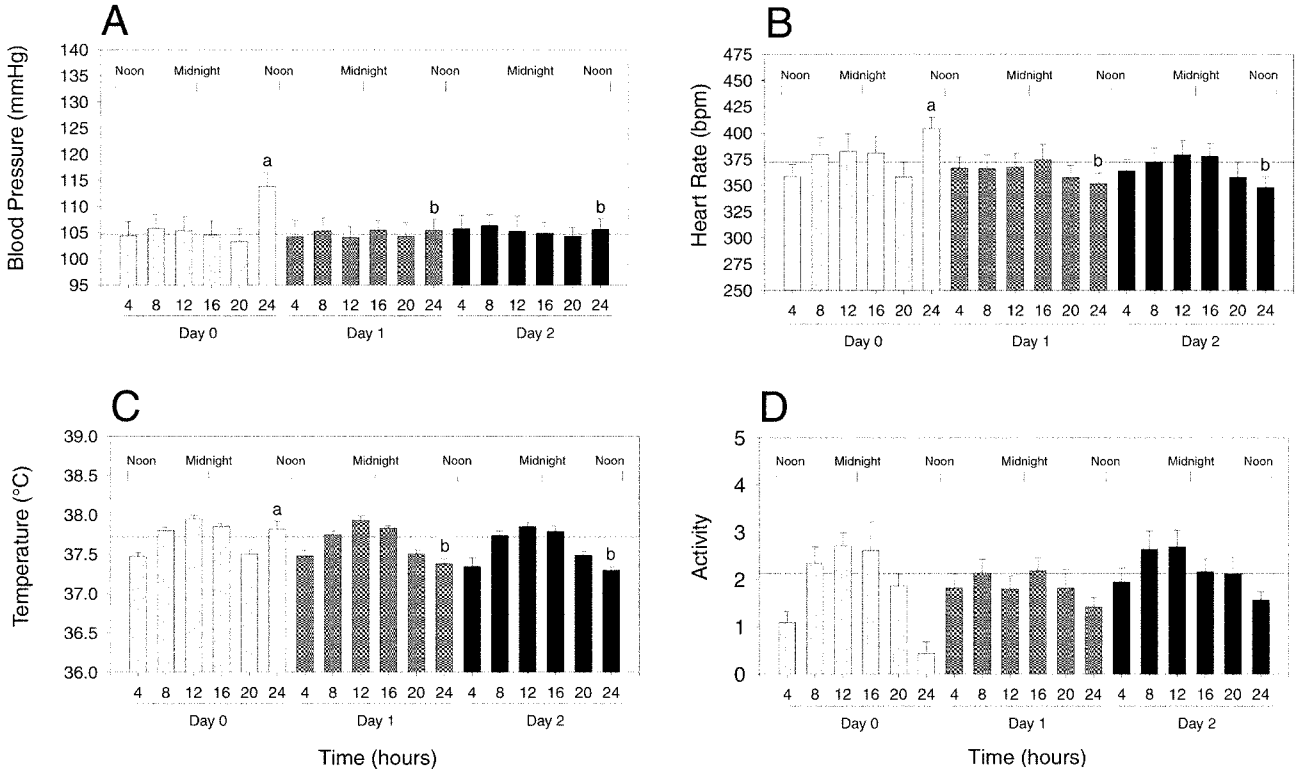

Figure 17. EHC-93L particles (49 mg/m³). (A) Mean blood pressure was elevated during exposure. (B) Heart rate was elevated during exposure. (C) Body temperature was elevated during exposure. (D) Activity of the animals was not significantly modified. For further explanation, see text on page 24.

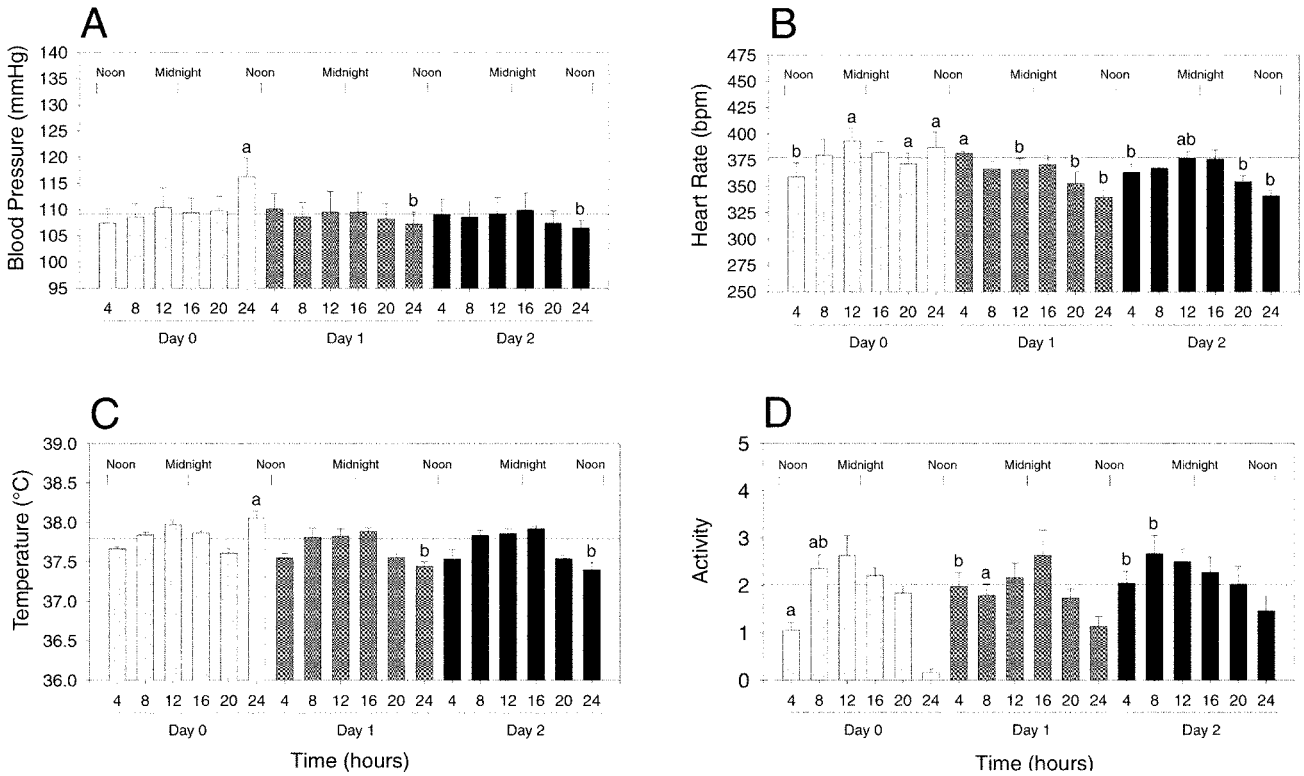

Figure 18. Carbon black particles (48 mg/m³). (A) Mean blood pressure was elevated during exposure. (B) Heart rate was elevated during and immediately after exposure, and reduced during time interval 12 (20:00–24:00) on day 1 compared with day 0. (C) Body temperature was elevated during exposure. (D) Comparing days 0, 1, and 2, activity of the animals was perturbed mainly during time intervals 4 and 8 (12:00–20:00). For further explanation, see text on page 24.

Table 6. Changes in Heart Rate After Exposures (18-Hour Average)

Treatment	Day Effect[a] (*P*)	Comparison[b]	Δ (bpm)	Tukey[c]
Clean air	0.046 (Rank)	D1 − D0	2.04	NS
		D2 − D0	6.64	$P < 0.05$
		D2 − D1	4.60	NS
EHC-93	0.092 (Rank)	D1 − D0	−1.61	NA
		D2 − D0	3.50	
		D2 − D1	5.10	
EHC-93L	0.138	D1 − D0	−7.20	NA
		D2 − D0	−0.64	
		D2 − D1	6.56	
Diesel soot	0.273	D1 − D0	1.90	NA
		D2 − D0	5.62	
		D2 − D1	3.76	
Carbon black	0.104	D1 − D0	−12.22	NA
		D2 − D0	−8.33	
		D2 − D1	3.89	

[a] *P* value assessed using a one-way repeated-measures ANOVA, except where "Rank" indicates the data were analyzed by rank using a Friedman RM ANOVA procedure.

[b] D0, D1, and D2 = Day 0, Day 1, and Day 2.

[c] Tukey multiple comparison procedure. NA = multiple comparison procedure not applied; NS = not significant ($P > 0.05$).

Table 7. Changes in Activity After Exposures (18-Hour Average)

Treatment	Day Effect[a] (*P*)	Comparison[b]	Δ (relative units)	Tukey[c]
Clean air	0.001	D1 − D0	0.21	NS
		D2 − D0	0.60	$P < 0.05$
		D2 − D1	0.39	$P < 0.05$
EHC-93	0.006 (Rank)	D1 − D0	0.00	NS
		D2 − D0	0.45	NS
		D2 − D1	0.45	$P < 0.05$
EHC-93L	0.090	D1 − D0	−0.32	NA
		D2 − D0	0.17	
		D2 − D1	0.50	
Diesel soot	0.035	D1 − D0	−0.15	NS
		D2 − D0	0.64	NS
		D2 − D1	0.79	$P < 0.05$
Carbon black	0.096	D1 − D0	−0.04	NA
		D2 − D0	0.34	
		D2 − D1	0.39	

[a] *P* value assessed using a one-way repeated-measures ANOVA, except where "Rank" indicates the data were analyzed by rank using a Friedman RM ANOVA procedure.

[b] D0, D1, and D2 = Day 0, Day 1, and Day 2.

[c] Tukey multiple comparison procedure. NA = multiple comparison procedure not applied; NS = not significant ($P > 0.05$).

Table 8. Changes in Body Temperature After Exposures (18-Hour Average)

Treatment	Day Effect[a] (*P*)	Comparison[b]	Δ (°C)	Tukey[c]
Clean air	0.846 (Rank)	D1 − D0	−0.01	NA
		D2 − D0	−0.06	
		D2 − D1	−0.05	
EHC-93	0.654	D1 − D0	−0.01	NA
		D2 − D0	0.01	
		D2 − D1	0.03	
EHC-93L	0.157	D1 − D0	−0.03	NA
		D2 − D0	−0.08	
		D2 − D1	−0.05	
Diesel soot	0.207	D1 − D0	−0.05	NA
		D2 − D0	−0.04	
		D2 − D1	0.01	
Carbon black	0.296	D1 − D0	−0.07	NA
		D2 − D0	−0.03	
		D2 − D1	0.03	

[a] *P* value assessed using a one-way repeated-measures ANOVA, except where "Rank" indicates the data were analyzed by rank using a Friedman RM ANOVA procedure.

[b] D0, D1, and D2 = Day 0, Day 1, and Day 2.

[c] Tukey multiple comparison procedure. NA = multiple comparison procedure not applied.

Table 9. Changes in Mean Blood Pressure After Exposures (18-Hour Average)

Treatment	Day Effect[a] (*P*)	Comparison[b]	Δ (mm Hg)	Tukey[c]
Clean air	0.395	D1 − D0	−0.29	NA
	(Rank)	D2 − D0	0.55	
		D2 − D1	0.84	
EHC-93	< 0.001	D1 − D0	1.25	NS
		D2 − D0	3.87	$P < 0.05$
		D2 − D1	2.61	$P < 0.05$
EHC-93L	0.539	D1 − D0	−0.18	NA
		D2 − D0	0.54	
		D2 − D1	0.72	
Diesel soot	0.214	D1 − D0	0.32	NA
		D2 − D0	1.04	
		D2 − D1	0.71	
Carbon black	0.988	D1 − D0	−0.26	NA
		D2 − D0	−0.18	
		D2 − D1	0.08	

[a] *P* value assessed using a one-way repeated-measures ANOVA, except where "Rank" indicates the data were analyzed by rank using a Friedman RM ANOVA procedure.

[b] D0, D1, and D2 = Day 0, Day 1, and Day 2.

[c] Tukey multiple comparison procedure. NA = multiple comparison procedure not applied; NS = not significant ($P > 0.05$).

Table 10. Changes in Systolic Blood Pressure After Exposures (18-Hour Average)

Treatment	Day Effect[a] (*P*)	Comparison[b]	Δ (mm Hg)	Tukey[c]
Clean air	0.865	D1 − D0	−0.16	NA
		D2 − D0	0.26	
		D2 − D1	0.41	
EHC-93	0.012	D1 − D0	1.62	NS
	(Rank)	D2 − D0	3.98	$P < 0.05$
		D2 − D1	2.36	NS
EHC-93L	0.323	D1 − D0	−0.02	NA
		D2 − D0	1.13	
		D2 − D1	1.15	
Diesel soot	0.232	D1 − D0	0.58	NA
		D2 − D0	1.19	
		D2 − D1	0.62	
Carbon black	0.978	D1 − D0	−0.15	NA
		D2 − D0	−0.38	
		D2 − D1	−0.23	

[a] *P* value assessed using a one-way repeated-measures ANOVA, except where "Rank" indicates the data were analyzed by rank using a Friedman RM ANOVA procedure.

[b] D0, D1, and D2 = Day 0, Day 1, and Day 2.

[c] Tukey multiple comparison procedure. NA = multiple comparison procedure not applied; NS = not significant ($P > 0.05$).

Table 11. Changes in Diastolic Blood Pressure After Exposures (18-Hour Average)

Treatment	Day Effect[a] (*P*)	Comparison[b]	Δ (mm Hg)	Tukey[c]
Clean air	0.120	D1 − D0	−0.58	NA
		D2 − D0	0.66	
		D2 − D1	1.24	
EHC-93	< 0.001	D1 − D0	0.60	NS
	(Rank)	D2 − D0	3.50	$P < 0.05$
		D2 − D1	2.90	$P < 0.05$
EHC-93L	0.845	D1 − D0	−0.36	NA
		D2 − D0	−0.20	
		D2 − D1	0.16	
Diesel soot	0.711	D1 − D0	−0.09	NA
		D2 − D0	0.53	
		D2 − D1	0.63	
Carbon black	0.486	D1 − D0	−0.51	NA
	(Rank)	D2 − D0	−0.11	
		D2 − D1	0.40	

[a] *P* value assessed using a one-way repeated-measures ANOVA, except where "Rank" indicates the data were analyzed by rank using a Friedman RM ANOVA procedure.

[b] D0, D1, and D2 = Day 0, Day 1, and Day 2.

[c] Tukey multiple comparison procedure. NA = multiple comparison procedure not applied; NS = not significant ($P > 0.05$).

changes in mean blood pressure occurred mainly during the night (Figure 15A). In contrast, the EHC-93L particles did not produce this elevation in blood pressure (Figure 17A and Table 9). Exposure to diesel soot (Figure 16A) or carbon black (Figure 18A) produced no detectable changes in blood pressure.

These results indicate that despite possible stress from the laboratory environment, nose-only exposure of the animals to clean air was not sufficient to cause an alteration of the hemodynamic endpoints during the 2 days after exposure. In contrast, inhalation of the urban dust EHC-93 caused a progressive increase of systemic blood pressure after recovery in clean air, although an identical internal dose of the modified EHC-93L particles was not sufficient to increase blood pressure. These data imply differences in the potency of these two particulate materials brought about by altering the physicochemical characteristics of the particles, presumably by extracting the water-soluble elements and polar organics that should be rapidly bioavailable after the particles are deposited on the extracellular lining of the lungs.

BIOCHEMICAL ENDPOINTS

Values for the concentrations of big ET-1, ET-1, ET-2, ET-3, nitrite, 3-nitrotyrosine, *m*-tyrosine, and *o*-tyrosine in all exposure groups are summarized in Figures 19 through 28. All data were first analyzed by two-way ANOVA with atmosphere (air, EHC-93, EHC-93L, diesel soot, carbon black) and time interval (preexposure basal value, and 2 hours, 24 hours, 36 hours, and 48 hours after exposure) as factors. Our database did not permit us to use repeated-measures statistical techniques. A significant atmosphere $\times$ time interval interaction was observed only for plasma ET-1 ($P = 0.0343$), ET-2 ($P = 0.0433$), and ET-3 ($P = 0.0001$). No significant two-way interactions were noted for plasma big ET-1 ($P = 0.2927$), blood nitrite ($P = 0.1045$), *m*-tyrosine ($P = 0.0880$), *o*-tyrosine ($P = 0.4403$), and 3-nitrotyrosine ($P = 0.8070$). These endpoints were not analyzed further for statistical significance and are described here only in qualitative terms. The data for ET-1, ET-2, and ET-3 were next examined within each exposure group by one-way ANOVA followed by multiple comparison procedures on time interval within the exposure groups to elucidate the patterns of significant effects.

No statistically significant ($P > 0.05$) changes in the biochemical values were observed in the air-exposed control animals at 2, 24, 32, or 48 hours after exposure when compared with basal levels (Figures 19 and 20).

Inhalation exposure to the EHC-93 particles (Figures 21 and 22) caused an elevation of ET-1 that was statistically significant 32 hours after exposure ($P < 0.05$; Figure 21B). The effect of inhalation exposure to EHC-93 on plasma ET-3 was rapid (Figure 21D), with an almost two-fold elevation of circulating ET-3 as early as 2 hours after exposure, and appeared to last for at least 48 hours. The plasma levels of *o*-tyrosine appeared to increase steadily after exposure to EHC-93 (Figure 22D), reaching close to 300% of the baseline value after 48 hours, although this change was not statistically significant by two-way ANOVA.

Exposing the animals to the modified EHC-93L particles (Figures 23 and 24), from which a significant portion of the water-soluble elements and polar organic species should have been removed, resulted in rapid transient elevation of the plasma endothelins and no changes in NO-related products. Both ET-1 (Figure 23B) and ET-2 (Figure 23C) were significantly elevated 2 hours after exposure ($P < 0.05$), but both endothelin forms returned to basal levels within 32 hours. The response pattern of ET-3 was different from that of ET-1 and ET-2, with an elevation ($\sim$100% at 2 and 24 hours) followed by a decline below the basal level ($\sim$50% at 32 and 48 hours). The changes in ET-3 were not statistically significant when comparing individual postexposure time points with the basal value. However, the Tukey multiple comparison procedure did confirm that ET-3 levels at 2 hours and 24 hours after exposure were statistically different from those at 32 and 48 hours after exposure ($P < 0.05$). The pattern for ET-3 suggested a possible compensatory response at the later time points.

Exposing the rats to the diesel soot aerosol (Figures 25 and 26) caused a significant elevation ($P < 0.05$) of ET-3 measured 32 hours after exposure. We saw a trend toward progressive increase of ET-1 and ET-2 (Figure 25), but the changes were not statistically significant ($P > 0.2$). Interestingly, inhaled diesel soot caused a pattern of effects on plasma endothelins that was qualitatively comparable to the pattern of effects induced by EHC-93, which was a late elevation of endothelins. Note that we did not find a significant atmosphere $\times$ time interaction for the NO-related products (Figure 26), and therefore those endpoints were not analyzed further. We also found no changes in the biochemical endpoints induced by exposure to carbon black (Figures 27 and 28).

As indicated earlier, cannula patency in our study was not optimal and led to incomplete time-series data. To increase statistical power, animals for both the cardiovascular and the lung histology assessments were cannulated (a total of 74 animals). In addition, because of poor cannula patency we added either tissue plasminogen activator or urokinase at some point to improve the useful life of the cannulas. To detect any possible bias from these experimental variables, the data for ET-1, ET-2, and ET-3 were

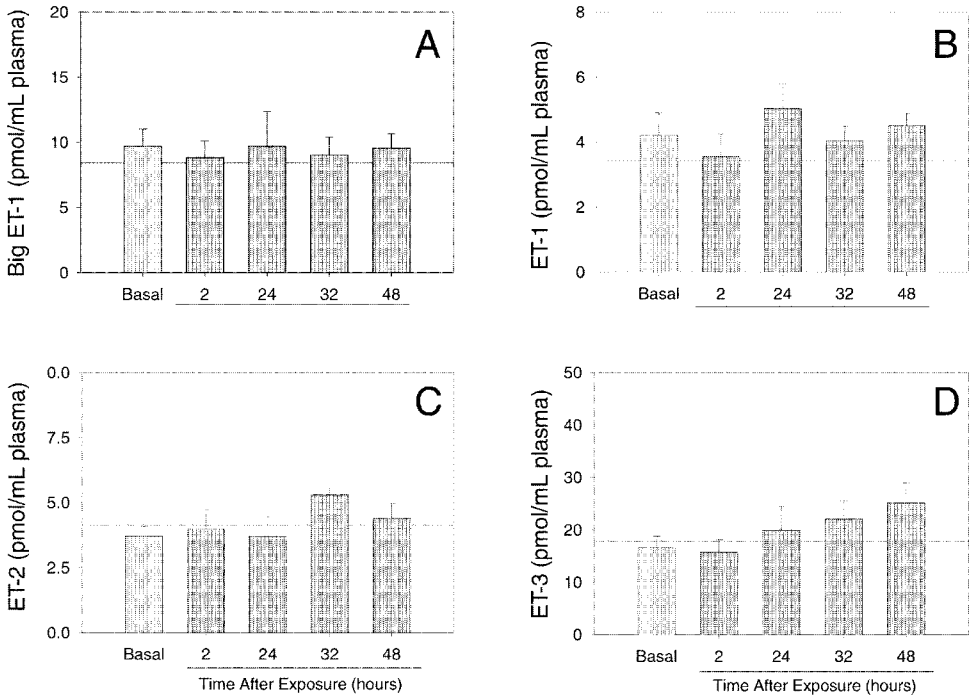

Figure 19. Levels of endothelins in rat plasma before and after inhalation exposure to clean air for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (n = 35–36). Basal = before exposure. Bars are means ± SE for n = 4–11 animals. The significant atmosphere × time interval interactions identified by two-way ANOVA were further tested by one-way ANOVA within each exposure group (ET-1, P = 0.608; ET-2, P = 0.235; ET-3, P = 0.286) and the pattern of significant effects was elucidated with the Dunnett and Tukey multiple comparison procedures (α = 0.05). No significant changes were observed in animals exposed to clean air for 4 hours.

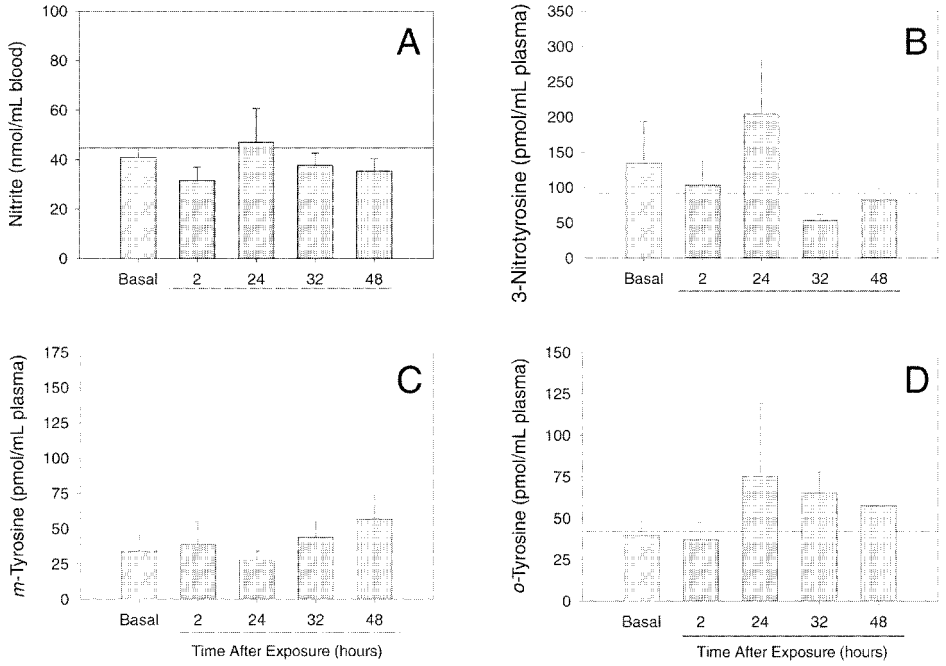

Figure 20. Levels of nitrite in blood and tyrosines in plasma of rats before and after inhalation exposure to clean air for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (nitrite, n = 41; tyrosines, n = 26). Basal = before exposure. Bars are means ± SE for n = 4–12 animals. We found no significant atmosphere × time interval interactions ($P > 0.05$); therefore, these endpoints were not tested further.

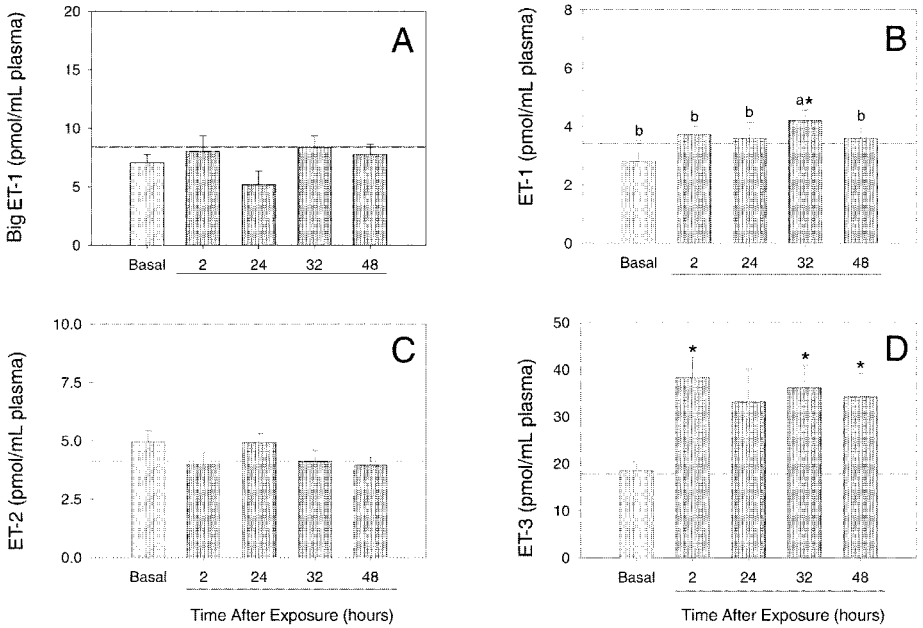

Figure 21. Levels of endothelins in plasma of rats before and after inhalation exposure to EHC-93 particles (48 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (n = 35–36). Basal = before exposure. Bars are means ± SE for n = 4–9 animals. The significant atmosphere × time interval interactions identified by two-way ANOVA were further tested by one-way ANOVA within each exposure group (ET-1, P = 0.057; ET-2, P = 0.301; ET-3, P = 0.037) and the pattern of significant effects was elucidated with the Dunnett and Tukey multiple comparison procedures. An asterisk (*) indicates a value significantly different from the basal value by the Dunnett procedure ($P < 0.05$). Bars with different letters (***a*** and ***b***) are statistically different by the Tukey procedure ($P < 0.05$).

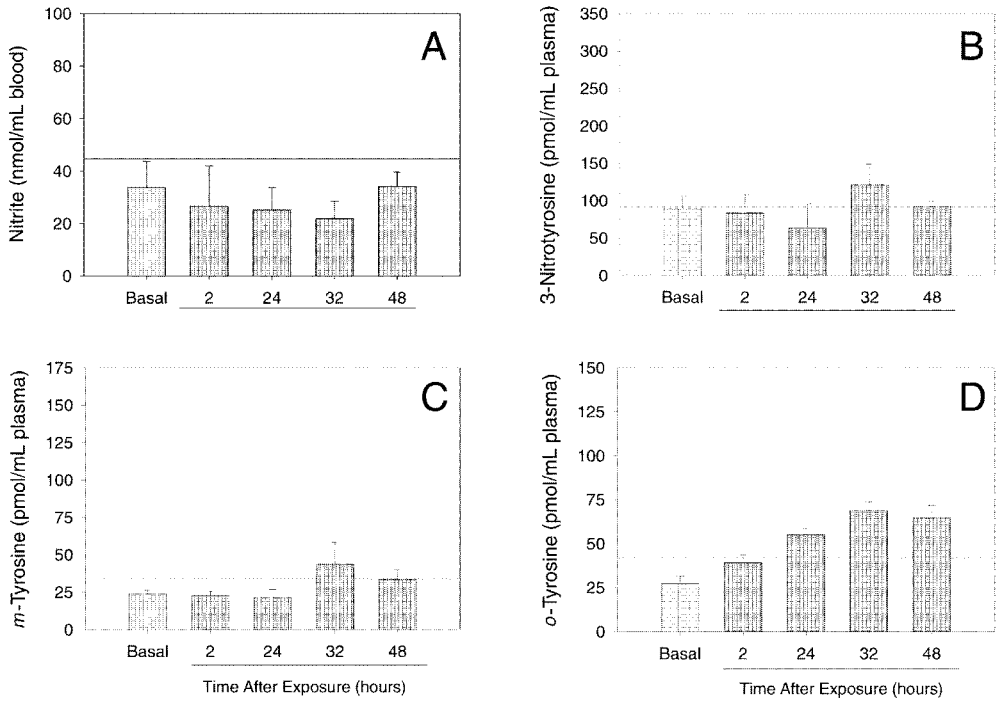

Figure 22. Levels of nitrite in blood and tyrosines in plasma of rats before and after exposure to EHC-93 particles (48 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (nitrite, n = 41; tyrosines, n = 26). Basal = before exposure. Bars are means ± SE for n = 2–9 animals. We found no significant atmosphere × time interval interactions ($P > 0.05$); therefore, these endpoints were not tested further.

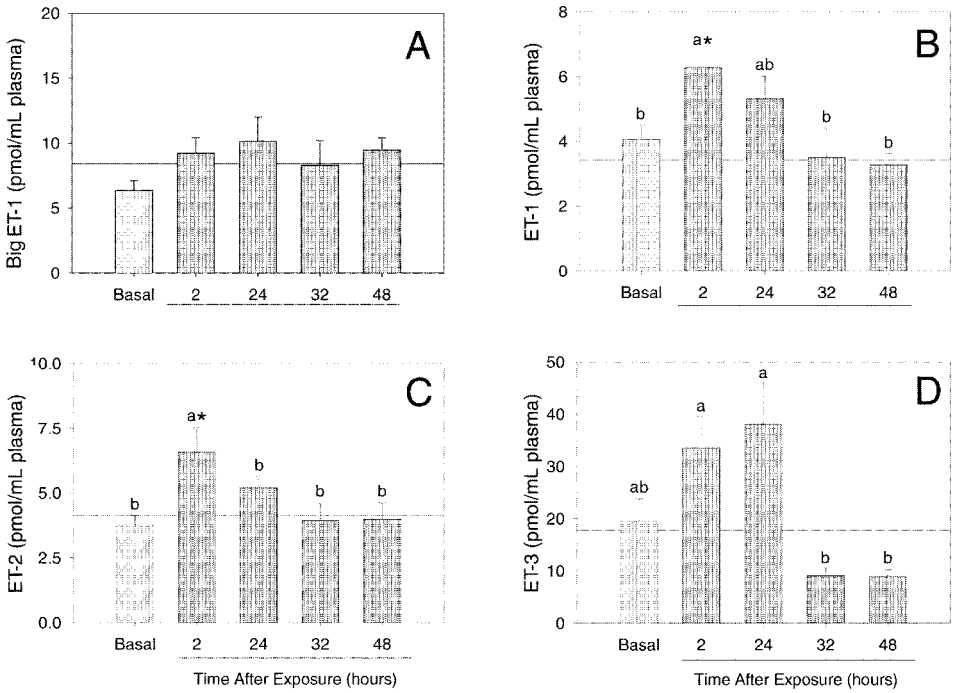

Figure 23. Levels of endothelins in rat plasma before and after exposure to EHC-93L particles (49 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (n = 35–36). Basal = before exposure. Bars are means ± SE for n = 4–9 animals. The significant atmosphere × time interval interactions identified by two-way ANOVA were further tested by one-way ANOVA within each exposure group (ET-1, $P = 0.004$; ET-2, $P = 0.012$; ET-3, $P < 0.001$) and the pattern of significant effects was elucidated with the Dunnett and Tukey multiple comparison procedures. An asterisk (*) indicates a value significantly different from the basal value by the Dunnett procedure ($P < 0.05$). Bars with different letters are statistically different by the Tukey procedure ($P < 0.05$).

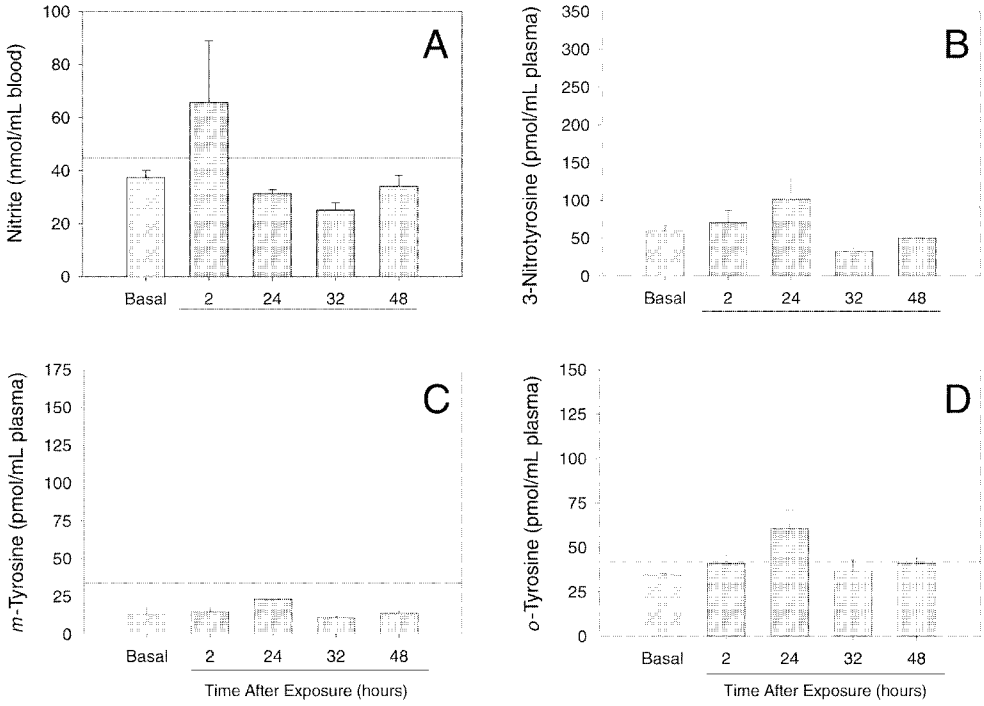

Figure 24. Levels of nitrite in blood and tyrosines in rat plasma before and after exposure to EHC-93L particles (49 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (nitrite, n = 41; tyrosines, n = 26). Basal = before exposure. Bars are means ± SE for n = 3–9 animals. We found no significant atmosphere × time interval interactions ($P > 0.05$); therefore, these endpoints were not tested further.

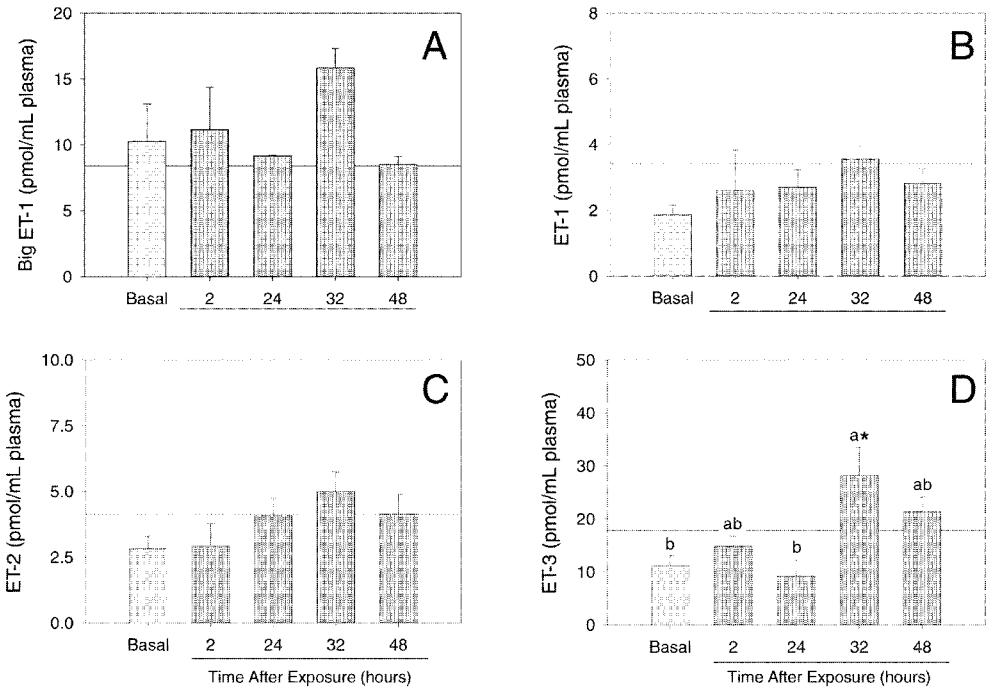

Figure 25. Levels of endothelins in rat plasma before and after exposure to diesel soot (4.2 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (n = 35–36). Basal = before exposure. Bars are means ± SE for n = 2–6 animals. The significant atmosphere × time interval interactions identified by two-way ANOVA were further tested by one-way ANOVA within each exposure group (ET-1, P = 0.239; ET-2, P = 0.317; ET-3, P = 0.017) and the pattern of significant effects was elucidated with the Dunnett and Tukey multiple comparison procedures. An asterisk (*) indicates a value significantly different from the basal value by the Dunnett procedure ($P <$ 0.05). Bars with different letters are statistically different by the Tukey procedure ($P < 0.05$).

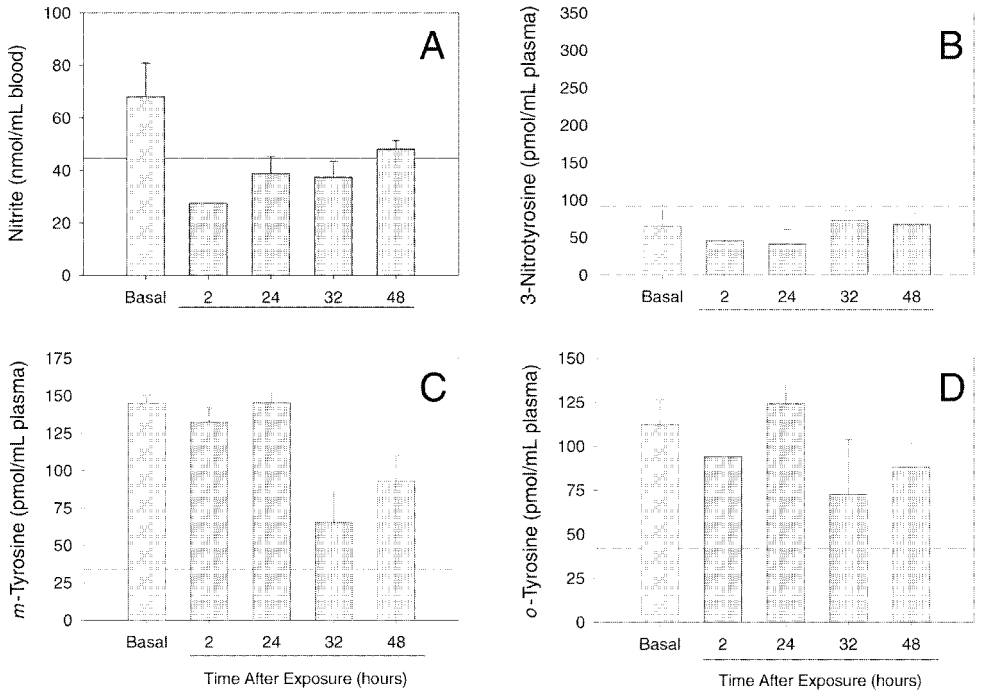

Figure 26. Levels of nitrite in blood and tyrosines in rat plasma before and after exposure to diesel soot (4.2 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (nitrite, n = 41; tyrosines, n = 26). Basal = before exposure. Bars are means ± SE for n = 2–6 animals, except for nitrate at 2 hours when n = 1. We found no significant atmosphere × time interval interactions ($P > 0.05$); therefore, these endpoints were not tested further.

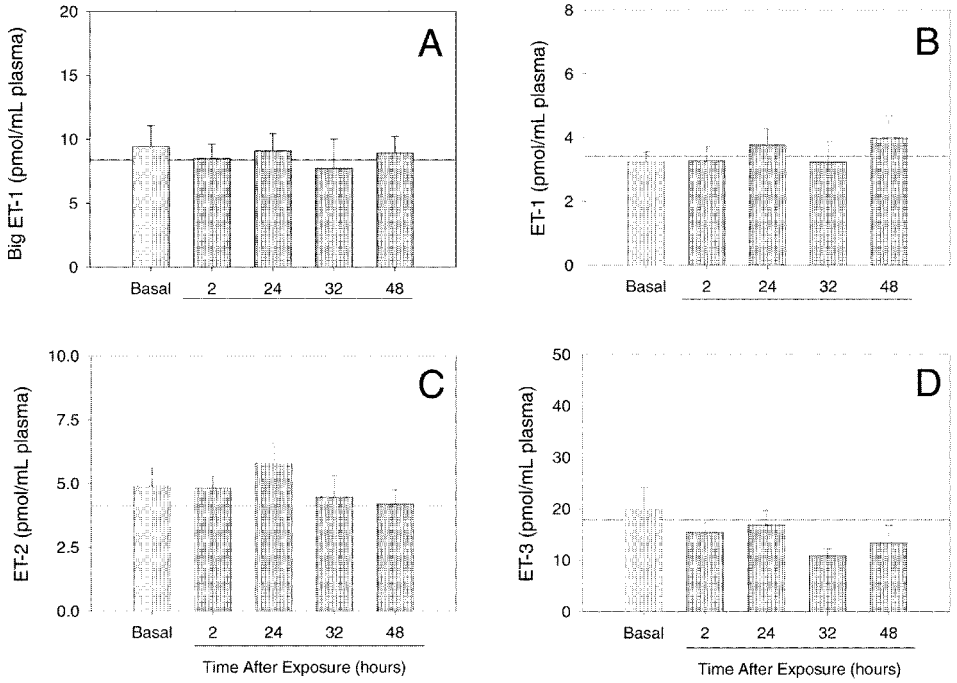

Figure 27. Levels of endothelins in rat plasma before and after exposure to carbon black particles (4.6 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (n = 35–36). Basal = before exposure. Bars are means ± SE for n = 4–10 animals. The significant atmosphere × time interval interactions identified by two-way ANOVA were further tested by one-way ANOVA within each exposure group (ET-1, P = 0.758; ET-2, P = 0.565; ET-3, P = 0.416). We found no significant atmosphere × time interval interactions ($P > 0.05$); therefore, these endpoints were not tested further.

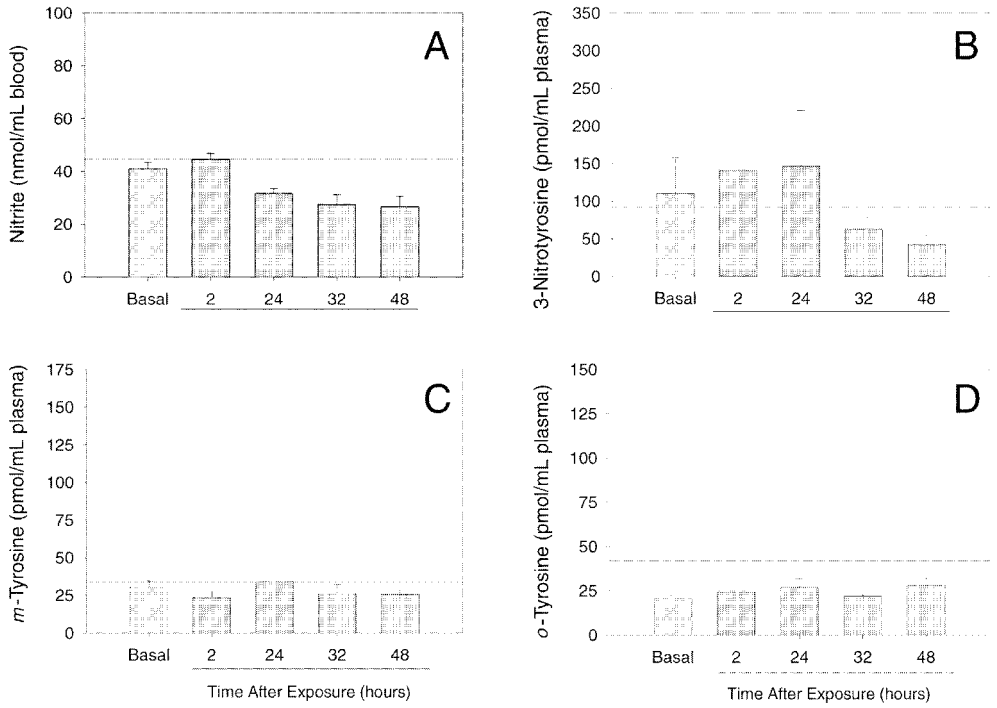

Figure 28. Levels of nitrite in blood and tyrosines in rat plasma before and after exposure to carbon black particles (4.6 mg/m³) for 4 hours. The reference line is the mean value before exposure for all animals analyzed in the study from which a preexposure blood sample could be obtained (nitrite, n = 41; tyrosines, n = 26). Basal = before exposure. Bars are means ± SE for n = 5–11 animals. We found no significant atmosphere × time interval interactions ($P > 0.05$); therefore, these endpoints were not tested further.

verified for normality and analyzed by three-way ANOVA with atmosphere (clean air, EHC-93, EHC-93L, diesel soot, carbon black), surgery (cannula alone, cannula plus radiotransmitter), and thrombolytic agent (none, tissue plasminogen activator or urokinase) as the factors, followed by the Tukey multiple comparison procedure ($\alpha = 0.05$). We found no statistically significant three-way or two-way factor interactions ($P > 0.05$) and no significant surgery or thrombolytic agent main effects ($P > 0.05$). The atmosphere main effect was statistically significant ($P < 0.05$). These analyses indicated that the surgery and thrombolytic agent variables did not confound data interpretation.

The results for the analyses of catecholamines are summarized in Table 12. We detected significant two-way interactions by ANOVA (atmosphere × time interval, $P < 0.05$) for the norepinephrine, dopamine, and L-DOPA endpoints. This indicated that for some of the exposure groups, the values of these endpoints changed over time. By examining the data and the results of the multiple pairwise comparisons, we were able to attribute this pattern of significant effects mainly to some elevated early values in the animals of the EHC-93 and diesel soot exposure groups. The basal values (preexposure) and the early time point values for norepinephrine in the animals exposed to EHC-93 and for dopamine and L-DOPA in the animals exposed to diesel soot were unusually high by comparison with (1) the values at the later time points or (2) the values of the other exposure groups at the early time points (eg, air-exposed control animals). Elevation of these catecholamines in the EHC-93 and diesel soot exposure groups was not attributed to particle effects because the basal preexposure values were also elevated. The changes were possibly related to an incomplete recovery from the surgery in a subset of the animals.

DOSIMETRIC MODELING

Elemental analyses of the EHC-93 particles (Table 13) sampled at the inhalation ports and recovered after size

Table 12. Catecholamines in Plasma of Rats After Exposure to Each Experimental Atmosphere[a]

		Hours After Exposure			
Endpoint	Basal	2	24	32	48
Clean Air					
n	8	7	4	10	12
Epinephrine	0.96 ± 0.14	0.91 ± 0.15	1.05 ± 0.17	0.95 ± 0.07	0.92 ± 0.09
Norepinephrine	0.32 ± 0.06	0.36 ± 0.07	0.33 ± 0.06	0.29 ± 0.04	0.22 ± 0.03
Dopamine	32.38 ± 9.90	48.23 ± 16.17	66.16 ± 31.48	71.64 ± 14.32	49.82 ± 7.71
L-DOPA	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
EHC-93 Particles					
n	3	4	2	6	9
Epinephrine	0.96 ± 0.23	1.00 ± 0.13	1.21 ± 0.07	0.95 ± 0.09	0.98 ± 0.05
Norepinephrine	0.64 ± 0.09	0.51 ± 0.09	0.40 ± 0.00	0.25 ± 0.01	0.24 ± 0.02
Dopamine	30.26 ± 1.31	46.99 ± 3.30	52.18 ± 0.67	50.70 ± 6.82	46.91 ± 6.46
L-DOPA	0.11 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.05 ± 0.01
EHC-93L Particles					
n	3	7	6	6	6
Epinephrine	0.73 ± 0.34	0.72 ± 0.17	0.69 ± 0.21	0.65 ± 0.12	0.77 ± 0.14
Norepinephrine	0.19 ± 0.01	0.20 ± 0.02	0.16 ± 0.03	0.12 ± 0.02	0.17 ± 0.01
Dopamine	17.20 ± 2.22	22.49 ± 3.10	32.57 ± 5.67	24.91 ± 6.17	31.84 ± 5.48
L-DOPA	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.05 ± 0.01
Diesel Soot					
n	2	2	2	3 or 4[b]	6
Epinephrine	1.12 ± 0.13	1.09 ± 0.00	1.38 ± 0.02	0.80 ± 0.20	1.05 ± 0.08
Norepinephrine	0.49 ± 0.04	0.52 ± 0.04	0.57 ± 0.03	0.27 ± 0.05	0.35 ± 0.05
Dopamine	131.09 ± 12.77	102.06 ± 3.77	116.51 ± 10.49	54.83 ± 27.96	84.02 ± 15.30
L-DOPA	0.13 ± 0.00	0.11 ± 0.02	0.12 ± 0.01	0.07 ± 0.02	0.06 ± 0.01
Carbon Black Particles					
n	9	5	6	6	7
Epinephrine	1.03 ± 0.02	0.97 ± 0.05	1.08 ± 0.06	0.95 ± 0.04	0.89 ± 0.02
Norepinephrine	0.28 ± 0.03	0.33 ± 0.04	0.31 ± 0.04	0.21 ± 0.02	0.17 ± 0.01
Dopamine	27.53 ± 3.13	26.72 ± 3.63	51.11 ± 17.68	45.14 ± 6.28	34.75 ± 6.20
L-DOPA	0.08 ± 0.00	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.06 ± 0.01

[a] Data are means ± SE.

[b] Three animals for norepinephrine and L-DOPA; four animals for epinephrine and dopamine.

fractionation in the cascade impactors were conducted to gain more insight into what elements were internally deposited in the animals. Aluminum, calcium, magnesium, silicon, and strontium were enriched in the bulk material or total port samples by comparison with the fine particles (having an effective cutoff diameter of 1.2 μm). In particular, the mass fraction of aluminum, magnesium, and silicon decreased by 50% from the bulk particles to the fine fraction; this was expected because of crustal material that contributes to the coarse fraction and the impaction and sedimentation of the larger particles in the inhalation system. Boron, cobalt, copper, lead, molybdenum, nickel, and vanadium were enriched in the fine particle fraction compared with the bulk preparation or the port samples of the resuspended particles.

Extraction of water soluble material from the EHC-93 particles to prepare the EHC-93L leached particles for inhalation studies did not significantly modify the relative amounts of most individual elements per unit of particle mass; exceptions were cadmium (−56%), calcium (−26%), sodium (−90%), and zinc (−47%) (Table 14). Sequential extraction of the particles in water for preparative purposes was done at a high solid-to-liquid ratio and the high ionic strength of the solution might have prevented full extraction of soluble elements. The analyses of elemental solubility on the various size fractions of the EHC-93 particles were done at increasing solid-to-liquid ratios (Figure 29). Extracting the highly soluble elements, such as zinc, was essentially complete (water-soluble yield, 11–12 mg/g, 95%) at very low solid-to-liquid ratios (Figure 29A). Thus, it is possible that only about half of the bioavailable zinc or cadmium might have been extracted during the bulk preparation of the EHC-93L material.

To simulate the elements being solubilized in the alveoli, which is a dynamic environment and for which we assume rapid diffusion of elements in a virtually unlimited volume, we estimated the solubility of each element by extrapolating the solubility curves to the Y-intercept (Table 15) and used these estimates in the dosimetric calculations. The concentration of each element in the three different size modes (Table 16) was calculated by attributing the mass of elements in the 1.2-μm ECD samples (Table 13) to mode 1 (1.3–1.6 μm MMAD), the mass of elements in the 3-μm ECD samples minus the 1.2-μm ECD samples to mode 2 (3.5–3.6 μm MMAD), and the mass of elements on the port filters minus the elements on the 1.2-μm ECD samples and on the 3-μm ECD samples to mode 3 (14–15 μm MMAD). These values were then used to calculate elemental depositions in the pulmonary compartment (Table 17) and in the tracheobronchial compartment (Table 18).

Comparative estimates of internal doses of the soluble elements in the rats from the present experiments and for extreme but plausible scenarios of human exposure to fine particulate mass are presented in Table 19. The experimental concentrations of the soluble elements in our experiments with rats were more than 3 orders of magnitude higher than the high ambient concentrations to which humans may be exposed (eg, the 95th percentile of ambient elemental concentrations associated with $PM_{2.5}$ in major Canadian cities [Burnett et al 2000]). Because lung deposition efficiencies in humans are 3- to 5-fold higher than in rats, the estimated doses of most soluble elements in the rats were 1 or 2 orders of magnitude higher than the human estimates. There were exceptions, however, and extreme values of internal doses such as soluble calcium, magnesium, and sodium could indicate a strong contribution of road salt to the chemistry of our EHC-93 particulate material. Calcium, magnesium, sodium, and zinc were

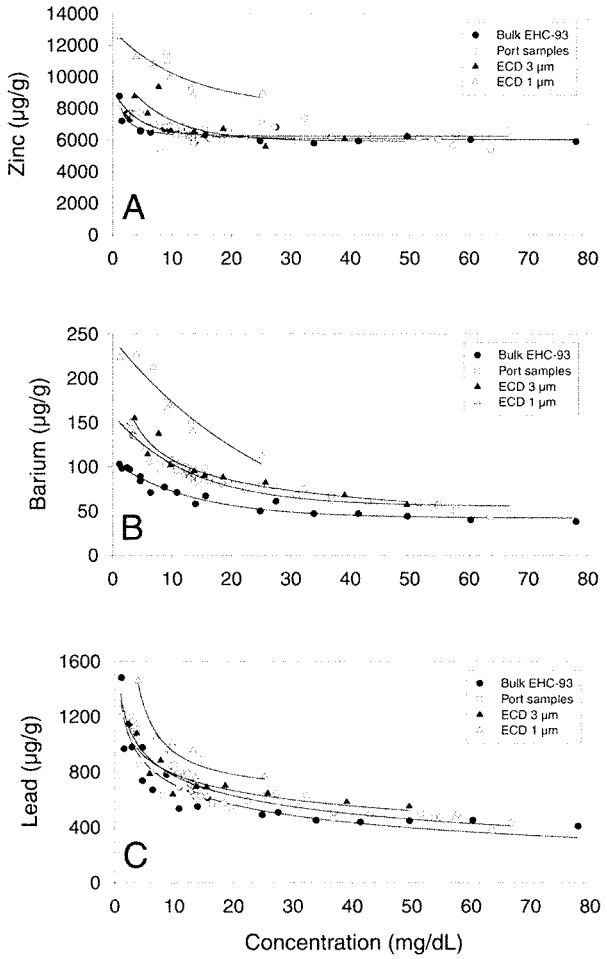

Figure 29. Solubility of elements. Data are presented as the relative mass of soluble recovered material (μg/g) as a function of the solid-to-liquid ratio (mg/dL).

Table 13. Elemental Content of EHC-93 Particles[a]

Element	Bulk EHC-93 (μg/g)	Port Filters (μg/g)	Effective Cutoff Diameter of 1.2 μm (μg/g)	Effective Cutoff Diameter of 3.0 μm (μg/g)
Aluminum	23.7×10^3	18.8×10^3	13.8×10^3	17.7×10^3
Barium	411	417	354	420
Boron	61	44	98	68
Cadmium	23	18	20	18
Calcium	122×10^3	103×10^3	91.3×10^3	99.3×10^3
Chromium	70	110	NQ	NQ
Cobalt	11	9	23	10
Copper	763	845	1423	960
Iron	20.2×10^3	14.8×10^3	15.0×10^3	14.8×10^3
Lead	6.78×10^3	6.71×10^3	8.2×10^3	7.48×10^3
Magnesium	15.2×10^3	11.5×10^3	7.12×10^3	9.3×10^3
Manganese	594	560	652	555
Molybdenum	19	15	32	15
Nickel	60	80	375	144
Silicon	95.4×10^3	70.3×10^3	40.1×10^3	85.0×10^3
Sodium	23.9×10^3	22.7×10^3	24.7×10^3	23.6×10^3
Strontium	363	307	228	285
Tin	1.23×10^3	764	826	881
Titanium	1.83×10^3	1.74×10^3	1.56×10^3	1.63×10^3
Vanadium	120	150	230	170
Zinc	11.2×10^3	9.22×10^3	11.5×10^3	9.93×10^3

[a] NQ = not quantified.

Table 14. Elemental Content of EHC-93L Particles[a]

Element	Bulk EHC-93L (μg/g)	Port Filters (μg/g)	Ratio of EHC-93L to EHC-93	
			Bulk	Port Filters
Aluminum	25.4×10^3	17.8×10^3	1.02	0.91
Barium	470	416	1.13	0.99
Boron	48	38	0.72	0.86
Cadmium	8	8	0.35	0.44
Calcium	114×10^3	77.6×10^3	0.91	0.73
Chromium	71	NQ	1.01	NQ
Cobalt	9	10	0.82	1.11
Copper	742	804	0.94	0.95
Iron	22.5×10^3	16.2×10^3	1.10	1.06
Lead	7.21×10^3	7.08×10^3	1.04	1.05
Magnesium	15.8×10^3	9.88×10^3	1.01	0.85
Manganese	584	478	0.97	0.84
Molybdenum	18	15	0.95	1.00
Nickel	66	75	1.17	0.97
Silicon	112×10^3	76.6×10^3	1.17	1.05
Sodium	6.48×10^3	2.37×10^3	0.29	0.10
Strontium	378	269	1.03	0.85
Tin	1.30×10^3	946	1.06	1.24
Titanium	2.01×10^3	1.80×10^3	1.10	0.97
Vanadium	140	172	1.13	1.15
Zinc	5.23×10^3	4.98×10^3	0.46	0.53

[a] NQ = not quantified.

Table 15. Water Solubility of Elements in EHC-93 Particles[a]

	Bulk EHC-93		Port Filters		Effective Cutoff Diameter of 1.2 μm	
Element	(μg/g)	(% soluble)	(μg/g)	(% soluble)	(μg/g)	(% soluble)
Aluminum	650	3	558	3	462	4
Barium	123	32	160	38	250	71
Boron	20	33	20	45	70	71
Cadmium	16	70	16	100	16	80
Calcium	65×10^3	53	79×10^3	74	92×10^3	97
Chromium	NQ	NQ	NQ	NQ	NQ	***1***
Cobalt	2	18	2	22	NQ	***20***
Copper	500	66	308	36	651	44
Iron	350	2	400	3	500	3
Lead	1.5×10^3	22	1.5×10^3	22	1.5×10^3	18
Magnesium	3×10^3	20	3×10^3	25	3×10^3	40
Manganese	220	37	210	37	240	35
Molybdenum	1	5	2	13	3	9
Nickel	20	33	25	31	35	9
Silicon	NQ	NQ	NQ	NQ	NQ	***1***
Sodium	19×10^3	78	19×10^3	82	20×10^3	73
Strontium	225	63	225	71	200	89
Tin	25	2	25	3	25	3
Titanium	10	0	10	1	10	1
Vanadium	20	17	25	17	40	17
Zinc	10×10^3	89	10×10^3	100	11×10^3	95

[a] NQ = not quantified. Values presented in bold italic type are estimated values.

Table 16. Distribution of Elements in the Size Distribution Modes of Resuspended EHC-93 Particles[a]

Element	EHC-93 Atmosphere (μg/g)	Mode 1 1.3–1.6 μm MMAD (μg/g)	Mode 2 3.5–3.6 μm MMAD (μg/g)	Mode 3 14–15 μm MMAD (μg/g)
Aluminum	18.8×10^3	13.8×10^3	19.9×10^3	20.2×10^3
Barium	417	354	457	414
Boron	44	98	51	14
Cadmium	18	20	17	18
Calcium	103×10^3	91.3×10^3	104×10^3	108×10^3
Chromium	110	***110***	***110***	***110***
Cobalt	9	23	3	8
Copper	845	1.42×10^3	700	701
Iron	14.8×10^3	15.0×10^3	14.7×10^3	14.8×10^3
Lead	6.71×10^3	8.20×10^3	7.08×10^3	5.76×10^3
Magnesium	11.5×10^3	7.12×10^3	10.5×10^3	14.6×10^3
Manganese	560	652	500	565
Molybdenum	15	32	5	15
Nickel	80	375	14	0
Silicon	70.3×10^3	40.1×10^3	110×10^3	52.6×10^3
Sodium	22.7×10^3	24.7×10^3	23.0×10^3	21.6×10^3
Strontium	307	228	317	334
Tin	764	826	912	621
Titanium	1.74×10^3	1.56×10^3	1.67×10^3	1.88×10^3
Vanadium	150	230	136	125
Zinc	9.22×10^3	11.5×10^3	9.05×10^3	8.34×10^3

[a] Values presented in bold italic type are estimated values.

Table 17. Modeling of Element Deposition in the Pulmonary Compartment[a]

	Total Mass		Soluble Mass		Insoluble Mass	
Element	ng	pg/cm²	ng	pg/cm²	ng	pg/cm²
Aluminum	5.1×10^3	1.5×10^3	203	60	4.9×10^3	1.4×10^3
Barium	124	36	88	26	36	11
Boron	26	7.5	18	5.3	7.4	2.2
Cadmium	6.0	1.8	4.8	1.4	1.2	0.4
Calcium	30×10^3	8.9×10^3	29×10^3	8.6×10^3	910	267
Chromium	***8.9***	***2.6***	***0.1***	***0.0***	***8.8***	***2.6***
Cobalt	4.9	1.4	***1.0***	***0.3***	***3.9***	***1.2***
Copper	366	108	160	47	205	60
Iron	4.7×10^3	1.4×10^3	141	41	4.6×10^3	1.3×10^3
Lead	2.4×10^3	723	443	130	2.0×10^3	593
Magnesium	2.6×10^3	778	1.1×10^3	311	1.6×10^3	467
Manganese	188	55	66	19	122	36
Molybdenum	7.0	2.1	0.6	0.2	6.4	1.9
Nickel	77	23	6.9	2.0	70	21
Silicon	21×10^3	6.1×10^3	***208***	***61***	***21 × 10³***	***6.1 × 10³***
Sodium	7.6×10^3	2.2×10^3	5.5×10^3	1.6×10^3	2.1×10^3	604
Strontium	82	24	73	22	9.1	2.3
Tin	271	80	8.1	2.4	263	77
Titanium	505	149	5.1	1.5	500	147
Vanadium	62	18	11	3.1	51	15
Zinc	3.3×10^3	985	3.2×10^3	935	167	49

[a] Values presented in bold italic type are estimated values.

Table 18. Modeling of Element Deposition in the Tracheobronchial Compartment[a]

	Total Mass		Soluble Mass		Insoluble Mass	
Element	ng	pg/cm²	ng	pg/cm²	ng	pg/cm²
Aluminum	3.6×10^3	160×10^3	144	6.4×10^3	3.5×10^3	154×10^3
Barium	87	3.9×10^3	62	2.7×10^3	25	1.1×10^3
Boron	16	714	11	507	4.7	207
Cadmium	4.0	176	3.2	141	0.8	35
Calcium	21×10^3	930×10^3	20×10^3	902×10^3	627	28×10^3
Chromium	***6.5***	***288***	***0.1***	***2.9***	***6.4***	***285***
Cobalt	2.8	124	***0.6***	***25***	***2.2***	***98***
Copper	229	10.2×10^3	101	4.5×10^3	128	5.7×10^3
Iron	3.2×10^3	142×10^3	96	4.2×10^3	3.1×10^3	137×10^3
Lead	1.6×10^3	73×10^3	295	13×10^3	1.3×10^3	60×10^3
Magnesium	1.9×10^3	84×10^3	756	34×10^3	1.1×10^3	50×10^3
Manganese	124	5.5×10^3	43	1.9×10^3	81	3.6×10^3
Molybdenum	4.1	180	0.4	16	3.7	164
Nickel	42	1.9×10^3	3.8	169	38	1.7×10^3
Silicon	16×10^3	713×10^3	***161***	***7.1 × 10³***	***16 × 10³***	***706 × 10³***
Sodium	5.1×10^3	228×10^3	3.7×10^3	166×10^3	1.4×10^3	61×10^3
Strontium	58	1.6×10^3	52	2.3×10^3	6.4	286
Tin	186	8.3×10^3	5.6	249	181	8.0×10^3
Titanium	347	15×10^3	3.5	154	343	15×10^3
Vanadium	39	1.8×10^3	6.7	298	33	1.5×10^3
Zinc	2.2×10^3	98×10^3	2.1×10^3	93×10^3	110	4.9×10^3

[a] Values presented in bold italic type are estimated values.

Table 19. Human Plausible Doses and Rat Experimental Doses for Soluble Elements[a]

Element	Fine Particle Mass Deposited in Humans During a 24-Hour Exposure (Dep P = 0.25, Dep TB = 0.05)[b]				EHC-93 Particles (50 mg/m^3) Deposited in Rats During a 4-Hour Exposure	
	95th Percentile[c]		Per 250 μg/m^3 [d]			
	Pulmonary (pg/cm^2)	Tracheobronchial (pg/cm^2)	Pulmonary (pg/cm^2)	Tracheobronchial (pg/cm^2)	Pulmonary (pg/cm^2)	Tracheobronchial (pg/cm^2)
Aluminum	7.6	256	4.6	155	60	6.4×10^3
Barium	0.1	4.1	1.2	39	26	2.7×10^3
Boron	NA	NA	9.2	311	5.3	507
Cadmium	0.0	1.4	0.0	1.2	1.4	141
Calcium	2.0	67	23	776	8.6×10^3	902×10^3
Chromium	0.1	1.9	0.1	1.6	***0.0***	***2.9***
Cobalt	0.0	0.7	0.0	0.2	***0.3***	***25***
Copper	0.2	7.3	1.2	39	47	4.5×10^3
Iron	2.1	72	4.6	155	41	4.2×10^3
Lead	1.6	53	0.6	19	130	13×10^3
Magnesium	0.7	23	2.3	78	311	34×10^3
Manganese	0.3	11	0.5	16	19	1.9×10^3
Molybdenum	0.0	0.6	0.0	0.8	0.2	16
Nickel	0.1	1.6	0.1	1.6	2.0	169
Silicon	3.1	104	1.8	62	***61***	***7.1×10^3***
Sodium	7.2	242	23	776	1.6×10^3	166×10^3
Strontium	0.0	0.4	0.1	3.9	22	2.3×10^3
Tin	0.1	4.2	0.0	0.8	2.4	249
Titanium	0.2	5.0	1.2	39	1.5	154
Vanadium	0.1	3.4	0.1	2.3	3.1	298
Zinc	0.8	28	2.3	78	935	93×10^3

[a] NA = not available. Values presented in bold italic type are estimated values.

[b] The fraction of inhaled material deposited in each lung compartment: 25% in pulmonary; 5% in tracheobronchial.

[c] Deposited dose estimated from the 95th percentile of mean daily $PM_{2.5}$ concentrations in eight major Canadian cities between 1986 and 1996 (Burnett et al 2000).

[d] Estimated for an assumed mean daily air concentration of 250 μg/m^3.

among the most abundant water-soluble elements in the EHC-93 preparation (Table 15).

DISCUSSION AND CONCLUSIONS

The question of the biologic plausibility of adverse health impacts of low levels of ambient respirable particles is a very complex one that requires a plurality of experimental models and investigative approaches. We implemented an approach that exposed animals by inhalation to resuspended ambient particles collected in batches. These particles collected and stored in dry form have likely been subject to some mixing of chemical species among size modes and to some level of physical aggregation, which would produce a larger MAD. The increased size may have reduced the number of particles that reached the respiratory compartment such that effects similar to those seen in our study could conceivably result from even lower environmental concentrations of ambient particulate matter.

We have no evidence that storage and resuspension of the EHC-93 particles has modified their overall chemical composition by creating new toxic species, although it is likely that short-lived reactive species produced from photochemical reactions in the atmosphere did not persist. At any rate, no current evidence suggests that adverse human health effects are actually driven by labile chemical species that are not represented in the resuspended EHC-93 material, or that the potency of the resuspended material

does not fall within the large span of toxic potencies of fresh environmental materials.

A number of critical aspects to the question of the biologic plausibility of adverse effects need to be addressed in an experimental framework; many of these aspects are not very sensitive to the issue of the chemical stability of the collected particles. Resuspended EHC-93 particles used in our previous work and in the current study are a reasonable alternative to fresh ambient material; they are a useful and reproducible tool for testing pathophysiologic and toxicologic paradigms and for identifying new effects endpoints and candidate mechanistic pathways of effects that can later be tested in human cohorts, in laboratory animals exposed to polluted environments, or in animals and human subjects exposed to concentrated ambient particles.

Ottawa urban particles EHC-93 have been shown to have cytotoxicity and biologic activity comparable to $PM_{2.5}$ and PM_{10} in cell cultures (Vincent et al 1997b). The EHC-93 material is directly toxic to the lungs by intratracheal instillation in saline (Adamson et al 1999a,b) and has a potency comparable to that of residual oil fly ash (Costa and Dreher 1997). In previous experiments, when healthy F344 rats inhaled EHC-93 particles simultaneously with ozone, the exposure resulted in severe structural changes in the lungs characterized by acute epithelial injury associated with a strong interstitial inflammatory response. The magnitude of these changes was several-fold higher than that measured after the rats inhaled ozone alone (Bouthillier et al 1998; Vincent et al 1997a). However, when healthy rats inhaled EHC-93 particles alone, only mild focal interstitial inflammatory changes were observed and no evidence of epithelial injury was apparent, although elevation of cytokine production could be documented in macrophages isolated by lung lavage (Bouthillier et al 1998; Vincent et al 1997a).

Despite this apparent innocuousness (based on absence of acute structural injury) of inhaled EHC-93 particles in the lungs of healthy F344 rats, a significant elevation of immunoreactive endothelins was detected in plasma 24 hours after exposure (Bouthillier et al 1998). That inhaled EHC-93 particles can mediate a systemic response characterized by an increase in circulating levels of endothelins without any evidence of acute structural injury or any apparent diffuse inflammatory changes in the lungs was verified in the present study conducted in Wistar rats.

The endothelin system consists of three isopeptides that have 21 amino acids (ET-1, ET-2, ET-3) that are coded by distinct genes (Yanagisawa et al 1988) and found in several cell types, including macrophages and endothelial, epithelial, and smooth muscle cells (Battistini et al 1993). Endothelins are synthesized first as preproendothelins that are cleaved by endoproteases to inactive precursors that have 38 to 41 amino acids (the big endothelins [big ET-1, big ET-2, big ET-3]), which in turn are converted into the mature and active 21–amino acid peptides by ECEs. The ECEs constitute a family of seven isoenzymes (ECE-1a, -1b, -1c, -1d, -2a, -2b, -3) associated with metalloproteases such as neutral endopeptidase (Corder 2001; Hasegawa et al 1998). The ECE-1 group is particularly important because it converts big ET-1 to ET-1 at the endothelial cell membrane surface (Turner and Murphy 1996; Turner and Tanzawa 1997).

Endothelial cells have been studied mainly with respect to the production of ET-1. Plasma levels of ET-1 are thought to reflect a spillover from endothelial secretion. The ET-3 isoform in the lungs has been localized in neuroepithelial bodies of the bronchioles and alveolar parenchyma (Seldeslagh and Lauweryns 1993), but inflammatory cells, smooth muscle cells, and epithelial cells may also contribute to the elevation of plasma ET-3. Endothelins affect the adjacent tissue in a paracrine fashion, being secreted basolaterally toward the underlying smooth muscle cells (Shigeno et al 1989; Wagner et al 1992). Endothelins also act as vasoactive hormones due to their luminal secretion or spillover into the blood; the half-lives of endothelins in plasma under normal conditions is 5 to 7 minutes (Anggard et al 1989; Shiba et al 1989; Kjekshus et al 2000).

Once released, endothelins exert their primary physiologic effects via specific receptors. Two major receptor subtypes have been cloned in mammalian tissues: ET_A and ET_B (Arai et al 1990; Sakurai et al 1990, 1992). The ET_A receptor subtype is selective for ET-1 and ET-2, whereas the ET_B receptor subtype has similar affinity for all three isopeptides. In the vasculature, the ETA receptor is localized on the vascular smooth muscle cells; binding of ET-1 and ET-2, and to a lesser extent of ET-3, to the ET_A receptor causes vasoconstriction. One ET_B receptor (ET_{B1} splice variant) is located on the endothelial cells; binding of endothelins to the ET_{B1} receptor stimulates the release of NO and prostacyclin, which are vasodilators. Another ET_B receptor (ET_{B2} splice variant) is located on the vascular smooth muscle cells; binding of endothelins to the ET_{B2} receptor causes vasoconstriction (Warner et al 1993).

In addition to being potent vasoconstrictors, endothelins are also potent bronchoconstrictors, promitogens, and inflammatory peptides; these characteristics reveal the importance of this humoral system in many cardiovascular diseases, such as congestive heart failure and hypertension (Teerlink et al 1994; Michael and Markewitz 1996; Kramer et al 1997; Moreau 1998; MacLean 1999; Schiffrin 2000; Spieker et al 2001; Giannessi et al 2001; Kiowski and

Sutsch 2001; Krum et al 2001; Lepailleur-Enouf et al 2001), vascular remodeling from atherosclerosis, post-angioplastic restenosis, and transplant vasculopathy (Barton 2000; Best and Lerman 2000; Tayara and Giaid 2000), and in asthma and other pulmonary disorders (Battistini and Warner 1997; Goldie and Henry 1999). Together with other vasoconstrictors such as the renin-angiotensin system (Brunner et al 1993) and eicosanoid-related lipids such as thromboxane A_2 and prostaglandin F_2 (Dusting et al 1979), the endothelins modulate vascular tone in opposition to vasorelaxants such as NO (Fields and Makhoul 1998), the atrial natriuretic peptide (Winquist 1987), and eicosanoid-related prostacyclin (Dusting et al 1979). Our observations reveal a novel pathway, independent of acute lung injury, by which inhaled particles alter the regulation of key effectors of cardiovascular homeostasis.

Based on ELISA determinations in earlier studies, we have reported a 15–20% increase of immunoreactive ET-1 in plasma of F344 rats 24 hours after inhalation exposure to EHC-93 (Bouthillier et al 1998). In the pilot phase of the present study, measurements of endothelins by HPLC indicated that inhalation of EHC-93 elevated ET-3 in F344 rats (not shown). In the main phase of the current study, plasma ET-1 increased on the order of 30% in Wistar rats 32 hours after inhalation exposure to EHC-93 particles, and plasma ET-3 increased on the order of 100% between 2 and 48 hours after exposure. Interestingly, exposure of Wistar rats to the EHC-93L leached particles appeared to cause only transient elevations of ET-1 and ET-2. The values of plasma ET-3 after EHC-93L exposure were not statistically different from the baseline values determined before exposure, but values at 2 and 24 hours after exposure were different from those at 36 and 48 hours; this confirms some time-dependent variations of the endothelin levels after exposure to the EHC-93L particles.

The NO levels in the Wistar rats were estimated from total nitrate, nitrite, and nitrosyl compounds (measured as nitrite) in blood, and from 3-nitrotyrosine in plasma. We did not detect significant changes of these NO endpoints in animals exposed to any of the particles. Nevertheless, even if the change was not statistically significant, of particular interest here was the progressive increase of *o*-tyrosine after exposure to EHC-93, reaching about 300% of baseline value after 48 hours. *o*-Tyrosine is produced by hydroxylation of phenylalanine and may indicate elevated hydroxyl radical flux from peroxynitrite degradation. Elevation of this endpoint may indicate an increased flux of superoxide and an increased level of oxidative stress in the lung capillary bed of the animals exposed to the EHC-93 particles, causing local endothelial dysfunction, depletion of NO, and enhanced production of 8-isoprostaglandin $F_{2\alpha}$ (Boulanger 1999, Drexler and Hornig 1999; Garcia-Palmieri 1997; Romero and Reckelhoff 1999).

In the present study, the levels of the catecholamines were somewhat variable and no significant treatment-dependent changes could be identified. Nevertheless, these endpoints should be useful in evaluating cardiovascular status in compromised animal models.

It is possible that EHC-93L leached particles deposited in the lungs caused some early changes in the regulation of endothelins, as suggested by the pattern of rapid but transient elevation of ET-1, ET-2, and ET-3. However, from the responses to the total EHC-93 material, it appears that those soluble components that had been leached from the EHC-93L particles were required for particles to cause a sustained elevation of plasma ET-3 and possibly of ET-1. Interestingly, inhalation of the diesel soot particles elevated plasma ET-3, and possibly ET-1 and ET-2, whereas the carbon black particles had no effect on plasma endothelins.

Of the four particulate matter preparations evaluated in this study, only EHC-93 particles produced a measurable increase in systemic blood pressure. This systemic response was statistically significant on the second day after exposure and coincided with elevation of plasma ET-1 and ET-3. We did not find strong correlations between the levels of endothelins in plasma and changes in systemic blood pressure. In normal rats, acute infusion of ET-3 in the jugular vein has been reported to cause an elevation of mean blood pressure, but the systemic effect disappeared after infusion continued for 3 days (Martinez et al 1996). One would expect that the vasopressor effect of ET-3 would be compensated by an elevation of NO production in these rats (Marshall and Johns 1999).

In human cardiac patients, elevated ET-3 is associated with systemic vasoconstriction (Cowburn et al 1999). Despite variability among humans and the large overlap of plasma endothelin levels between healthy and compromised individuals, some prognostic value of endothelins has been reported. For example, a 25% elevation of plasma ET-1 has a high predictive value for chronic heart failure whereas a 20% decrease of ET-1 in chronic heart failure patients is associated with improvement of symptoms (Galatius-Jensen et al 1996; Tsumamoto et al 1994, 1995). Plasma ET-1 is also a predictor of mortality 1 year after an acute myocardial infarct and it is known that acute increases of endothelins can be directly cardiotoxic by promoting infarct size and by altering heart electrophysiology (Alexiou et al 1998; Maxwell et al 1992; Omland et al 1994).

Changes in the homeostasis of endothelins and an ensuing vasopressor response after human subjects inhale ambient particles may be relevant not only to cardiac

pathophysiology but also to a number of other vascular conditions. For example, a significant negative correlation has been found between maternal plasma endothelin levels within 1 week before labor and birth weights in normal and preeclamptic pregnancies, presumably due to a pressor effect of the elevated endothelin and a decrease of uteroplacental blood flow (Furuhashi et al 1995). Interestingly, particulate air pollution has been associated with low birth weight in human populations (Bobak 2000).

More generally, ET-3 is a ligand of the ET_B receptor, which predominates in human lungs and airways (ET_A at 45% vs ET_B at 55%; Henry 1999). Therefore, in addition to cardiovascular pathophysiology, changes in the systemic levels of ET-3 could also be relevant to exacerbation of pathologic symptoms in both the lung parenchyma and the airways, or in other tissues where cell physiology and proliferation could be regulated by the ET_B receptor (Cambray et al 1994).

The increase of plasma ET-1 and ET-3 and the vasopressor response measured in the animals in this study may reveal only one aspect of a series of molecular mechanisms initiated by ambient particles. The functions of the heart and of vascular vessels are strongly regulated by complex and interactive short-term and long-term mechanisms geared to maintain blood pressure and an adequate delivery of oxygen to the body. These regulatory mechanisms include the sympathetic nervous system, the renin-angiotensin system, endothelins, vasopressin, prostaglandins, NO, bradykinin, and natriuretic peptides. In cardiovascular pathologic conditions, the balance between vasopressor and vasodilator factors is often offset, although the resulting blood pressure may be within normal limits. When overactivated, these regulatory systems have several detrimental long-term consequences on the heart and vascular vessels, affecting the way the entire cardiovascular system may respond to stimulation. For example, in patients with congestive heart failure, neurohormonal systems (eg, sympathetic nervous system, renin-angiotensin-aldosterone balance, endothelins, vasopressin) are activated, which results in a greater risk of arrhythmia, vasoconstriction, vascular remodeling, endothelial dysfunction, fluid and salt retention, and decreased baroreflex.

In turn, endothelial dysfunction (which is also common to other pathologic conditions such as atherosclerosis) may result in an impairment in endothelium-dependent relaxations, which could be due to either a decreased release or action of NO and other vasodilators (such as prostacyclin, atrial natriuretic peptide, or bradykinin), or a greater ability to evoke endothelium-dependent contractions, or both (Schriffin et al 1997, 1998; Vanhoutte 1997). The same stimuli, therefore, could elicit different responses depending on the neurohormonal levels or the pathologic process. In addition, changes in the level of one neurohormonal factor are most often associated with changes in the levels of other factors. It is often difficult to isolate these effects and to determine whether a change is the cause of a symptom, a reactive process, a feedback mechanism, or a combination of these. More work lies ahead to elucidate the full pathophysiologic significance of our observations; in particular, more in-depth studies of the effects of particle subfractions need to be conducted in animals with compromised cardiovascular conditions (such as hypertension, congestive heart failure, or renal failure).

Notwithstanding the limitations of our present study, the following patterns of effects are emerging clearly from our assessment in healthy rats: (1) inhaled urban particles can cause disregulation of endothelins in the lungs, and (2) modifying the chemical profile of the particles also modifies the pattern of response of the endothelin isoforms.

Leaching the EHC-93 particles in water removed a large fraction of the water-soluble elements and reduced the potency of the particles to cause hemodynamic effects. The elements cadmium, calcium, sodium, and zinc were notably reduced by the leaching process. Interestingly, we have verified that zinc in the EHC-93 particles can account for the acute lung injury caused by an intratracheal injection of the soluble fraction of EHC-93 (Adamson et al 1999a, 2000). Recent epidemiologic work also implicates iron, nickel, and zinc as elements more strongly associated with cardiac and pulmonary death (Burnett et al 2000) and these metals are known pneumotoxicants (Nemery 1990). Inhalation of zinc chloride aerosol is notoriously toxic to the lungs, causing at high dose a progressive clinical course resembling adult respiratory distress syndrome, although this toxicity has been attributed to the caustic effect of zinc chloride through the formation of zinc oxychloride in the respiratory tract (Evans 1945; Hjortso et al 1988; Macauley and Mant 1964). Zinc also plays a central role in gene regulation and in the activation of metalloproteases and endopeptidases; it is conceivable that deposition of free, ionized zinc (eg, as zinc sulfate) in the alveoli could create local cellular loads of the metal that interfere with cell regulation pathways (Ellerbroek and Stack 1999; Klug 1999; Palacek et al 1999; Samet et al 1998).

Remarkably, the endothelin-converting enzymes that cleave big ET-1 or big ET-3 into the potent peptides ET-1 and ET-3 are zinc-dependent endopeptidases (Lee et al 1999; Takahashi et al 1993). Zinc, administered as zinc sulfate to rats or as Zn^{2+} added to rat aortic smooth muscle cells and bovine pulmonary artery endothelial cells in culture, has been shown to inhibit NO formation, apparently

through an effect on NO synthase activity (Abou-Mohamed et al 1998). It is tempting to speculate that zinc in EHC-93 particles is a potency determinant with regard to the sustained elevation of ET-3 and the vasopressor response in the Wistar rats in this study. We expect that other elements and other chemical species are involved in the cardiovascular effects of particles and our observations begin to provide some insight into potential chemical determinants of the potency of ambient particles.

The exposure concentrations in the present study are high compared with ambient conditions. However, in evaluating the data, the internal dose of particles must be considered as more important than the exposure levels (Miller et al 1995). Rats are obligate nasal breathers and particles deposit in the nasopharyngeal compartment with very high efficiency. Furthermore, particles larger than 5 μm MAD do not penetrate to any significant extent into the pulmonary region in rats. The EHC-93 exposure atmospheres were characterized by two respirable size modes, with the finer mode at 1.3–1.6 μm MAD having a higher mass fraction of bioavailable toxic metals than the coarse mode (14–15 μm MAD). Our deposition calculations indicated that the alveolar doses (expressed as mass per unit of surface area) of a number of soluble elements of toxicologic relevance were within 1 or 2 orders of magnitude of the doses estimated for plausible human exposure scenarios: aluminum, barium, chromium, iron, nickel, and vanadium. Furthermore, the doses of some metals in the rats were 3 orders of magnitude higher than what could be expected from a high-level human exposure scenario. However, if we adopt a conservative approach and consider a 10-fold uncertainty factor for extrapolating our low-adverse-effect-level to a no-adverse-effect-level and another 10-fold uncertainty factor for interspecies extrapolation of doses and effects to human subjects with compromised cardiovascular systems, then the toxicologic doses in our experiments are within the boundaries of what is conventionally considered as relevant to human health.

Of course, more research is needed to confirm a relevance of the endothelin shifts in human subjects and in animals with compromised cardiovascular conditions, to conduct a dose-response assessment, and to clarify the identity of the agents of effects. Toward that end, our current findings provide a strong basis for assessing the biologic plausibility of human health impacts of ambient particulate matter.

Various epidemiologic investigations of respiratory and cardiovascular hospital admissions and mortality have shown that daily ambient particulate levels have acute impacts on health. It seems inconceivable that exposure to such low concentrations of particles could, over a 24-hour period, initiate in healthy individuals a primary lesion followed by a series of biologic events that could cascade into angina symptoms, arrhythmias, or a cardiac infarct. One could argue that if particles had such a high toxic potency, a gradation of clinical manifestations should be apparent in the general population during high-level pollution episodes.

Also, the epidemiologic evidence strongly implies that ambient particles act as nonthreshold contaminants. According to classical toxicologic concepts, there should be a level of exposure to contaminants, or a critical internal dose, at which defense and repair mechanisms compensate adequately for the low rate of molecular or biologic lesions. The difficulty in explaining in biologic terms the phenomenon of acute health impacts of low levels of ambient air contaminants may be due in part to an assumption that the contaminants are acting as direct toxicants, causing discrete structural or molecular lesions in target tissues with consequences that can be defined as clinically adverse. Seaton and colleagues (1995) have broken out of this classical toxicologic paradigm. They contend particles may not need to cause direct injuries with loss of organ function in order to cause adverse biologic effects. Rather, particles may interfere with biologic processes that are typically dependent on signaling pathways and may do so in a manner that could increase the probability of an adverse outcome. To illustrate their hypothesis, Seaton and associates have presented a case for a link between inflammatory events induced or amplified in the lungs by inhaled particles and the coagulation cascade, which would conceivably result in a higher incidence of cardiovascular accidents following an air pollution episode. According to this scheme, cytokines produced by cells in response to the particle or chemical stimuli enhance blood coagulation pathways, a process that could disseminate systemically. This process of enhanced blood coagulation may then be impacting adversely on health.

Whether or not ambient air contaminants actually promote blood coagulation to a significant level remains to be determined; but the basic concept is sound: that particles may disrupt physiologic regulatory mechanisms, causing adverse consequences when this occurs within a pathophysiologic process. Similar mechanisms can be imagined for pulmonary effects of particles, such as amplifying the production of cytokines and eicosanoid cascades in subjects with asthma.

Regulation of endothelins could conceivably be altered in humans by components of inhaled particles. In toxicologic terms, the changes we report in plasma ET-1 and ET-3 and the vasopressor response after inhalation of EHC-93

have the required characteristics of critical toxicologic endpoints for assessing inhaled ambient particles. Our current data indicate with reasonable certainty that the deposition of particles from ambient air in the lungs can modify the homeostasis of ET-1 and ET-3 and cause a vasopressor response in Wistar rats. As discussed above, the pathophysiologic significance of elevated plasma ET-1 and ET-3 has been established and elevation of endothelins can be detrimental to an individual with a compromised cardiovascular system (Teerlink et al 1994; Michael and Markewitz 1996; Kramer et al 1997). If we translate the biologic effects we observed in our animal model into a potential mechanism operative in the lungs of healthy human subjects, then particles might alter the regulation of ET-1 in the lung capillary bed and ET-3 in the parenchyma, which would be compensated by homeostatic regulation with little immediately perceptible consequences on health. In cardiac patients, the same particles would affect regulation of ET-1 and ET-3, but in the context of the pathophysiologic condition of a cardiovascular disease and precarious homeostasis. This elevation of ET-1 and ET-3, coupled with local oxidative stress enhancing endothelial dysfunction and local NO deficit, would next set the stage for a cascade of events on the paths to such outcomes as heart ischemia, infarct extension, or altered electrophysiology, any of which would increase the probability of hospital admission or death.

Our current study did not assess all stages of this event cascade. However, in light of the evidence presented here, it appears to us that it is not possible anymore to discount epidemiologic evidence of an association between inhalation of ambient particulate matter and acute cardiovascular outcomes on the basis of lack of evidence for biologically plausible pathways of effects. Our data clearly identify a plausible biologic pathway for such effects. The data from the present study could be seen as describing a corollary for the pattern of population health impacts and elucidating why inhalation of particles may be associated with such severe health outcomes as morbidity and mortality in a few individuals although apparently innocuous to most. The answer could be the interaction between intrinsic toxicity of the particles and individual sensitivity factors.

IMPLICATIONS OF THE FINDINGS

Human epidemiologic investigations over the past 20 years have provided strong evidence of acute health effects of low concentrations of ambient air particulate matter. Cardiac morbidity and mortality have been associated with daily variations in the concentrations of ambient particles lower than 30–50 $\mu g/m^3$. The exact biologic and toxicologic bases for the health impacts remain, for the most part, unclear. Our previous experimental work indicated that potency of respirable particles does not equate with high biologic reactivity or cytotoxicity and that direct induction of structural lung injury by ambient particles is not essential for causing adverse biologic responses. Our current data extend this concept and support these conclusions:

- Systemic distribution of toxicants may not be essential for an adverse impact on the heart from inhalation of ambient particulate matter.
- Particles act essentially by paracrine-endocrine disruption.
- The deposition of particles from ambient air in the lungs of rats can modify the homeostasis of ET-1 and ET-3 and cause a vasopressor response.
- Modifying the physicochemical characteristics of the particles modified their potency toward causing changes in cardiovascular endpoints.

More work needs to fully define the pathophysiologic significance of our observations. Nevertheless, from a perspective of public health protection, it is no longer possible to discount epidemiologic evidence of an association between inhalation of ambient particulate matter and acute cardiovascular outcomes on the basis of the lack of evidence for biologically plausible pathways of effects. The findings from this study contribute to the weight of evidence in favor of a biologic plausibility of health impacts at low-level exposures to ambient particles.

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ABBREVIATIONS AND OTHER TERMS

ACGIH	American Conference of Governmental Industrial Hygienists
ANOVA	analysis of variance
CAPs	concentrated ambient particles
ECD	effective cutoff diameter
ECE	endothelin-converting enzyme
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EHC-93	Ottawa ambient particles
EHC-93L	water-leached EHC-93 particles
ELISA	enzyme-linked immunosorbent assay
EPA	US Environmental Protection Agency
ET	endothelin
GSD	geometric standard deviation
HEPA	high-efficiency particulate air [filter]
HF	hydrofluoric acid
HNO_3	nitric acid
HPLC	high-pressure liquid chromatography
L-DOPA	3-(3,4-dihydroxyphenyl)-L-alanine
MAD	median aerodynamic diameter
MMAD	mass median aerodynamic diameter
NAAQS	National Ambient Air Quality Standard (US)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
NIST	National Institute of Standards and Technology (US)
NO	nitric oxide
NWRI	National Water Research Institute
$PM_{2.5}$	particulate matter 2.5 μm or smaller in aerodynamic diameter
PM_{10}	particulate matter 10 μm or smaller in aerodynamic diameter
RM ANOVA	repeated-measures analysis of variance
SRM	Standard Reference Material
TFA	trifluoroacetic acid

INTRODUCTION

Particulate matter (PM*) in ambient air is a complex mixture containing particles of different sizes and chemical composition. Epidemiologic studies have described an association between increases in cardiovascular morbidity and mortality and short-term increases in ambient PM levels (for a review, see US Environmental Protection Agency [EPA] 1996). However, the biologic mechanisms by which PM may affect cardiovascular events and the role of particle size and composition in PM toxicity are not well understood. Assessing the effects of particles of different composition in appropriate animal models is critical in understanding how PM may exert adverse health effects.

In 1998, HEI issued RFA 98-1, "Characterization of Exposure to and Health Effects of Particulate Matter", inviting proposals to investigate the health effects of PM. In response, Dr Renaud Vincent and colleagues at Health Canada proposed to study the cardiovascular response of rats to urban particles, alone or in combination with ozone, and to washed urban particles from which the water-soluble components had been removed. In addition to determining concentration-response information for particle exposure with and without ozone in normal rats, the authors proposed to test different animal models that were thought to be more sensitive to inhaled particles: senescent rats and cardiomyopathic hamsters. The biologic responses to be measured included acute lung injury, changes in heart function, and changes in vasoregulators in the blood. The exposures would be conducted with resuspended urban particles that had been collected previously on air filters at the Environmental Health Centre in Ottawa, Canada. The HEI Health Research Committee asked the investigators to conduct a one-year study to look at the cardiovascular effects of the particles alone, without ozone exposure. The investigators then proposed to study a single concentration of urban particles and washed particles, and compare their effects with those of an equivalent concentration of diesel soot and carbon particles; this plan would address how chemical composition could change the effects of PM on the cardiovascular system. HEI funded the proposal because the Health Research Committee thought the evaluation of the proposed cardiovascular endpoints was a novel and interesting approach to studying the possible mechanisms of PM's effects.†

This Commentary is intended to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations, and placing the report into scientific perspective.

BACKGROUND

The association between increased levels of PM and increased morbidity and mortality observed in epidemiologic studies conducted in different places is relatively consistent, but many questions remain about these results, including the nature of the pathobiologic mechanisms that might cause such effects. Toxicologic studies using many animal models and many types of particles have been initiated in the last 5 years. Particles from different sources have been investigated, including residual oil fly ash, diesel soot, carbon black, coal dust, metal oxides, and concentrated ambient particles (see for example Becker et al 1996; Costa and Dreher 1997; Nikula et al 1997; Murphy et al 1998). These particles differ substantially in their core composition and surface characteristics, and whether sulfates, transition metals, organic pollutants, or other toxic and nontoxic compounds have been adsorbed onto their surfaces. Whether particles show toxic effects in vitro or in vivo depends on, among other factors, their size and composition, which may be altered during storage and handling.

Some recent animal studies have shown that exposure to particles might produce pulmonary inflammation (Killingsworth et al 1997; Kodavanti et al 1997) or alterations in cardiac function (Godleski et al 2000). Individuals with preexisting lung or heart disease are hypothesized to be more susceptible to the health effects of particle exposure. Some recent epidemiologic studies have pointed in that direction (Burnett et al 1995; Peters et al 1997; Goldberg et al 2000), and toxicologic studies have begun to explore susceptibility in animal models of cardiopulmonary

*A list of abbreviations and other terms appears at the end of the Investigators' Report.

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†Dr Renaud Vincent's 13-month study, *Inhalation Toxicology of Resuspended Urban Particulate Matter*, began in December 1998. Total expenditures were $163,603. The draft Investigators' Report from Dr Vincent and colleagues was received for review in January 2000. A revised report received in June was accepted for publication in July 2000. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Commentary.

disease (see Kodavanti et al 1998). For instance, after intratracheal instillation of residual oil fly ash in rats with pulmonary vasculitis and hypertension, researchers observed significant mortality and enhanced airway responsiveness; the inflamed lungs in these rats showed amplified metal-mediated oxidant chemistry (Costa and Dreher 1997). In another study, significant pulmonary inflammation and an increase in tidal volume and peak expiratory flow were observed in animals with chronic bronchitis after exposure to concentrated ambient particles (Clarke et al 1999).

One of the outstanding questions is whether the induction of acute lung injury or inflammation is required for particles to have an effect on the cardiovascular system. Changes in cardiac and pulmonary responses were observed in the absence of significant airway inflammation in dogs exposed to concentrated ambient particles (Godleski et al 2000). Because lung tissue is heavily interspersed with capillary blood vessels for oxygen transport, compounds entering the lungs may easily reach and have an effect on the vascular tissue. In response to some stimuli, the vascular endothelium is known to secrete vasodilators, such as nitric oxide (NO), and vasoconstrictors, such as endothelins. Increased endothelin levels in blood could cause coronary artery constriction and thereby exacerbate preexisting heart conditions (see Parris and Webb 1997; Kjekshus et al 2000).

Endothelins belong to a recently discovered family of peptides, which are potent vasoconstrictors (Yanagisawa et al 1988). All the known active endothelins (ET-1 through ET-3) are generated by cleavage of larger peptides, called "big" endothelins (Zellers and Vanhoutte 1991). Endothelin-1 and ET-3 are principally produced by the vascular endothelium; ET-2 is produced in intestinal mucosa, lung, and kidney (Cunningham et al 1997). Endothelin-1 contributes to the maintenance of basal vascular tone and blood pressure by activating vascular smooth-muscle receptors and, at the same time, acts through endothelial receptors to stimulate formation of NO (Haynes and Webb 1998). Because NO is a vasodilator, its production counteracts the effects of endothelins. Thus, the NO and endothelin systems are in a homeostatic relation to maintain the integrity of the vascular system (Warner 1999). This makes the endothelin and NO systems prime candidates for evaluating PM's effects on cardiovascular health.

In a previous study, Vincent found that exposure to ozone, but not to urban particles, led to acute lung injury in rats (Vincent et al 1997). In contrast, exposure to urban particles, but not to ozone, caused an increase in ET-1 in blood (Bouthillier et al 1998). Vincent and colleagues hypothesized that after particles are deposited in the alveoli, soluble components such as metals would diffuse to the capillary bed and affect endothelin regulation. Vincent proposed to further investigate the effects of urban particles on the endothelin system and other vasoactive substances and to determine whether the soluble components of the urban particles were responsible for the observed effects. The effects of urban particles and washed urban particles would be compared with the effects of diesel soot and carbon black. In addition, the investigators would study whether acute lung injury was necessary for the cardiac system to be affected and whether these particles caused acute changes in heart physiology.

TECHNICAL EVALUATION

AIMS AND OBJECTIVES

The aim of the study was to test the hypothesis that urban PM has adverse health effects on the cardiovascular system by causing changes in blood vasoactive substances. The investigators compared the effects of urban PM, washed urban PM, diesel soot, and carbon black exposure in rats on heart physiology, lung tissue injury, and levels of vascular substances in blood.

STUDY DESIGN

Wistar rats were exposed in nose-only chambers for 4 hours to clean air or one of four types of resuspended particles: ambient urban PM that had been collected in 1993 and stored at −80°C, urban PM washed in water to remove soluble components, diesel soot, or carbon black. For each type of particle, the experimental groups consisted of six rats for physiologic measurements and six rats for histologic measurements. The investigators measured several endpoints in blood plasma (endothelins, NO products, and catecholamines), physiological endpoints (electrocardiogram [ECG], heart rate, blood pressure, and general activity), and lung tissue injury. Using surgical techniques, the investigators implanted rats with telemetry devices for continuous measurement of cardiac function endpoints and with indwelling catheters for repeated blood sampling. Resuspended particles rather than fresh particles were used to ensure that the particle concentrations and chemical compositions were identical among experiments.

METHODS

Particle Exposure Conditions

Ambient urban PM was recovered from air filters in 1993 and stored until used. Dust was mechanically sieved

to a size of less than 36 mm in diameter. Part of the recovered PM was suspended in water and washed four times to remove soluble components (washed urban PM). Diesel soot and carbon black were obtained from external sources. The particles were dispersed and directed to the nose-only exposure manifold. Particle counts and size measurements were taken during exposure sessions and particle samples were collected for chemical analysis. Concentrations reached 48 mg/m^3 for urban PM, 49 mg/m^3 for washed urban PM, 4.2 mg/m^3 for diesel soot, and 4.6 mg/m^3 for carbon black. The investigators based the target concentration of 50 mg/m^3 for washed and urban PM on a previous toxicologic study in their laboratory (Bouthillier et al 1998). Diesel soot and carbon black were tested at 5 mg/m^3 due to limited availability of diesel particles.

Animals and Surgical Procedures

Rats were housed in cages with filtered air systems. Separate groups of six rats were exposed to one of the four kinds of particles; control rats were exposed to filtered air during each particle exposure session. A telemetry transmitter was implanted in the abdomen of each animal in the cardiovascular group for continuous monitoring of cardiac endpoints; an indwelling catheter was simultaneously inserted in the jugular vein for repeated blood sampling. After recovery from surgery for 5 to 8 days, animals were adapted to the nose-only exposure chamber for 5 days before the exposure sessions started. Additional groups of six rats were exposed to measure histologic endpoints.

Endpoints

The investigators measured four endothelins in blood plasma before and at 2, 24, 32, and 48 hours after exposure. To measure the effect of particles on the homeostatic balance between endothelins and NO, the investigators estimated NO (which is highly reactive and cannot be measured directly) from blood compounds that contain NO. The investigators also measured protein oxidation markers to monitor the integrity of the vascular endothelium and plasma catecholamines, which indicate whether the animals were stressed during and after exposure. The investigators used the implanted telemetry transmitters to continuously monitor heart rate, ECG, blood pressure, body temperature, and the animal's general activity. Activity was measured by the relative position of the transmitter over the antenna receiver board underneath the cage and was a general indicator of movement. Injury to lung tissue was evaluated histologically and by ^{3}H-thymidine incorporation into airway cells.

Statistical Analysis

Values obtained at different time points after exposure were compared with preexposure values for each individual animal. Telemetry data were summarized as 18-hour averages, tested for normality, and tested for significance for each exposure type separately using a one-way repeated-measures analysis of variance (ANOVA) with day (day before exposure, or day 1 or day 2 after exposure) as the factor. Biochemistry data were analyzed by two-way ANOVA with exposure type (clean air, urban particles, washed particles, diesel soot, or carbon black) and time (before, 2, 24, 32 or 48 hours after exposure) as factors. A significant two-way interaction would indicate a difference between particle exposures over the time period tested, but would not specify which exposure condition or time point was driving the significance. Therefore, additional one-way ANOVAs were performed for each particle type with time as the factor. Direct comparisons among exposure conditions were not performed. Due to cannula blockage over time, the data sets for blood measurements contained many missing values, which complicated the statistical analysis. Also, animals were added that had a cannula but no transmitter implanted. Possible confounding by surgery (with or without radiotransmitter) and addition of a thrombolytic agent to extend cannula patency was analyzed using a three-way ANOVA with particle type, surgery, and thrombolytic agent as factors.

RESULTS: COMMENTS ON KEY FINDINGS

A summary of the findings is presented in Commentary Table 1. The main findings were an increase in plasma ET-1 at 32 hours, ET-3 between 2 and 48 hours, and blood pressure on the second day after exposure to urban particles. The effect on blood pressure disappeared when washed urban particles were used, which suggests that one or more of the soluble components on the urban particles may be responsible for the effects on blood pressure.

Exposure to washed urban particles caused a transient increase in ET-1 and ET-2 at 2 hours after exposure and an increase in ET-3 at 2 and 24 hours after exposure that was followed by a decrease at 32 and 48 hours.

Exposure to diesel soot caused an increase in ET-3 at 32 hours and a trend toward an increase in ET-1 and ET-2 at 32 hours, but no change in blood pressure. Carbon black exposure had no significant effect on any parameters measured.

Other changes were observed such as an increase in activity after exposure to filtered air, urban PM, and washed urban PM, as well as a trend toward an increase in

Commentary Table 1. Summary of Key Findings

Endpoint	Exposure Atmosphere[a]				
	Filtered Air (0 mg/m³)	Urban PM (48 mg/m³)	Leached Urban PM (49 mg/m³)	Diesel Soot (4.2 mg/m³)	Carbon Black (4.6 mg/m³)
Telemetry Measurements					
Heart rate	(↑ Day 2)[b]	—[c]	—	—	—
Mean blood pressure	—	↑ Day 2[d]	—	—	—
Systolic blood pressure	—	↑ Day 2	—	—	—
Diastolic blood pressure	—	↑ Day 2	—	—	—
QA interval	—	—	—	—	—
Body temperature	—	—	—	—	—
Activity	↑ Day 2	↑ Day 2	—	↑ Day 2	—
Blood Measurements					
Endothelins					
Big endothelin-1	—	—	—	—	—
Endothelin-1	—	↑ 32 hours	↑ 2 hours	(↑ 32 hours)	—
Endothelin-2	—	—	↑ 2 hours	(↑ 32 hours)	—
Endothelin-3	—	↑ 2, 32, 48 hours	↑ 2, 24/↓ 32, 48 hours[e]	↑ 32 hours	—
Nitric Oxide Compounds					
Nitrite	—	—	—	—	—
3-Nitrotyrosine	—	—	—	—	—
m-Tyrosine	—	—	—	Basal[f]	—
o-Tyrosine	—	(↑ 24, 32, 48 hours)	—	Basal	—
Catecholamines					
Epinephrine	—	—	—	—	—
Norepinephrine	—	Basal	—	—	—
Dopamine	(↑ 32 hours)	(↑ 2, 24, 32, 48 hours)	—	Basal	(↑ 24, 32 hours)
L-DOPA	—	—	—	Basal	—
Lung Histology					
Autoradiography	—	—	—	—	—
Morphology	—	—	—	—	—

[a] Particle concentrations are given below each atmosphere. Animals were exposed once for 4 hours by nose-only inhalation.

[b] Time points in parentheses indicate a trend towards an increase that was either not significant or not formally tested.

[c] A dash (—) indicates no significant change.

[d] A significant change is indicated by an arrow and the time point (in hours or days) after exposure when the endpoint was significantly changed compared with the preexposure value.

[e] Time points separated by a slash (/) indicate a significant increase followed by a significant decrease.

[f] Basal = an increased baseline value (before exposure) compared with the filtered-air control group (not formally tested).

heart rate after exposure to filtered air. None of the four types of particles caused changes in lung tissue structure or cell proliferation. No significant changes were observed in levels of NO-containing compounds or catecholamines. Some preexposure basal levels of NO-containing compounds and catecholamines were higher in two of the particle-exposed groups than in the control group: norepinephrine in the urban PM exposure group and *m*-tyrosine, *o*-tyrosine, dopamine, and L-DOPA in the diesel soot exposure group.

DISCUSSION

The HEI Health Review Committee found the investigators' findings of a possible link between exposure to PM and the endothelin system novel and interesting. The comparison of several classes of particles and the array of cardiovascular endpoints evaluated were impressive features of this carefully conducted study. The observed difference between urban PM and washed urban PM in their effects

on blood pressure strengthens the evidence that particle composition affects toxicity. In washed urban PM, the soluble components such as certain metal compounds had been removed, which led to reduced toxicity in this study. This interesting result warrants further research into which soluble components of urban PM might be causing adverse health effects. The investigators suggested that zinc is one candidate component because an enzyme that contains zinc, endothelin-converting enzyme, catalyzes the formation of the biologically active form of endothelins from the big endothelin precursors (see Turner and Tanzawa 1997). Whether zinc is one of the causative components needs to be investigated further. If confirmed, these results could open an avenue of research into the mechanism of PM health effects.

In light of the potential importance and the need to replicate the findings, the HEI Health Review Committee identified some issues that may be further explored in future studies.

ENDOTHELINS

The finding that urban PM and diesel soot had their greatest effect on ET-3 strengthens the confidence in these data. The observed effect on ET-1 after exposure to urban PM confirms previous findings by the investigators (Bouthillier et al 1998).

The conclusion that exposure to urban PM changes endothelin levels could be strengthened by using several PM concentrations to obtain exposure-response information, which had been proposed originally by the investigators. Future studies should explore dose-response relations of the effects of particles on endothelins. Another possibility would be to conduct reconstitution experiments in which urban PM would be reconstituted from washed particles; this could demonstrate whether or not adding the soluble components to the washed particles would restore the biologic effects or determine which soluble components restore the activity. Such experiments could also explore which components of the soluble fraction might be responsible for the effects on endothelins.

PARTICLES

The use of resuspended particles rather than fresh particles was justified because resuspension enhances the consistence of the administered dose and the chemical composition of the particles among experiments. In addition, the toxicity of this urban PM mixture had been demonstrated in both cell culture and in vivo experiments (Vincent et al 1997; Goegan et al 1998). The relevance of the effects of resuspended particles to those obtained with fresh particles is in question, however, because storage most likely modified both chemical physical properties. Due to aggregation, these particles are presumably larger than fresh particles and it is unclear whether the larger particles penetrate the deep lung. The investigators were aware of these issues and characterized the particles both chemically and physically to address the problem.

Future experiments should explore whether fresh particles, such as concentrated ambient particles or fresh diesel exhaust, would cause similar changes in endothelins as the resuspended urban PM and diesel soot. In addition, the current results need to be confirmed with lower concentrations of urban PM. The investigators had originally proposed to also test urban PM at 0.5 and 5 mg/m^3 but were requested by HEI to focus their efforts and test only one concentration. The lowest proposed concentration approaches environmental levels; for example, the 24-hour National Ambient Air Quality Standard for PM_{10} is 0.15 mg/m^3 (US EPA 1998).

ANIMALS AND SURGERY

The investigators were successful in implementing an animal model with combined telemetry and blood sampling devices. Keeping catheters patent for repeated blood sampling is increasingly difficult as time progresses; thus it is understandable that the investigators opted for the shortest possible recovery time after surgery. However, surgeries as extensive as those performed on these animals require extensive recovery time. Some physiologic parameters may not return to normal until 2 to 3 weeks after such surgery (Harper et al 1996). The short recovery time after surgery, which varied in length among animals, may have produced increased variability in baseline levels or a drifting baseline for the telemetry measurements during the experiment. This might have reduced the chance that significant changes could be detected because the effects of PM were likely to be subtle. Whether this was the case in the current study remains unclear.

BLOOD MEASUREMENTS

The selection of several endothelins and NO compounds was clearly justified for this study. Many other vasoactive substances could be investigated in future experiments. For example, prostacyclin is an important pulmonary vasodilator similar to NO that is also produced by the epithelium (Zellers and Vanhoutte 1991). Other candidates would include proinflammatory cytokines and chemokines, angiotensin II, thrombomodulin, and bradykinin, all of which are involved in vasoregulation. The investigators did not specify why they selected big ET-1

for measurement and not big ET-2 or ET-3. Because ET-3 changed in response to three of the four exposure atmospheres, it may be especially worthwhile to measure big ET-3.

TELEMETRY MEASUREMENTS

The increase in blood pressure after exposure to urban PM was small and its significance is unclear because the stress caused in the animals by handling and surgery may have been substantial as reflected in the variability of the baseline physiologic parameters and the high levels of blood catecholamines.

Continuous telemetry via implanted radiotransmitters has clear advantages because the animals remain undisturbed during measurements. Thousands of data points per second were captured continuously over 6 to 7 days for heart rate, blood pressure, ECG waveforms, body temperature, and animals' activity. Out of the data collected, the investigators selected for analysis only 10 seconds from every 5-minute interval on the days each animal was monitored. Given the wealth of unanalyzed data, it would be interesting to further analyze heart rate and blood pressure data with a focus on variations in daily patterns. Evidence of additional transient changes in these physiologic parameters after exposure to PM would strengthen the case that PM may have adverse effects on the cardiovascular system. Furthermore, it would be interesting to see the results of the ECG measurements, which were not included in this report, in a future publication.

LUNG INJURY ASSESSMENT

The investigators were interested in assessing lung injury after particle exposure. They used one particular assay, ^{3}H-thymidine incorporation in lung tissue, to assess changes in lung structure and possible injury. This assay measures cell proliferation several days after exposure; the investigators found no evidence of changes in the structure of the epithelial lung tissue or of cell proliferation for any of the particles they evaluated. However, lung inflammation is a complex process that can be monitored by changes in inflammatory markers in lung lavage fluid over the course of hours to days after exposure. In fact, previous studies by Vincent and colleagues with the urban particles used in the current study found increased levels of some inflammatory markers in lung lavage fluid, such as macrophage inflammatory protein-2 (Bouthillier et al 1998), macrophages, and polymorphonuclear leukocytes (Adamson et al 1999). The current study could not include such assays due to time limitations. It is possible, however, that exposure to washed urban particles, diesel soot, or carbon black might also have affected these markers of lung inflammation.

STATISTICS

The investigators used repeated-measures ANOVAs when individual animals could be followed over time, as with the telemetry data.

Measurements of vascular substances in blood were taken at different time points in groups of rats of various sizes; some groups had only 2 or 3 rats, which reduced the power of the statistical testing. In addition, the preexposure baseline levels of several blood parameters were higher than in control groups. These two factors together may have reduced the possibility of finding significant results in the vascular substances.

The fact that catheters tended to clog over time resulted in many missing values in the database. Estimated least-squares means were used to fill in the gaps during statistical testing. This approach to handling missing data underestimates the true variance in the datasets; the impact of this approach on the conclusions is not known.

SUMMARY AND CONCLUSIONS

The investigators found consistent increases in ET-1, ET-3, and blood pressure after exposure to resuspended ambient particles. Smaller, transient effects were observed in ET-1, ET-2, and ET-3 after exposure to washed urban particles, but no change in blood pressure was noted. A similar increase in ET-3, a trend toward an increase in ET-1 and ET-2, and a lack of effect on blood pressure were found after exposure to diesel soot. No effect on endothelins or blood pressure was observed after exposure to carbon black. No effect of any particle type was observed on big ET-1, NO-containing compounds, heart rate, or body temperature. Some changes in heart rate and general activity were observed after exposure to filtered air, most likely due to the exposure conditions and the stress of handling. The observation that high concentrations of resuspended urban particles and diesel soot caused changes in endothelins and cardiovascular parameters opens an interesting avenue to further explore the possible mechanisms by which PM may cause adverse cardiovascular events. More research is needed to assess whether changes in endothelin levels would occur in humans exposed to ambient levels of particulate matter, and whether these changes may contribute to the adverse cardiovascular events that have been associated with air pollution episodes in epidemiology studies.

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