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Pulmonary Effects of Inhaled Diesel Exhaust in Young and Old Mice: A Pilot Project

Debra L. Laskin, Gediminas Mainelis, Barbara J. Turpin, Kinal J. Patel, and Vasanthi R. Sunil

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with a Critique by the HEI Health Review Committee

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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI research and analyses to public and private decision makers.

HEI receives half of its core funds from the U.S. Environmental Protection Agency and half from the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or certain research programs. HEI has funded more than 280 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in the peer-reviewed literature and in more than 200 comprehensive reports published by HEI.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Health Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Health Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Health Review Committee are widely disseminated through HEI's Web site (*www.healtheffects.org*), printed reports, newsletters and other publications, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 151, *Pulmonary Effects of Inhaled Diesel Exhaust in Young and Old Mice: A Pilot Project*, presents a research project funded by the Health Effects Institute and conducted by Dr. Debra L. Laskin, of the Department of Pharmacology and Toxicology, Rutgers University, and the Environmental and Occupational Health Sciences Institute, Piscataway, New Jersey, and her colleagues. This report contains three main sections.

The HEI Statement, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Health Review Committee's comments on the study.

The Investigators' Report, prepared by Laskin and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

The Critique is prepared by members of the Health Review Committee with the assistance of HEI staff; it places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Health Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.

HEI STATEMENT

Synopsis of Research Report 151

Biologic Effects of Inhaled Diesel Exhaust in Young and Old Mice

BACKGROUND

Exposure to particulate matter (PM) has been associated with increases in cardiopulmonary morbidity and mortality, with elderly people particularly susceptible. However, biologic pathways that might explain why the elderly are more susceptible than younger people to the effects of PM have not been examined extensively. In response to HEI's Request for Preliminary Applications 05-3, issued in 2005, Dr. Debra L. Laskin of Rutgers University and the Environmental and Occupational Health Sciences Institute and colleagues submitted an application for a study to explore possible differences in the responses of young and old mice exposed to diesel exhaust. The investigators proposed to evaluate the hypothesis that the increased susceptibility of elderly animals to PM results from impairment of the capacity of lung cells — alveolar macrophages, specifically — to produce the cytokine tumor necrosis factor α (TNF-α), as compared with lung-cell production of TNF-α by young animals. Laskin and colleagues reasoned that although TNF-α is proinflammatory — that is, it plays a central role in the induction of oxidative stress (a pathway emerging as a plausible mechanism to explain the adverse effects of exposure to PM) and inflammatory responses — TNF-α is also thought to induce protective, antioxidant defenses and tissue-repair mechanisms, and thus it may play a role in limiting the extent of inflammatory responses and injury.

The investigators proposed to test this hypothesis by comparing the production of TNF-α, other markers of the inflammatory response, and molecules involved in antioxidant defenses in young and elderly mice exposed to diesel exhaust emissions, a component of PM found in urban air.

APPROACHES

Laskin and colleagues developed and characterized an animal inhalation exposure system, using diesel exhaust from a Yanmar 406-cc diesel-powered electric generator operated with diesel fuel containing < 500 ppm sulfur and 40-weight motor oil. Young (2-month-old) and old (18-month-old) male CB6F1 mice were exposed for 3 hours on one day ("single exposure") or for 3 hours on each of three consecutive days ("repeated exposure") to diesel exhaust at a concentration of 300 μg/m^3 or 1000 μg/m^3 PM or to filtered air.

The investigators assessed the lungs of mice histologically immediately after and 24 hours after the end of exposure for qualitative changes in markers of inflammation (such as edema and numbers of macrophages and neutrophils) and expression of the manganese-dependent isoform of superoxide dismutase, an enzyme involved in antioxidant defenses. They also measured messenger RNA (mRNA) and protein levels of TNF-α and several molecules associated with inflammation and injury in lung tissue and bronchoalveolar-lavage fluid. These molecules included interleukin-8 (a cytokine that recruits neutrophils into tissues in response to an inflammatory stimulus), interleukin-6 (an acute-phase protein, a component of the rapid systemic response to infectious or other agents), cyclooxygenase-2 (an enzyme that synthesizes prostaglandins, which are involved in inflammatory responses), and lipocalin 24p3 (another acute-phase protein). They also measured levels of TNF-α in the blood.

RESULTS AND INTERPRETATION

The original hypothesis of the study was that TNF-α production would be impaired in old animals

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Debra L. Laskin of the Department of Pharmacology and Toxicology, Rutgers University, Piscataway, New Jersey, and colleagues. Research Report 151 contains both the detailed Investigators' Report and a Critique of the study prepared by the Institute's Health Review Committee.

exposed to diesel exhaust. In their final submitted report, the investigators extended their hypothesis to suggest that exposure to diesel exhaust may differentially affect molecules involved in inflammation and protective antioxidant pathways in young and old mice. However, after single and repeated exposures of young and old mice to diesel exhaust, the pattern of changes in TNF-α levels did not differ between the young and old mice in a straightforward, easily interpreted way. Some qualitative differences were found in inflammatory endpoints measured histologically in lung tissue, with more changes detected in the old than the young mice. However, this pattern of inflammatory changes in the lung was not consistent with the pattern of changes in bronchoalveolar-lavage fluid. The investigators also found that the effects of diesel exhaust exposure on expression of the manganese-dependent isoform of superoxide dismutase differed between young and old mice, as did mRNA levels of cyclooxygenase-2. The mRNA expression of lipocalin 24p3 in the lung increased in more diesel exposure scenarios in old than in young mice.

In its independent evaluation of the study, the HEI Review Committee thought that Laskin and colleagues had succeeded in creating a diesel exposure system and generating preliminary data on multiple endpoints in response to the inhalation of diesel exhaust in young and old mice. The Committee further thought that the investigators' hypothesis to explain the greater susceptibility of the elderly to adverse cardiovascular effects after exposure to PM was novel and interesting. However, the original hypothesis — that TNF-α production would be impaired in elderly animals exposed to diesel exhaust — was not supported by the findings, and the complexity of the pattern of changes in other endpoints in young and old animals exposed to diesel exhaust made it challenging to interpret the study findings in a clear-cut fashion. Thus, it was difficult to make links between changes in expression of the molecules measured in the study and age-related changes in susceptibility to diesel exhaust exposure. The Review Committee further noted that whereas the investigators interpreted the increases in lipocalin 24p3 mRNA expression in the lungs of old mice as representing an increase in oxidative-stress responses, the Committee regarded this molecule as being only peripherally related to oxidative stress. Finally, the Review Committee noted that strides have been made in diesel particulate control technology and that the sulfur content of diesel fuel has been reduced very substantially in recent years. Thus, future emissions of PM from diesel engines are expected to be much lower than those found in the past. Hence, the relevance of the emissions derived from the diesel generator used in the current study to emissions from future diesel engines is uncertain. Further studies are needed to assess hypotheses and biologic response pathways that may explain why the elderly are more susceptible to exposure to PM than the young and healthy.

INVESTIGATORS' REPORT

Pulmonary Effects of Inhaled Diesel Exhaust in Young and Old Mice: A Pilot Project

Debra L. Laskin, Gediminas Mainelis, Barbara J. Turpin, Kinal J. Patel, and Vasanthi R. Sunil

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ABSTRACT

It is well established that exposure to ambient fine particulate matter (PM*), defined as PM ≤ 2.5 μm in aerodynamic diameter ($PM_{2.5}$), is associated with increased cardiovascular morbidity and mortality and that elderly persons are particularly susceptible to these effects. We speculated that the increased susceptibility of elderly persons to PM is due to altered production of inflammatory mediators and antioxidants in the lung. We performed pilot studies in an animal model to test this hypothesis. For these studies we used diesel exhaust (DE), a major component of urban PM, as a model. Two groups of male CB6F1 mice, 2 months and 18 months old, (referred to in this report as young and old mice, respectively) were exposed to DE at 300 or 1000 μg/m^3 PM (referred to as low- or high-dose DE, respectively), or to filtered air as a control, for one 3-hour period (single exposure) or for 3 hours on each of three consecutive days (repeated exposure). Mice were killed and bronchoalveolar lavage (BAL) fluid, serum, and lung tissue were collected immediately after exposure (0 hours) and 24 hours after the final exposure. After single or repeated exposure to DE, persistent structural alterations and inflammation were observed in the lungs of old mice. These changes consisted of patchy thickening of alveolar septa and an increase in the number of neutrophils and macrophages in alveolar spaces. In the young mice, in contrast, no major alterations in lung histology were noted. In old but not in young mice, significant increases in messenger RNA (mRNA) expression of the oxidative-stress marker lipocalin 24p3 were also observed. In both young and old mice, exposure to DE was associated with increased expression of tumor necrosis factor α (TNF-α) mRNA in the lung. However, this response was attenuated in old mice. Exposure to high-dose DE resulted in significant increases in interleukin (IL)-6 and IL-8 mRNA expression in the lungs of old animals; these increases persisted for 24 hours. Whereas IL-6 was also up-regulated in young mice after DE exposure, no major effects were evident on the expression of IL-8 mRNA. Expression of the antioxidant enzyme manganese superoxide dismutase (MnSOD) was decreased in lung tissue from young animals after single or repeated exposure to DE. In contrast, constitutive expression of MnSOD was not evident in lungs of old mice, and DE had no effect on the expression of this antioxidant. These preliminary data confirm that old mice are more sensitive to DE than young mice and that increased sensitivity is associated with altered expression of inflammatory cytokines and the antioxidant MnSOD. These aberrations may contribute to the increased susceptibility of old mice to inhaled PM.

This Investigators' Report is one part of Health Effects Institute Research Report 151, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Debra L. Laskin, Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ 08854; laskin@eohsi.rutgers.edu.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83234701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

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

INTRODUCTION

Epidemiologic studies have demonstrated strong associations between hourly or daily changes in air pollution and cardiovascular morbidity and mortality (Dockery et al. 1992; Pope et al. 1992, 2004; Mauderly 2000; Samet et al. 2000; Peters et al. 2001, 2004; Brunekreef and Holgate, 2002; U.S. Environmental Protection Agency 2004; Forastiere et al. 2005; Park et al. 2005; Rich et al. 2005; Schwartz et al. 2005;

Vermylen et al. 2005). Of particular concern are ambient fine PM as a causal agent and gases such as ozone (O_3), carbon monoxide (CO), sulfur dioxide (SO_2), and nitrogen dioxide (NO_2). One of the most sensitive populations is elderly persons, (those over 65 years of age). Although underlying cardiopulmonary disease in this group is no doubt an important factor, the precise mechanisms mediating increases in susceptibility to the adverse effects of inhaled PM are unknown. Our research focused on the reasons for such heightened susceptibility. In previous studies using model PM mixtures, we found that lung injury in rodents is associated with increased production of TNF-α in the tissue (Morio et al. 2001; Laskin et al. 2003; Sunil et al. 2007a). Interestingly, this response was significantly attenuated in older animals (Sunil et al. 2007a). Although cytokines such as TNF-α, IL-1, IL-6, and IL-8 have classically been considered proinflammatory (that is, produced rapidly in response to tissue injury), recent evidence suggests that they also play an important role in initiating tissue repair later in the inflammatory process and in regulating inflammatory-cell trafficking into tissues (Murtaugh et al. 1996; Wong et al. 1996; Geiser et al. 2001; Chiu et al. 2003a, 2003b; Geiser 2003; McClintock et al. 2008). Thus, aberrant production of cytokines may be a factor in the enhanced susceptibility of the elderly to PM.

Since oxidative stress appears to be a common mechanism underlying the biologic effects of PM and other air pollutants, another important contributory factor may be age-related alterations in lung antioxidants (Kelly et al. 2003). These changes include reduced levels of superoxide dismutase (SOD) and vitamin C, and dysregulation of vitamin B_{12} and folic acid metabolism in the lung (Ischiropoulos et al. 1990; van der Loo et al. 2003; Wolters et al. 2004). Aging is also associated with decreased generation (as compared with that in the young and healthy) of reactive oxygen and nitrogen species by macrophages, resulting in increased oxidative and nitrosative stress (Tasat et al. 2003).

Our overall hypothesis was that the production of inflammatory mediators by alveolar macrophages and antioxidant defense mechanisms in the lung are altered in the elderly exposed to fine PM and that these alterations increase oxidative stress and susceptibility to cardiopulmonary damage. In order to carry out mechanistic experiments aimed at testing this hypothesis, we performed a series of pilot studies using inhaled DE in a rodent model with the goal of generating supportive preliminary data.

SPECIFIC AIMS

Our study had the following three specific aims:

1. *Develop and Characterize a DE Animal Exposure System.* Total particle number and mass distribution were evaluated at two DE mass concentrations (300 and 1000 μg/m^3 PM) in a 17-L whole-body animal exposure chamber. Measurements of PM characteristics and copollutant levels were performed. These included particle-size distribution and particle mass concentration measurements, as well as analysis of nitrogen monoxide (NO), NO_2, nitrogen oxides (NO_x), CO, carbon dioxide (CO_2), elemental carbon (EC), and organic carbon (OC).
2. *Determine Whether Old Mice Are More Susceptible to the Adverse Pulmonary Effects of Inhaled DE Than Young Mice.* The effects of exposure to inhaled DE were compared in young mice (2 months old) and old mice (18 months old). Lungs of treated animals were examined histologically for evidence of structural alterations and inflammatory-cell accumulation. BAL fluid was collected and measurements made of protein, lactate dehydrogenase (LDH), and number of cells, as well as expression of lipocalin 24p3 mRNA. Dose–response studies were performed using filtered air (0 μg/m^3 PM, as a control) and DE at 300 and 1000 μg/m^3 mass concentrations. The effects of exposure to DE for one 3-hour period (single exposure) or for 3 hours per day for three consecutive days (repeated exposure) were evaluated immediately (0 hours) and 24 hours after the final exposure.
3. *Determine Whether Any Increase in the Susceptibility of Old Mice to Inhaled DE is Associated with Altered Expression of Inflammatory Mediators and Antioxidants.* Differences in mRNA expression of TNF-α, IL-6, IL-8, and cyclooxygenase-2 (COX-2) and expression of MnSOD in the lung and of TNF-α in serum from young and old mice were compared after exposure to DE.

METHODS

EXPOSURE SYSTEM

A Yanmar (model YDG 5500E) 5.5 kW, 406-cc, one-cylinder, two-cycle, diesel-powered electric generator was used as the source of diesel emissions. The generator is located in the penthouse of the Environmental and Occupational Health Sciences Institute (EOHSI) building of Rutgers University–University of Medicine and Dentistry of New Jersey (UMDNJ)–Robert Wood Johnson Medical School, directly above the ceiling of the animal exposure laboratory. The generator was operated using premium low-sulfur diesel fuel (< 500 ppm sulfur; Petro Inc., Somerset, NJ) and 40-weight motor oil (Proline HD40). The generator's load module consisted of a bank of ceramic heaters

(Maxi Heat Inc.) monitored by an ammeter (model 1357, Simpson Inc.) to dissipate 5500 W of generated electricity (100% load). An engine silencer (URB 2 muffler, Cummins Inc.) was installed after the tailpipe to reduce noise.

A schematic diagram of the diesel dilution-and-delivery system used in this pilot study is shown in Figure 1. The components of the overall system can be separated into two parts: one is used for animal exposures (left of the dotted line) and the other used for a controlled-exposure facility (right of the dotted line) in human studies. The system for animal exposures includes a two-stage mass-reduction device. The first stage contains a 10-position butterfly control valve (Control Valve 1 in Figure 1) that can split the DE between the laboratory stream and the waste exhaust pipe in 10 different ratios. The emissions diverted to the animal exposure chamber were aged in the delivery system and were then diluted with high-efficiency particulate air (HEPA)-filtered air in the main pipe (Point A in Figure 1), before the DE was isokinetically drawn into the exposure chamber (Point B in Figure 1). Five- to ten-fold dilution ratios were used, depending on the desired diesel concentration and the setting of Control Valve 1. Under steady-state operating conditions, it took the DE about 5 seconds to travel from the exhaust to the dilution point (Point A) and then about 1 additional second to reach the animal exposure chamber. To minimize cooling, the pipe carrying

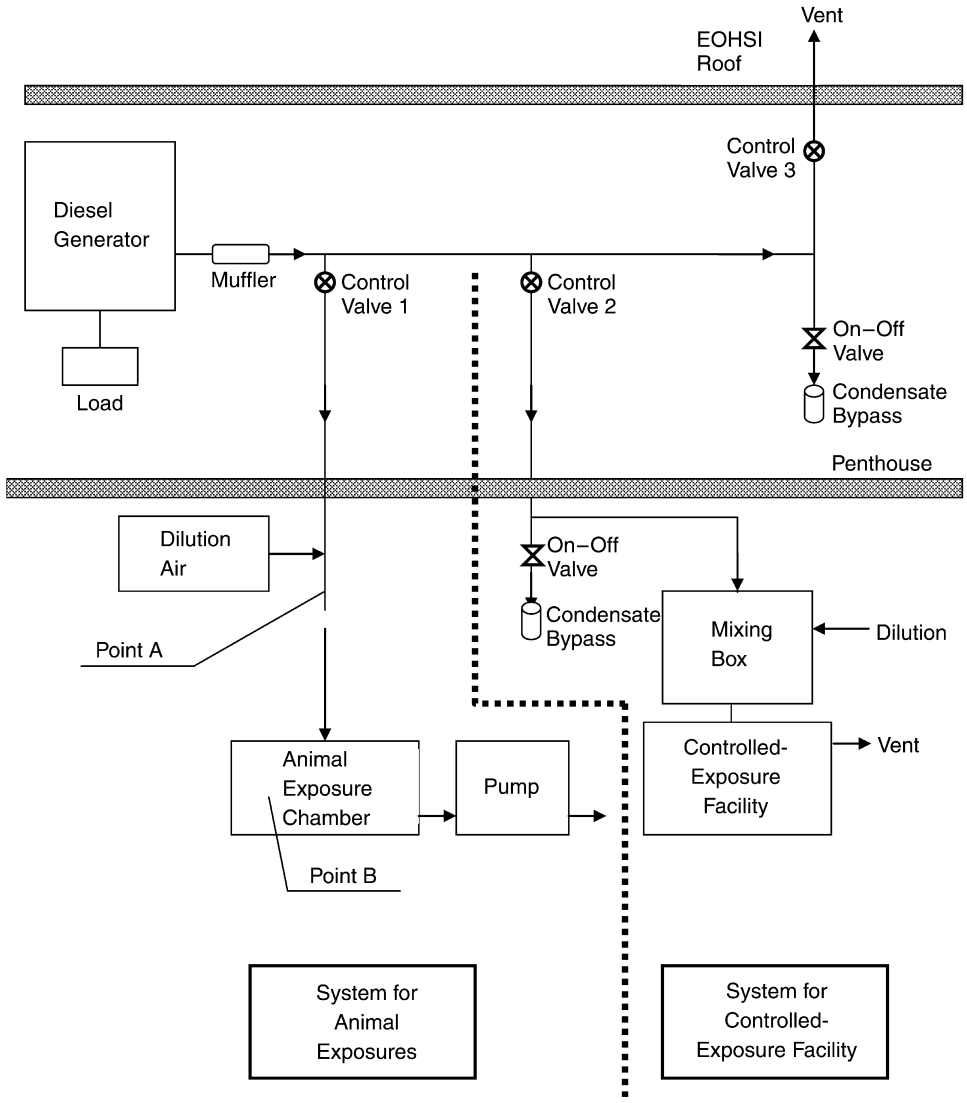

Figure 1. Schematic diagram of exposure system. The area to the left of the dotted line is used for animal exposures, and the area to the right for controlled human exposures. For a detailed description of the system, see the text (Methods/Exposure System).

the exhaust from the diesel generator was insulated up to the dilution point. Before animal exposure sessions, the engine was operated for 15 to 20 minutes to achieve a stable aerosol mass concentration.

Animals were exposed to DE in a 17-L whole-body Plexiglas divided enclosure (16"L × 8"W × 8"H) with a removable lid. During the exposures, the inside and outside of the lid were sealed with plastic to ensure that the chamber would be air tight. To improve the distribution of the particles (i.e., mixing), two small fans were installed and operated inside the chamber. In addition to inlet and outlet ports for the DE, there were six sampling ports available to monitor various characteristics of the exposure aerosol. The flow of the DE through the chamber was controlled by a pump downstream of the chamber, which maintained air flow at 5 L/min. Air flow was monitored using a rotameter. The DE mass concentration in the chamber was controlled by adjusting the fraction of total DE directed toward the chamber and by the amount of air used for dilution. During the exposures, the pressure inside the chamber was approximately 1" H_2O lower than the room pressure.

EXPOSURE CHARACTERIZATION

The particle mass concentration in the chamber was measured in real time by a SidePak personal aerosol mass monitor (TSI Inc.), which was calibrated against gravimetric (filter) measurements of the DE. Particle numbers and mass distributions from 13 nm to 20 μm were obtained with a Scanning Mobility Particle Sizer (SMPS; model 3936, TSI Inc.) and an Aerodynamic Particle Sizer (APS; model 3321, TSI Inc.). The SMPS uses an electrostatic classifier in combination with a water-based condensation particle counter (CPC; model 3786), whereas the APS is based on the time-of-flight principle and provides high-resolution real-time sizing of particles from 0.5 to 20 μm at a sampling flow rate of 1 L/min. The data from the SMPS and the APS were combined using the Data Merge Software Module (TSI Inc.). When merging the data, electrical mobility diameter (the diameter of a spherical particle carrying one elementary electrical charge that has the same electrical mobility) was used for the SMPS data, and aerodynamic diameter was used for the APS data. As a control, total particle number concentration was monitored in real time by a CPC (model 3007, TSI Inc.), which measured the concentration of particles larger than 10 nm. Levels of copollutant gases (e.g., NO, NO_2, CO, and CO_2) were assessed using an indoor-air-quality (IAQ) RAE gas monitor with multigas sensors (RAE Systems). To determine the specific concentrations of EC, OC, and total carbon (TC, measured as EC plus OC), DE PM was collected on 25-mm quartz-fiber filters that had been prefired to remove organic impurities (Pall Corp.-Life Sciences). The air was drawn through the filter and a PM_{10} ($PM \leq 10$ μm in aerodynamic diameter) preclassifier (A.D.E. Inc., Naples, ME) at a flow rate of 4 L/min by a BGI personal pump for 1 hour. After collection of the samples, the filters were sent to Sunset Laboratory, Inc. (Tigard, OR) for analysis of EC, OC, and TC.

Particle-number distribution was measured inside the exhaust pipe (from which DE was drawn into the chamber) and inside the animal exposure chamber for both mass concentrations of DE (300 and 1000 $\mu g/m^3$). The initial measurements inside the chamber were performed with the SMPS, which uses a water-based CPC (model 3786) to count particles in each individual size channel. By adding particle concentrations from all the size channels, the total particle concentration for the entire size range was obtained. Comparison of the total number provided by the SMPS and the total concentration from the control measurements made with the CPC 3007 revealed that the water-based CPC 3786 of the SMPS undercounted the number of particles. The manufacturer of the CPC, TSI, suggested that the undercounting was likely to be due to slightly negative pressure inside the exposure chamber, which reduced the water-vapor condensation on the diesel particles. TSI subsequently provided a butanol-based CPC (model 3025) as a control. Thus, the DE characterization was performed with a SMPS equipped with both the water-based model 3786 and the butanol-based model 3025 CPC. Characterization of DE particle-size distribution and measurements of copollutant gases were performed before the animal exposures took place; mass concentration was measured both before and during the animal exposures.

ANIMALS

We used male CB6F1 mice in the exposure studies. Two groups of mice were acquired: 2-month-old mice (average weight, 24 g; Harlan, Indianapolis, IN) and 18-month-old mice (average weight, 41 g; National Institute on Aging, Bethesda, MD). These groups are referred to in this report as young and old mice, respectively. Animals were housed in micro-isolator cages and maintained on sterile food (Picolab Rodent Diet 20, Fisher Scientific, Pittsburgh, PA) and pyrogen-free water ad libitum except during exposures. Animals were acclimated to housing conditions for 7 to 10 days before the study was initiated. Mice were weighed and randomly assigned to exposure groups. The animal rooms were maintained at 20 ± 2°C with 45% to 55% relative humidity on a 12-hour light–dark cycle. All animals received humane care in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals, published by the National

Institutes of Health. Animal care personnel followed all applicable standard operating procedures specified by the Association for Assessment and Accreditation of Laboratory Animal Care International.

ANIMAL EXPOSURES

Animals were exposed either for one 3-hour period (single exposure) or for 3 hours per day on three consecutive days (repeated exposure) to filtered air (Messer Gas, Allentown, PA) as a control, or to DE at 300 μg/m^3 or 1000 μg/m^3 $PM_{2.5}$. Exposures were carried out in a single partitioned chamber without a floor grid. During exposure sessions, a total of 12 animals could be exposed (6 per exposure group on each side of the divided chamber).

SAMPLE COLLECTION

Groups of six animals per exposure group were killed immediately (0 hours) or 24 hours after exposure. Although we originally proposed to collect samples only at 24 hours after exposure, recent findings by our laboratory suggested that injury and inflammatory changes in the lung induced by fine PM may occur earlier than 24 hours after exposure (Sunil et al. 2007a). We therefore included a 0-hour postexposure sampling time in our analysis, as a way to evaluate inflammatory alterations that occur immediately after exposure, in addition to the 24-hour sampling time, which was intended to identify the persistence of early inflammatory changes and the later appearance of proinflammatory markers and markers of early tissue repair. Nembutal (200 mg/kg body weight) was injected intraperitoneally using a 26½-gauge needle to euthanize the animals. The body cavity was opened and blood collected from the right ventricle using a 27½-gauge needle. The volume of blood recovered was recorded, and the tubes were maintained at room temperature until clots formed. Serum samples were centrifuged at 1000×g for 10 minutes at 4°C, divided into aliquots, and stored at −80°C.

The thoracic cavity was then opened and the trachea and lungs exposed. The largest lobe was clamped at the bronchus and reserved for histologic and immunohistochemical analyses and analysis of RNA (as described below). The remaining lobes were lavaged three times through the trachea with 1 mL sterile, pyrogen-free, ice-cold phosphate-buffered saline (PBS). The volume of recovered BAL fluid was recorded, and the fluid was transferred to tubes containing 5 mM diethylenetriaminepentaacetic acid. Tubes were centrifuged at 300×g for 8 minutes at 4°C, after which the supernatants were transferred to fresh tubes for analysis of protein and LDH activity. Cell pellets were resuspended in 50 μL of PBS. Ten μL of each sample was stained with trypan blue and analyzed with a hemocytometer to determine cell number and viability. PBS (160 μL) was then added to the cells, which were cytocentrifuged (at 600×g for 8 minutes, at room temperature; Shannon Southern Products, Cheshire, England) onto slides. Slides were stained with Giemsa (Lab Chem Inc., Pittsburgh, PA) and analyzed at ×400 by light microscopy. A total of 300 cells per sample was counted and used to determine the percentage of alveolar macrophages, monocytes, neutrophils, and lymphocytes. For all exposure groups, more than 98% of the cells were identified as alveolar macrophages and 1% to 2% as other mononuclear cells (data not shown).

For RNA analysis (performed in three of each group of six mice), the largest lobe was removed and stored at −80°C in tubes containing 200 μL of RNALater (Sigma-Aldrich Corp., St Louis, MO). For histologic and immunohistochemical analyses (three of the six mice per group were analyzed histologically and the other three immunohistochemically), the largest lobe was instilled with 3% cold paraformaldehyde in PBS, removed, and placed on ice for 4 hours. Samples were then transferred to 50% ethanol.

MEASUREMENT OF PROTEIN IN BAL FLUID

Total protein content was quantified in cell-free preparations of BAL fluid using a BCA (bicinchoninic acid) Protein Assay kit (Pierce Biotechnologies Inc., Rockford, IL) with bovine serum albumin as the standard. Ten μL of undiluted sample from each animal was analyzed in triplicate by measuring fluorescence intensity at 540 nm. Data were normalized to the volume of BAL fluid collected. Samples from five to six animals per exposure group were assayed.

MEASUREMENT OF ALBUMIN IN BAL FLUID

A mouse albumin quantitative enzyme-linked immunosorbent assay (ELISA) (Bethyl Laboratories, Montgomery, TX) was used to determine albumin content in BAL fluid. For the assay, 100 μL of red-blood-cell–free undiluted BAL fluid was analyzed in duplicate at 450 nm. Data were normalized to the volume of BAL fluid collected. Samples from five to six animals per exposure group were assayed.

MEASUREMENT OF LDH ACTIVITY IN BAL FLUID

LDH activity in BAL fluid was determined using the CytoTox 96 nonradioactive cytotoxicity assay kit from Promega Corporation (Madison, WI). Each sample (50 μL of undiluted BAL fluid) was analyzed in triplicate at 490 nm. Data were normalized to the volume of BAL fluid collected. Samples from six animals per exposure group were assayed.

HISTOLOGIC ANALYSIS

Histologic sections (4 μm) were stained with hematoxylin and eosin. Specimens were analyzed by light microscopy using ProgRes Capture Pro version 2.5 software. The extent of inflammatory changes, including accumulation of alveolar macrophages and neutrophils, alterations in alveolar epithelial barriers, and edema, were assessed by a veterinary pathologist (Sherritta Ridgely, D.V.M., Ph.D., clinical laboratory animal veterinarian, Laboratory Animal Services, Rutgers University). Three mice per exposure group were analyzed.

IMMUNOHISTOCHEMICAL ANALYSIS

Tissue sections (4 μm) were deparaffinized, blocked in 100% serum (room temperature, 3 to 4 hours), and incubated overnight at 4°C with rabbit immunoglobulin G (IgG) or anti-MnSOD antibody (1:400, Stressgen Biotechnologies Inc., San Diego, CA), followed by 30 minutes of incubation with biotinylated secondary antibody (Vector Labs, Burlingame, CA). Binding was visualized using a Peroxidase Substrate Kit DAB (Vector Labs). Three mice per exposure group were analyzed.

MEASUREMENT OF TNF-α IN SERUM

TNF-α levels in serum were quantified using a Quantikine immunoassay kit (R&D Systems, Minneapolis, MN). Serum (50 μL) from each sample was diluted twofold and analyzed in duplicate. Plates were analyzed at 450 nm and 540 nm (to correct for optical imperfections in the plate), and a standard curve was generated using a four parametric logistic (4-PL SoftMax Pro, version 4, software, R&D Systems) curve fit. Samples from two to four animals per exposure group were assayed.

QUANTITATIVE REAL-TIME POLYMERASE-CHAIN-REACTION ANALYSIS

Total mRNA was isolated from lung tissue using the RNeasy Mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed using the High Capacity cDNA (complementary DNA) Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Standard curves were generated using serial dilutions from pooled cDNA samples. Real-time polymerase-chain-reaction (PCR) analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler according to the manufacturer's protocol. All PCR primers were generated using Primer Express 3.0 (Applied Biosystems). Samples from two to three animals per exposure group were analyzed and results presented relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Primer sequences were as follows:

24p3 Forward: 5′ AGG AAC GTT TCA CCC GCT TT 3′
24p3 Reverse: 5′ TGT TGT CGT CCT TGA GGC C 3′

TNF-α Forward: 5′ AGG GAT GAG AAG TTC CCA AAT G 3′
TNF-α Reverse: 5′ TGT GAG GGT CTG GCG CAT A 3′

IL-6 Forward: 5′ CCA CGG CCT TCC CTA CTT C 3′
IL-6 Reverse: 5′ GTT GGG AGT GGT ATC CTC TGT GA 3′

IL-8 Forward: 5′ CAG CTG CCT TAA CCC CAT CA 3′
IL-8 Reverse: 5′ CTT GAG AAG TCC ATG GCG AAA 3′

COX-2 Forward: 5′ CAT TCT TTG CCC AGC ACT TCA C 3′
COX-2 Reverse: 5′ GAC CAG GCA CCA GAC CAA AGA C 3′

GAPDH Forward: 5′ TGA AGC AGG CAT CTG AGG G 3′
GAPDH Reverse: 5′ CGA AGG TGG AAG AGT GGG AG 3′

STATISTICAL METHODS AND DATA ANALYSIS

Statistical design and analysis were performed in consultation with Rebecka Jornsten, Ph.D., of the Department of Statistics, Rutgers University. Separate data sets were constructed for each exposure regimen (single or repeated) and for each postexposure sampling time (0 hours or 24 hours). For each separate data set, a two-way analysis of variance (ANOVA) was used to test the impact of age (2 months vs. 18 months) on each response variable (BAL-fluid volume, cell number, etc.), at each exposure concentration (0, 300, and 1000 $\mu g/m^3$). Thus, the two common factors in each model were age and exposure concentration. Two-way ANOVA models were fit to each data set using SAS software (a licensed software product). The validity of each model was carefully assessed using residual diagnostics. Specifically, the underlying modeling assumption was examined using normal quantile–quantile plots. Extreme observations (i.e. outliers) were identified from the quantile–quantile plots. All values are presented for all assays with the exception of total protein in BAL fluid, for which outliers were excluded before analysis. Single outliers were excluded from the following groups: young mice assigned to a single exposure to DE at 1000 $\mu g/m^3$ at the 0-hour and 24-hour sampling times; young mice assigned to repeated exposure to DE at 0 $\mu g/m^3$ at the 0-hour sampling time; young mice assigned to repeated exposure to DE at 1000 $\mu g/m^3$ at the 0-hour sampling time; and old mice assigned to a single exposure to DE at 0 $\mu g/m^3$ at the 0-hour and 24-hour sampling times. In addition, two outliers were excluded from the group of young mice assigned to repeated exposure to DE at 300 $\mu g/m^3$ at the 24-hour sampling time.

Specific hypotheses that were tested included the effect of age at each exposure regimen. *P* values for the effect of age at each exposure concentration (0, 300, and 1000 μg/m^3 PM) are reported for each separate data set. To examine the impact of exposure concentration on the outcome for each exposure regimen, sampling time, and age group, one-way ANOVA models were used. Modeling assumptions were examined using quantile–quantile plots, and outliers were removed before hypothesis testing. Specific hypotheses that were tested for young (2-month-old) and old (18-month-old) animals included the following: (1) that 0 μg/m^3 and 300 μg/m^3 PM result in the same expected outcome; (2) that 0 μg/m^3 and 1000 μg/m^3 PM result in the same outcome; (3) that 300 μg/m^3 and 1000 μg/m^3 PM result in the same outcome when each was compared with the air control (0 μg/m^3). Separate *P* values are reported for each of these hypotheses. A *P* value of ≤ 0.05 was considered to indicate statistical significance. Changes in gene expression of more than twofold were considered biologically significant.

RESULTS

CHARACTERIZATION OF THE DE ANIMAL EXPOSURE SYSTEM

The first part of our study was designed to assess the stability of the DE mass concentrations within the chamber over the planned 3-hour experimental exposure period. We found that particle mass concentrations from 300 to 1000 μg/m^3 $PM_{2.5}$ remained stable over several hours with a coefficient of variation $\leq 15\%$. In contrast, under the conditions used, we were unable to maintain stable particle mass concentrations of 100 μg/m^3 in the animal exposure chamber (see "Problems Encountered During the Study" in the Discussion section below). This inability was most likely due to the high sensitivity of the diesel exhaust fraction diverted toward the animal chamber to engine variations and wind drafts. Therefore, in further pilot characterization and exposure studies, we limited our analysis to DE concentrations of 300 and 1000 μg/m^3. The particle-size distributions by number at these two different mass concentrations are shown in Figures 2 and 3. A summary of measured atmosphere characteristics is presented in Table 1. Based on the particle-size-distribution analysis with the butanol-based CPC placed inside the animal chamber, at the 300 μg/m^3 mass concentration, the median diameter was approximately 255 nm; the mean, approximately 273 nm; and the mode, approximately 246 nm, with a total particle concentration of approximately 3.6×10^4/cm^3. For the 1000 μg/m^3 mass concentration, the median diameter was approximately 231 nm; the mean, approximately 242 nm; and the mode, approximately 246 nm, with a total particle concentration of $> 1 \times 10^5$/cm^3. These values are greater than those reported previously by McDonald and colleagues (2004); the discrepancy is most likely due to differences in the design of the diesel-exposure systems. In our system, it took a few seconds for the DE to reach the dilution point. This time may be sufficient for nanosized particles to agglomerate, causing a shift in the median particle diameter.

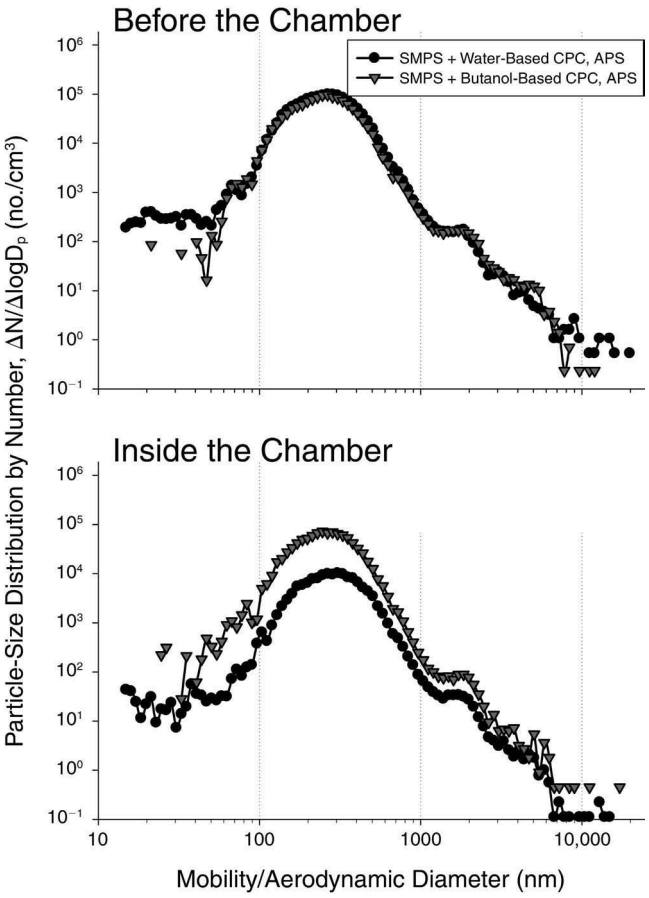

Figure 2. DE particle-size distributions by number at 300 μg/m^3 mass concentration ($PM_{2.5}$). The upper panel shows results for sampling in the exhaust pipe before the exposure chamber, and the lower panel results for sampling inside the chamber. $\Delta N/\Delta \log D_p$ denotes the number of particles within a size channel normalized to the channel width.

As shown in Figure 2, at the DE concentration of 300 μg/m^3, the data obtained from the exhaust pipe with the water-based CPC and the butanol-based CPC were similar; however, the water-based CPC underestimated the concentration of particles inside the exposure chamber when compared with the butanol-based CPC, especially from 100 to 500 nm. Figure 3, shows that at the DE concentration of 100 μg/m^3, data from both CPCs at both sampling

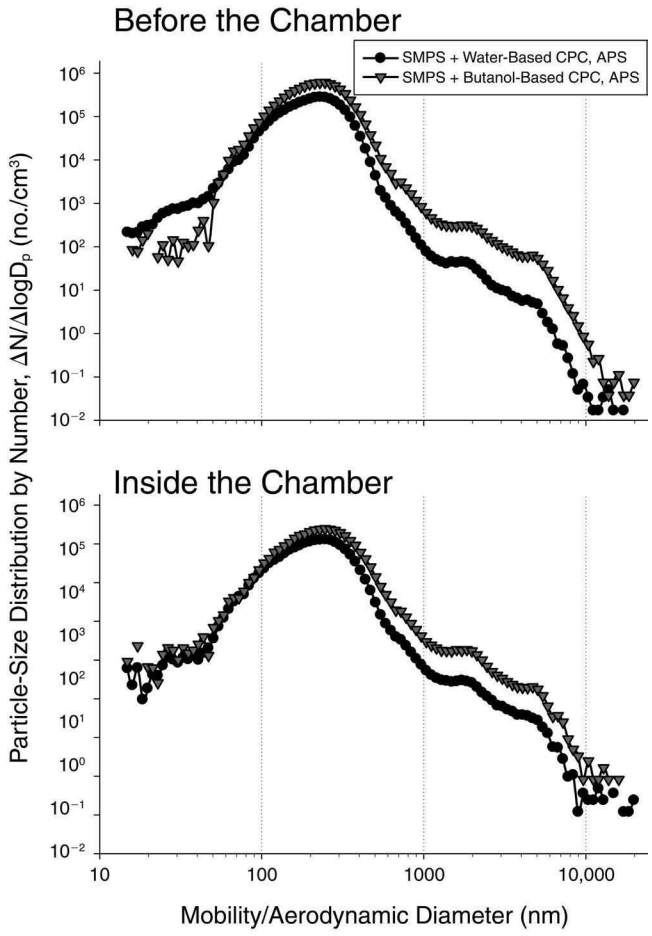

Figure 3. DE particle-size distributions by number at 1000 µg/m³ mass concentration ($PM_{2.5}$). The upper panel shows results for sampling in the exhaust pipe before the exposure chamber, and the lower panel results for sampling inside the chamber. $\Delta N/\Delta \log D_p$ denotes the number of particles within a size channel normalized to the channel width.

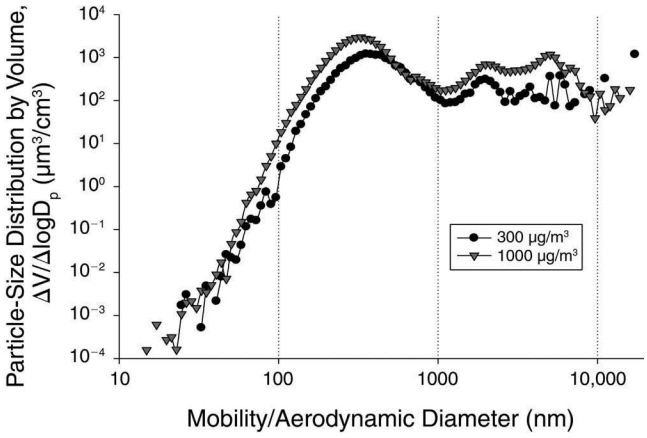

Figure 4. Particle-size distributions by volume inside the exposure chamber at 300 and 1000 µg/m³ mass concentrations ($PM_{2.5}$) as determined by an SMPS with a butanol-based CPC and APS. $\Delta N/\Delta \log D_p$ denotes the number of particles within a size channel normalized to the channel width.

points were reasonably close in the range from 60 to 400 nm, then the concentrations diverged somewhat for larger particles. For all distributions, the geometric standard deviation (SD) was 1.5. Aerosols with geometric SDs < 2 are considered monodisperse. According to the particle-volume data shown in Figure 4, the median mass diameter was 487 nm and 411 nm for the 300 µg/m³ and the 1000 µg/m³ mass concentrations, respectively, and the mean mass diameter was approximately 2200 nm and 1676 nm. Taken together, these results indicate that the number and mass concentration in the animal exposure chamber can be varied, within the range of particle mass concentrations used in this study, without substantially changing the particle-size distribution.

An example of changes in DE mass concentration in the exposure chamber over time is presented in Figure 5. For both particle mass concentrations (300 and 1000 µg/m³), the coefficient of variation over a 3-hour period was ≤ 15%, which indicates stable aerosol concentration during the exposures.

We also measured gas concentrations inside the chamber (Table 1). Since gaseous copollutants are generated by combustion, it was expected that their concentrations would change in the same ratio as the DE mass concentration, owing to the introduction of air for dilution. As the PM concentration increased from 300 µg/m³ to 1000 µg/m³, the concentration of NO_x ($NO + NO_2$) increased by a factor of 3.4, as expected, from 4.3 ppm to 14.5 ppm. The increase was largely due to an increase in NO_2 (from 3.9 to 14.0 ppm), while the concentration of NO remained relatively stable.

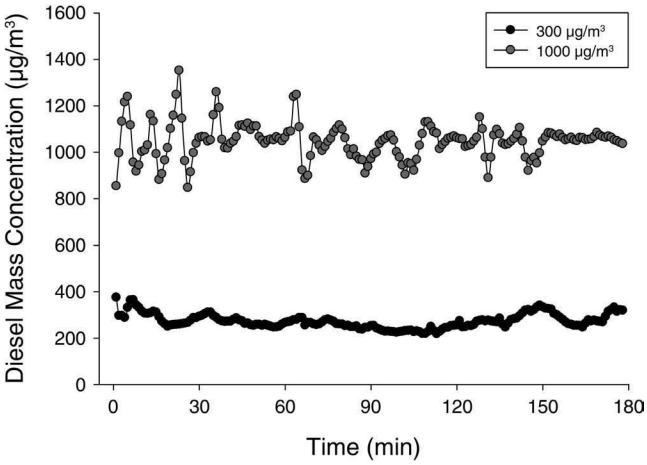

Figure 5. Examples of variations in PM mass concentration in the exposure chamber over time at target mass concentrations of 300 and 1000 µg/m³ PM.

Table 1. Summary of DE Characteristics in the Exposure Chamber

DE Concentration[a] and Characteristics of Particle-Size Distribution by Number[b]	Measurement in the Exhaust Pipe		Measurement in the Chamber					
	SMPS + Water-Based CPC	SMPS + Butanol-Based CPC	SMPS + Water-Based CPC	SMPS + Butanol-Based CPC	CO	CO_2	NO	NO_2
300 μg/m^3								
Gases (ppm)					4.0	1196	0.4	3.9
Number median diameter (nm)	253.8	249.0	280.0	254.9				
Number mean diameter (nm)	272.0	266.3	302.1	272.7				
Number mode diameter (nm)	264.2	264.2	305.1	245.8				
Geometric mean (nm)	249.2	245.4	276.7	252.0				
Geometric SD (nm)	1.5	1.5	1.5	1.5				
Total concentration[c]	4.4×10^4	3.9×10^4	4.4×10^3	2.9×10^4				
Total particle concentration[d]	1.0×10^5	4.4×10^4	7.8×10^3	3.6×10^4				
1000 μg/m^3								
Gases (ppm)					6.6	2510	0.5	14.0
Number median diameter (nm)	210.2	217.3	218.1	231.1				
Number mean diameter (nm)	216.5	227.4	225.4	242.1				
Number mode diameter (nm)	228.8	245.8	245.8	245.8				
Geometric mean (nm)	201.6	211.7	210.4	225.2				
Geometric SD (nm)	1.5	1.5	1.5	1.5				
Total particle concentration[c]	1.1×10^5	2.4×10^5	5.1×10^4	9.5×10^4				
Total particle concentration[d]	1.5×10^5	$> 1 \times 10^5$	5.8×10^4	$> 1 \times 10^5$				

[a] Concentration inside the chamber at 100% engine load.
[b] SD denotes standard deviation.
[c] Measured by SMPS and CPC.
[d] Measured by CPC alone.

The CO concentration was found to increase from 4.0 ppm at a PM concentration of 300 μg/m^3 to 6.6 ppm at 1000 μg/m^3, which is less than a factor of 2. The CO_2 concentration increased by more than a factor of 2, from approximately 1200 to approximately 2500 ppm, as the PM concentration increased. By comparison, the CO concentration in the air used for dilution was < 1 ppm, the CO_2 concentration approximately 350 ppm, and the NO_x concentration < 0.02 ppm (data not shown).

Levels of EC, OC, and TC in DE were quantified by a commercial laboratory. Preliminary results for the mass concentration at 300 μg/m^3 indicated an OC concentration of 124 μg/m^3, an EC concentration of 115 μg/m^3, and a resulting TC concentration of 239 μg/m^3. These values are in reasonable agreement with OC/EC ratios reported in diesel vehicle emissions studies, which are quite variable (Zielinska et al. 2004; Fujita et al. 2007). The carbon-content analysis at the 1000 μg/m^3 mass concentration was not performed owing to damage to filters during handling.

EFFECTS OF INHALED DE ON MARKERS OF LUNG INJURY IN YOUNG AND OLD MICE

Lung tissue was evaluated histologically for structural and morphologic alterations. Additional markers of lung injury that were assessed included cell number, protein and albumin content, and LDH activity in BAL fluid and mRNA expression of the acute-phase protein lipocalin 24p3, a marker of oxidative stress. For the purposes of clarity, data on single and repeated exposure to DE are described separately.

Effects of DE on Histologic Findings in the Lung

Single Exposure A single acute exposure of young mice to either low- or high-dose DE (300 μg/m^3 or 1000 μg/m^3, respectively) had no significant statistical effects on histologic features of the lung (Figure 6, panels B, C, H, and I). In old animals, however, after low-dose DE exposure, an immediate increase in neutrophils was noted in alveolar

spaces and capillaries, which became more pronounced after 24 hours (Figure 6, panels E and K). Inflammatory changes were more marked in old mice exposed to high-dose DE and included greater numbers of neutrophils in alveolar spaces and capillaries and focal inflammatory infiltrates consisting predominantly of plasma cells and macrophages; these changes became more prominent 24 hours after exposure to high-dose DE (Figure 6, panels F and L).

Repeated Exposure In old animals, repeated exposure to low-dose DE resulted in patchy thickening of alveolar septa, cytomegaly in alveolar septal walls, and an increase in the number of macrophages in alveolar spaces immediately after exposure (Figure 7, panel E). By 24 hours after exposure, focal thickening of alveolar septal walls and increased numbers of neutrophils and erythrocytes were evident (Figure 7, panel K). Structural alterations in young

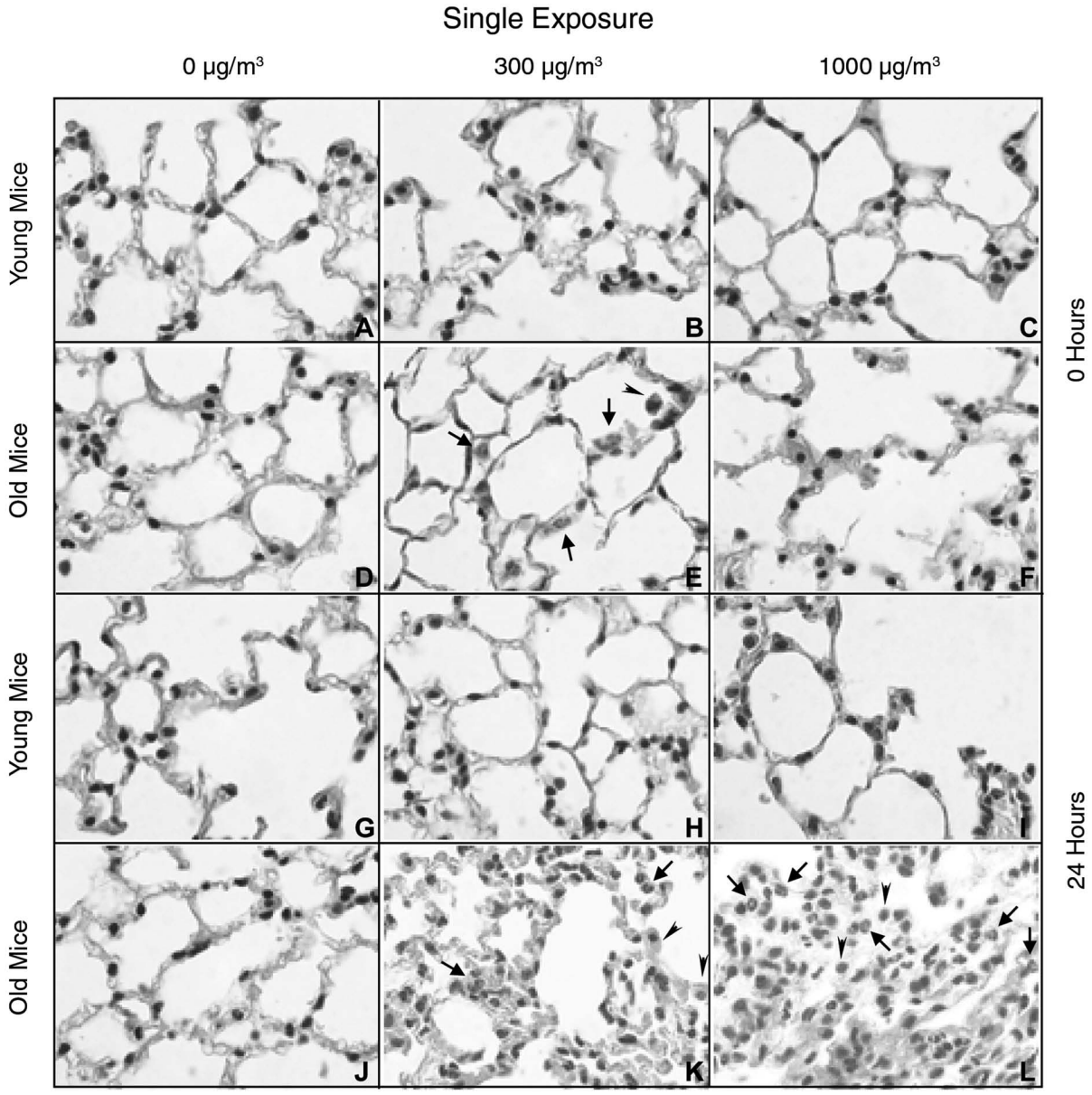

Figure 6. Effects of single exposure to DE on histologic features of the lung. Lung sections (4 µm) prepared from young (2-month-old) and old (18-month-old) mice immediately (0 hours) and 24 hours after a single exposure to DE (0 [air control], 300, or 1000 µg/m^3) were stained with hematoxylin and eosin. One representative section from each exposure group (n = 3 mice per exposure group) is shown (magnification, ×100). Arrows indicate neutrophils, and arrowheads, alveolar macrophages.

mice after low-dose exposure to DE were minimal when compared with changes observed in old animals (Figure 7, panels B and H). However, the most significant and consistent inflammatory changes were noted in old animals exposed repeatedly to high-dose DE. Thus, in old animals, greater numbers of neutrophils were observed in alveolar spaces and capillaries. Focal infiltrates consisting predominantly of plasma cells and macrophages were also increased in old animals (Figure 7, panels F and L). Unlike the effects of a single exposure to high-dose DE, histologic changes in lungs of old animals exposed to repeated DE were more prominent immediately after exposure.

Effects of DE on Cell Numbers in BAL Fluid

Single Exposure In further studies, we compared the number of cells recovered in BAL fluid from young and old mice after a single acute exposure to low- or high-dose DE

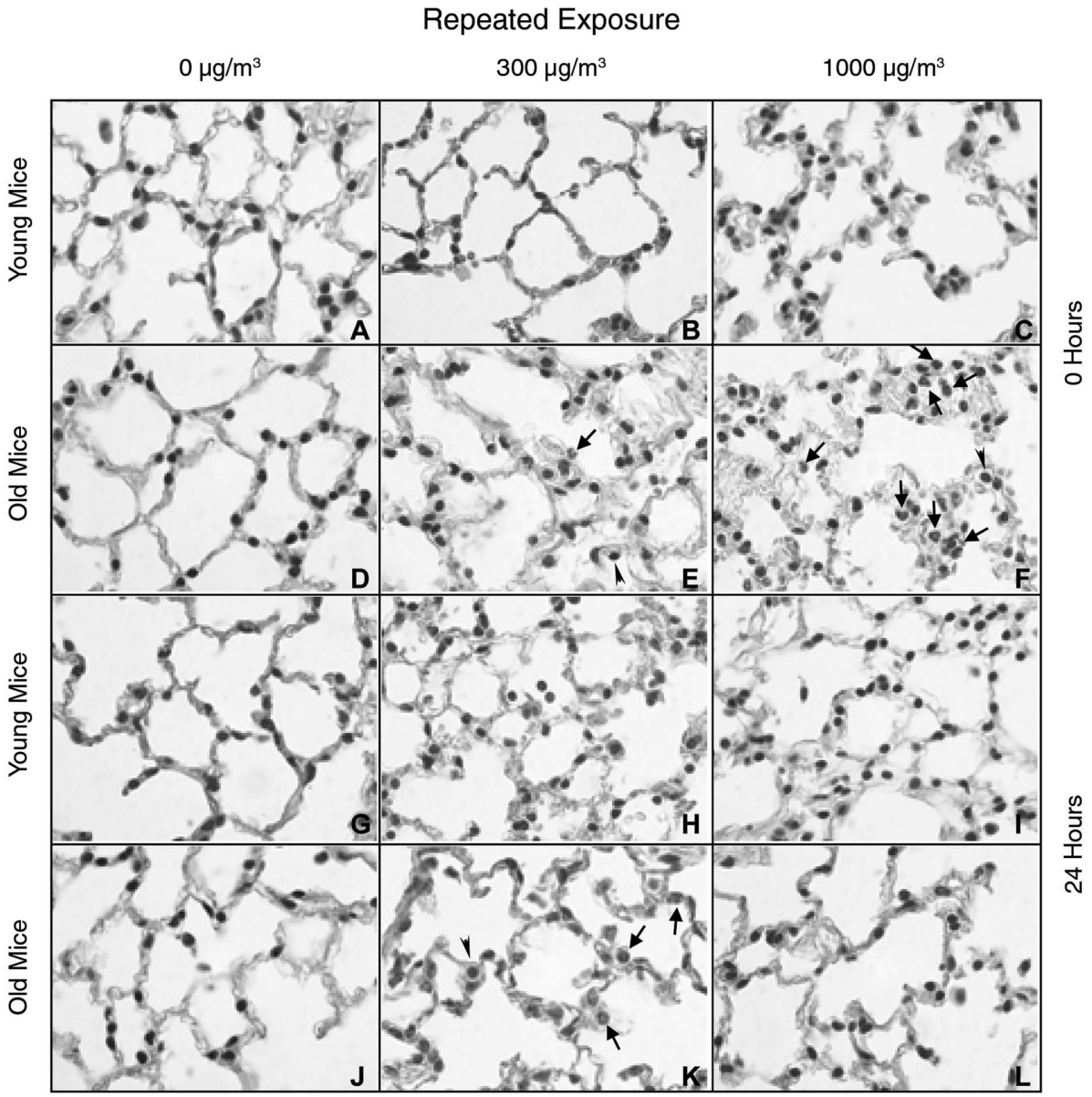

Figure 7. Effects of repeated exposure to DE on histologic features of the lung. Lung sections (4 µm) prepared from young (2-month-old) and old (18-month-old) mice immediately (0 hours) and 24 hours after repeated exposure to DE (0 [air control], 300, or 1000 µg/m^3) were stained with hematoxylin and eosin. One representative section from each exposure group (n = 3 mice per exposure group) is shown (magnification, ×100). Arrows indicate neutrophils, and arrowheads, alveolar macrophages.

and the air control. In general, these values were highly variable, probably owing to variations in the quantities of BAL fluid recovered from the mice. Similar numbers of cells were recovered from young and old animals immediately after exposure to the air control (Figure 8, upper panel). Whereas no significant effects were observed in young animals immediately after exposure to low- or high-dose DE, 24 hours after low-dose exposure, a small but significant increase in cell number was noted. In contrast, a decrease in cell number was observed in the old animals exposed to low-dose DE.

Repeated Exposure Repeated exposure of young animals to high-dose DE resulted in an immediate increase in BAL-fluid cell number, which persisted for at least 24 hours. In old animals, as observed with single DE exposure, cell numbers decreased after repeated exposure to DE (Figure 8, lower panel).

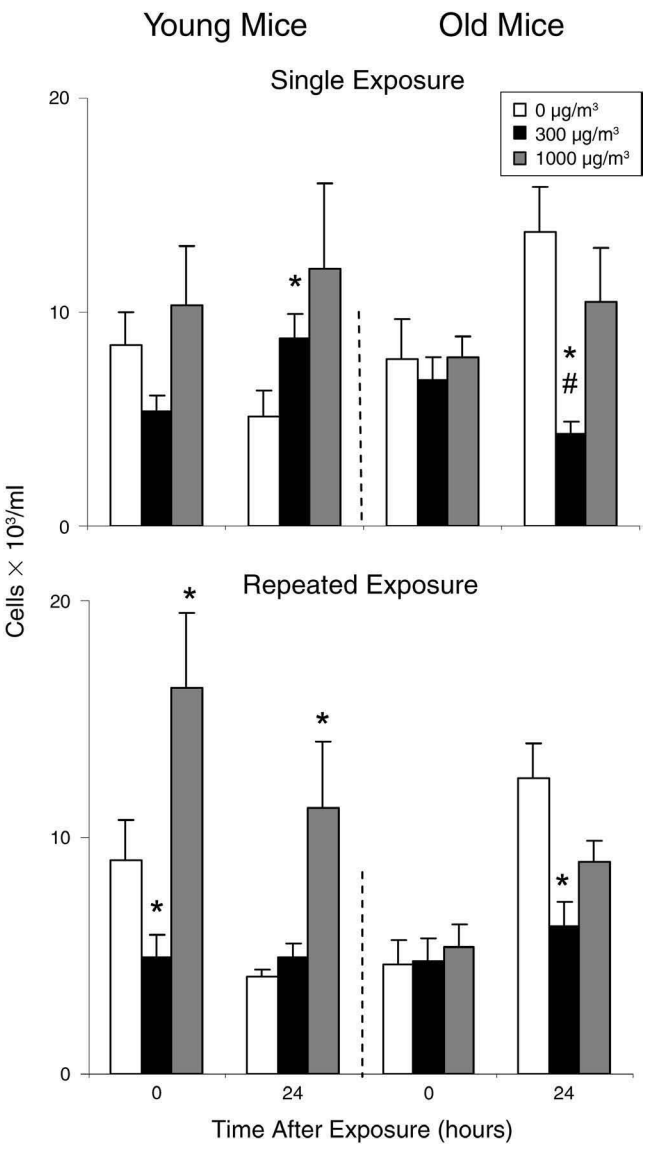

Figure 8. Effects of DE on cell numbers in BAL fluid. BAL fluid was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. Viable cells in BAL fluid were enumerated by trypan blue dye exclusion. Each bar is the mean + standard error (SE) (n = 6 mice). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

Effects of DE on Protein Levels in BAL Fluid

Single Exposure Increased BAL-fluid protein content indicates enhanced alveolar epithelial permeability and is a marker of acute injury to the lower lung (Bhalla 1999). Relatively low levels of protein were detected in BAL fluid from both young and old animals. Whereas a single exposure to high-dose DE increased BAL-fluid protein in young animals, no significant effects were noted in old animals (Figure 9, upper panel).

Repeated Exposure As compared with levels in mice exposed only to air control, increases in BAL-fluid protein were observed in young animals after repeated exposures to low-dose DE. In general, this trend persisted for at least 24 hours (Figure 9, lower panel). In contrast, no significant changes were noted in BAL-fluid protein in old animals after repeated exposure to DE.

We also quantified albumin levels in BAL fluid from young and old mice after single and repeated exposures to DE. No significant effects were observed at either 300 or 1000 µg/m^3 PM (data not shown).

Effects of DE on LDH Activity in BAL Fluid

Single Exposure In our next set of experiments, we quantified LDH activity in BAL fluid as a marker of cytotoxicity. Constitutive LDH activity was detected in BAL fluid from both young and old mice (Figure 10, upper panel). Whereas exposure of young mice to low-dose DE resulted in a small decrease in LDH activity at 24 hours, no significant effect was seen in old animals.

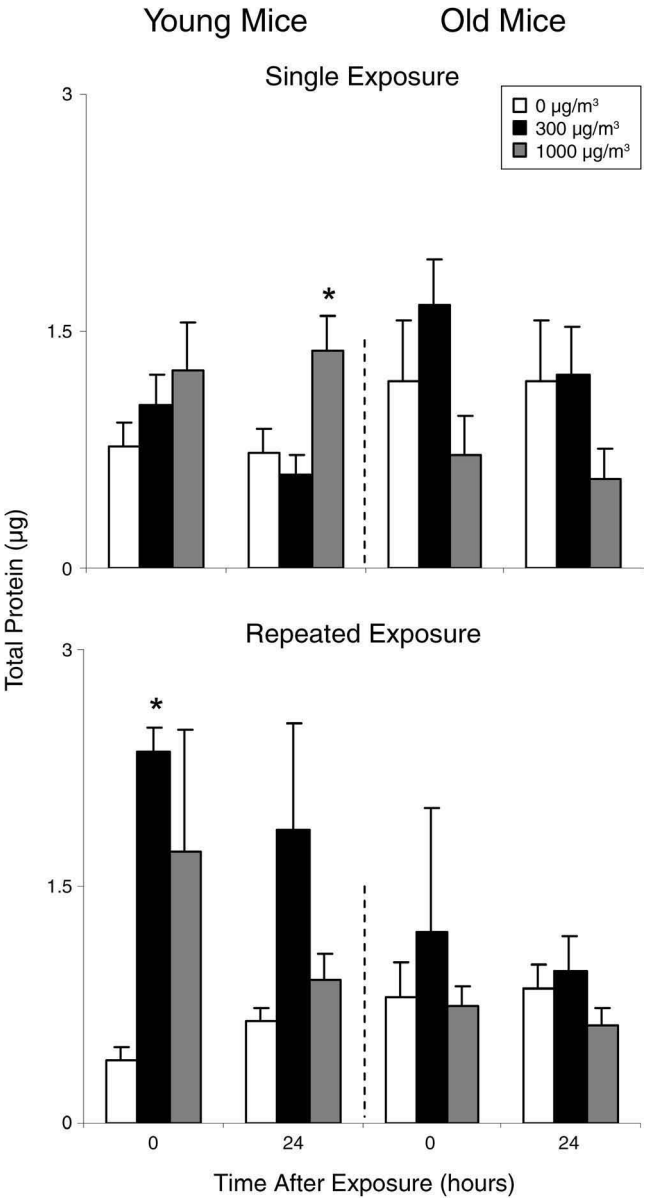

Figure 9. Effects of DE on protein content in BAL fluid. BAL fluid was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. Total protein was determined using a BCA Protein Assay kit (Pierce Biotechnologies Inc.) Samples collected from five to six animals per exposure group were assayed in triplicate. Each bar is the mean + SE (n = 15–18 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

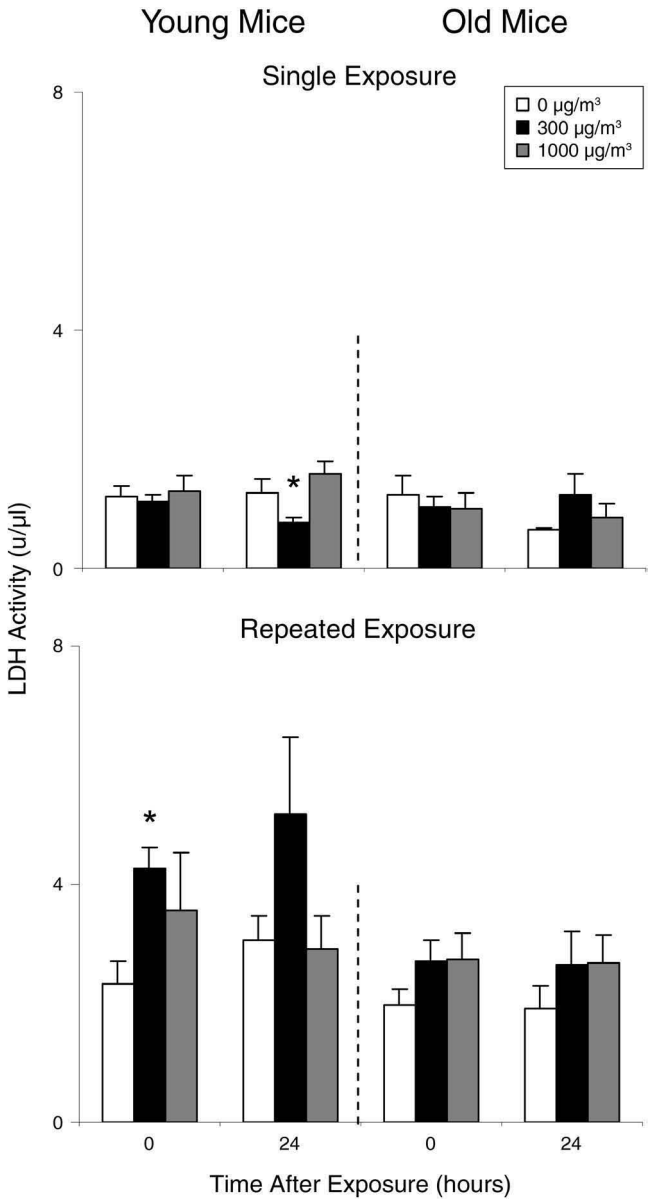

Figure 10. Effects of DE on LDH activity in BAL fluid. BAL fluid was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3 and assayed for LDH activity (expressed in arbitrary units provided by kit manufacturer). Samples from six animals per exposure group were assayed in triplicate. Each bar is the mean + SE (n = 18 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

Repeated Exposure In young mice, in contrast to results observed after a single DE exposure, an increase in LDH activity was noted after repeated exposure to low-dose DE (Figure 10, lower panel). However, these changes were not observed in old mice.

Effects of DE on Expression of Lipocalin 24p3 mRNA in the Lung

Acute-phase proteins such as lipocalin 24p3 are rapidly produced in response to oxidative stress and tissue injury (Roudkenar et al. 2007). In further studies, we analyzed the effects of DE on expression of 24p3 mRNA in the lung.

Single Exposure A single acute exposure to DE caused a dose-related increase in lipocalin 24p3 mRNA expression in the lung, but only in old animals (Figure 11, upper panel). The effects of DE were most pronounced immediately after exposure.

Repeated Exposure In young animals, repeated exposure to DE resulted in a small but transient increase in 24p3 mRNA expression (Figure 11, lower panel). By comparison, in old mice, repeated exposure to high-dose DE caused an increase of approximately threefold in expression of this acute-phase protein. This effect was observed immediately after exposure and persisted for at least 24 hours.

EFFECTS OF INHALED DE ON EXPRESSION OF INFLAMMATORY MEDIATORS AND ANTIOXIDANTS IN THE LUNGS OF YOUNG AND OLD MICE

According to our hypothesis, increases in the sensitivity of the elderly to inhaled DE are due to age-related alterations in the expression of inflammatory mediators such as TNF-α and antioxidants. We addressed this issue in our next series of pilot studies. Although we originally proposed to evaluate the effects of DE on TNF receptor 1 (TNFR1) using ELISA, we were unable to complete these studies since we did not have sufficient quantities of BAL fluid and serum to perform the assays. We were also unable to assess expression of TNF-α or TNFR1 protein in the lung owing to technical difficulties (discussed under "Problems Encountered During the Study," below). As an alternative, we analyzed the effects of DE on TNF-α, IL-6, IL-8, and COX-2 mRNA expression in the lung by real-time PCR and TNF-α protein in serum by ELISA.

Effects of DE on TNF-α Expression

Single Exposure Both young and old animals constitutively expressed low levels of TNF-α mRNA in the lung. Exposure of young mice to DE had no effect on TNF-α expression immediately after exposure (Figure 12, upper panel). In contrast, a roughly fivefold increase in lung

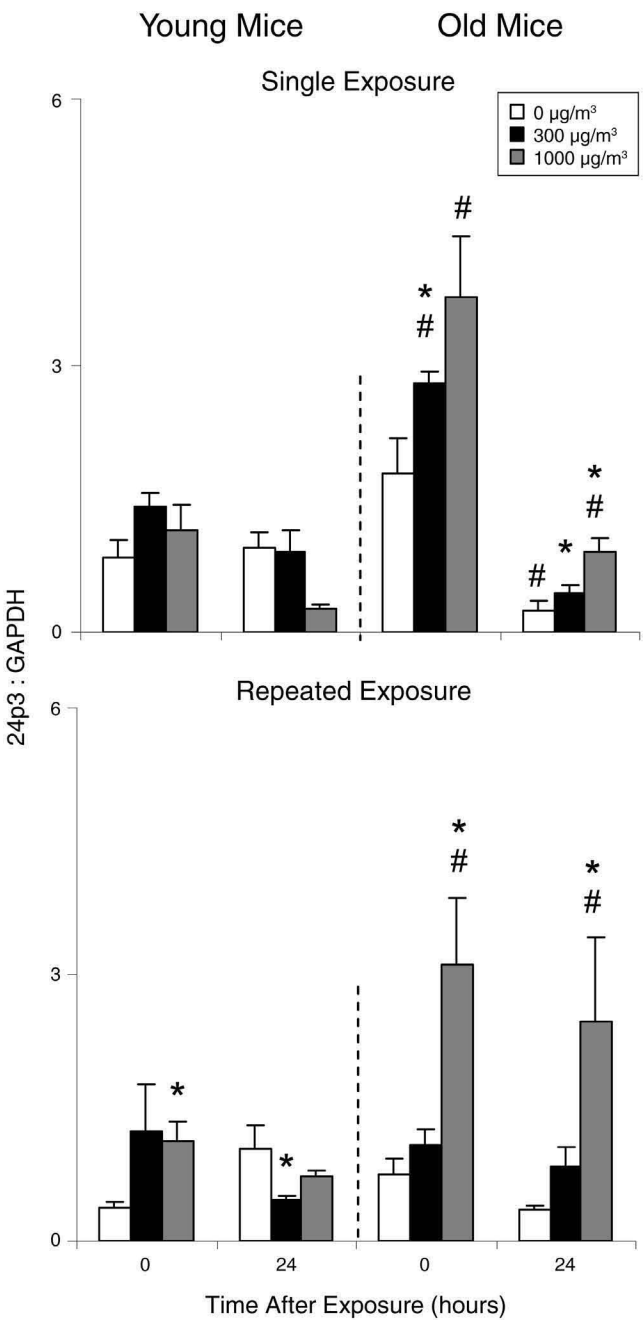

Figure 11. Effects of DE on lipocalin 24p3 mRNA expression. Lung tissue was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. mRNA was extracted from the tissue and analyzed by real-time quantitative PCR for lipocalin 24p3. Data are presented relative to GAPDH mRNA expression. Samples from two to three animals per exposure group were assayed in triplicate. Each bar is the mean + SE (n = 6–9 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

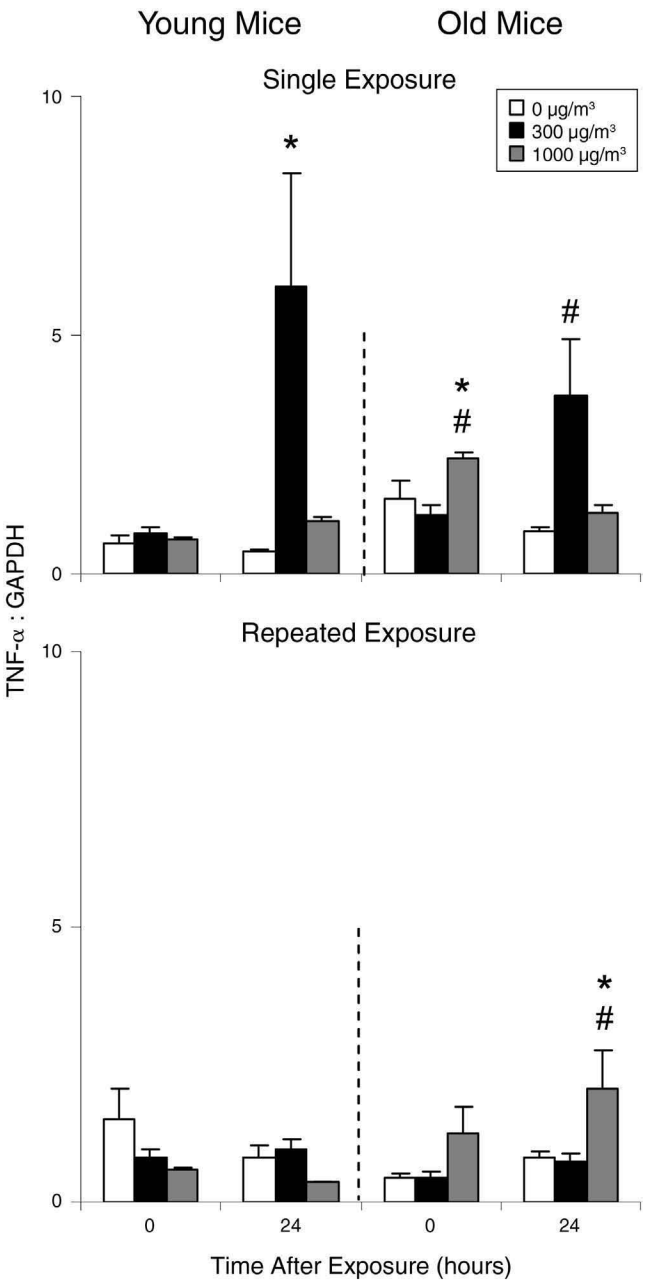

Figure 12. Effects of DE on TNF-α mRNA expression. Lung tissue was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. mRNA was extracted from the tissue and analyzed by real-time quantitative PCR for TNF-α. Data are presented relative to GAPDH mRNA expression. Samples from two to three animals per exposure group were assayed in triplicate. Each bar is the mean + SE (n = 6–9 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

TNF-α was noted 24 hours after exposure to low-dose DE. Although increases in TNF-α mRNA were also observed at 24 hours in old animals after exposure to low-dose DE, this response was significantly attenuated as compared with that in young mice.

Repeated Exposure No significant change in TNF-α expression was noted in young animals after repeated exposure to DE. In old animals, however, a small but significant increase in TNF-α mRNA expression was observed after high-dose DE exposure, but this was only evident after 24 hours (Figure 12, lower panel).

Effects of DE on Serum TNF-α Levels

Single Exposure We next assessed the effects of DE on serum TNF-α levels. Low basal levels of TNF-α were detected in serum from both young and old animals (Figure 13, upper panel). High-dose DE caused a marked increase in serum TNF-α in old, but not young animals, an effect that persisted for 24 hours.

Repeated Exposure In general, the effects of repeated exposure of young and old mice to DE were quite modest. Thus, although a small but significant increase in serum TNF-α was noted in young mice 24 hours after high-dose DE exposure, serum TNF-α levels decreased slightly in old mice (Figure 13, lower panel).

Effects of DE on IL-6, IL-8, and COX-2 mRNA Expression

In further studies we investigated whether exposure of young and old mice to DE differentially modified expression of other inflammatory proteins known to be important in the lung response to pulmonary irritants. These included IL-6, IL-8, and COX-2.

Single Exposure High-dose exposure to DE resulted in significant increases in IL-6 and IL-8 mRNA expression in lungs of old animals, which persisted for 24 hours (Figure 14, upper panels). Although IL-6 was also up-regulated in young mice after a single high-dose DE exposure, no major changes were noted in IL-8 mRNA expression. A single low-dose exposure to DE in young animals also resulted in increased COX-2 mRNA expression, which was observed immediately after exposure (Figure 15, upper panel). In contrast, COX-2 mRNA expression decreased in lungs of old animals exposed to a single dose of DE.

Repeated Exposure Generally similar effects of DE on IL-6 and IL-8 expression were observed after repeated exposure relative to single exposure (Figure 14, lower panels). In contrast, repeated exposure to DE had no consistent effects on COX-2 expression in the lung (Figure 15, lower panel).

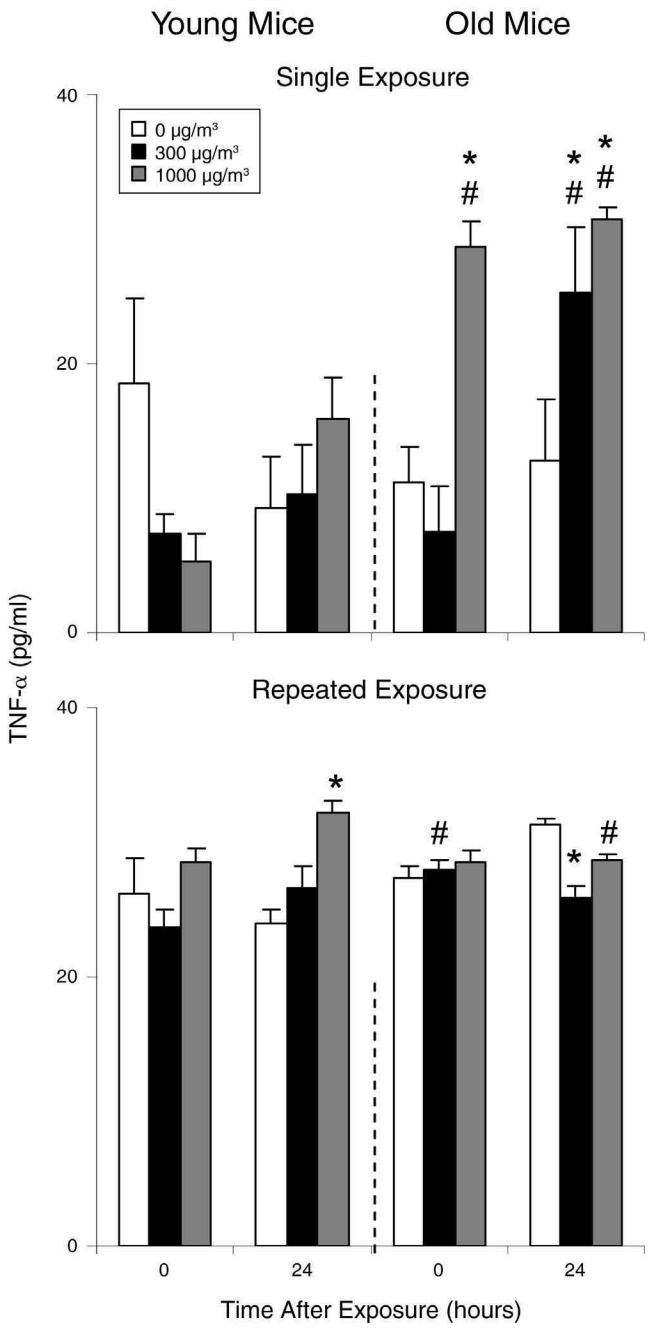

Figure 13. Effects of DE on TNF-α in serum. Serum was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. TNF-α levels were determined by ELISA. Samples from two to four animals per exposure group were assayed in duplicate. Each bar is the mean + SE (n = 4–8 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

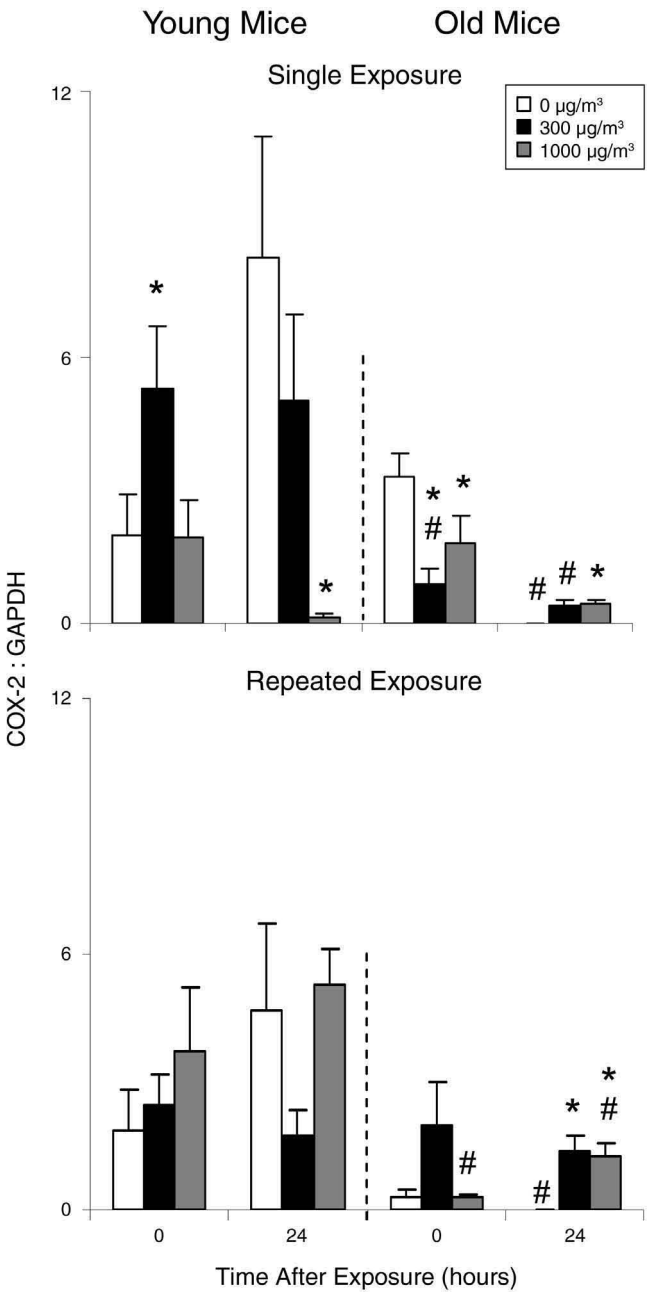

Figure 15. Effects of DE on COX-2 mRNA expression. Lung tissue was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. mRNA was extracted from the tissue and analyzed by quantitative real-time PCR for COX-2 expression. Data are presented relative to GAPDH mRNA expression. Samples from two to three animals per exposure group were assayed in triplicate. Each bar is the mean + SE (n = 6–9 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

Figure 14 appears on next page.

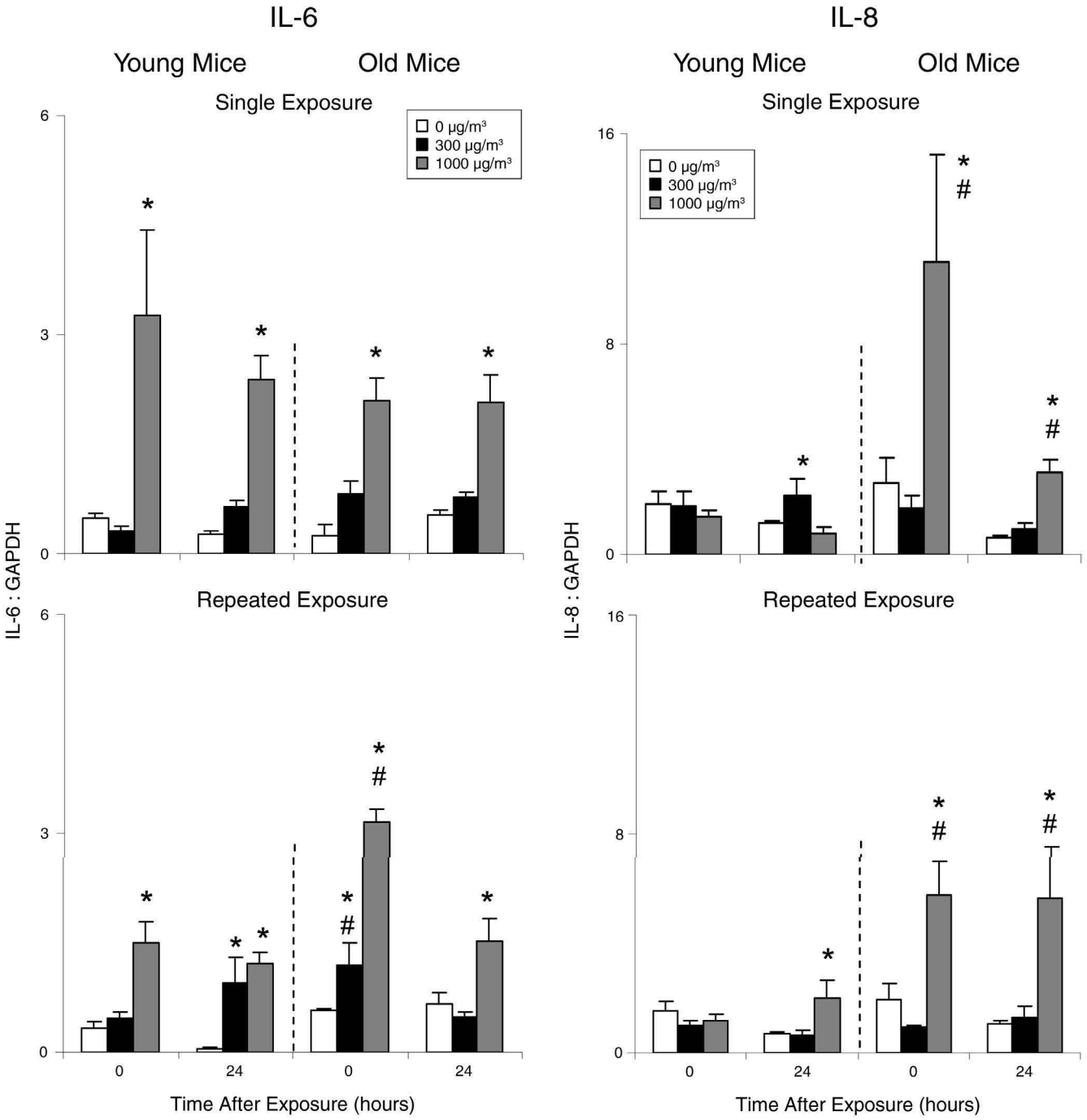

Figure 14. Effects of DE on IL-6 and IL-8 mRNA expression. Lung tissue was collected immediately (0 hours) or 24 hours after a single exposure (upper panel) or repeated exposure (lower panel) of young (2-month-old) and old (18-month-old) mice to air (0 µg/m^3) or to DE at 300 or 1000 µg/m^3. mRNA was extracted from the tissue and analyzed by quantitative real-time PCR for IL-6 and IL-8 expression (left and right panels, respectively). Data are presented relative to GAPDH mRNA expression. Samples from two to three animals per exposure group were assayed in triplicate. Each bar is the mean + SE (n = 6–9 values). The asterisk indicates a significant difference ($P \leq 0.05$) from air-exposed animals; the number sign, a significant difference ($P \leq 0.05$) from the young animals.

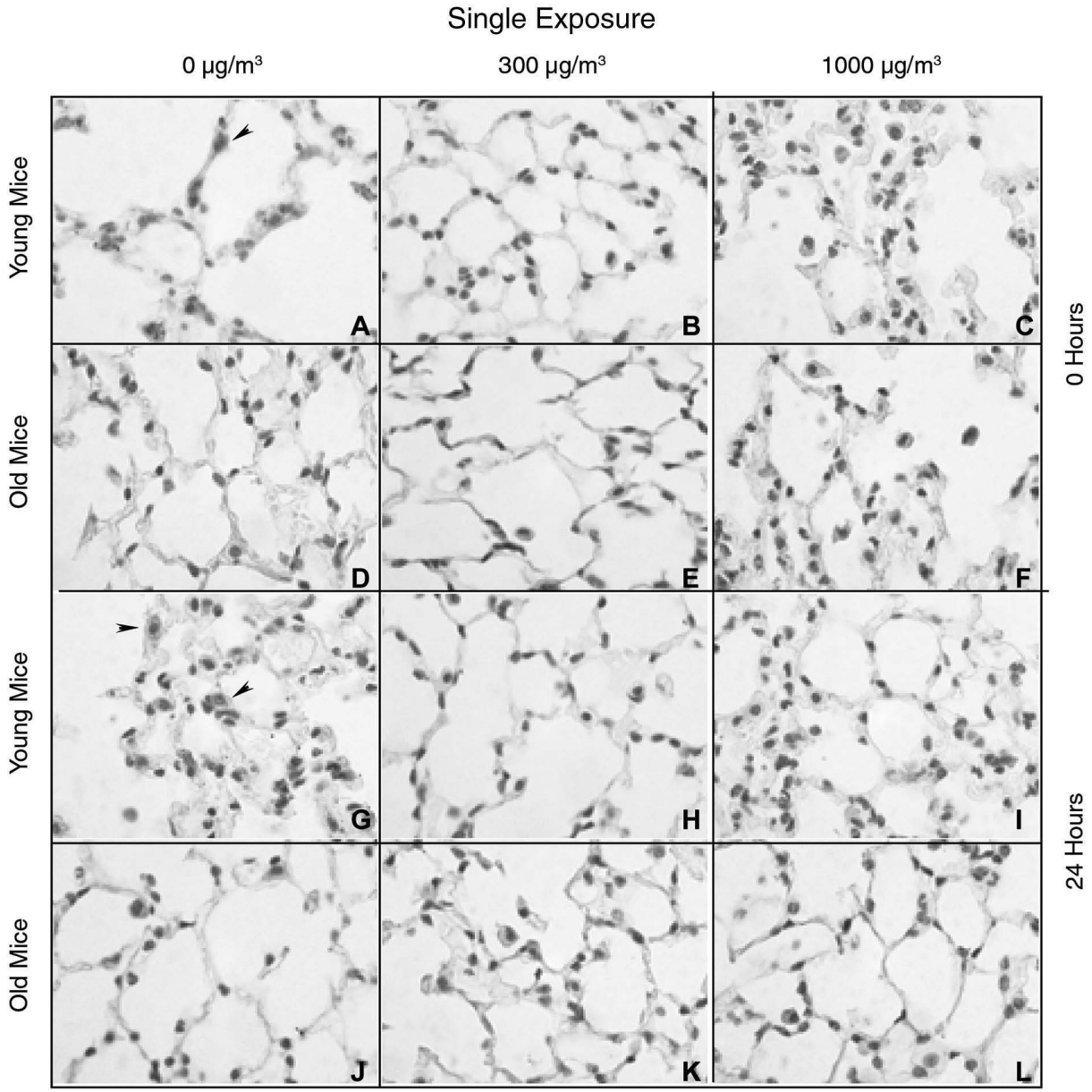

Figure 16. Effects of single DE exposure on MnSOD expression. Lung sections (4 µm), prepared from young (2-month-old) and old (18-month-old) mice immediately (0 hours) and 24 hours after a single exposure to DE (0 [air control], 300, or 1000 µg/m^3), were stained with antibody to MnSOD. One representative section from each exposure group is shown (magnification, ×100). Arrowheads indicate alveolar macrophages.

Effects of DE on MnSOD Expression

Single Exposure Immunohistochemical analysis revealed that young but not old mice, constitutively expressed MnSOD (Figure 16, panels A, and G). Such expression was most prominent in alveolar macrophages (indicated by arrowheads in panels A and G). Whereas exposure to DE at both 300 and 1000 µg/m^3 resulted in a persistent decrease in MnSOD expression in young animals (at both 0 and 24 hours; Figure 16, panels B, C, H and I), no changes were seen in old animals (Figure 16, panels E, F, K, and L).

Repeated Exposure A similar antioxidant response was noted in young mice after repeated exposure to DE. Figure 17 shows a persistent decrease in constitutive expression of MnSOD in these mice, seen at 0 and 24 hours, after repeated exposure to DE (panels A, B, C, G, H, and I). In contrast, MnSOD levels were initially undetectable and

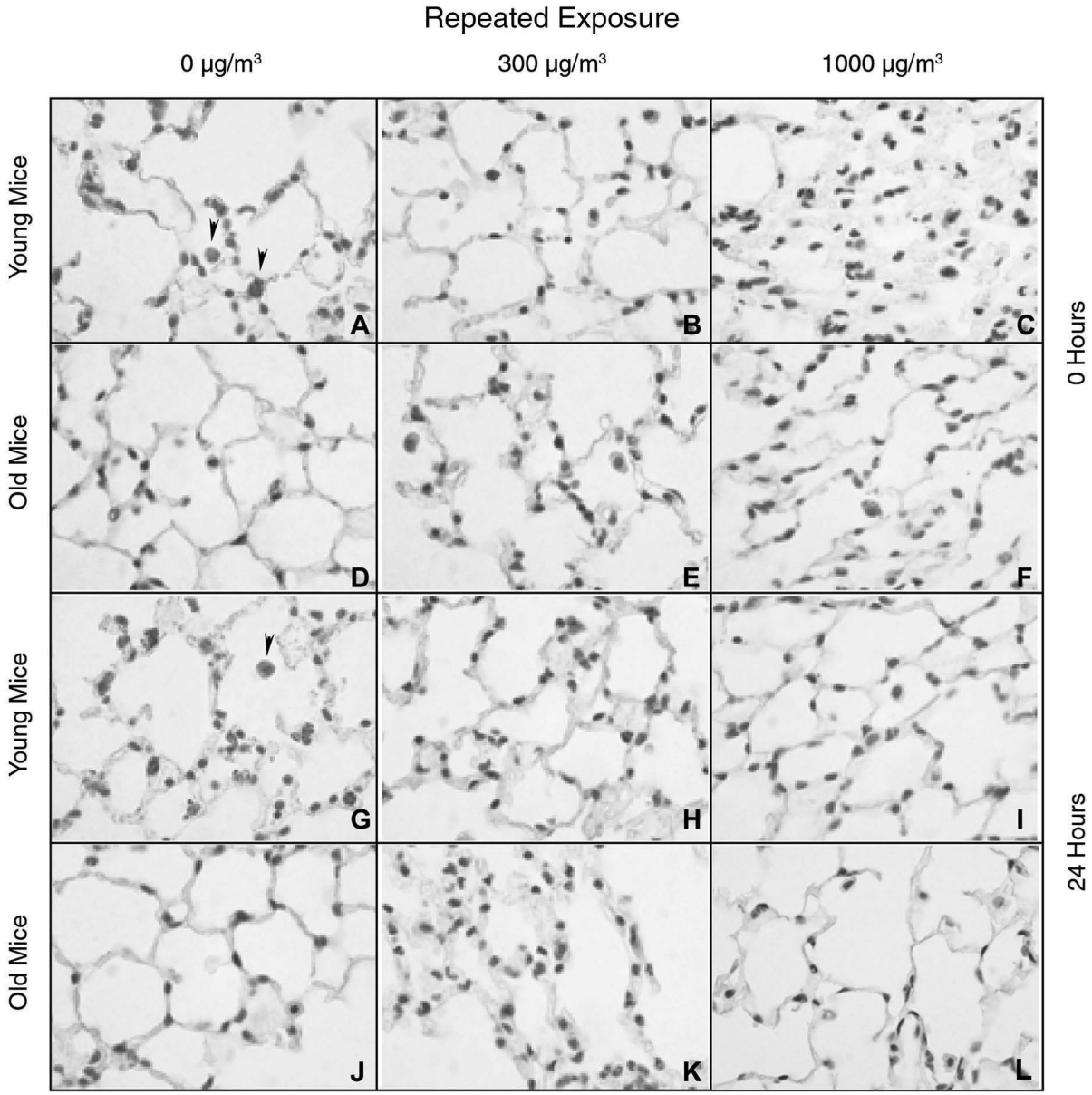

Figure 17. Effects of repeated DE exposure on MnSOD expression. Lung sections (4 µm), prepared from young (2-month-old) and old (18-month-old) mice immediately (0 hours) and 24 hours after repeated exposures to DE (0 [air control], 300, or 1000 µg/m^3), were stained with antibody to MnSOD. One representative section from each exposure group is shown (magnification, ×100). Arrowheads indicate alveolar macrophages.

remained so in old animals after repeated exposure to DE (Figure 17, panels D, E, H, J, K, and L).

DISCUSSION

The aims of this study were to develop and characterize a DE animal exposure system and to compare the effects of inhaled DE on young and old mice. We hypothesized that older animals have increased susceptibility to the adverse cardiopulmonary effects of inhaled DE and that this increase in susceptibility is due to altered expression of inflammatory mediators and antioxidants in the lung. Our overall goal in these pilot studies was to generate preliminary data to support this hypothesis.

The animal exposure chamber that was constructed and used in these studies consisted of a 17-L whole-body Plexiglas divided enclosure that allowed simultaneous exposure of 12 mice (2 groups of six mice on each side of the

enclosure). Using a SidePak personal aerosol mass monitor, we found that particle mass concentrations from 300 μg/m^3 to 1000 μg/m^3 could be maintained stably in the chamber during the 3-hour animal exposure period. Moreover, the mass concentrations in the chamber could be varied without substantially changing the particle-size distribution. The particle-size median diameter distribution within the chamber was found to be approximately 200 to 300 nm, which is larger than previously reported (McDonald et al. 2004). This discrepancy may be due to differences in the animal exposure systems. It is possible that the larger particle-size distribution of our DE resulted in lower deposition in the lung. Copollutant measurements assessed using an IAQ RAE gas monitor revealed relatively low levels of CO and NO in the chamber and markedly higher concentrations of CO_2 and NO_2. Although concentrations of NO remained relatively stable, CO_2 and NO_2 levels increased two- to almost fourfold, respectively, as PM mass increased from 300 to 1000 μg/m^3. These increases are probably due to the fact that a larger fraction of the primary DE was directed into the delivery system to achieve the desired 1000 μg/m^3 mass concentration than was the case with the 300 μg/m^3 concentration, rather than to the presence of contaminants in the air used for dilution.

SUMMARY OF FINDINGS

For our animal studies, three different exposure concentrations (0 μg/m^3 PM [air control], 300 μg/m^3 PM, and 1000 μg/m^3 PM), two exposure regimens (single and repeated), and two postexposure sampling times (0 and 24 hours) were evaluated. (Table 2 summarizes these findings.) In our first series of experiments, we determined whether old mice were more susceptible to the adverse pulmonary effects of DE than young mice. Markers of lung injury (structural and morphologic alterations, BAL-fluid cell number, protein and albumin content, and LDH activity, and lung expression of lipocalin 24p3 mRNA) were assessed. In old but not young mice, exposure to DE was associated with rapid and progressive morphologic and structural alterations in the lung. These included neutrophil accumulation in alveolar spaces and capillaries, as well as focal infiltrates consisting predominantly of plasma cells and macrophages, patchy thickening of alveolar septa, and an increase in the number of macrophages in alveolar spaces. Similar morphologic changes have previously been described in older animals after exposure to PM (Sunil et al. 2007a). The effects of DE on pathological findings in the lung in old mice were dose-related and more pronounced 24 hours after exposure. Interestingly, no major differences were noted between single and repeated exposure to DE, suggesting that the effects of DE on lung morphology are not cumulative. The fact that substantial changes in lung pathology were not observed in young mice is consistent with our hypothesis that old mice are more susceptible to the toxic effects of inhaled DE.

Characteristic features of injury to the lower lung are an accumulation of protein and inflammatory cells in BAL fluid and an increase in LDH activity (Bhalla 1999). Despite substantial evidence of pathological changes in histologic sections of lung tissue, no changes were noted in levels of protein or LDH activity in BAL fluid from old mice. The absence of such changes may reflect the relative insensitivity of

Table 2. Summary of Findings[a]

	Single Exposure		Repeated Exposure	
Marker	Young Mice	Old Mice	Young Mice	Old Mice
Lung histology (neutrophils, macrophages)	No change	Increase	No change	Increase
BAL-fluid cells	Increase	Decrease	Increase	Decrease
BAL-fluid protein	Increase	No change	Increase	No change
BAL-fluid LDH activity	Decrease	No change	Increase	No change
Lung lipocalin 24p3 expression	No change	Increase	Small increase	Increase
Lung TNF-α expression	Increase	Small increase	No change	Small increase
Serum TNF-α	Decrease	Increase	Small increase	Small decrease
Lung MnSOD expression				
Constitutive	+	−	+	−
After DE exposure	−	−	−	−
Lung IL-6 and IL-8 expression	Increase	Increase	Increase	Increase

[a] Plus signs denote the presence of MnSOD expression, and minus signs its absence.

these measures as markers of lung injury and inflammation (Li et al. 1999). Alternatively, DE-induced injury may not be prominent in the lower lung in old mice. In contrast, some alterations in cell number, protein level, and LDH activity in BAL fluid were detected in young animals. In general, these were greater after repeated than after single DE exposure and — for protein and LDH activity — were more pronounced at the 300 μg/m^3 dose of DE. However, no major structural alterations were noted in the lungs of young mice. These findings further indicate the limitations of the use of BAL-fluid protein and cell number as general markers of acute lung injury. Our studies suggest that DE induces a distinct spectrum of toxic effects in young as compared with old mice. A notable finding was that BAL-fluid cell number was reduced in old mice 24 hours after single or repeated DE exposure, as compared with findings in mice exposed to low-dose DE. This reduction is probably due to increased adherence of inflammatory cells to alveolar epithelium, which is consistent with our histologic findings.

Lipocalin 24p3 (also known as Lcn2 and NGAL) is a 25-kDa member of the lipocalin family of proteins. Originally identified as an acute-phase protein produced in the liver (Liu and Nilsen-Hamilton 1995), it has since been demonstrated that 24p3 is up-regulated in a number of disease states including cancer, acute endotoxemia, ischemia–reperfusion injury, β-thalassemia, infection, inflammation, and kidney, lung, and heart injury, as well as burn- and radiation-induced injury (Nielsen et al. 1996; Friedl et al. 1999; Mishra et al. 2004, 2006; Missiaglia et al. 2004; Vemula et al. 2004; Giordano, 2005; Roudkenar et al. 2007; Sunil et al. 2007b; Stevens et al. 2008). The fact that each of these conditions is characterized by excessive production of reactive oxygen species has led to the suggestion that 24p3 is important in the response of cells and tissues to oxidative stress (Roudkenar et al. 2007). This concept is supported by findings that hydrogen peroxide–induced 24p3 expression is inhibited by antioxidants (Roudkenar et al. 2007). Our current study findings demonstrate that exposure of old mice to DE is associated with a rapid dose-related induction of 24p3 expression in the lung. Increased expression occurred immediately after single or repeated exposure to DE and persisted for 24 hours, although at reduced levels. These data suggest that 24p3 may be a highly sensitive biomarker of early oxidative stress in the lung. In contrast to findings in old mice, increases in 24p3 expression in young animals were relatively small and were noted only immediately after repeated exposure to high-dose DE. Similar, relatively small increases in 24p3 expression have also been reported in the lungs of young mice after exposure to the combination of DE and lipopolysaccharide (Yanagisawa et al. 2004). These data provide additional evidence that older mice are more sensitive than younger mice to inhaled DE.

In our next series of studies, we analyzed potential mechanisms underlying the increased susceptibility of old mice to the adverse effects of inhaled DE. We speculated that, after DE exposure, altered production of inflammatory cytokines such as TNF-α, IL-6, and IL-8 and reduced expression of antioxidants such as SOD may underlie this response. TNF-α is a macrophage-derived cytokine implicated in the pathogenesis of lung injury induced by a number of air pollutants (Kelley 1990; Dinarello 1997; Schins and Borm 1999; Gowdy et al. 2008). Although TNF-α possesses pro-inflammatory and cytotoxic activity, it also plays an important role in initiating tissue repair. The latter role is thought to be due to the up-regulation of antioxidants, such as SOD, catalase, and heme oxygenase, and stimulation of epithelial-cell proliferation and extracellular-matrix turnover (Tsan et al. 1990; Sasaki et al. 2000; Ryter et al. 2002; Alam and Cook 2003; Chiu et al. 2003b; Guo et al. 2003). In accordance with previous studies (Saber et al. 2006; Gowdy et al. 2008), we found significant increases in expression of TNF-α in the lungs of young animals 24 hours after exposure to DE. Interestingly, this response was most notable after exposure of young mice to 300 μg/m^3 PM, which is consistent with our findings of increases in BAL-fluid cells and LDH activity. It may be that higher doses of DE cause suppression of inflammatory responses in the lung (Yin et al. 2002).

Although TNF-α expression also increased in lungs of old animals 24 hours after exposure to DE at 300 μg/m^3 PM, this response was attenuated relative to that in young mice. A similar attenuation of TNF-α in lung or BAL fluid has previously been described in older animals after induction of endotoxin shock and after exposure to silica or terpene oxidation products (Corsini et al. 2003, 2004; Ito et al. 2007; Sunil et al. 2007a). Age-related decreases in production of TNF-α have also been described in isolated monocytes and macrophages (Lloberas and Celada 2002; Renshaw et al. 2002; Boehmer et al. 2004). Notably, this response appears to be tissue-specific, with the lung showing the greatest decrease in TNF-α activity (Bradley et al. 1989; Higashimoto et al. 1993; Shimada and Ito 1996). It remains to be determined whether these aberrations in TNF-α production in older mice are important in their susceptibility to inhaled DE.

In contrast to the effects of DE on TNF-α expression in the lung, serum TNF-α levels were increased in old mice, but not young mice, after a single DE exposure. Whereas at the higher dose of DE, this effect was observed immediately after exposure, at the lower dose the response was delayed for 24 hours. The origin of TNF-α in serum from old mice is unknown. It may reflect TNF-α rapidly released in the lung after DE exposure. Alternatively, TNF-α

may be released by extrapulmonary tissues responding to DE-induced oxidative stress. Additional studies are required to explore these possibilities.

IL-6 and IL-8 are known to be important in regulating inflammatory-cell trafficking into injured tissues (Murtaugh et al. 1996; McClintock et al. 2008). Previous studies have described DE-induced increases in IL-6 and IL-8 in rodent lung (Gowdy et al. 2008) and in cultured bronchial epithelial cells (Steerenberg et al. 1998). Similarly, we noted significant increases in IL-6 and IL-8 mRNA expression in lungs of young and old animals after both single and repeated exposure to DE. Whereas the kinetics of IL-6 mRNA expression appeared to be independent of age, IL-8 expression differed markedly between young and old animals: IL-8 levels increased in old animals immediately after DE exposure, but expression of this cytokine was delayed for 24 hours in young animals. A number of studies have described increased production of IL-8 with advancing age (Esposito et al. 1989; Himi et al. 1997; Pulsatelli et al. 2000). Our finding that IL-8 is generated more rapidly in the lung in old mice after DE exposure accords with these studies. IL-8 is a potent chemoattractant for neutrophils (Kafoury and Kelley 2005; Matsuzaki et al. 2006). These cells are known to be a major source of reactive oxygen species. Our findings of increased IL-8 in old mice after exposure to DE are consistent with our histologic data showing increased accumulation of neutrophils in the lung. These cells may contribute to excessive oxidative stress in the elderly in response to inhaled DE.

We also analyzed expression of COX-2, an inducible enzyme that mediates the production of prostaglandins during inflammation and immune responses (Park and Christman 2006). COX-2 expression has been reported to be induced in lung cells after exposure to DE particles (Inoue et al. 2004; Cao et al. 2007; Ahn et al. 2008). Similarly, we found that a single DE exposure was associated with up-regulation of COX-2 expression in young mice. In contrast, COX-2 expression decreased in old animals after exposure to DE. These results were surprising, since COX-2 expression and prostaglandin E_2 production have been reported to be up-regulated in elderly animals (Go et al. 2007; Meydani and Wu 2007; Wu et al. 2007; Tang and Vanhoutte 2008). These findings suggest that, in our current studies with DE in mice, the proinflammatory activity of COX-2 may not play a major role in determining susceptibility to DE; however, this remains to be determined.

Li and colleagues (2004) have suggested that oxidative stress is a key mechanism by which ambient PM induces adverse health effects. According to these investigators, PM-induced oxidative stress is a multitier response, in which cytoprotective responses transition to injurious effects as the level of oxidative stress increases. Antioxidants play a critical role in host defense by scavenging and detoxifying oxidants. Thus, they minimize tissue damage and the development of diseases associated with oxidative stress (Heffner and Repine 1991; Nel et al. 1998; Dhalla et al. 2000; Lang et al. 2002). One antioxidant that plays a key role in protecting cells and tissues from oxidative stress is SOD. Three isoforms of SOD have been identified, MnSOD, copper–zinc SOD (CuZnSOD) and extracellular SOD (EC-SOD), each with different structures and tissue distributions (Kinnula and Crapo 2003). The important protective role of SOD is most evident in studies demonstrating reduced mortality and tissue injury after treatment with hypoxia, O_3, PM, radiation, or bleomycin in transgenic mice that overexpress CuZnSOD or EC-SOD (Janssen et al. 1993; Weinberger et al. 1998; Fakhrzadeh et al. 2004). With increasing age, constitutive levels of tissue antioxidants and the capacity to respond to oxidative stress decline (Lykkesfeldt and Ames 1999; Squier 2001; Thomas and Mallis 2001; Servais et al. 2005). Thus, whereas constitutive expression of antioxidant enzymes such as SOD rapidly decreases in younger animals as it is utilized after exposure to PM, in older mice this response is reduced or absent (Elsayed et al. 1982; Sagai et al. 1993; Lim et al. 1998; Ghio et al. 2002; Sunil et al. 2007a). We found that young mice constitutively expressed MnSOD, which was predominantly localized in alveolar macrophages. Single or repeated exposure of young mice to DE resulted in a rapid reduction in MnSOD expression, which persisted for at least 24 hours. In contrast, constitutive expression of MnSOD was not evident in lungs of old mice, and DE did not alter its expression. These findings are consistent with our hypothesis that the increased susceptibility of older mice to DE is due to aberrant antioxidant defense. Further studies are necessary in order to elucidate mechanisms regulating expression of antioxidants such as SOD in the lung.

In summary, the data obtained in this pilot study provide strong support for our hypothesis that there are major aberrations in the production of inflammatory mediators and antioxidant defenses in the lungs of old mice in response to inhaled DE. Further studies are needed to directly test our hypothesis that these alterations underlie increases in susceptibility of elderly persons to PM.

PROBLEMS ENCOUNTERED DURING THE STUDY

In conducting our study, we encountered some technical difficulties that required us to make adjustments to our proposed approach.

First, in the original project plan, we proposed to evaluate the effect of DE in young and old mice using four test atmospheres (i.e., filtered air, 100 $\mu g/m^3$, 300 $\mu g/m^3$, and 1000 $\mu g/m^3$ PM) and one postexposure sampling time (24 hours). Using the existing DE-generation system at

EOHSI, which was modified for animal exposures, we found that we were unable to maintain stable particle mass concentrations of 100 μg/m^3 in the animal exposure chamber. The instability in the concentrations probably reflects the fact that, at this concentration, only a small fraction of the total DE is directed toward the animal exposure system, relative to the amount that is diverted to the exhaust vent (see Figure 1). As a consequence, the exhaust from the animal exposure chamber became highly susceptible to variations in the concentration in the engine exhaust, as well as to the effects of wind drafts at the vent. Because we could not overcome this difficulty within the time frame and budget constraints of the pilot project, we were forced to limit our studies to the exposure concentrations of 300 and 1000 μg/m^3 DE. However, we included an additional postexposure sampling time of 0 hours (immediately after the exposure), a time at which we had previously found that some biologic effects could be detected.

Second, we originally proposed to assess thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxidation in BAL fluid, as well as TNFR1 in serum using ELISA. However, because insufficient quantities of BAL fluid and serum were recovered from some of the mice, and because some of the samples were contaminated with red blood cells, we were unable to perform these assays in sufficient replicates to permit statistical analysis of the data.

Third, in the original project plan, we proposed to assess TNF-α and TNFR1 protein expression in the lung. Unfortunately, we encountered technical problems and were unable to measure these proteins in lung tissue by immunostaining or by Western blotting. The reason for these technical problems is unclear. It may be that the commercial antibodies that we tested (from three different vendors) did not recognize the exposed antigenic determinants in mouse lung. Alternatively, the postexposure analysis times that we chose (0 hours and 24 hours) may not have been appropriate for the detection of changes in these proteins. It is also possible that exposure to DE may have caused the release of TNF-α from the cells. Immunohistochemical analysis and Western blotting are not suitable techniques for the detection of secreted proteins. Thus, in future studies, we may need to use an ELISA.

FUTURE STUDIES

The overall hypothesis underlying our research is that production of inflammatory proteins such as TNF-α, IL-6, IL-8, and COX-2 by alveolar macrophages and antioxidant defense mechanisms in the lung are altered in the elderly and that this change increases susceptibility to oxidative stress and tissue injury induced by fine PM. The studies described in this report provide preliminary data that support this hypothesis. In this study, after mice were exposed to DE, we found reduced expression of TNF-α mRNA and increased expression of IL-6 and IL-8 mRNA in lungs of old mice relative to that in young mice; moreover, antioxidant defense responses (e.g., MnSOD expression) were impaired in the old mice. These preliminary data provide a strong basis and rationale for continuing studies aimed at testing our hypothesis. For example, such studies might include assessment of the injurious and inflammatory effects of DE on the lung, as well as the heart, at lower concentrations than those we studied (e.g., 30 and 100 μg/m^3) and at additional postexposure sampling times (e.g., 6 hours, 48 hours, 72 hours, 7 days, 14 days). In addition to structural evaluation by light and electron microscopy, biochemical markers of tissue injury could be assessed, including troponin C and troponin I in the heart, as well as levels of immunoglobulin M and fibrin in the lung, which are potentially more specific markers of alveolar epithelial injury than used in the current study.

It would also be of interest to assess expression of 24p3 mRNA and protein in the lung and heart as an early marker of oxidative stress. The expression of various cytokines (TNF-α, TNFR1, IL-1, IL-6, IL-8, IL-10), chemokines (monocyte chemotactic protein 1 [MCP-1], macrophage inflammatory protein 2 [MIP-2]), and antioxidants (SOD, ascorbate, glutathione *S*-transferase [GST], nicotinamide adenine dinucleotide phosphate [NADPH], quinine oxidoreductase, catalase, glutathione peroxidase, and heme oxygenase) in lungs and hearts of young and old mice might be evaluated after DE exposure to determine whether these substances are important in the increased susceptibility of the elderly to PM. A unique aspect of future studies might be a comparative analysis of the effects of DE in a transgenic animal model of chronic emphysema (surfactant protein D–deficient mice). Such studies would be particularly relevant, since chronic emphysema is a common lung disease observed in elderly persons, particularly in those who have smoked cigarettes. We predict that in mice that lack the gene for surfactant protein D, older animals that have developed emphysema will be more susceptible to DE than their younger counterparts or than wild-type mice.

The results of such additional studies are likely to be important, since they should provide mechanistic data that may be useful in designing efficacious strategies aimed at preventing or reducing cardiopulmonary morbidity and mortality in the geriatric population.

IMPLICATIONS OF FINDINGS

The mechanisms underlying increases in the susceptibility of elderly persons to the adverse effects of inhaled PM are unknown. Data generated in our pilot studies suggest

that, after initial exposure to DE, aberrant production of inflammatory cytokines such as TNF-α, IL-6, and IL-8 and alterations in downstream events such as the expression of antioxidants may be key determinants of susceptibility. Further studies are necessary to define the precise relationship between inflammatory mediators and antioxidant defenses in the lung and possibly the heart in elderly persons. Results from these further studies should provide mechanistic insights into the pathogenesis of toxicity that may be useful in designing effective strategies for treatment or prevention that can limit PM-induced cardiopulmonary morbidity and mortality in susceptible populations.

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ABBREVIATIONS AND OTHER TERMS

ANOVA	analysis of variance
APS	Aerodynamic Particle Sizer
BAL	bronchoalveolar lavage
BCA	bicinchoninic acid
cDNA	complementary DNA
CO	carbon monoxide
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
CPC	condensation particle counter
CuZnSOD	copper–zinc superoxide dismutase
DE	diesel exhaust
EC	elemental carbon
EC-SOD	extracellular SOD
ELISA	enzyme-linked immunosorbent assay
EOHSI	Environmental and Occupational Health Sciences Institute
fine PM	particulate matter ≤ 2.5 μm in aerodynamic diameter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione *S*-transferase
HEPA	high-efficiency particulate air
IAQ	indoor air quality
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	intereukin-10
LDH	lactate dehydrogenase
MCP-1	monocyte chemotactic protein 1
MIP-2	macrophage inflammatory protein 2
MnSOD	manganese superoxide dismutase
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain enhancer of activated B cells
NO	nitrogen monoxide
NO_2	nitrogen dioxide
NO_x	nitrogen oxide
O_3	ozone
OC	organic carbon
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PM	particulate matter
$PM_{2.5}$	particulate matter ≤ 2.5 μm in aerodynamic diameter
PM_{10}	particulate matter ≤ 10 μm in aerodynamic diameter
RFPA	request for preliminary applications
SD	standard deviation
SE	standard error
SMPS	Scanning Mobility Particle Sizer
SO_2	sulfur dioxide
SOD	superoxide dismutase
TC	total carbon
TNF-α	tumor necrosis factor α
TNFR1	TNF receptor 1
Ultrafine particles	particles < 0.1 μm in aerodynamic diameter
UMDNJ	University of Medicine and Dentistry of New Jersey

Research Report 151, *Pulmonary Effects of Inhaled Diesel Exhaust in Young and Old Mice: A Pilot Project*, D.L. Laskin et al.

INTRODUCTION

HEI periodically issues a request for preliminary applications (RFPA*) for novel research on the health effects of air pollutants derived from motor vehicle emissions. In response to RFPA 05-3, "Health Effects of Air Pollution," issued in 2005, Dr. Debra L. Laskin, of Rutgers University and the Environmental and Occupational Health Sciences Institute, and colleagues submitted an application for an animal study entitled "Cardiopulmonary Effects of Inhaled Diesel Exhaust in the Elderly." The reasoning underlying the proposal was that exposure to particulate matter (PM) has been associated with increases in cardiopulmonary morbidity and mortality, with elderly people particularly susceptible (reviewed in U.S. Environmental Protection Agency 2004); however, biologic pathways to explain why elderly people might be more susceptible than younger people to the effects of PM have not been examined extensively. On the basis of her group's preliminary data and for further reasons described below under "Scientific Background," Laskin extended this reasoning to study another mammalian species, mice. She and her colleagues hypothesized that greater susceptibility to PM resulted from impaired production of the cytokine tumor necrosis factor α (TNF-α) by alveolar macrophages in elderly animals, as compared with young animals. The group proposed to test this hypothesis by exposing young and elderly mice to diesel exhaust (DE) emissions, a component of PM found in urban air. They further hypothesized that this impairment of TNF-α production in lung cells would in turn lead to impairment of protective antioxidant defense mechanisms in the heart.

The HEI Research Committee thought that Laskin and colleagues' preliminary data were intriguing and the proposed focus on the role of TNF-α in mediating responses to DE was novel and likely to produce interesting results. Laskin's group had proposed a 3-year study, but the Research Committee decided to delay consideration of funding for this full period. The Committee wanted the investigators first to establish whether the lungs of young and elderly mice differed in their ability to synthesize TNF-α. After Laskin and colleagues submitted a revised application, HEI approved a 1-year pilot study to evaluate this hypothesis. The Investigators' Report and this Critique describe the results of the pilot study by Laskin et al.

SCIENTIFIC BACKGROUND

CHARACTERISTICS AND EFFECTS OF DE

DE is an important component of air pollution in urban environments. DE from older engines contributes large amounts of nitrogen oxides (NO_x) and primary PM (that is, PM from sources that emit directly into the atmosphere) to urban air, although most urban PM is derived from city and regional background. DE is a complex mixture of particulate and gaseous compounds that vary with engine type. DE particles are composed of a carbon core to which several compounds — nitrate, sulfate, metals, and organic compounds — adhere. The particles emitted by diesel engines (as well as by gasoline engines) fall into a bimodal size distribution: nuclei mode (particles < 50 nm in aerodynamic diameter), which accounts for most of the particles by number, and accumulation mode (particles from 50 nm to approximately 250 nm in aerodynamic diameter, thus falling in the category of PM ≤ 2.5 μm in aerodynamic diameter [$PM_{2.5}$]), which accounts for most of the particles by mass. The principal gaseous components of DE are carbon dioxide (CO_2), carbon monoxide (CO), NO_x, sulfur oxides, and low-molecular-weight hydrocarbons.

Epidemiologic studies published since the 1990s suggested that short-term exposure to traffic-related pollution in urban areas is associated with exacerbation of respiratory symptoms in people (especially children) with asthma (HEI Panel on the Health Effects of Traffic-Related

Dr. Debra L. Laskin's 1-year study, "Cardiopulmonary Effects of Inhaled Diesel Exhaust in the Elderly," began in October 2006. Total expenditures were $112,480. The draft Investigators' Report from Laskin and colleagues was received for review in May 2008. A revised report, received in September 2008, was accepted for publication in October 2008. 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 the Review Committee's Critique.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

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

Air Pollution 2010). In these studies, DE was likely to have been a contributor to the pollution mix. In addition, some controlled-exposure studies involving healthy and asthmatic volunteers exposed to DE had found small changes in inflammatory and immunologic endpoints (Rudell et al. 1999; Nightingale et al. 2000; Nordenhäll et al. 2001; Salvi et al. 1999, 2000). Further experimental studies suggested that intranasal administration of DE particles affected the nasal immune response of people with asthma (Diaz-Sanchez et al. 1994, 1997). Instillation of DE elicited pulmonary immune responses in rodent models of allergic airway disease (Fujimaki et al. 1997; Takano et al. 1997; Miyabara et al. 1998).

OXIDATIVE STRESS IN THE RESPONSE TO DE AND OTHER PARTICLES

As described above, several studies indicate that exposure to PM such as DE results in the development of an inflammatory response, a key component of the body's defense mechanisms against outside agents (HEI 2002). How do these inflammatory responses develop? A voluminous literature, particularly from studies in rodents and in vitro, plausibly links inflammation that develops after exposure to DE or components of DE to the generation of reactive oxygen species and the development of oxidative stress inside cells (e.g., Li et al. 2002). Oxidative stress results from an imbalance between the synthesis of oxidants—reactive oxygen species such as superoxide and oxygen-based free radicals, together with hydrogen peroxide—and the activity of antioxidant enzymes and levels of nonenzymatic antioxidants (such as glutathione) that detoxify (clear) the oxidants. Antioxidant enzymes include superoxide dismutase (SOD), heme oxygenase, catalase, and glutathione peroxidase. Oxidative stress activates intracellular signal-transduction pathways that activate the expression of "proinflammatory" molecules, cytokines including TNF-α, and chemokines such as interleukin (IL)-8 that recruit inflammatory cells into tissues (Rahman et al. 2002; Kaur et al. 2006).

TNF-α PRODUCTION AND INFLAMMATORY AND ANTIOXIDANT RESPONSES IN YOUNG AND OLD ANIMALS

Production of TNF-α by airway cells is thought to play a central role in contributing to lung inflammation and injury (reviewed in Samet and Ghio 2007). (We have followed the investigators' use of the term "production," rather than "synthesis," because some evidence exists [e.g., Coward et al. 2002] that TNF-α released by lung cells such as mast cells may be preformed rather than newly synthesized.) TNF-α activates the transcription factor NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells), which in turn activates the transcription of genes whose products play a role in the inflammatory response. In addition, NF-κB activates the transcription of protective genes such as the manganese-dependent isoform of SOD (MnSOD), as well as other antioxidants (Rahman et al. 2002, Kaur et al. 2006). Thus, although TNF-α may play a role in initiating damage to tissues, it may also help limit these effects through upregulation of expression of antioxidants, initiation of tissue repair, and regulation of inflammatory-cell trafficking into tissues in response to damage (Wong et al. 1996, Chiu et al. 2003a,b).

Some studies have suggested that components of the inflammatory response are diminished in elderly humans and rodents compared with the inflammatory responses generated in younger individuals. For example, some functions of neutrophils and macrophages, key cells in the inflammatory response, are impaired in older humans and rodents. Neutrophils from older people generated less superoxide anion than did neutrophils from younger people and showed reduced chemotaxis (movement toward a chemical stimulus) (Biasi et al. 1996; Di Lorenzo et al. 1999; Fulop et al. 2004). In addition, phagocytosis and respiratory burst activity in alveolar macrophages from old animals were reduced compared with those in cells from young animals (Higashimoto et al. 1993; Plackett et al. 2004). Furthermore, decreased production of TNF-α by macrophages, particularly from the lung, has also been noted with increasing age (Shimada and Ito 1996; Lloberas and Celada 2002; Renshaw et al. 2002; Boehmer et al. 2004) and in older animals after exposure to infectious agents or terpene oxidation products (Renshaw et al. 2002; Sunil et al. 2007a).

Studies have also suggested that levels of antioxidants and the ability to respond to oxidative injury decline with age (Lykkesfeldt and Ames 1999; Squier 2001; Servais et al. 2005). For example, antioxidant enzymes such as SOD and catalase are rapidly induced in younger rodents after exposure to hypoxia, ozone, or certain types of PM, but this induction does not occur in older animals (Mustafa et al. 1985; Montgomery et al. 1987; Oberdörster et al. 2000).

In the current study, Laskin and colleagues sought to evaluate whether the production of TNF-α, changes in levels of molecules associated with inflammation and injury, and oxidative stress and protective antioxidant responses differed in old and young mice exposed to DE.

STUDY AIMS

The objective of this pilot study was to determine whether impairments in TNF-α production by alveolar macrophages and TNF-α–induced antioxidant defense mechanisms in

elderly mice exposed to DE are associated with greater susceptibility to PM-induced oxidative stress and enhanced lung injury. To meet these objectives, Laskin and colleagues originally proposed the following specific aims:

1. Develop and characterize a DE animal exposure system.
2. Determine whether old mice are more susceptible to the adverse pulmonary effects of inhaled DE than young mice.

The investigators proposed to assess endpoints associated with inflammation and oxidative stress in the lungs (by histopathologic analysis of lung sections and assessment of changes in levels of molecules in bronchoalveolar-lavage [BAL] fluid):

3. Determine whether the increased sensitivity of old mice to inhaled DE is associated with impaired expression of TNF-α, TNF receptor 1 (TNFR1), and antioxidants.

The investigators proposed to focus on expression of TNF-α, TNFR1, and SOD in the lung and on levels of TNF-α and soluble TNFR1 proteins in serum.

In the final version of the report that Laskin and colleagues submitted, Specific Aim 3 was modified, in response to the Review Committee's initial comments, to read as follows: "Determine whether any increase in the susceptibility of old mice to inhaled DE is associated with altered expression of inflammatory mediators and antioxidants." In addition to TNF-α and SOD, the investigators evaluated the expression of cytokines and cyclooxygenase-2 (COX-2) in lung tissue.

As Laskin et al. explain in the Investigators' Report, the team was unable to measure levels of TNFR1 and TNF-α protein in lung tissue or TNFR1 in serum, so no TNFR1 data are included in the report. The lack of success in making these measurements in lung tissue was attributed to technical problems in the use of the antibodies available to detect the proteins; the lack of success in measuring TNFR1 in serum was attributed to the fact that the quantities of serum recovered in some mice were too small for the investigators to be able to perform sufficient replicates of the assays.

METHODS

EXPOSURE SYSTEM

Laskin and colleagues developed an exposure system using a Yanmar 406-cc diesel-powered electric generator and a dilution-and-delivery system (Figure 1 in the Investigators' Report). The investigators operated the generator using diesel fuel with a sulfur content of < 500 ppm and 40-weight motor oil.

EXPOSURE CHARACTERIZATION

The investigators measured number-based and mass (volume)-based particle-size distributions using a Scanning Mobility Particle Sizer and an Aerodynamic Particle Sizer, respectively. In addition, the investigators measured PM content after collecting samples on quartz-fiber filters. Laskin and colleagues performed characterization of DE inside both the exhaust pipe and the exposure chamber using water-based and butanol-based condensation particle counters.

Animal Exposures

Young (2-month-old) and old (18-month-old) male CB6F1 mice were exposed for 3 hours on one day ("single exposure") or for 3 hours on each of three consecutive days ("repeated exposure") to DE at a concentration of 300 $\mu g/m^3$ or 1000 $\mu g/m^3$ PM, or to filtered air (0 $\mu g/m^3$ PM).

Biologic Endpoints

Laskin and colleagues killed mice either immediately (0 hours) or 24 hours after exposure. They collected blood samples from the heart (to obtain serum TNF-α levels) and removed the largest lobe of the lung for histologic, immunohistochemical, and RNA analysis. Inflammatory changes in lung tissue were measured histologically by qualitatively assessing numbers of alveolar macrophages and neutrophils, alterations in alveolar epithelial barriers, and degree of edema. Laskin and colleagues isolated and reverse-transcribed lung-tissue messenger RNA (mRNA) to evaluate mRNA expression in the lung of the cytokines TNF-α, IL-8 (a cytokine that recruits neutrophils into tissues in response to an inflammatory stimulus), and IL-6 (an acute-phase protein, a component of the rapid systemic response to infectious and other agents); COX-2 (an enzyme that synthesizes prostaglandins, which are involved in inflammatory responses); and lipocalin 24p3 (another acute-phase protein). The function of lipocalin 24p3 is discussed further in the HEI Review Committee Evaluation section below.

The investigators also lavaged the remaining lobes of each mouse's lungs and determined total protein and albumin content and lactate dehydrogenase activity in BAL fluid, as markers of vascular permeability and of lung injury.

STATISTICAL METHODS AND DATA ANALYSIS

Laskin and colleagues used two-way analysis of variance (ANOVA) to test the impact of age on each response variable (e.g., BAL-fluid protein level or cell number) at

each exposure concentration. The investigators fitted two-way ANOVA models to each data set and examined the underlying modeling assumption of each model using normal quantile–quantile plots. Extreme observations (i.e., outliers) were removed before hypothesis testing.

In addition, the investigators used one-way ANOVA models to test the impact of the exposure concentration (0, 300, or 1000 μg/m^3 PM) on the outcome for each exposure regimen (single or repeated), sampling time (0 or 24 hours), and age group (2 months or 18 months). Again, modeling assumptions were examined using quantile–quantile plots, and extreme observations removed before hypothesis testing. For each pair of exposure concentrations (0 vs. 300, 0 vs. 1000, and 300 vs. 1000 μg/m^3 PM), the investigators tested the hypothesis that exposure resulted in the same outcome.

RESULTS

CHARACTERISTICS OF PARTICLES AND GASES GENERATED

Critique Table 1 summarizes the characteristics of the particles and gases generated by the DE exposure system and as measured in the exposure chamber at the two levels of DE used, 300 and 1000 μg/m^3 PM. The 300 μg/m^3 PM mass concentration had a total carbon concentration of 239 μg/m^3, of which the organic carbon (OC) concentration was 124 μg/m^3 and the elemental carbon (EC) concentration was 115 μg/m^3.

BIOLOGIC EFFECTS OF EXPOSURE TO DE

The biologic effects of exposure to DE are described in the section below and shown in Critique Table 2.

Critique Table 1. Gas and Particle Characteristics of DE

	Exposure Atmospheres		
	Air	300 μg/m^3 PM	1000 μg/m^3 PM
Mean diameter (nm)	—	273	242
Total particle number/cm^3	—	3.6×10^4	$> 1 \times 10^5$
Median mass diameter (nm)	—	487	411
Mean mass diameter (nm)	—	2200	1676
Gases (ppm)			
NO_x	< 0.02	4.3	14.5
NO_2	—	3.9	14.0
CO	< 1	4.0	6.6
CO_2	350	1196	2510

HEI REVIEW COMMITTEE EVALUATION OF THE STUDY

In its independent review of the study, the HEI Health Review Committee thought that Dr. Laskin and colleagues had succeeded in creating a DE exposure system and in generating preliminary data on multiple endpoints in response to the inhalation of DE in young and old mice. In addition, the Committee thought the hypothesis for the study was novel and interesting and that the study had provided preliminary support, particularly from the limited and qualitative histologic data in lung tissue, for the hypothesis that the acute response to DE differed in the lungs of old and young mice.

However, although the Review Committee found the results interesting, it noted that the findings showed a more complex and confusing pattern than reported in the investigators' summary of findings in Table 2 of the Investigators' Report. That table presented changes in only selected endpoints and reported changes even if they were detected at only one exposure level or at one timepoint. Critique Table 2 provides a more complete view of all the results obtained.

As Critique Table 2 indicates, single or repeated exposure to DE at high or low levels (1000 or 300 μg/m^3 PM, respectively) did not affect many of the endpoints measured in young and old mice. Of the endpoints that were affected by DE exposure, the pattern of changes was complex: some changes were noted after low-dose but not after high-dose DE exposure, and other changes after single but not repeated exposure. These findings present a complicated picture of the differences between responses to DE exposure in young and old mice. The Committee also noted that there was additional uncertainty about how *P* values should be interpreted, because the investigators excluded outliers in some statistical analyses without any adjustment to the statistical tests used.

The investigators had hypothesized that production of TNF-α would differ in young and old mice after exposure to DE. However, the Review Committee found little basis to support this hypothesis. Expression of TNF-α mRNA in lung tissue differed between young and old mice under only a few exposure conditions (see Critique Table 2). In addition, although changes in serum levels of TNF-α after exposure differed between young and old mice in some exposure scenarios, the changes were complex. For example, there was a decrease in TNF-α in young mice and an increase in old mice after a single exposure, but a small increase in young mice and a small decrease in old mice after repeated exposure. As Laskin and colleagues acknowledge, the changes in serum levels of TNF-α are difficult to

Critique Table 2. Summary of Results: Comparison of Endpoints in DE-Exposed Versus Control Animals[a]

	mRNA Expression in Lung Tissue						Immunohisto-chemistry		BAL Fluid		
Group	TNF-α	IL-6	IL-8	COX-2	Lipocalin 24p3	Serum TNF-α	Mn SOD	Neutro-phils	Cell Number	LDH	Protein
Young Mice											
Single exposure											
300 μg/m^3 PM											
0 hours	=	=	=	↑	=	↓	↓	=	=	=	=
24 hours	↑	=	↑	=	=	=	↓	=	↑	↓	=
1000 μg/m^3 PM											
0 hours	=	↑	=	=	=	↓	↓	=	=	=	=
24 hours	=	↑	=	↓	=	=	↓	=	=	=	↑
Repeated exposure											
300 μg/m^3 PM											
0 hours	=	=	=	=	=	=	↓	=	↓	↑	↑
24 hours	=	↑	=	=	↑	=	↓	=	=	=	=
1000 μg/m^3 PM											
0 hours	=	↑	=	=	↑	=	↓	=	↑	=	=
24 hours	=	↑	↑	=	=	↑	↓	=	↑	=	=
Old Mice											
Single exposure											
300 μg/m^3 PM											
0 hours	=	=	=	↓	↑	=	=[b]	↑	=	=	=
24 hours	=	=	=	=	↑	↑	=[b]	↑	↓	=	=
1000 μg/m^3 PM											
0 hours	↑	↑	↑	↓	=	↑	=[b]	↑	=	=	=
24 hours	=	↑	↑	↑	↑	↑	=[b]	↑	=	=	=
Repeated exposure											
300 μg/m^3 PM											
0 hours	=	↑	=	=	=	=	=[b]	=	=	=	=
24 hours	=	=	=	↑	=	↓	=[b]	↑	↓	=	=
1000 μg/m^3 PM											
0 hours	=	↑	↑	=	↑	=	=[b]	↑	=	=	=
24 hours	↑	↑	↑	↑	↑	=	=[b]	↑	=	=	=

[a] For all endpoints except immunohistochemistry, ↑ or ↓ indicates a significant increase or decrease ($P \leq 0.05$) relative to the response in animals exposed to air. For immunohistochemistry, ↑ or ↓ indicates an increase or decrease by nonquantitative assessment of tissue. = indicates no significant effect relative to animals exposed to air.

[b] No MnSOD expression was observed in old mice exposed to air or DE.

interpret. Taking all these TNF-α findings together, the Committee was not convinced that the original hypothesis of the study was supported by the results.

The Committee agreed with the investigators that the most convincing evidence of differences in response to DE exposure between young and old mice came from a nonquantitative histologic examination of lung-tissue sections; in particular, an influx of neutrophils and other inflammatory-cell types (macrophages and plasma cells) was observed in old but not young mice after single or repeated DE exposure. These findings will need to be confirmed in future experiments. The Committee expected that a similar pattern of inflammatory changes would be obtained in BAL fluid, because findings are generally consistent between BAL-fluid and lung tissue, but this was not the case. Of particular note, endpoints indicative of inflammation and airway injury in BAL fluid (total cell number and protein levels, respectively) were *not* increased in old mice by exposure to DE. The authors suggest that the lack of consistency between BAL-fluid and lung histologic

findings may be the result of increased adherence of inflammatory cells to alveolar epithelium and thus the fact that the cells were not removed and not detected in lavage fluid. Nonetheless, the Committee believed that caution must be used in interpreting the effects of DE exposure on inflammatory endpoints in the current report because this discrepancy has not been observed in previous studies.

The Committee agreed with the investigators that there were some differences between young and old mice after exposure to DE, particularly in the expression of antioxidant molecules and molecules involved in oxidative stress; however, the Committee was not convinced that a clear-cut pattern emerged from these findings. For example, generally fewer changes were noted in mRNA expression of COX-2 (a molecule involved in synthesis of prostaglandins, which are involved in inflammatory responses) after DE exposure in young than in old mice, but the changes noted in the old mice were complex: decreased expression after a single low-dose exposure at a sampling time of 0 hours, but not at 24 hours, and decreased expression after single high-dose exposure at 0 hours but an increase after 24 hours. In addition, baseline expression of MnSOD, determined histologically, differed between young and old animals (it was detectable in young but not old animals) and expression of MnSOD in young animals decreased after exposure to DE. COX-2 and MnSOD are components of inflammatory and antioxidant responses, so the implications of these complex patterns of response to DE in young and old animals are not clear.

The Committee agreed with the investigators that lung mRNA expression of lipocalin 24p3 increased in more exposure scenarios in old than in young mice. This was an interesting finding, but the Committee was not convinced that lipocalin 24p3 is a sensitive marker of oxidative stress; although Laskin and colleagues cite a publication by Roudkenar et al. (2007) that supports this assertion, the Committee considered lipocalin 24p3 (also known as Lcn2 and NGAL) to be an acute-phase protein involved in innate immune defenses, particularly in response to bacteria, and only peripherally related to oxidative-stress pathways (e.g., Chan et al. 2009). A prior publication from Laskin's group (Sunil et al. 2007b) supports this interpretation. The Committee thought that further evidence was needed to support or refute the suggestion that changes in expression of lipocalin 24p3 are directly relevant to oxidative-stress pathways.

A further concern pertained to the real-world applicability of the DE exposures in the study to emissions derived from the current or future diesel-powered fleet. The characteristics of the DE generated by the diesel generator used in the current study (see Critique Table 1) differed markedly from those reported for DE produced by diesel-powered engines. For example, DE particles from diesel engines used in other animal exposure studies are typically in the ultrafine range (< 0.1 µm in aerodynamic diameter) (Reed et al. 2004, Campen et al. 2003), whereas the particles generated in the current study were much larger, approximately 250 nm in mean diameter. The data in Critique Table 1 also indicate that higher levels of nitrogen dioxide (NO_2), and a very high percentage of NO_x as NO_2, were found in the DE emissions in the current study than in studies using diesel engines (Reed et al. 2004, Campen et al. 2003). In addition, the OC/EC ratio reported in the current study was 1.1, whereas in other studies (Reed et al. 2004, Gottipolu et al. 2009), OC/EC ratios were close to 0.3. These findings suggest that the diesel atmosphere in the current study differed considerably from the components of diesel atmospheres in other studies that used engines from motor vehicles. Thus, the relevance of the DE exposures in the current study to DE exposures derived from diesel-powered vehicles is uncertain. In addition, improvements have been made in diesel particulate control technology, and new engines on the market — especially those for automotive applications — are increasingly being outfitted with such devices. The sulfur content of diesel fuel has also been reduced very significantly in recent years. Thus, future emissions of PM from diesel engines are expected to be substantially lower than those found in the past.

SUMMARY AND CONCLUSIONS

The original hypothesis of the study was that TNF-α production would be impaired in old animals exposed to DE. In their final submitted report, the investigators extended their hypothesis to suggest that exposure to DE may differentially affect molecules involved in inflammation and protective antioxidant pathways in young and old mice. However, after single and repeated exposure of young and old mice to DE, the pattern of changes in TNF-α levels did not differ between the young and old mice in a straightforward, easily interpreted way. Some qualitative differences were found in inflammatory endpoints measured histologically in lung tissue, with more changes detected in the old than the young mice. However, this pattern of inflammatory changes in the lung was not consistent with the pattern of changes in BAL fluid. The investigators also found that the effects of DE exposure on expression of MnSOD differed between young and old mice, as did mRNA levels of COX-2. The mRNA expression of lipocalin 24p3 in the lung increased in more diesel exposure scenarios in old than in young mice.

In its independent evaluation of the study, the HEI Review Committee thought that Laskin and colleagues had

succeeded in creating a diesel exposure system and generating preliminary data on multiple endpoints in response to the inhalation of DE in young and old mice. The Committee further thought that the investigators' hypothesis to explain the greater susceptibility of the elderly to adverse cardiovascular effects after exposure to PM was novel and interesting. However, the original hypothesis — that TNF-α production would be impaired in elderly animals exposed to DE — was not supported by the findings, and the complexity of the pattern of changes in other endpoints in young and old animals exposed to DE made it challenging to interpret the study findings in a clear-cut fashion. Thus, it was difficult to make links between changes in expression of the molecules measured in the study and age-related changes in susceptibility to DE exposure. The Review Committee further noted that whereas the investigators interpreted the increases in lipocalin 24p3 mRNA expression in the lungs of old mice as representing an increase in oxidative-stress responses, the Committee regarded this molecule as being only peripherally related to oxidative stress. Finally, the Review Committee noted that strides have been made in diesel particulate control technology and that the sulfur content of diesel fuel has been reduced very substantially in recent years. Thus, future emissions of PM from diesel engines are expected to be much lower than those found in the past. Hence, the relevance of the emissions derived from the diesel generator used in the current study to emissions from future diesel engines is uncertain. Further studies are needed to assess hypotheses and biologic response pathways that may explain why the elderly are more susceptible to exposure to PM than the young and healthy.

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