

Health Effects of Subchronic Inhalation Exposure to Gasoline Engine Exhaust

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Gasoline engine emissions are a ubiquitous source of exposure to complex mixtures of particulate matter (PM) and non-PM pollutants; yet their health hazards have received little study in comparison with those of diesel emissions. As a component of the National Environmental Respiratory Center (NERC) multipollutant research program, F344 and SHR rats and A/J, C57BL/6, and BALBc mice were exposed 6 h/day, 7 days/week for 1 week to 6 months to exhaust from 1996 General Motors 4.3-L engines burning national average fuel on a simulated urban operating cycle. Exposure groups included whole exhaust diluted 1:10, 1:15, or 1:90, filtered exhaust at the 1:10 dilution, or clean air controls. Evaluations included organ weight, histopathology, hematology, serum chemistry, bronchoalveolar lavage, cardiac electrophysiology, micronuclei in circulating cells, DNA methylation and oxidative injury, clearance of *Pseudomonas aeruginosa* from the lung, and development of respiratory allergic responses to ovalbumin. Among the 120 outcome variables, only 20 demonstrated significant exposure effects. Several statistically significant effects appeared isolated and were not supported by related variables. The most coherent and consistent effects were those related to increased red blood cells, interpreted as likely to have resulted from exposure to 13–107 ppm carbon monoxide. Other effects supported by multiple variables included mild lung irritation and depression of oxidant production by alveolar macrophages. The lowest exposure level caused no significant effects. Because only 6 of the 20 significant effects appeared to be substantially reversed by PM filtration, the majority of effects were apparently caused by non-PM components of exhaust.

INTRODUCTION

Inhalation exposures to emissions from gasoline-powered vehicles and other gasoline engines are ubiquitous throughout the developed world. All segments of the populations of developed countries are exposed to both fresh and aged gasoline emissions and their reaction products. A considerable and growing literature demonstrates relationships between adverse health impacts and the proximity to traffic of residences (Boothe and Shendell, 2008; Finkelstein et al., 2004; Hoek et al., 2002) and schools (Janssen et al., 2003), or time spent on roadways (Peters et al.,

2004; Riediker et al., 2004). Although emissions are commonly assumed to be the principal cause, the studies have had little ability to determine the causal pollutants (Lipfert and Wyzga, 2008). Studies in some locations have shown stronger statistical associations between health effects and truck traffic than with automobile traffic (Brunekreef et al., 1997; Janssen et al., 2003), and the particulate matter (PM) and nitrogen oxide levels associated with some effects often are assumed to be dominated by diesel exhaust (Janssen et al., 2003). Nevertheless, exhaust from gasoline engines, and especially those from older or malfunctioning engines (McDonald et al., 2004a; Seagrave et al., 2002), certainly contribute strongly to near-roadway pollution. Moreover, gasoline exhaust would likely dominate exposures to volatile organic compounds, which are not routinely measured, and thus seldom implicated in health studies.

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In contrast to widespread concern shown for the health effects of diesel exhaust over the past few decades and a correspondingly large literature from myriad epidemiology and laboratory studies (Hesterberg et al., 2006; Mauderly and Garshick, 2008), relatively little attention has been directed toward the health effects of gasoline exhaust. A recent review (McDonald et al., 2007) cited only seven inhalation studies of gasoline exhaust, all conducted prior to the mid 1980s, and most using 1970s and earlier engines and fuels. None of the gasoline emission health research publications presented detailed analysis of the exposure atmospheres, and many health outcomes of current interest were not examined. There is clearly a need for a more contemporary, more detailed evaluation of the contribution of gasoline exhaust to traffic-related health effects.

The present study was conducted by the National Environmental Respiratory Center (NERC) program, which is funded by government and industry to determine the pollutants and combinations causing the respiratory and cardiovascular health effects of complex pollution mixtures (www.nercenter.org). The fundamental NERC strategy is to create a new composition-concentration-effect database by similarly designed studies of different mixtures, with the goal of using multivariate analysis to reveal components that covary most closely with different health outcomes. By using common source emissions as the study mixtures, the NERC program also provides insight into the contributions of different sources to the pollution-related health burden. Studies of diesel exhaust (Campen et al., 2003; Harrod et al., 2005; McDonald et al., 2004b; Reed et al., 2004; Seagrave et al., 2005) and hardwood smoke (Barrett et al., 2006; McDonald et al., 2006; Reed et al., 2006; Seagrave et al., 2005) were completed. Gasoline exhaust was selected for the next study both because it is common and because it presents a pollution mixture having a composition that overlaps with those of diesel exhaust and hardwood smoke but also differs in several ways.

As for previous NERC exposures, a workshop of academic, industry, and government experts was convened to develop consensus recommendations for generating the exhaust and setting exposure levels. The methods described below followed those recommendations. The exposures began in late 2004 and ended in early 2006. Although the longest single exposure was 6 months, it was necessary to stagger exposures over a longer period to accommodate the different response models. The general exposure methods were described earlier (McDonald et al., 2007), and detailed information on the generation, measurement, and composition of the exposure atmospheres is presented in an accompanying paper by McDonald et al. (2008). A previous paper reported the effects of exposure on vascular responses in ApoE^{-/-} mice (Lund et al., 2007), and another accompanying paper reports the exacerbation of pre-existing respiratory allergic responses in BALBc mice (Day et al., 2008). The experimental design and remainder of the health effects study is reported here.

MATERIALS AND METHODS

Animals, Treatment Groups, and Maintenance

The allocation of animals to the different health assays and the ages of animals at the beginning of exposure are presented in Table 1. Male and female CDF (F344)/CrIBR rats and spontaneously hypertensive (SHR) rats and male C57BL/6 mice were purchased from Charles River Laboratories (Raleigh, NC). F344 rats were used for assessment of body weight, organ weight, histology, clinical chemistry, hematology, bronchoalveolar lavage, and lung DNA damage. SHR rats were used for assessment of heart rate, electrocardiogram waveforms, and heart and vessel histology. C57BL/6 mice were used for assessment of the clearance of bacteria from the lung and associated histopathology. Strain A/J mice were purchased from Jackson Laboratories (Bar Harbor, MA) and used for assessment of organ weight, body weight, micronucleus formation, and lung DNA damage. BALBc mice were purchased from Charles River Laboratories (Wilmington, MA) and used for assessment of the effects of *in utero* and postnatal exposure on development of respiratory allergic responses. Individual animals were assigned to treatment groups randomly by body weight using a computerized algorithm (Path/Tox System[®] 4.2.2, Xybion, Cedar Knolls, NJ). The rats were identified by tail tattoo, and the mice were identified by microchip (Midfinger Print, Finedon, Northants, UK), using an alphanumeric code linking each individual uniquely to its study and experimental group.

The animals were housed in 2-m³ inhalation exposure chambers (H-2000, Hazleton Systems, Maywood, NJ) for a 2-week quarantine period and throughout exposures. The chambers were maintained at 30–60% relative humidity and 20–24°C. Light was on a 12-h on-off cycle (on 0600–1800 h daily), and windows to continuously lighted hallways were lined with red film to avoid disruption of the light cycle. Excreta trays under wire cages were lined with paper that was changed twice daily. Chambers and caging were washed and sanitized weekly. Mice held after exposure for bacterial clearance tests were housed in shoebox cages overnight. All animals were fed Teklad[™] certified rodent diet (Harlan Teklad, Madison, WI) *ad libitum* except during the 6-h daily exposure period. Water was available *ad libitum* throughout the study.

A few animals from each experimental group were tested serologically for common rodent pathogens after quarantine (BioReliance, Rockville, MD), and sera from portions of the animals sacrificed after 6 months of exposure were tested upon the conclusion of the study. Rat sera were tested for CAR bacillus (CARB), Kilham rat virus (KRV), H-1 virus (H-1), lymphocytic choriomeningitis virus (LCM), *Mycoplasma pulmonis* (*M. pul.*), rat parvovirus (RPV), pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis virus (RCV/SDA), reovirus (Reo 1, 2, 3), and Sendai virus (Sendai). Mouse sera were tested for CARB, epizootic diarrhea of infant mice (EDIM), Theiler's mouse encephalomyelitis virus (TMEV/GDVII), LCM, mouse

TABLE 1
Assignment of animals

Exposure duration Gender	Organ and body weights, histology, clinical chemistry, hematology, clotting factors, bronchoalveolar lavage, lung DNA damage				Organ and body weights, micronucleus formation, histology, and lung DNA damage				Development of respiratory allergic response				Heart rate, ECG, heart and vessel histology				Bacterial clearance				Serum markers of vascular injury and clotting factors			
	F344 Rats				A/J Mice				BALBc mice (offspring) 3 wk gestation, 4 wk after birth				SHR ^a Rats				C57BL/6 mice				SHR rats			
	1 wk				1 wk				1 wk				1 wk				1 wk				1 wk			
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Control (C)	12	12	12	12	10	10	10	10	20	20	20	20	6	6	6	6	16	16	16	16	4	4	4	4
Low (L)	12	12	12	12	10	10	10	10	20	20	20	20	6	6	6	6	16	16	16	16	4	4	4	4
Medium (M)	12	12	12	12	10	10	10	10	20	20	20	20	6	6	6	6	16	16	16	16	4	4	4	4
High (H)	12	12	12	12	10	10	10	10	20	20	20	20	6	6	6	6	16	16	16	16	4	4	4	4
High-filtered (HF)	12	12	12	12	10	10	10	10	20	20	20	20	6	6	6	6	16	16	16	16	4	4	4	4

Note: ECG = electrocardiogram; SHR = spontaneously hypertensive rats.

hepatitis virus (MHV), *M. pul.*, mouse parvovirus (MPV, MVM), PVM, and Sendai. Rats and C57BL/6 mice were negative for all pathogens throughout the study.

Exposures

Animals were exposed 6 h/day (0800–1400 h), 7 days/wk, for periods ranging from 3 days to 6 months to one of three concentrations of whole, diluted gasoline engine exhaust; to the highest concentration with PM removed by filtration; or to clean air as controls. The engines, fuel, operating cycle, analytical methods, and characteristics of the exposure atmospheres are summarized here and described in greater detail in McDonald et al. (2008). Exhaust was generated by two 1996 General Motors 4.3-L V-6 engines equipped with stock exhaust systems, including mufflers and catalytic converters. The engines were removed from in-use, mid-mileage vehicles purchased after verification of normal operation and exhaust. The engines were connected to eddy current dynamometers and operated on continuous repeats of the variable-load California Unified Driving Cycle adapted for test stand use. Each engine was operated for 3 h daily in order to incorporate two cold (room temperature) starts in each 6-h exposure day. The fuel was regular, unleaded, non-oxygenated, non-reformulated gasoline blended to the US average composition for summer 2001 and winter 2001–2002 (Specialty Fuels Division, Chevron-Phillips Chemical Company, Borger, TX). The crankcase lubricating oil (10W-30, Pennzoil Products Company, Houston, TX) and oil filter were changed every 122 h, which approximated 3,000 miles.

Whole exhaust was diluted with conditioned, charcoal-filtered, ambient air to approximately 1:10, 1:15, and 1:90 to produce the high (H), mid (M) and low (L) exposure levels, respectively, and controls (C) were exposed to diluting air. A “high-filtered” (HF) exposure group was exposed to the 1:10 dilution after filtration with a high-efficiency particulate air (HEPA) filter to remove exhaust PM. Removal of exhaust PM was verified by both mass and PM number measurements, but all chambers contained $\sim 2 \mu\text{g PM}/\text{m}^3$ generated by the animals themselves. The concentrations of PM, nitrogen oxides (NO_x, including nitric oxide (NO) and nitrogen dioxide (NO₂)), carbon monoxide (CO), and non-methane vapor-phase hydrocarbons (NMHC) were measured daily. NO_x was used for real-time monitoring of dilution ratios. PM mass was measured gravimetrically using Teflon® membrane filters and PM size distribution was measured by fast mobility particle sizer (FMPS, TSI, St. Paul, MN). NO_x was measured by chemiluminescence, CO was measured by a photoacoustic infrared method, NMHC was measured by flame ionization, and sulfur dioxide (SO₂) and ammonia (NH₃) were measured by ion chromatography.

Health Responses

Clinical Observations and Body Weight

All animals were examined daily to document the onset and progression of any abnormal clinical signs. More detailed clinical

examinations were performed during body weight measurements prior to the beginning of exposure, weekly for the first month of exposure, and monthly thereafter. Body weight also was recorded at necropsy. Body weights and clinical findings were recorded using a computerized database (Path/Tox System).

Necropsy

The necropsy and histopathology methods were identical to those in previous NERC subchronic studies (see Reed et al., 2004 and 2006 for F344 rats and Strain A/J mice; Campen et al., 2003 for SHR rats; and Harrod et al., 2003 and 2005 for C57BL/6 mice). Animals were euthanized by intraperitoneal injection of barbiturate. Necropsies consisted of gross examination of all organ systems, weighing of major organs (A/J mice and F344 rats), fixation of the respiratory tract (all animals), and fixation of major non-respiratory organs (A/J mice and F344 rats; lung, heart and aorta only for SHR rats). The lungs of C57BL/6 mice were removed aseptically; the left lung was preserved for histopathology, and the right lung was processed for bacterial plating. Blood was obtained from the inferior vena cava of F344 and SHR rats for clinical chemistry, hematology, and clotting factors. Blood was obtained from A/J mice by cardiac puncture for micronucleus formation assays. Major organs of F344 rats and A/J mice were weighed, and tissues were collected and fixed in 10% neutral buffered formalin (NBF). The extrapulmonary left lung bronchus of F344 rats was clamped temporarily, and the right lung was lavaged. The right lungs of F344 rats and A/J mice were snap frozen and stored at -80°C . The left lungs were fixed at a constant pressure of 25 cm fixative for 2 h, followed by immersion in 10% NBF for at least 48 h. The volumes of the left lungs of F344 rats and A/J mice were determined by water displacement. For SHR rats, a transverse section (1/3) of the dorsal heart was snap frozen, and the remainder of the heart was fixed in NBF. Necropsy data were managed using the Path/Tox System.

Histopathology

Tissues were sectioned at $5 \mu\text{m}$, stained with hematoxylin and eosin (H&E), and examined by a board certified pathologist (A.G.) using light microscopy. All gross abnormalities were examined. F344 rat lung, bronchial lymph nodes, nasopharyngeal tissue, trachea, heart, aorta, liver, spleen, kidneys, thymus, and adrenals were examined. A/J mouse lung, bronchial lymph nodes, nasopharyngeal tissue, liver, spleen, kidneys, heart, and thymus were examined. SHR rat lung, heart, and aorta were examined. A “read-down” approach was employed for F344 and SHR rats and A/J mice, in which tissues from the C and H exposure groups were examined, and tissues from other exposure groups were examined only if abnormalities were found at the high level. A semi-quantitative scale of 0–4 was used to score the severity of abnormalities. Inflammation scores were pooled

to develop summary histopathology scores. The presence of black/brown particulate material within alveolar macrophages was assessed, although this was an expected result of the exposure. Tissues from all groups were examined, and lungs were scored for suppurative alveolitis, perivascular hemorrhage, perivascular cuffing, and bronchiolitis and/or peribronchiolar cuffing. Histological findings were recorded using the Path/Tox System.

Serum Chemistry, Hematology, and Clotting Factors

Whole blood, serum, and plasma obtained from F344 rats exposed 1 week or 6 months and fasted overnight were analyzed for serum chemistry, hematology, and clotting parameters. Serum chemistry analyses were performed by automated analyzer (Hitachi 911, Roche Diagnostics, Indianapolis, IN). Parameters included alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, glucose, blood urea nitrogen, creatinine, total protein, albumin, total globulin, albumin/globulin, total bilirubin, cholesterol, sodium, potassium, chloride, calcium, and inorganic phosphorus. Hematology analyses were performed by automated analyzer (Advia 120, Bayer Corp., Tarrytown, NY). Parameters included red blood cell count, hemoglobin, hematocrit, platelet count, white blood cell count, and differential white blood cell count. Clotting factors included thrombin-antithrombin (TAT) complex, plasma fibrinogen, and Factor VII. Factor VII analyses were conducted by automated analyzer (Diagnostica Stago, STA, Asnières, France) and were expressed as a percentage of the activity in male and female plasma standards. Plasma fibrinogen was measured by heat precipitation (Desvignes and Bonnet, 1981). TAT was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Enzygmosa[®] TAT, Dade Behring, San Jose, CA) (Laudes et al., 2002).

Micronucleus Formation

Micronuclei in peripheral blood from A/J mice exposed 6 months were counted by flow cytometry (Torous et al., 2001). Approximately 100 μ L of whole blood was added to anticoagulant solution (MicroFlow[®], Litron Laboratories, Rochester, NY). A 100- μ L aliquot of blood plus fixative was added to methanol and chilled to -80°C . Samples were shipped on dry ice overnight to Litron Labs for analysis. Reticulocytes and micronucleated cells were counted based on the analysis of at least 200,000 total erythrocytes and approximately 20,000 reticulocytes.

DNA Methylation and Oxidative Damage

Right lung lobes of F344 rats and A/J mice were snap frozen at necropsy after 1 week or 6 months of exposure. DNA was extracted using the methods described by Barbin et al. (2003), modified by the addition of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, Aldrich, St. Louis, MO) to reduce oxidation during the

procedure. The frozen right lung lobes were thawed and homogenized in phosphate buffered saline (PBS, pH 7.4) containing 20 mM TEMPO using a Tehran homogenizer (Wheaton Instruments, Millville, NJ). Following centrifugation at $1,700 \times g$ for 5 min, the nuclear pellets were incubated overnight at 4°C in lysis buffer containing 20 mM TEMPO (Gentra, Minneapolis, MN) and proteinase K (500 mg/mL, Gentra). DNA was then extracted twice with a mixture of phenol, chloroform, and water (Applied Biosystems, Foster City, CA) containing 20 mM TEMPO, and once with 24:1, chloroform (Sigma, St. Louis, MO) and isopentyl alcohol (Aldrich) with 20 mM TEMPO, followed by ethanol precipitation. The extracted DNA was incubated at 37°C for 30 min in PBS containing RNase T1 (50 units/mL), RNase A (100 mg/mL), and 20 mM TEMPO. After DNA precipitation with cold ethanol, the pellet was re-suspended in sterilized distilled water with 1 mM TEMPO. The DNA solution was stored at -80°C until assayed.

Genomic methylation levels were determined using a methyl acceptor assay previously described (Belinsky et al., 1996). Briefly, DNA concentrations were determined spectrophotometrically using a DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA). DNA (150 ng) was incubated at 37°C for 4 h with 4 units of M.SssI CpG methylase (New England Biolabs, Beverly, MA) in the presence of ^3H -labeled S-adenosylmethionine (Amersham, Van Nuys, CA), and the reaction was stopped by adding non-radioactive S-adenosylmethionine (New England Biolabs). Samples were spotted on Whatman filters (Brentford, Middlesex, England), dried, washed with 5% trichloroacetic acid followed by 70% ethanol, and placed in scintillation cocktail to determine radioactivity. Controls included reactions without either DNA or enzyme. All samples were assayed in duplicate, and values were obtained as dpm/ng of DNA. Because incorporation of ^3H -methyl groups into the DNA was inversely proportional to the original CpG methylation level, increases and decreases in incorporated radioactivity compared to control were interpreted as hypomethylation and hypermethylation, respectively.

The concentration of abasic (apurinic and apyrimidinic, AP) sites was measured in DNA from F344 rats and A/J mice in the C, H, and HF exposure groups after 6 months of exposure, using methods described by Nakamura and Swenberg (1999). In the absence of significant differences between the C and H exposure groups, DNA from other groups was not analyzed. Three μg of DNA in 50 μL of PBS was incubated at 37°C for 10 min in 3 mM aldehyde reactive, biotin-tagged probe (ARP, Dojindo Molecular Technologies, Gaithersburg, MD). After precipitation in cold ethanol, DNA was washed three times with 70% ethanol and resuspended in Tris EDTA (TE) buffer (10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA). An aliquot containing 250 ng DNA in TE buffer was heat denatured at 99°C for 11 min, placed on ice, and loaded on a nitrocellulose membrane (NCM) at 110 ng DNA/slot. The NCM was soaked with $5 \times \text{SSC}$ (0.75 M NaCl, 75 mM trisodium citrate) and then baked in a vacuum oven at 80°C for 30 min. The NCM was pre-incubated with Tris-HCl buffer containing bovine serum albumin, Tween-20, and casein

(all from Sigma) at room temperature for 15 min and then incubated in the same solution containing streptavidin-conjugated horseradish peroxidase (BioGenix, San Ramon, CA) at room temperature for 1 h. After rinsing the NCM, the enzymatic activity on the membrane was visualized by enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL). The NCM was exposed to X-ray film (XAR 5, Kodak, Rochester, NY), and the exposed film was analyzed using a Kodak Image Station 440. Quantitation was based on comparisons to internal standard DNA containing known amounts of AP sites.

Bronchoalveolar Lavage

The methods were described previously in detail (Seagrave et al., 2005). Right lungs of F344 rats necropsied after 1 week or 6 months of exposure were lavaged twice with Dulbecco's phosphate-buffered saline at 1.67 mL/100 g body weight. Recovered fluid was pooled and an aliquot was used for total and differential cell counts. Remaining fluid was centrifuged and divided into multiple aliquots. One aliquot was used for analysis of total protein, lactate dehydrogenase (LDH), and alkaline phosphatase (Hitachi Modular Analytics Clinical Chemistry System 911, Roche Diagnostics). Another aliquot was used for analysis of β -glucuronidase by a colorimetric method (Henderson et al., 1985). A third aliquot was added to sulfosalicylic acid, centrifuged, and analyzed for total and oxidized glutathione (Tietze, 1969). Additional aliquots were analyzed for interleukin 1 β , tumor necrosis factor α , and macrophage inflammatory protein 2 (MIP-2) using ELISA kits (Biosource International, Camarillo, CA).

The cell pellet was resuspended in RPMI 1640 medium with fetal bovine serum, penicillin, streptomycin, and glutamine, and the cells were placed in 96-well tissue culture plates at 10^5 cells/well to measure production of superoxide and peroxide. The cells were allowed to adhere to the plates for 1 h, and then were gently washed with HEPES-buffered Earle's salts. Production of superoxide using reduction of acetylated cytochrome C (Nasrallah et al., 1983; Pick, 1986) and peroxide using peroxidase-catalyzed luminescence of luminol (Pick, 1986) was measured in the presence and absence of 200 μ g/mL opsonized zymosan ("stimulated" and "unstimulated," respectively). To define the specificity of the reactions, responses were measured in the presence and absence of enzymatic catalysts (10^4 U/mL superoxide dismutase to eliminate superoxide, and 3,200 U/mL catalase to eliminate peroxide).

Cardiovascular

The methods used were described previously in detail (Campen et al., 2003). SHR rats were anesthetized with isoflurane and shaved, and the incision sites were scrubbed with sterilant. A midline incision was made, and the body of a telemeter (Model TA11CTA-F40, Data Sciences International, St. Paul, MN) was positioned in the peritoneal cavity. Electrocardiogram

(ECG) electrodes were extruded through the abdominal muscle wall and secured subcutaneously in positions that approximate Lead II of the standard clinical ECG. All procedures were performed aseptically. Animals were allowed 4 weeks to recover from surgery prior to exposure. ECG data were obtained every 15 min beginning 3 days prior to exposures, throughout the 7-day exposure, and for 4 days following exposures. Heart rate was measured. ECG waveform (10 s in duration) analyses were conducted by using an automated detector (Data Sciences International) to determine waveform intervals (PR, QRS, and QT). Given the circadian behavioral patterns of rats, data for morning, afternoon (both during daily exposures), and evening (after daily exposures) periods were analyzed separately.

Serum from separate groups of male and female SHR rats was analyzed by ELISA for markers of vascular injury, including endothelin-1 (Quantiglo R&D Systems, Minneapolis, MN), angiotensin II (Bachem International, Torrance, CA) following solid-phase extraction, and haptoglobin (Biosource International, Camarillo, CA). Serum was also analyzed for markers of oxidative stress, including thiobarbituric acid substances (TBARS; Rhoden et al., 2005) and lipid peroxides (Oxis International, Foster City, CA) and clotting factors, including platelets, thrombin-antithrombin complex (ELISA, Enzygmosa[®] TAT; Laudes et al., 2002), Factor VII, and fibrinogen (AMAX Destiny Plus, Trinity Biotech, Jamestown, NY).

Clearance of Bacteria

The methods used were described previously (Harrod et al., 2005). Immediately following 1 week of exposure, C57BL/6 mice were anesthetized with isoflurane in 50:50 nitrous oxide and oxygen. 1×10^9 colony forming units of *Pseudomonas aeruginosa* suspended in 100 μ L of PBS were instilled intratracheally. The *P. aeruginosa* was generated from a frozen culture of a clinical isolate. Eighteen hours after instillation, the mice were euthanized, and the lungs were removed under sterile conditions. Homogenates of the right apical and cardiac lobes were serially diluted to three dilutions and plated onto 2 $\times$ YT agar plates, which were incubated at 37°C overnight. Colonies were counted for each dilution, and the bacterial concentration per mL of lung homogenate and lung weight was calculated. Clearance and histopathology data were screened prior to statistical analyses. A few animals having no lung bacterial counts and no histological evidence of infection were excluded under the assumption that they were not successfully dosed with *P. aeruginosa*.

Development of Respiratory Allergic Responses

Male and female BALB/c mice were conditioned to exposure chambers and mated. Pregnant females were exposed beginning on gestational Day 1 (based on observation of vaginal plugs) and throughout gestation. The females and litters were provided with nesting material and housed in open-top plastic shoebox

cages placed in the exposure chambers. Exposures of the offspring continued until 4 weeks of age, after which they were housed in cages placed in clean air. One-half were sensitized to ovalbumin (OVA, chicken egg, grade V, Sigma, St. Louis, MO) by intranasal instillation of 50 μ g at 4, 5, 6, 7, 8, and 9 weeks of age, and the other half were sham-sensitized by instillation with saline. The mice were tested for airway reactivity by methacholine challenge 48 h after the last instillation and euthanized for bronchoalveolar lavage, serum collection, and histopathology, using methods described in detail previously (Barrett et al., 2006). Mice were anesthetized (Avertin[®], Winthrop Laboratories, Rensselaer, NY); the trachea was cannulated by dissection; and the cannula was connected to a positive-pressure ventilator with a capability for measuring compliance and resistance and administering methacholine aerosol (Flexi-Vent[®], SCIREQ, Montreal, QC). After administering a respiratory paralytic (doxycurium, 0.5 mg/kg), increasing doses of methacholine (1, 3, 6, 12, and 25 mg/mL nebulizer solution) were administered in steps, and compliance and resistance were measured. Following the last measurement, blood was sampled from the renal artery, and the mice were sacrificed by injection of barbiturate (Euthasol[®], Virbac, Fort Worth, TX). Lungs were removed and lavaged three times with 0.8 mL PBS. An aliquot of recovered fluid was used for cell counts and differentials. Both lavage fluid supernatant and serum were analyzed for total immunoglobulin E (IgE) and OVA-specific IgG₁, IgG_{2a}, and IgE. Lavage fluid supernatant also was analyzed for protein, interleukins 2, 4, 5, and 13, and interferon gamma by ELISA.

Statistics

Terminal body weight and organ weight data were analyzed using analysis of variance (ANOVA). When the ANOVA indicated significant ($p < 0.05$) differences in mean responses across experimental groups, Dunnett's multiple comparison procedure (Dunnett, 1955; 1980) was performed to compare exposed group means to those of controls. The linear term of the ANOVA was used to assess exposure-related trends in response across controls and groups exposed to unfiltered gasoline exhaust (coded as 0, 1, 2, and 3).

Most clinical chemistry, hematology, clotting, DNA, and micronucleus data exhibited skewed (non-normal) distributions and were analyzed using non-parametric statistical methods. Dunn's multiple comparison procedure (Dunn, 1964) was employed to assess pair-wise differences in median values between control and exposed groups, and the Jonckheere-Terpstra test (Jonckheere, 1954) was utilized to evaluate the significance of exposure-related trends across all treatment groups. Endpoints for results that encompassed fewer than 10 discrete values (albumin, bilirubin, creatinine, gamma-glutamyl transferase (GGT), chloride, sodium) were analyzed with weighted least squares ANOVA for categorical data (Grizzle et al., 1969). Pair-wise comparisons of mean scores in exposed versus control groups were adjusted for multiple comparisons using the modified Bon-

ferroni procedure devised by Hochberg (1988). The linear term of the ANOVA was used to assess exposure-related trends across treatment groups (coded as 0, 1, 2, and 3).

Bacterial count data, which exhibited standard deviations that scaled with sample means, were log transformed after adding 0.5 to each count (in order to include zero counts in the analysis). An ANOVA was performed on the log-transformed data followed by Dunnett's multiple comparison procedure (Dunnett, 1955; 1980). Bacterial histopathology data were scaled according to severity. Weighted least squares analysis of variance (Grizzle et al., 1969) was applied to the graded response data.

For terminal body weight, clinical chemistry, hematology, clotting factors, DNA, micronucleus, C57/BL6 bacterial clearance, C57BL6 bacterial histopathology, and ECG analyses, a biological effect was judged to be exposure-related if a statistically significant difference from control occurred at one of the two highest exposure levels and there was a statistically significant trend across the C-H exposure groups. In some cases, statistically significant trends were reported to corroborate exposure-related effects.

Heart rate and ECG interval data were averaged by exposure groups and analyzed by ANOVA. A repeated measured ANOVA was employed to accommodate the multiple observations on each animal. In conjunction with these analyses, mean values in the exposed groups were compared to the controls in each experimental period with Dunnett's multiple comparison test, using the mean square for the nested effect of animals within the exposure group as the error variance. Exposure-related trends across concentration groups were evaluated by using linear exposure terms in the ANOVA regression models.

The statistical significance of pair-wise comparisons and trend tests was assessed at $p < 0.05$. A response was judged to be a "significant exposure-related effect" when both: a) the trend among the C, L, M, and H exposure groups was significant at $p < 0.05$; and b) the responses of either the M or H exposure group, or both, were also significantly different from control at $p < 0.05$. Values for the HF exposure group were not included in trend tests, but the difference of that group from control was tested by pair-wise comparison. In cases of significant exposure-related effect, the role of PM was evaluated by comparing the group mean (or median) that deviated most substantially from controls with that of the HF exposure group. Statistical significance of these comparisons was assessed at $p < 0.1$. Calculations were performed using the SAS software system (Cary, NC).

RESULTS

Exposure Atmospheres

The compositions of the exposure atmospheres are summarized briefly in Table 2 and in greater detail in the accompanying paper (McDonald et al., 2008). Table 2 includes the major components, except for carbon dioxide, water vapor, and methane. Among the listed components of the unfiltered H exposure atmosphere, CO contributed 72.5% of the total mass; NO contributed

TABLE 2
Major components of the exposure atmosphere

Analyte	Units	C Mean $\pm$ error	L Mean $\pm$ error	M Mean $\pm$ error	H Mean $\pm$ error	HF Mean $\pm$ error
PM mass	$\mu\text{g}/\text{m}^3$	2.5 ± 2.9	6.6 ± 3.7	30.3 ± 11.8	59.1 ± 28.3	2.3 ± 2.6
PM number	$\#/\text{cm}^3$	1.5×10^3	2.6×10^4	2.6×10^5	5.0×10^5	1.1×10^4
CO	mg/m^3	0.0 ± 0.2	12.2 ± 5.3	70.1 ± 16.3	102.8 ± 33.0	99.5 ± 1.6
	(ppm)	(nd)	(12.8)	(73.2)	(107.3)	(103.9)
NO	mg/m^3	0.04 ± 0.1	2.1 ± 0.5	12.2 ± 1.1	18.4 ± 2.8	17.2 ± 1.9
	(ppm)	(0.04)	(2.0)	(11.9)	(17.9)	(16.7)
NO ₂	mg/m^3	0.0 ± 0.0	0.13 ± 0.0	0.7 ± 0.1	1.4 ± 0.3	1.7 ± 0.2
	(ppm)	(nd)	(0.1)	(0.5)	(0.9)	(1.1)
SO ₂	$\mu\text{g}/\text{m}^3$	1.1 ± 0.1	193.5 ± 7.9	862.6 ± 35.3	1366.8 ± 56.0	1051.1 ± 43.0
	(ppm)	(nd)	(0.09)	(0.38)	(0.62)	(0.47)
NH ₃	$\mu\text{g}/\text{m}^3$	20.0 ± 0.1	219.1 ± 0.9	1149.5 ± 6.0	1957.7 ± 8.1	1241.5 ± 6.1
	(ppm)	(0.03)	(0.38)	(1.98)	(3.37)	(2.14)
NMHC	mg/m^3	0.1	1.9	10.9	15.9	25.9

Note. C = control; L = low; M = mid; H = high; HF = high=filtered exposure groups, error = standard deviation for NOx, PM, CO; root mean square of measurement uncertainty for other analytes, nd = not detected; below level of reliable quantitation.

13.0%; NMHC contributed 11.2%; NH₃ contributed 1.4%. NO₂, and SO₂ each contributed 1%, and PM contributed 0.04%. The PM mass concentrations in the HF and C exposure atmospheres were nearly identical, reflecting the absence of emission PM after filtration (a low concentration of PM is generated by the animals themselves). The mass median diameter of PM was approximately 150 nm, with the greatest number of particles in the 5–20 nm range. The concentrations of gases were similar in the H and HF exposure atmospheres; the differences most likely resulted from differences in reactions among the components due to the lack of PM and perhaps some effect of the HEPA filter.

Effects of Exposure to Whole Exhaust

Survival, Clinical Signs, Body Weight, and Microbiological Status

The animals tolerated the exposures well, and there were no visible indications of exposure-related stress, injury, or illness. There was no exposure-related mortality. Body weight was not affected significantly by exposure of either rats or mice. All serum samples submitted for detection of pathogens were negative.

Organ Weight and Excised Lung Volume in F344 Rats and A/J Mice

There were no significant effects of exposure on organ weights of either F344 rats or A/J mice at 1 week. At 6 months, three organ weights met the criteria for significant exposure-related effects. The most robust organ-weight response was an increase in heart weight of male rats (Figure 1) expressed as absolute weight (12% in M exposure group, trend $p = 0.01$)

or heart-to-body weight ratio (12% in H exposure group, trend $p = 0.001$). The heart weight of female rats also was increased in an exposure-related manner, but to a lesser degree that did not meet the significance criteria (8% in H group, trend $p = 0.09$). The other two organ weight effects that met the criteria were less robust. The seminal vesicle weight of male rats was decreased (16% in M exposure group); and the kidney weight of female A/J mice was decreased (10% in M exposure group). There was no significant effect of exposure on excised lung weights or volumes (Table 3).

Histopathology in F344 Rats and A/J Mice

There were no visible exposure-related tissue or inflammatory changes that merited scoring in F344 rats or A/J mice after either 1 week or 6 months of exposure. The only evidence of exposure at 1 week was PM in a very few alveolar macrophages of both species. The number of macrophages containing PM had increased by 6 months in both species; but even then, particle loading in the H exposure groups was minor and merited only the minimum score. Thus, although there was evidence of exposure by light microscopy, there were no alterations other than PM accumulation after 6 months that generated significant quantitative differences from control.

Serum Chemistry in F344 Rats

No significant exposure-related effects were observed in F344 rats exposed for 1 week (Table 4). Three significant effects were observed in females at 6 months, but no significant effects were observed in males. Serum alanine aminotransferase (28% in H exposure group), aspartate aminotransferase (23%,

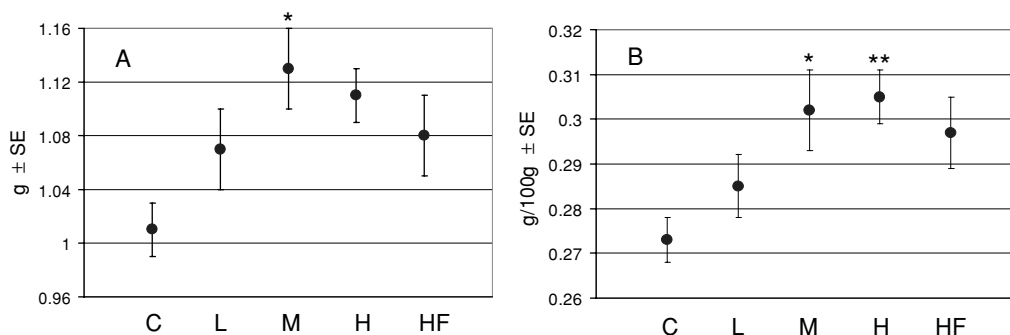


FIG. 1. Effect of exposure on heart weight in male F344 rats after 6 months of exposure, expressed as absolute weight (A) and heart-to-body-weight ratio (B) (means $\pm$ SE). The trends across the control (C), low (L), mid (M), and high (H) exposure groups were significant at $p = 0.01$ and $p = 0.001$, respectively. Asterisks indicate significance of pair-wise differences from C (* $p = 0.05$, ** $p = 0.01$).

M and H exposure groups), and phosphorus (8% in M exposure group) were all decreased in females.

Hematology in F344 Rats

Significant exposure-related effects in F344 rats were limited to hematocrit, red blood cell count, and hemoglobin (Table 4), which were increased at both 1 week and 6 months. Hemat-

ocrit was increased 4% and 6% in H females and males, respectively, at 1 week, and 7% and 7% in H exposure group females and males, respectively, at 6 months. The red blood cell count was increased 3% and 6% in H exposure group females and males, respectively, at 1 week, and 6% and 4% in M exposure group females and males at 6 months, respectively (Figure 2). Hemoglobin was increased 5% in H exposure group males at 1 week and 7% and 6% in M and H exposure group females

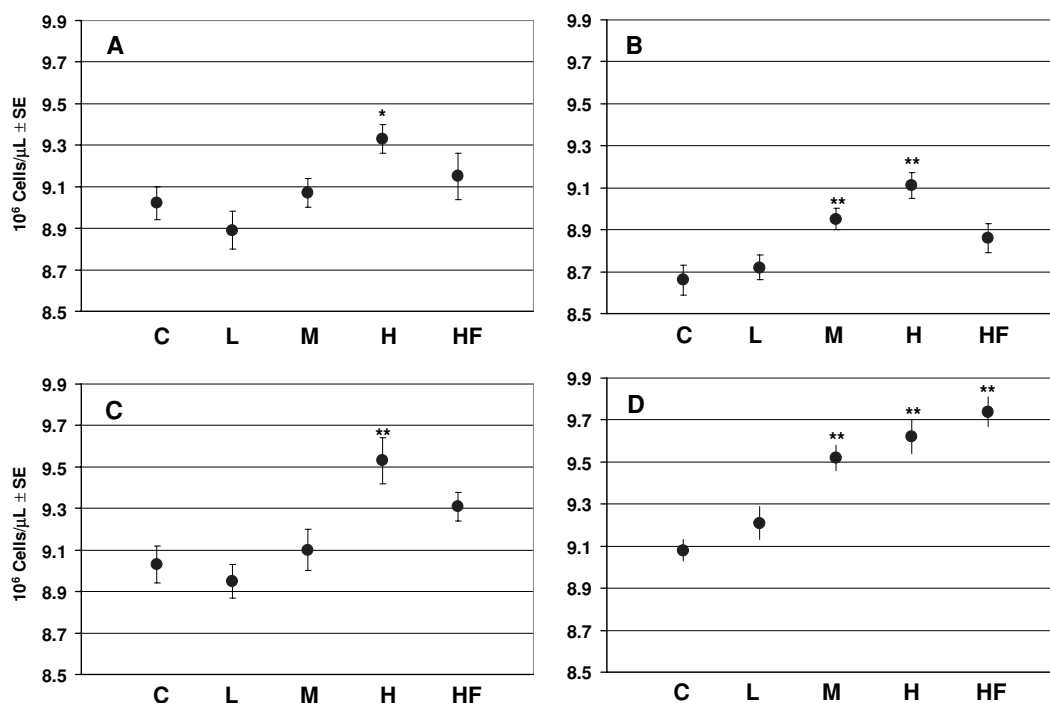


FIG. 2. Effect of exposure on red blood cell counts of female F344 rats after 1 week (A) and 6 months (B) of exposure, and male F344 rats after 1 week (C) and 6 months (D) after exposure (means $\pm$ SE). The trends across the control (C), low (L), mid (M), and high (H) exposure groups were significant. Asterisks indicate significance of pair-wise differences from C (* $p = 0.05$, ** $p = 0.01$).

TABLE 3
Significant effects of exposure on organ weights

Gender	F344 Rats				A/J Mice			
	1 wk		6 mo		1 wk		6 mo	
	Female	Male	Female	Male	Female	Male	Female	Male
Heart				↑M (12%)				
Kidney							↓M (10%)	
Seminal vesicle				↓H (16%)				

Note. Arrows indicate increases (↑) or decreases (↓) meeting the dual criteria for a significant exposure effect ($p < 0.05$ for trend across the control (C), low (L), mid (M), and high (H) exposure groups, and also for pair-wise difference between either the M or H groups and C). Values in parentheses are the largest significant percentage differences between exposed and C group means or medians.

and males, respectively at 6 months. The trend toward increased hemoglobin was significant for females at 1 week, but pair-wise differences from C were not significant.

Clotting Factors in F344 Rats (Table 4)

The only significant effect of exposure on clotting factors in F344 rats was an increase in plasma fibrinogen in males at 1 week (24% in M exposure group).

Micronucleus in A/J Mice

There were no significant effects of exposure on the concentrations of micronuclei in peripheral blood of A/J mice (data not shown).

Lung DNA Damage in F344 Rats and A/J Mice

There was no significant effect of exposure on genomic methylation of lung DNA in F344 rats or A/J mice at 1 week.

Hypermethylation occurred in male rats at 6 months (Figure 3A), but there was no significant effect in females. Methylation in both the M and H exposure groups of males was increased approximately 20% above the C mean, but the increase was only significant in the M exposure group due to variability in the H exposure group. Methylation levels were similar in the HF and C exposure groups. The only significant effect in mice was hypomethylation in females, but not males, at 1 week (Figure 3B). Methylation levels in all exposed female groups were reduced 30–40% from C, but there was not a clear dose-response. Genomic AP site levels were measured in DNA from rats and mice in only the C, H, and HF exposure groups at 6 months, and there were no significant differences from C in either species.

Bronchoalveolar Lavage of F344 Rats

LDH was increased 47% and 29% in H exposure group females and males, respectively at 6 months (Figure 4), but LDH

TABLE 4
Significant effects of exposure on serum chemistry, hematology, and clotting variables of F344 rats

	1 wk		6 mo	
	Females	Males	Females	Males
Alanine aminotransferase			↓H (28%)	
Aspartate aminotransferase			↓M, H (23%)	
Phosphorus			↓M (8%)	
Hematocrit	↑H (4%)	↑H (6%)	↑H (7%)	↑H (7%)
Red blood cell count	↑H (3%)	↑H (6%)	↑M, H (6%)	↑M, H (4%)
Hemoglobin	+	↑H (5%)	↑M, H (7%)	↑M, H (6%)
Fibrinogen		↑M (24%)		

Arrows indicate increases (↑) or decreases (↓) meeting the dual criteria for a significant exposure effect ($p < 0.05$ for trend across the control (C), low (L), mid (M), and high (H) exposure groups, and also for pair-wise difference between either the M or H groups and C). Values in parentheses are the largest significant percentage differences between exposed and C group means or medians. + indicates significant positive trend, but failure to meet the pair-wise criterion.

TABLE 5
Significant effects of exposure on bronchoalveolar lavage variables of F344 rats

	1 wk		6 mo	
	Females	Males	Females	Males
LDH			↑H (47%)	↑H (29%)
MIP-2	+		↑H (58%)	↑H (52%)
Unstimulated macrophage production of hydrogen peroxide	↓H (38%)		↓H (57%)	↓H (64%)
Unstimulated macrophage production of superoxide			↓M (60%), H (48%)	
Stimulated macrophage production of superoxide	↓H (71%)	–		

Note. Arrows indicate increases (↑) or decreases (↓) meeting the dual criteria for a significant exposure effect ($p < 0.05$ for trend across the control (C), low (L), mid (M), and high (H) exposure groups, and also for pair-wise difference between either the M or H groups and C). Values in parentheses are the largest significant percentage differences between exposed and C group medians. + and – indicate significant positive and negative trends, respectively, but failure to meet the pair-wise criterion.

was not increased at 1 week. There was a significant trend toward an increase in MIP-2 of females at 1 week, but not a significant difference from C. At 6 months, however, MIP-2 was significantly increased 58% and 52% in H exposure group females and males, respectively. Exposure depressed the production of reactive oxygen species by alveolar macrophages at both 1 week and 6 months. The production of hydrogen peroxide by unstimulated macrophages was reduced (38% in H exposure group) in females but not in males at 1 week and was reduced 57% and 64% (in H exposure group) at 6 months in females and males, respectively. The production of superoxide by unstimulated macrophages was reduced 48–60% in M and H exposure group males but not females at 6 months. The production of

superoxide in stimulated macrophages was reduced 71% in H exposure group females at 1 week but not at 6 months. There was a significant trend toward reduced production in males at 1 week, but not a significant difference from C (Table 5).

Cardiovascular Effects in SHR Rats

Exposure did not significantly affect heart rate or ECG parameters in SHR rats. Exposure significantly increased the concentrations of lipid peroxides in males (2-fold increase in the H exposure group; no trend in females) and decreased the concentration of TAT complex in females (43% in the H exposure group; similar trend in males but no significant difference from C) (Figure 5). Other serum markers of vascular injury, oxidative stress, and clotting factors were not significantly affected.

Clearance of Bacteria in C57BL/6 Mice

The clearance of *P. aeruginosa* from the lung was unaffected by exposure when expressed either as colony forming units per gram of lung weight or colony forming units per unit of lung protein (data not shown).

Development of Respiratory Allergic Responses in BALBc Mice

There was little evidence that exposure affected the propensity for developing a respiratory allergic response to OVA. The most striking effect was an increase in serum total IgE (Figure 6), for which the exposure-related trend was highly significant and the mean value for the H exposure group was over 2-fold greater than the C mean. Both OVA-specific serum IgE and OVA-specific IgG1 gave slight evidence of an exposure-related effect, but neither met the significance criteria. There

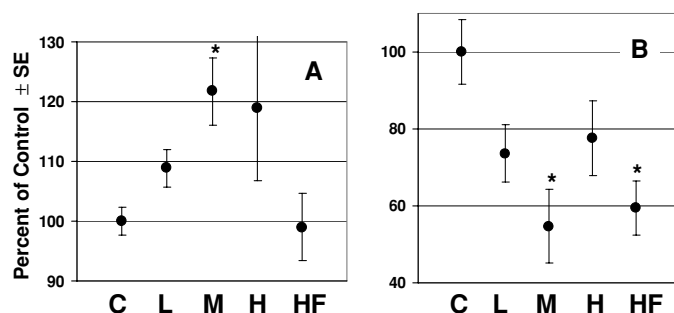


FIG. 3. Effect of exposure on methylation levels in genomic lung DNA from male F344 rats after 6 months of exposure (A) and female A/J mice after 1 week of exposure (B). Group mean values ($\pm$ SE) are expressed as percentages of control values. The trends across the control (C), low (L), mid (M), and high (H) exposure groups were significant and values indicated by asterisks (*) differed significantly from C at $p = 0.05$.

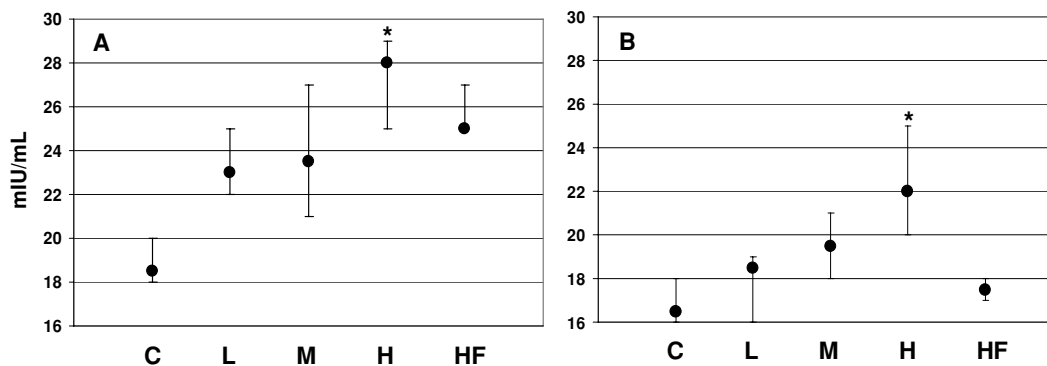


FIG. 4. Effect of exposure on LDH in female (A) and male (B) F344 rats after 6 months of exposure (medians $\pm$ interquartile ranges). The trends across the control (C), low (L), mid (M), and high (H) exposure groups were significant, and values indicated by asterisks (*) differed significantly from C at $p = 0.05$.

were no significant exposure-related effects on cytokine or cell concentrations in bronchoalveolar lavage fluid, although there were slight trends toward increased numbers of neutrophils and eosinophils. Exposure had no significant effect on airway reactivity.

Impact of Removal of Emission PM on Responses to Exposure

As described above, a total of 20 measured variables reflected significant exposure-related effects by the dual criteria of a significant trend across the C, L, M, and H exposure groups combined with a significant pair-wise difference from C at either the M or H exposure levels (Table 6). Among these 20 variables, only the six indicated in Table 6 gave evidence that removal of exhaust PM may have substantially mitigated the exposure effect; and thus, that PM was likely an important factor in causing the response. These included increased seminal vesicle weight in male rats at 6 months, decreased kidney weight in female mice at 6 months, increased red blood cell counts in female rats at 6 months (Figure 2B), increased LDH in bronchoalveo-

lar lavage fluid of both genders of rats at 6 months (Figure 4), increased lipid peroxides in male SHR rats at 1 week (Figure 5A), and increased methylation in male rats at 6 months (Figure 3A). These variables met the exploratory criterion of $p < 0.10$ for the difference between the HF exposure group and the M or H exposure groups differing most significantly from C. The other 14 variables gave little or no evidence that the responses of the HF exposure group were less than the responses of the H exposure group.

The variability of responses within all exposure groups, and the variations in mean or median responses among the groups exposed to the unfiltered atmospheres, made it difficult to judge the importance of exhaust PM to most effects. This difficulty was illustrated by the hypermethylation of lung DNA in male F344 rats (Figure 3A). Because the mean values of the M and H exposure groups were increased 20% above the C mean and the values of the HF and C exposure groups were nearly identical, this was perhaps the most convincing example of a likely PM effect. The variability of response in the H exposure group, however, prevented the difference between the H and HF exposure

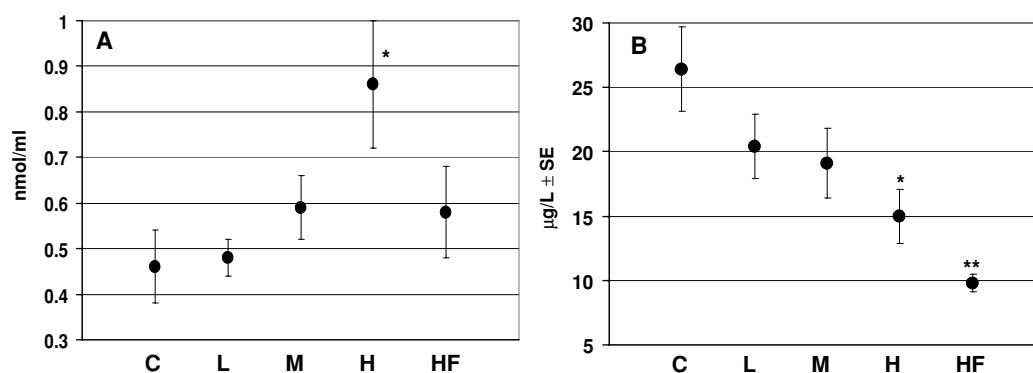


FIG. 5. Effect of exposure on lipid peroxide concentrations in serum of male SHR rats (A) and TAT concentrations in serum of female SHR rats (B) after 1 week of exposure (means $\pm$ SE). The trends across the control (C), low (L), mid (M), and high (H) exposure groups were significant. Asterisks indicate significance of pair-wise differences from C (* $p = 0.05$, ** $p = 0.01$).

TABLE 6
Impact of removal of gasoline emissions PM on significant exposure-related effects

Significant exposure effects	Effects showing evidence for at least partial causality by particles
Organ weight in F344 rats and A/J mice, 1 wk and 6 mo	
↑heart, male rats, 6 mo	—
↑seminal vesicle, male rats, 6 mo	✓
↓kidney, female mice, 6 mo	✓
Serum chemistry in F344 rats, 1 wk and 6 mo	
↓alanine aminotransferase, females, 6 mo	—
↓aspartate aminotransferase, females, 6 mo	—
↓phosphorus, males, 6 mo	—
Hematology and clotting factors in F344 rats, 1 wk and 6 mo	
↑hematocrit, females and males, 6 mo	—
↑red blood cell count, females and males, 1 wk and 6 mo	✓ (6 mo f only)
↑hemoglobin, males, 1 wk; females and males, 6 mo	—
↑fibrinogen, males, 1 wk	—
Bronchoalveolar lavage in F344 rats, 1 wk and 6 mo	
↑LDH, females and males, 6 mo	✓
↑MIP-2, females and males, 6 mo	—
↓unstim. macrophage peroxide, females, 1 wk; females and males, 6 mo	—
↓unstim. macrophage superoxide, males, 6 mo	—
↓stim. macrophage superoxide, females, 1 wk	—
Serum factors in SHR rats, 1 wk	
↓thrombin-antithrombin complex, females	—
↑lipid peroxides, males	✓
DNA damage in F344 rats and A/J mice, 1 wk and 6 mo	
↓methylation, female mice, 1 wk	—
↑methylation, male rats, 6 mo	✓
Development of respiratory allergic responses in BALBc mice	
↑serum total IgE	—

Note. Only measured variables meeting 2-stage criteria for significance: 1) trend across the control (C), low (L), mid (M), and high (H) exposure groups significant at $p < 0.05$; and 2) pair-wise difference from C significant at $p < 0.05$ for M or H exposure group. Arrows indicate direction of significant differences; ↑ and ↓ indicate increases or decreases from control values, respectively. Responses marked with ✓ differed at $p = 0.10$ or less between the high-filtered (HF) exposure group and either the M or H exposure group differing most significantly from C.

groups from reaching even $p = 0.10$. The red blood cell counts provided another example of the difficulty of judging PM effects. Although the difference between the HF and H exposure groups of female rats at 6 months (Figure 2B) met the exploratory criterion of $p < 0.10$, removal of PM did not reduce the response of males at the same time (Figure 2D), and the differences between the HF and H exposure groups of both genders at 1 week did not meet the exploratory criterion (Figures 2A, 2C). Moreover (as discussed below), there is a much stronger rationale for ascribing the effect to CO rather than to PM.

DISCUSSION

Overall, the responses to exposure were modest and few were significant, even though the exposures involved higher concentrations of gasoline combustion-derived pollutants than are

widespread in the environment. Concentrations at the L exposure level approximated those encountered in many on-road or roadside locations; certainly, the CO, PM, and NO_x concentrations at that level were within the range of those environments (EPA, 2000; 2004; 2007). The 9-fold higher concentrations at the H exposure level far exceeded environmental and occupational exposure levels. None of the variables included in this report demonstrated significant differences from C at the L exposure level. The NERC protocol includes two additional health assays that were performed as part of this study, but reported separately: cardiovascular effects in ApoE^{-/-} mice (Lund et al., 2007) and exacerbation of pre-existing respiratory allergic responses in BALBc mice (Day et al., 2008). In those assays, only TBARS in the proximal aorta of ApoE^{-/-} mice were significantly different from C at the L exposure level. With this

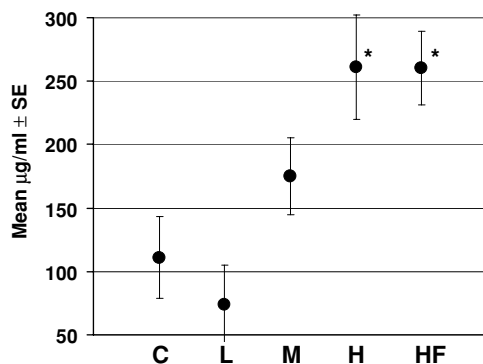


FIG. 6. Effect of exposure on serum total IgE in BALBc mice exposed *in utero* and 4 week after birth, followed by sensitization with OVA (means $\pm$ SE). The trend across the control (C), low (L), mid (M), and high (H) exposure groups was significant and values for both the H and high-filtered (HF) exposure groups differed significantly from C at $p = 0.05$.

exception therefore, the L exposure level was a “no effects” level within the limitations of the study.

Only 20 (17%) of the 120 measured and calculated variables demonstrated effects that were significant by pre-established statistical criteria. These criteria were identical to those applied to the same variables in preceding NERC studies of diesel exhaust (Campen et al., 2003; Harrod et al., 2005; Reed et al., 2004; Seagrave et al., 2005) and hardwood smoke (Reed et al., 2006; Seagrave et al., 2005), both of which demonstrated larger numbers of significant effects. The criteria assume that important health effects are related to exposure concentrations in a monotonic (although not necessarily linear) manner. Although non-monotonic effects are possible, the criteria were considered acceptable because only a few responses in the NERC program to date have presented strong evidence for significant deviation from monotonicity, and monotonic relationships are assumed in all contemporary hazard assessments. For some variables, the greater significance at the M exposure level than at the H exposure level resulted from greater variability at the H exposure level rather than from a reversal of the exposure effect between the M and H exposure levels.

The large number of response variables, combined with evaluation of effects in males and females separately for many variables (conventional practice for those variables in bioassays), resulted in over 200 individual statistical tests of exposure effect. Using $p < 0.05$ as the criterion for significance, it might be expected that several indications of significant effects might have occurred by chance, despite the stringency of the dual criteria. Confidence that an effect is real is bolstered by significant responses in both genders and in both species for variables measured in two species. None of the organ weight effects were significant in both genders or in both rats and mice. Among the 12 significant responses of rats listed in Tables 4 and 5 and the two responses in serum of SHR rats, only 6 were significant in

both genders. Only the increase in red blood cells in F344 rats demonstrated coherence among both genders and measurement times (discussed further below). Regardless of the statistical uncertainties, we take the indications of significant exposure effects in this study at face value. The quality of the information gained would not be improved by reducing the number of comparisons by reporting this large study as a series of smaller studies, as sometimes occurs in large research efforts.

In the following discussion, several of the effects of gasoline exhaust are not only compared to the effects of gasoline exhaust in previous studies, but also to the effects of exposure to diesel exhaust. Although the comparisons are useful, the compositions of diesel and gasoline exhaust are quite different. At the same dilution used for the highest concentration in the present study, for example, the NERC diesel exposure atmosphere (McDonald et al., 2004b) contained much higher concentrations of PM ($1,005 \mu\text{g}/\text{m}^3$), NO (50 ppm), and NO₂ (7 ppm), and much lower concentrations of CO (31 ppm) and NMHC ($1.6 \text{ mg}/\text{m}^3$). Particle size was similar to that in the present study (150 nm mass median aerodynamic diameter). Despite the greater PM concentration however, PM in the diesel study comprised a portion of total exposure mass very similar to that in the present study.

The most coherent effects of exposure were the interrelated increases in red blood cell count, hematocrit, and hemoglobin. These effects in M exposure level rats at 1 week and both M and H exposure level rats at 6 months most likely occurred in response to the high levels of CO and were consistent with effects reported by others. Brightwell et al. (1986) observed increases in red blood cell count, hematocrit, and hemoglobin in F344 rats and Syrian hamsters exposed at 21 ppm (with catalyst) or 224 ppm (without catalyst) CO 16 h/day, 5 days/week for 18–24 months (rats) or 16 months (hamsters). The heart/body weight ratio of the rats was also markedly increased by long-term exposure at the higher CO level. Roggendorf et al. (1981) also observed increased hemoglobin and a slight increase in heart/body weight ratio in male Sprague-Dawley rats exposed 8 h/day, 5 days/week for several months to gasoline exhaust at 250 ppm, but did not report other hematological variables. In the present study, there also was evidence of an exposure-related increase in heart weight in male rats after 6 months of exposure at 73 ppm CO. It can be speculated that the heart weight of mice or female rats might have been significantly increased with more prolonged exposure. We did not observe significant effects on heart weight, red blood cell count, hematocrit, or hemoglobin in the NERC diesel study at 30 ppm CO (Reed et al., 2004). Together, these findings indicate that subchronic or chronic exposure to extreme concentrations of gasoline exhaust can induce compensatory responses in the oxygen transport system. We agree with previous authors that the effect is most likely attributable to the high concentrations of CO.

A general depression of the production of reactive oxygen species by both stimulated and unstimulated alveolar macrophages from F344 rats was reflected by reductions in hydrogen peroxide and superoxide. These changes might act

to reduce the effectiveness with which macrophages inactivate bacteria or other particles. Although the protocol did not include bacterial challenges of rats, exposures were not found to significantly reduce the ability of mice to clear bacteria from the lungs. No previous gasoline exhaust study included measures of macrophage function; thus, it is not known if this is a characteristic effect of extreme or more prolonged exposures. Production of hydrogen peroxide by unstimulated macrophages was decreased to a similar degree in H exposure level female but not male F344 rats after 6 months exposure to a similar dilution of diesel exhaust in the earlier NERC study (Reed et al., 2004), but production of other reactive oxygen species was not significantly affected.

The strongest indications of an adverse effect on the lung were the significant increases in LDH and MIP-2 in bronchoalveolar lavage fluid of both female and male rats after 6 months of exposure at the H exposure level. These increases in LDH and MIP-2 were not accompanied by increases in leukocyte counts or other pro-inflammatory cytokines or by histological evidence of inflammation. The only previous report of lavage responses in animals exposed to gasoline exhaust was that of Muhle et al. (1984), who exposed Wistar rats and Syrian hamsters 19 h/day, 5 days/week for 91 weeks at two dilutions containing 73 or 35 $\mu\text{g PM}/\text{m}^3$, 155 or 306 ppm CO (no catalyst), 11.6 or 23.8 ppm NO_x, and 18.3 or 36.5 ppm NMHC. Although levels of significance were not reported by Muhle and colleagues, bar graphs indicated that no significant increases were observed in LDH, leukocytes, or other measured variables in either species; pro-inflammatory cytokines were not measured. Together, these findings suggest that subchronic or chronic exposure to extreme concentrations of gasoline exhaust causes little inflammation in the lung. It is tempting to speculate that the high concentration of CO in the exposure acted to suppress lung inflammatory responses, based on the known suppression by endogenous and exogenous CO of experimental and clinical lung oxidative injury and inflammation (Hoetzel et al., 2007; Kaczorowski and Zuckerbraun, 2007; Ryter et al., 2007). However, exposure to a similar dilution at the H exposure level in the NERC diesel study, but containing only 30 ppm CO (Seagrave et al., 2005) also increased LDH and had little effect on other variables, but differed from the response to gasoline exhaust in that MIP-2 was not increased. As might be expected, the amount of PM in alveolar macrophages was greater in diesel-exposed animals than in gasoline-exposed animals.

The responses of blood clotting factors in F344 and SHR rats were inconsistent, and the impact of exposure on blood clotting was equivocal overall. The increased fibrinogen in male F344 rats at 1 week suggested an enhanced tendency toward clotting, but the finding did not occur in females at 1 week, in either gender at 6 months, or in either gender of SHR rats after 1 week of exposure. The reduced TAT complex in female SHR rats at 1 week did not support an enhanced tendency toward clotting, and the effect was not observed in males or in F344 rats of either gender. Exposure to diesel exhaust at a similar dilution

(Reed et al., 2004) reduced the TAT complex in male F344 rats, and also reduced Factor VII in females and males at both 1 week and 6 months. It is unclear why TAT would be reduced by these exposures. Impairment of synthesis of TAT, or increased degradation/utilization could explain the effects, but how engine exhaust might interfere with normal TAT homeostasis remains uncertain. Together, these findings suggest that exposure to extreme concentrations of diesel and gasoline exhaust does not enhance the tendency toward blood clotting in rodents.

The lack of effect on the heart rate or ECG of SHR rats was consistent with the finding by Stupfel et al. (1973) that chronic exposure of Sprague-Dawley rats to gasoline exhaust at dilutions containing 0.2 or 23 ppm NO_x and 50 ppm CO (CO held constant) reduced heart rate significantly at only 4 of 22 measurement times and had no effect on the amplitude or incidence of abnormalities of the QRS complex. In contrast, the NERC exposure to diesel exhaust at the same dilution as the H exposure level in the present study reduced heart rate and prolonged the P-Q interval (Campen et al., 2003).

The lack of effect on clearance of *P. aeruginosa* from the lungs of mice suggests that defenses against gram negative bacteria and macrophage function were not severely depressed. No previous study of gasoline exhaust included this response model. The finding appears in contrast with that of Coffin and Blommer (1967) who exposed Swiss white mice for 4 h to irradiated exhaust from an early 1960s engine burning leaded fuel, followed by exposure to aerosolized *Streptococcus* and observation of increased mortality. They reported that exposure at concentrations containing between 25 and 100 ppm CO increased mortality in a dose-related manner (PM concentration not reported). Differences between this and the Coffin and Blommer study in the response models and exposure parameters prevent a direct comparison. The present results do not eliminate the possibility that exposure to high concentrations of gasoline exhaust might impair defenses against other bacteria or viruses. In previous NERC studies using the same model, clearance of *P.a.* was retarded by diesel exhaust (Harrod et al., 2005) but not by hardwood smoke (Reed et al., 2006). Clearance of respiratory syncytial virus was retarded by diesel exhaust in a pilot study (Harrod et al., 2003), but not in a follow-up NERC study (Reed and Berger, 2006).

There are no previous reports of the effects of inhaled gasoline exhaust on the frequency of micronuclei in peripheral blood. Micronuclei were not increased in the same model in previous NERC studies of diesel exhaust (Reed et al., 2004) or hardwood smoke (Reed et al., 2006). Either these combustion emissions have little clastogenic effect, or this model was not sensitive to the effect.

Although the mutagenic content of engine exhaust has received much attention (Schuetzle and Lewtas, 1986; Mauderly and Garshick, 2008), there are no published reports of the effects of inhaled engine exhaust on DNA global methylation levels or oxidative DNA injury. Alteration of methylation levels is an epigenetic hallmark of cancer, and is an early finding in cancer cells (Feinberg and Tycko, 2004). The seemingly conflicting findings

of reduced methylation levels in female mice at 1 week and increased methylation levels in male rats at 6 months are of uncertain interpretation, but may have reflected disturbances to the normal control of methylation levels. Regardless, failure to find the effects in both genders, or in both species, suggests caution in interpretation. Unpublished results from the NERC diesel study (Divine, in preparation) indicated increased methylation in rats and decreased methylation in mice at both 1 week and 6 months at intermediate but not H exposure levels (dilutions similar to those in the present study). Together, these findings suggest that exposure to engine exhaust can influence DNA methylation levels, but that the effects of extreme exposures to gasoline exhaust are not striking and of uncertain interpretation.

The finding that only serum total IgE demonstrated a significant effect of *in utero* and postnatal exposure on respiratory allergic responses developed in response to sensitization after 7 weeks of age makes the implications of the finding somewhat uncertain. The absence of significant effects on serum OVA-specific IgE, other antibodies, pro-allergic cytokines, or airway responsiveness suggests that the exposures may not have altered the likelihood of sensitization or strength of the allergic response in a clinically meaningful manner. This response model was not used in previous studies of gasoline or diesel exhaust, and because this was the first application of the model in a NERC study, there are few results for comparison. A similar model used at LRRRI demonstrated enhanced responses in BALBc mice exposed to cigarette smoke and sensitized to *Aspergillus fumigatus* extract (Singh et al., 2003). Watanabe and Ohsawa (2002) reported that exposure to diesel exhaust *in utero* increased responses of rats to sensitization with Japanese cedar pollen.

One of the more interesting aspects of this study was the direct comparison of effects in the H and HF exposure level groups, a potentially straightforward test of the importance of exhaust PM to the effects. Within the limitations of the experimental design, it was not straightforward to assign a portion of the effect to PM for variables in which filtration appeared to mitigate the effects somewhat, but not completely. However, because only 6 of the 20 significant responses appeared likely to have been substantially influenced by PM, it can be stated with confidence that many effects reported in this paper were apparently driven by the non-PM components. The majority of the cardiovascular effects in atherosclerosis-prone ApoE^{-/-} mice reported earlier by Lund et al. (2007) were also apparently caused by non-PM components. The experimental design did not allow identification of the specific gas or vapor species (or their combinations) that might have caused the effects. Based largely on proportional mass and somewhat on potential mechanisms for some responses, we speculate (but certainly cannot prove) that CO, NO_x, and NMHC are the most likely candidates, and that the lower concentrations of NH₃ and SO₂ are less likely to have been involved.

It is noteworthy however, that PM apparently did drive, or substantially contribute to, some effects. This occurred despite the facts that PM comprised only 0.04% of the exposure mass,

and the concentration was not far above the upper end of the ambient PM_{2.5} concentration range. It is possible, but can not be speculated on the basis of this study, that PM may exert greater relative effects if the exposures were extended for a longer period. Regardless, it is certainly plausible that gasoline PM contributes some portion of the health burden associated with traffic PM.

Although this was the first comparison of the effects of unfiltered and filtered gasoline exhaust, several studies of inhaled diesel exhaust have produced similar findings for both cancer and non-cancer responses. Japanese studies of rodents exposed to whole or filtered exhaust have shown that all, or the majority, of effects on amplification of allergic responses from *in utero* or postnatal exposures (Maejima et al., 2001; Watanabe and Ohsawa, 2002) and organ development (Watanabe and Oonuki, 1999; Watanabe and Kurita, 2001; Watanabe, 2005) were attributable to non-particulate components. It also was demonstrated in two studies that filtration eliminated the lung tumor response of rodents to diesel exhaust (Heinrich et al., 1986; Brightwell et al., 1989). There is some evidence from experimental human exposures that non-PM components of engine exhaust can drive some responses. Rudell et al. (1999) found that adding a ceramic particle trap only reduced lung inflammatory responses of humans to single exposures by approximately 20%, although most of the DPM mass was removed. Results of other studies of animals and humans exposed to diesel exhaust have indicated the importance of PM to some responses.

In summary, repeated inhalation exposure of rodents to exhaust from catalyst-equipped gasoline engines operated on a simulated urban cycle caused several significant effects at the higher exposure levels, but none (among the variables included in this report) at the lowest exposure level. Although only a portion of the response variables examined in this study were included in the few previous studies of gasoline exhaust, responses that could be compared were generally similar. No previous study used engines of more recent vintage than the 1980s, and only two (Pepelko et al., 1979; Brightwell et al., 1986, 1989) used catalyst-equipped engines burning unleaded fuel. The engines in the present study were not of the most recent ultra-low emission vintage. The 1996 engines burning fuel representative of average USA composition in the early 2000s were intended to be more typical of the USA fleet average at the time this study was designed. It must also be noted that only fresh exhaust was studied, and the exposure atmospheres had a residence time in the animal chambers of only a few minutes. People are certainly exposed to fresh exhaust on roadways but also are exposed to aged exhaust that has reacted with other air contaminants and sunlight. It is not known how the results of this study might pertain to exposures to aged exhaust more distant from the source.

An important outcome of the study was the finding that the majority of significant responses were apparently not caused by exhaust PM. This, of course, does not mean that PM in gasoline exhaust is unimportant. Rather, this finding serves as a strong reminder that PM constitutes a small portion of the

total mass emitted by gasoline engines and most other combustion sources, and effects of such complex mixtures cannot be ascribed to PM or any other physical-chemical class unless the experimental design provides for differentiation among components.

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