TOXICOLOGICAL SCIENCES **71,** 237–245 (2003)

Inhaled Environmental Combustion Particles Cause Myocardial Injury in the Wistar Kyoto Rat

Urmila P. Kodavanti,*[,1] Carolyn F. Moyer,† Allen D. Ledbetter,* Mette C. Schladweiler,* Daniel L. Costa,* Russ Hauser,‡ David C. Christiani,‡ and Abraham Nyska§

**Pulmonary Toxicology Branch, Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27709; †Pathology Associates, Raleigh, North Carolina; ‡Harvard School of Public Health, Boston, Massachusetts 02115; and §Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709*

Received July 29, 2002; accepted November 6, 2002

Epidemiologists have associated particulate matter (PM) air pollution with cardiovascular morbidity and premature mortality worldwide. However, experimental evidence demonstrating causality and pathogenesis of particulate matter (PM)-induced cardiovascular damage has been insufficient. We hypothesized that protracted, repeated inhalation by rats of oil combustion-derived, fugitive emission PM (EPM), similar in metal composition to selected sources of urban air PM, causes exposure duration- and dose-dependent myocardial injury in susceptible rat strains. Zinc was the only primary water-leachable/bioavailable element of this EPM. Male Sprague-Dawley (SD), Wistar Kyoto (WKY), and spontaneously hypertensive (SH) rats were exposed nose-only to EPM (2, 5, or 10 mg/m^3, 6 h/day for 4 consecutive days or 10 mg/m^3, 6 h/day, 1 day/week for 4 or 16 consecutive weeks). Two days following the last EPM exposure, cardiac and pulmonary tissues were examined histologically. The results showed that particle-laden alveolar macrophages were the only pulmonary lesions observed in all three rat strains. However, WKY rats exposed to EPM (10 mg/m^3 6 h/day, 1 day/week for 16 weeks) demonstrated cardiac lesions with inflammation and degeneration. To further characterize the nature of EPM-associated lesions, more rigorous histopathological and histochemical techniques were employed for WKY and SD rats. We examined the hearts for myocardial degeneration, inflammation, fibrosis, calcium deposits, apoptosis, and the presence of mast cells. Decreased numbers of granulated mast cells, and multifocal myocardial degeneration, chronic-active inflammation, and fibrosis were present in 5 of 6 WKY rats exposed to EPM for 16 weeks. None of these lesions were present in WKY exposed to clean air. EPM-related cardiac lesions were indistinguishable from air-exposed controls in SD and SH rats. This study demonstrates that long-term inhalation exposures to environmentally relevant PM containing bioavailable zinc can cause myocardial injury in sensitive rats. These findings provide supportive evidence for the epidemiological associations of cardiovascular morbidity and ambient PM.

Key Words: **inhaled particulate matter; myocardial injury; Wistar Kyoto rats; bioavailable zinc; cardiovascular disease.**

Epidemiological studies have associated cardiovascular and respiratory morbidity and mortality with particulate matter (PM) air pollution (reviewed in Dockery 2001; Morris, 2001; Pope, 2001; Schwartz, 2001), particularly in susceptible humans with concurrent cardiovascular and pulmonary diseases (Pope, 2001). Recently, an increased incidence of myocardial infarctions in previously infarcted patients has been linked to acute episodes of ambient PM (Peters *et al.*, 2001). Laboratory studies addressing PM-induced cardiovascular effects are rapidly emerging. Most of these studies have focused on the impact of PM on cardiophysiology (Campen *et al.*, 2001; Vincent *et al.*, 2001; Watkinson *et al.*, 1998; Wellenius *et al.*, 2002) but not on histopathology or biochemical alterations. In retrospective analyses of cardiac tissues from studies conducted by the National Toxicology Program, we have demonstrated coronary arteritis in mice following life-long inhalation exposure to several metallic aerosols (Moyer *et al.*, 2002); however, to date, causality has not been established with ambient PM.

Several pathophysiological mechanisms have been proposed for the induction of PM-induced cardiovascular effects. One involves the concept that metallic substances bound to PM, that are water-soluble and thus bioavailable, pass directly into the pulmonary circulation, migrate to the heart, and induce cardiac injury. Another hypothesis asserts that PM exposure leads to local pulmonary vascular inflammation/microvascular thrombosis and systemic endothelial changes resulting in altered myocardial contractility (Frampton, 2001). Unfortunately, at-

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

[1] To whom correspondence should be addressed at Pulmonary Toxicology Branch, MD B143-02, Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, 109 T. W. Alexander Drive, Research Triangle Park, NC 27709. Fax: (919) 541-0026. E mail kodavanti.urmila@epa.gov.

tempts to evaluate fully these proposed mechanisms have been hampered by a variety of problems: (1) Appropriately susceptible animal models that develop myocardial effects at environmentally relevant PM exposures are unavailable. (2) Clinically useful plasma markers of cardiac injury are less sensitive to subtle inflammatory and degenerative changes in the heart. Histological evaluation of cardiac tissue using a variety of staining techniques remains the most accurate method of detecting subtle cardiac toxicity. Unfortunately, these techniques are not used routinely in air pollution studies. (3) Environmental PM contains diverse causative constituents; therefore, without having sufficient knowledge of constituent-specific cardiac tissue effects, it has been difficult to define appropriate PM samples with which to study cardiovascular effects. Based on an understanding of these shortcomings, we retrospectively analyzed cardiac histopathology that included acute and long-term exposures of three rat strains to a combustion emission particle (EPM) sample containing zinc but minimal bioavailable vanadium, nickel, and iron. The EPM sample selected for this study was distinct from other extensively studied residual oil fly ashes (ROFAs) that contain high levels of iron, vanadium, and nickel (Dreher *et al*., 1997; Kodavanti *et al*., 1997, 1998, 2002). Evidently the elemental and organic composition of this EPM was similar to ambient PM composition from select urban locations (Adamson *et al*, 2000; Balachandran *et al.*, 2000; Dye *et al.*, 2001; Harrison and Yin, 2000). We hypothesized that exposure of the most susceptible rat strain to this EPM would be associated with time- and dose-dependant cardiac injury.

MATERIALS AND METHODS

Animals. Specific pathogen-free, 11–13-week-old, healthy male Sprague-Dawley (SD) rats, normotensive Wistar Kyoto (WKY) rats, and spontaneously hypertensive (SH, SHR/NCrlBR) rats (derived from WKY rats by phenotypic segregation of the hypertensive trait and inbreeding) were purchased from Charles River Laboratories (Raleigh, NC), with the exception of the WKY rats used for the 4-day consecutive exposures (Harlan Sprague-Dawley, Indianapolis, IN or Taconic, Germantown, NY), due to the lack of concurrent availability of age-matched rats from Charles River Laboratories. These rats were maintained in an isolated animal room in an AAALAC-approved animal facility (21 ± 1°C, 50 ± 5% relative humidity, 12-h light/dark cycle) for a quarantine of one week and for nonexposure periods. The rats were housed singly in plastic cages with beta-chip bedding changed three times per week. All animals received standard (5001) Purina rat chow (Brentwood, MO) and water *ad libitum,* except during nose-only exposure periods of 6 h. The protocol for the use of rats in nose-only inhalation was approved by the EPA Animal Care and Use Committee.

Emission PM (EPM) source and composition analysis. The EPM used in this study was collected in Boston, MA in 1997 in a sterile, 50-ml polyethylene tube from a stack of a power plant burning residual oil (#6). These particles were released into the ambient air from an emission device. Because the elements that readily leach off in the water are likely to be absorbed by lung cells and in circulation within a short period of time after inhalation, the leachable elements of ROFAs have been responsible for the majority of injuries (Dreher *et al.*, 1997; Kodavanti *et al.*, 1997, 1998). However, the components not readily leached off may not be bioavailable, especially at distant sites. To better ascertain the role of individual components of EPM, and to relate the composition of this EPM to other residual oil fly ashes and ambient PM studied earlier by us and other investigators (Adamson *et al*, 2000, Dye *et al.*, 2001; Kodavanti *et al.*, 1997, 1998, 2000a), the water-leachable and 1 M HCl-leachable metals and sulfate, as well as total carbon content, were determined and recently reported by our laboratory (Kodavanti *et al.*, 2002b). In brief, the bulk sample was ground, sieved, and analyzed for size using a TSI Model 3310A Aerodynamic Particle Sizer (TSI, Inc., St. Paul, MN), assuming a log-normal distribution as described earlier (Kodavanti *et al.,* 1998). The particles were made airborne with a TSI Model 3433 small-scale powder dispenser for size determination. EPM was extracted in double distilled water or 1.0 M HCl as described (Kodavanti *et al*, 1998, 2002b). Supernatants resulting from water and 1.0 M HCl were analyzed for the presence of sulfate (SO_4), zinc (Zn), nickel (Ni), vanadium (V), iron (Fe), manganese (Mn), and copper (Cu), which were presumed to be the predominant causative metals of this combustion source emission based on previous studies (Kodavanti *et al.*, 1997; 1998). The elemental analysis of the extracts was done using inductively coupled plasma-atomic emission spectroscopy (ICP-AES), as described in Kodavanti *et al.* (2002b).

Nose-only inhalation exposure protocol. Since this study involved retrospective analysis of cardiac tissues from our recently conducted EPM inhalation study (Kodavanti *et al*., 2002b), the detailed inhalation exposure protocol and the experimental design are described in that paper. In brief, three rat strains were selected for EPM inhalation exposure. SD rats were evaluated for comparison to a healthy control rat strain that is extensively used in PM studies (Adamson *et al*, 2000; Campen *et al.*, 2001; Dreher *et al.*, 1997; Dye *et al.*, 2001; Kodavanti *et al.*, 1997, 1998, 1999). WKY rats were employed for their propensity to develop hypertrophic cardiomyopathy while remaining normotensive (Kuribayashi, 1987), and SH rats for their predilection to develop hypertension and cardiomyopathy (Kuribayashi, 1987). SD, WKY, and SH rats were randomized into air control and EPM groups based on body weights (n = 6/group for SD and WKY and 8/group for SH rats). A larger group size (n = 8) was selected for SH rats because these rats have yielded more variable responses to given exposures relative to SD and WKY rats (Kodavanti *et al.*, 2000b, 2002b). The selection of exposure concentrations/scenarios was based on the presumption that acute exposures will result in mild to moderate dose-dependent cardiopulmonary injury, and will represent scenarios included in our previous studies with different particles (Kodavanti *et al*., 2000a,b; Schladweiler *et al*., 2002) as well as extreme environmental PM episodes. Inclusion of 4 consecutive days of exposure versus episodic exposure over 4 or 16 weeks was to understand the cumulative nature of injury based on our previous study where episodic ROFA exposure over 4 weeks resulted in progressive lung injury while systemic effects occurred only after acute exposures (Kodavanti *et al*., 2002a). Rats were exposed by nose-only inhalation to either filtered air or a dry aerosol of EPM at 2, 5, or 10 mg/m^3 for 6 h/day on 4 consecutive days, or 10 mg/m^3, 6 h/day, 1day/week, for 4 or 16 weeks (Kodavanti *et al.*, 2002b; Ledbetter *et al.*, 1998). As reported recently (Kodavanti *et al*., 2002b), aerosolized particles were aerodynamically size-separated through a cyclone impactor at ≤2.5 micrometer and introduced into the mixing chamber. Chamber aerosol concentrations were determined gravimetrically and by a real-time aerosol monitor (RAM-1, GCA Corp., Bedford, MA). Determination of aerosol-size distribution was performed at least once per exposure, using a 7-stage cascade impactor (Intox Products, Albuquerque, NM). Chamber temperature, relative humidity, airflow, and pressure were monitored continuously and maintained at constant levels (Ledbetter *et al.*, 1998).

Necropsy and histology. Previous studies have indicated that following an inhalation exposure of 2–4 days, pulmonary injury and systemic effects remain persistent for at least up to 4 days with regard to inflammation, protein leakage, and pathology (Kodavanti *et al.*, 2002b). Therefore, we decided to evaluate injury/pathology at two days following the last exposure. Two days after the final exposure, rats were weighed and anesthetized with sodium pentobarbital (100–150 mg/kg, ip). The trachea was cannulated and the right lung tied; the left lung was inflated with filtered 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) at the total lung capacity (28 ml/kg body weight). The

TABLE 1
Semiquantitative Criteria Used for the Assessment of Cardiac Pathology in Rats

Pathology	Score	Description
Inflammation		
	1	Few scattered inflammatory cells
	2	Minimal inflammatory infiltrates
	3	Small localized multiple foci of inflammatory cells involving more than one area
	4	Diffuse severe inflammatory infiltrates
Degeneration		
	1.0	<5%
	1.5	5–15%
	2.0	16–25%
	2.5	26–35%
	3.0	>35%
Fibrosis		
	1	Minimal fibrosis in ventricles, septum or papillary muscles
	2	Small foci of fibrosis involving small foci at multiple locations
	3	Multiple foci of fibrosis involving more than one area
	4	Large diffuse fibrosis area involving ventricular septum and left ventricular papillary muscles
Calcium deposition		
	1	Occasional calcium deposits in ventricles, septum, or papillary muscles
	2	Calcium deposits involving more than one of above locations
	3	Calcium deposits involving more than one of above locations
	4	Large diffuse area of calcium deposition involving ventricular septum and left ventricular papillary muscles
Apoptosis		
	1	Single myocytes randomly distributed in ventricles, septum, or papillary muscles
	2	Singe foci consisting of a few myocytes involving more than one of above locations
	3	Small localized, multiple foci of myocytic apoptosis involving more than one area
	4	Large diffuse area of myocytic apoptosis involving ventricular septum and left ventricular papillary muscles

Note. The severity of apoptosis, inflammation, fibrosis, and calcium deposition were based on semiquantitative criteria outlined by Herman *et al* (1996, 2000). The severity of myocardial fibrillar loss was established from criteria according to the semiquantitative methods described by Billingham (1991). The score for myocardial degeneration refers to myocardial fibrillar loss and/or vacuolation (based on the % of cells exhibiting fibrillar loss). Note that the mast cells were counted from toluidine blue-stained slides according to the description provided in Materials and Methods (category not shown in table).

heart was removed and cut longitudinally to obtain representative portions of right and left ventricles. After fixation, the lung and heart tissues were embedded in paraffin (Experimental Pathology Laboratory, Research Triangle Park, NC).

Heart and lung sections from SD, WKY, and SH rats were cut at 5 micron, stained with hematoxylin and eosin, and examined microscopically. Based on the EPM-related strain differences in the presence or absence of cardiac lesions, further evaluation using a variety of staining techniques of the cardiac tissues was performed in WKY rats. SD rats were also evaluated further for comparison to a healthy control rat strain that is extensively used in PM studies (Adamson *et al.*, 2000; Campen *et al.*, 2001; Dreher *et al.*, 1997; Kodavanti *et al.*, 1997, 1998, 1999). These evaluations were performed for the 16-week-exposure group as this long-term exposure scenario only depicted cardiac pathology. Replicate 5-micron heart sections from WKY and SD rats were stained with periodic acid Schiff (PAS) for the detection of myocardial glycoprotein and polysaccharide, Masson's trichrome (MT) for collagen, toluidine blue (TB) for mast cells, phosphotungstic acid hematoxylin (PTAH) for evaluation of myofibrilar loss, von Kossa for calcium deposition, and Movat's pentachrome for distinction of myocyte and myofibroblasts. The ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Intergen, Purchase, NY) was used to detect apoptosis. Histological examinations of the left ventricle, right ventricle, septum, and large coronary vessels were conducted. The lesion severity was assessed based on semiquantitative criteria (Table 1) previously outlined by Herman *et al* (1996, 2000) and Billingham (1991). Mast cells were identified by their large metachromatic, coarse granules in abundant cytoplasm in toluidine blue-stained histologic sections. The number of mast cells from three randomly selected fields per ventricular site was counted. These data were expressed as the mean ± the standard deviation of mast cells/×40 field (0.05 mm^2).

Statistical analysis. The pathology severity scores were analyzed by the Kruskal-Wallis test. The two levels of the independent variable were concentrations of 0.0 mg/m^3 and 10 mg/m^3. Each response was analyzed separately. A differing response between the concentrations was considered significant if the p value was less than 0.05.

RESULTS

EPM Composition. The elemental and organic composition of EPM has been recently reported (Kodavanti *et al*., 2002b) and depicted in Figure 1. This EPM differed in many regards from other ROFAs used in previous studies (Dreher *et al.*, 1997; Kodavanti *et al.*, 1998). It contained smaller quantities of total bioavailable metals (wt/wt), and especially low levels of iron, vanadium, and nickel commonly found in such materials (Kodavanti *et al*, 1998). The unique composition of this EPM revealed the presence of bioavailable zinc, which was found to be similar to that of a select air pollution PM from urban locations (Adamson *et al*, 2000; Balachandran *et al.*, 2000; Dye *et al.*, 2001; Harrison and Yin, 2000). Zinc was the

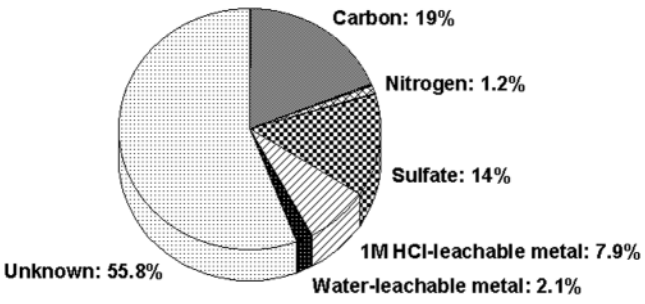

FIG. 1. EPM composition. Note that the major leachable metal fraction of this EPM contained primarily zinc with a small quantity of nickel, whereas the insoluble but acid-leachable fraction contained vanadium and iron.

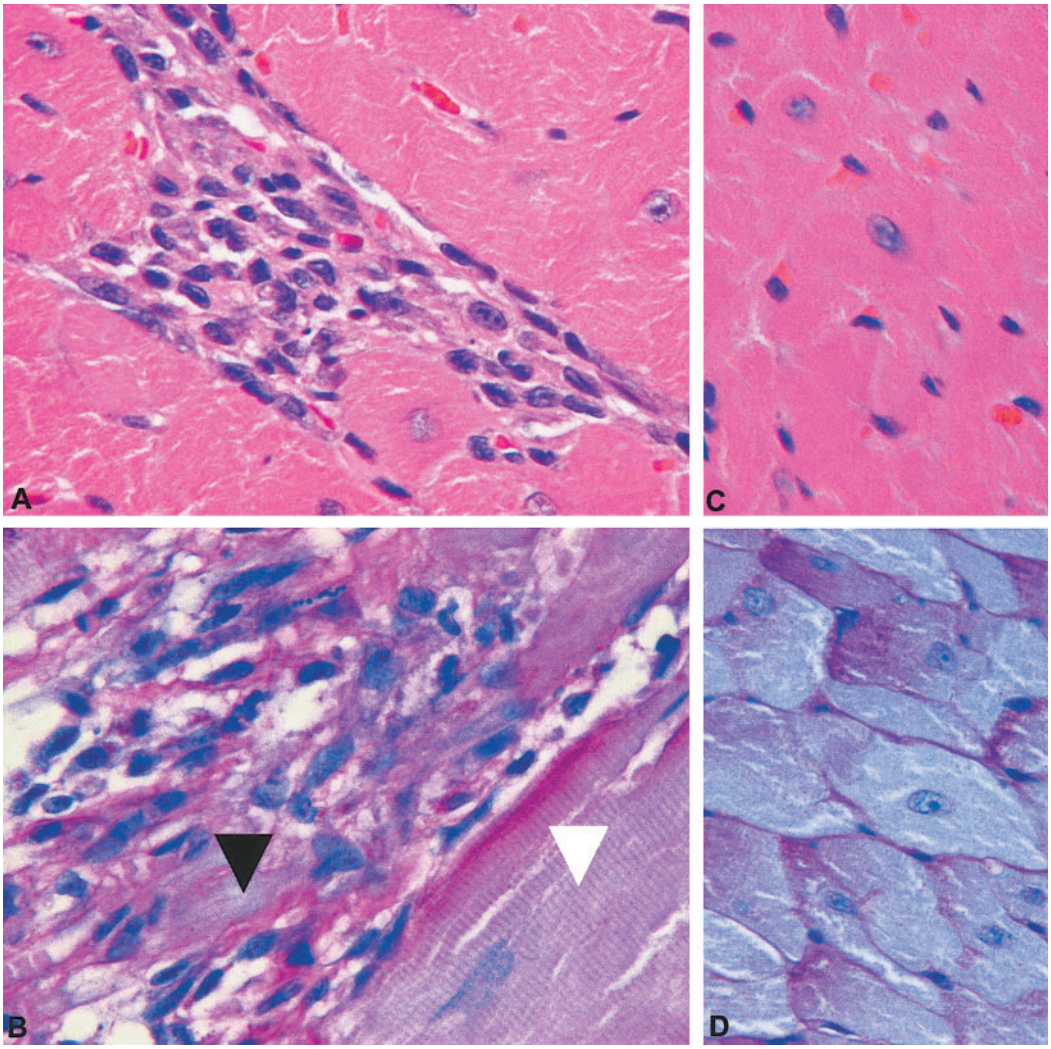

FIG. 2. (A) Myocardial inflammation (H&E, original magnification ×100), and (B) degeneration in WKY hearts (PAS, original magnification ×100) following inhalation of EPM. Notice the fragments of degenerating myocardial cells within the inflammatory cell focus (black arrow). A longitudinal section of normal striated myocardium is noted immediately adjacent to the degenerating muscle fragment (white arrow). Compare these staining features with (C), the normal H&E staining of myocardium (H&E, original magnification ×100) and (D) normal PAS staining of myocardial cells in cross-section (PAS, original magnification ×100) in an air-exposed control rat.

primary water-leachable metal (1.45% of the total mass) with small amounts of nickel (0.3%) and iron (0.25%). Water-leachable vanadium, copper, cobalt, and manganese were minimal (<0.004%) (Kodavanti *et al*., 2002b). As previously reported by our laboratory (Kodavanti *et al*., 2002b), iron and vanadium concentrations were detectable only in the 1 M HCl-leachable fraction of this EPM, however, these metals are less likely to be bioavailable because of their lack of solubility in water. The mass median aerodynamic diameter (MMAD) of this EPM was 1.2 μm and the geometric standard deviation (GSD, σ_g), 2.6. The endotoxin content was negligible and would likely not result in lung inflammation (0.26 EU/mg EPM). EPM contained 18.8% carbon, 1.2% nitrogen, and <0.5% hydrogen (wt/wt) (Kodavanti *et al*., 2002b). The remainder of the insoluble EPM mass was not analyzed (Fig. 1).

Histopathology of heart and lung lesions. EPM dose- and time-dependent pulmonary accumulation of particle-laden alveolar macrophages with no associated alveolar fibrosis or neutrophilic cell infiltrates was apparent in all three rat strains evaluated (data not shown). Furthermore, examination of cardiac tissues showed that the hearts from WKY rats exposed to episodic long-term EPM for 16 weeks developed randomly distributed foci of inflammation and fibrosis throughout the ventricles and the interventricular septum compared to the hearts from WKY rats exposed to air only (Fig. 2A compared to Fig. 2C, Table 2). The inflammatory infiltrate was composed of a mixed population of neutrophils, lymphocytes, and macrophages suggestive of an ongoing chronic-active inflammatory process. In direct contrast to the incidence of cardiac lesions observed in WKY rats, no such differences in the

TABLE 2
Qualitative Mean Pathology Scores in WKY Rats Exposed to Air or EPM

Pathology	Staining method	Severity scores (mean ± SD)	
		Air	EPM
Myocardial degeneration			
	H&E	0.00 ± 0.00	0.83 ± 0.37*
	PAS	0.00 ± 0.00	0.90 ± 0.49*
Inflammation	H&E	0.33 ± 0.75	2.80 ± 0.40*
Fibrosis	Masson's trichrome	0.00 ± 0.00	0.83 ± 0.69*
Degeneration and inflammation	All stains (mean)	0.08 ± 0.08	1.30 ± 0.96*
Apoptosis	TUNEL	0.83 ± 0.37	0.67 ± 0.47
Granulated mast cells	Toluidine blue	0.91 ± 1.80[a]	0.39 ± 0.71

Note. Severity score of 0–4 assigned to each animal indicates increasing pathology, based on published criteria (Herman *et al.*, 1996, 2000; Billingham, 1991). The mean severity was derived by adding the score of each animal in the air or EPM group and dividing that number by the number of animals.

[a]The score for mast cells represents the number of granulated cells present in a given ×40 field under the microscope.

*A significant increase in pathology at $p \leq 0.05$.

severity or character of cardiac lesions were observed between air- and EPM-exposed SD and SH rats. The randomly distributed inflammatory foci and fibrotic regions were readily apparent in control air-exposed SH rats as an underlying hypertension-associated complication, and therefore, the effect of EPM exposure was not distinguishable histologically from that of control animals. Such foci were less frequent in control SD and absent in control WKY rats. In addition, no EPM-related cardiac effects were observed in any rat strain exposed acutely to EPM, including rats exposed for 4 consecutive weeks (1 day/week), suggesting that long-term exposure was necessary for discernible lesions to develop in the sensitive strain of WKY rats. This is expected, based on the composition of EPM employed and the exposure scenario (10 mg/m^3, 6 h/day, 1 day/week for 16 weeks). Because no detectable differences in cardiac lesions were observed at 10 mg/m^3 exposure concentrations in 4-day and 4-week (1day/week) exposure groups, histologic evaluation of the hearts from rats exposed for 4 days at lower concentrations (5 or 2 mg/m^3) were not conducted.

Because EPM-associated inflammatory and degenerative changes were apparent in hearts from WKY rats following exposure to 10 mg/m^3 (6 h/day, 1 day/week for 16 weeks), further lesion characterization was carried out in EPM- and air-exposed WKY rats. Air- and EPM-exposed SD rats were also evaluated for comparative purposes, as these rats are commonly used in combustion and ambient source PM studies. In hearts of WKY rats exposed to EPM, inflammatory sites were frequently associated with myocardial degeneration, confirmed by PAS and PTAH staining (Fig. 2B, note the residual, degenerating myocyte indicated by a black arrow, and compare it to the surrounding healthy myocytes shown longitudinally in the adjacent area and in cross section in Fig. 2D).

Histological examination of EPM-exposed WKY rat hearts stained by the Movat's Pentachrome and Masson's Trichrome stains revealed increased collagen deposition/myocardial fibrosis within degenerative foci (Fig. 3A). Fibrotic changes were not apparent in control clean-air-exposed WKY rats. In addition, heart sections stained with toluidine blue to identify mast cell numbers and granulation demonstrated no increase in the number of mast cells among ventricular sites in EPM-exposed WKY or SD rats. Conversely, decreased numbers of granulated mast cells were noted in EPM-exposed WKY rats (Figs. 3C and 3D, Table 2), suggesting mast cell degranulation and its possible involvement in the process of myocardial degeneration/inflammation. Presence of mast cells within degenerating foci was also apparent in selected hematoxylin and eosin-stained heart tissue sections of WKY rats (Fig. 3B), but a granulation pattern was not clearly visible. To determine if myocardial degeneration was associated with increased apoptotic cardiomyocytes and/or inflammatory cells, TUNEL staining was carried out for the heart tissue sections from control and EPM-exposed WKY and SD rats. No increase in apoptosis was detected in cardiomyocytes from EPM-exposed WKY and SD rats over air-exposed WKY rats. Finally, no calcium deposition, as determined by von Kossa staining, was noted in hearts from either EPM- or air-exposed WKY rats.

DISCUSSION

This study shows cardiac injury in normotensive WKY rats following episodic, long-term inhalation of ambient-like combustion PM. While the EPM concentration was high relative to an ambient PM exposure, it was episodic (one 6-h exposure per week), allowing time for clearance and "recovery" between exposures. The EPM-induced myocardial injury, unique to the WKY rats, was characterized predominantly by degenerating cardiomyocytes, active inflammatory foci, and accumulation of collagen in the ventricles and interventricular septa. Injury was apparent at exposure concentrations that resulted in minimal pulmonary toxicity. The composition of this EPM resembled

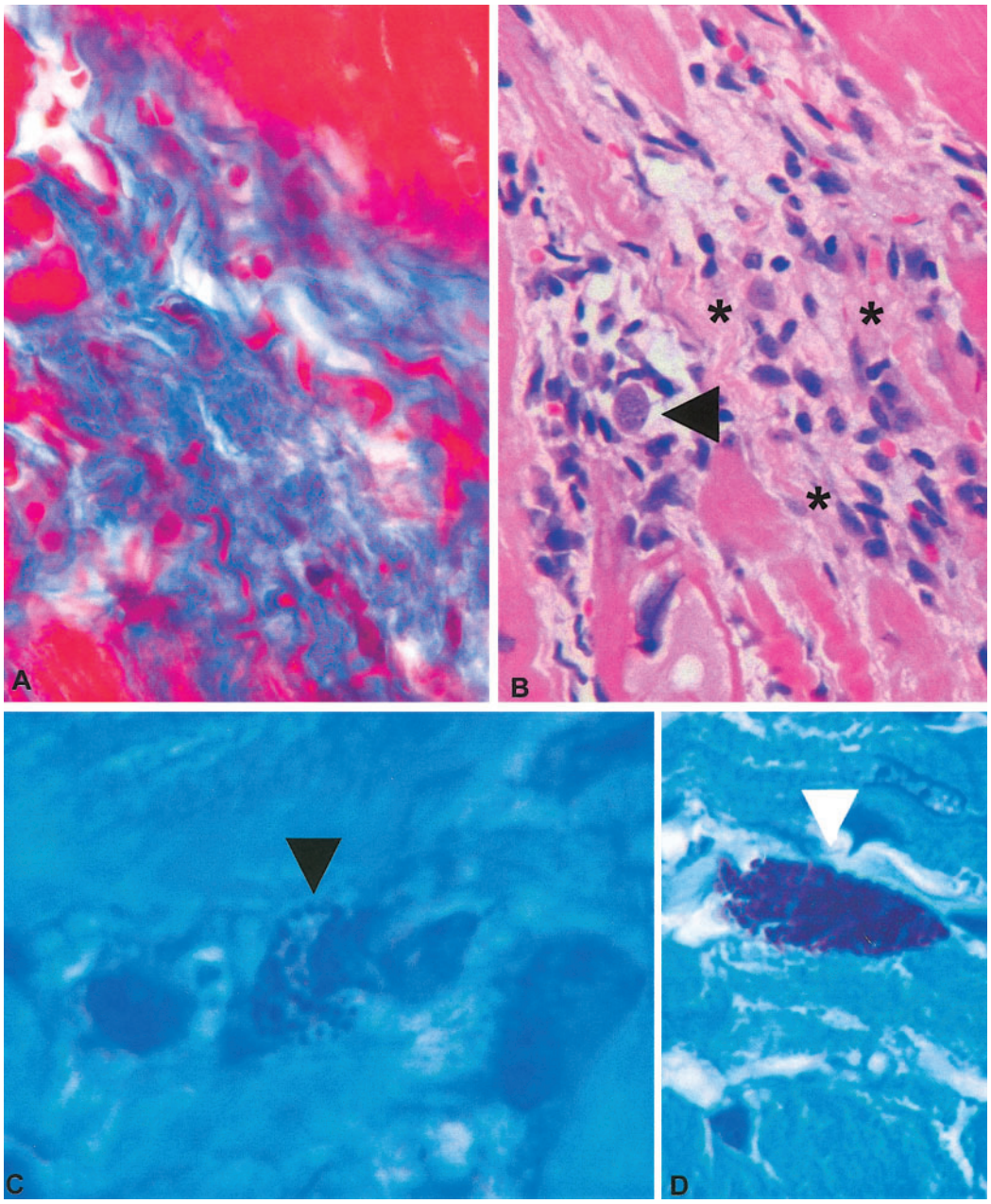

FIG. 3. (A) Myocardial fibrosis in hearts of WKY rats exposed to EPM as shown by staining of cardiac sections with Masson's trichrome (collagen, blue; myocardial fibers, red; MT, original magnification ×100). Compare this staining to (B), the same lesion stained by H&E. The arrow points to a mast cell. Asterisks indicate collagen deposition (original magnification ×100). (C) Representative example of partial mast cell degranulation (black arrow) near an inflammatory focus in a WKY rat exposed to EPM (toluidine blue, original magnification ×250). Notice that this mast cell contains few granules within the cytoplasm in comparison to (D) a fully granulated, normal mast cell (white arrow; stained with toluidine blue, original magnification ×250).

that of ambient PM in that the causative combustion-derived metal concentrations and predominance of bioavailable zinc were similar to those of selected, known ambient PM samples (Adamson *et al.*, 2000; Dye *et al.*, 2001; Harrison and Yin, 2000; Vincent *et al.*, 1997).

Among the three rat strains evaluated, only WKY rats demonstrated marked EPM-induced pathology. Whether this susceptibility is related to systemic or local myocardial changes is unclear. Strain predilection for development of cardiac disease has previously been described in WKY and SH rats. Normotensive, and therefore often used as negative controls to the SH, WKY rats develop spontaneous cardiomyopathy in the absence of alterations in blood pressure (Kuribayashi, 1987; Leenen and Yuan, 1998). Under the experimental conditions outlined in this study, EPM-induced inflammatory cardiac disease may have developed as a result of this predilection. In contrast, SD

and SH rats developed indistinguishable incidences of myocardial disease seen in a comparison of the two exposure groups. The SH rats, although they are desirable as models of human cardiomyopathy, develop spontaneous inflammatory myocardial lesions and myocardial degeneration. Because the basal inflammatory disease was expressed almost identically in the air- and EPM-exposed groups, a clear separation of the potential contribution of EPM exposure from the onset or severity of myocardial inflammation in these rats was likely made impossible. Thus, the usefulness of the SH rat for this type of study may be limited. The current findings suggest, therefore, that the WKY rat may be a highly sensitive and desirable model system with which to study incipient, PM-induced morphological lesions in the myocardium.

There has been limited research on the cardiotoxicity of inhaled pollutants. Most of the studies have focused on cardiophysiological changes (Campen *et al*., 2001; Watkinson *et al*., 1998; Wellenius *et al*., 2002). However, in the field of cardiotoxicology, histopathology and molecular techniques have proven highly effective in the study of cardiotoxic agents such as doxorubicin and monoxidil (Herman *et al*., 1996, 2000). Histopathology has been considered the most accurate method of detecting subtle cardiotoxicty, as analysis of clinical plasma markers is often insensitive to small changes. We applied a panel of histochemical stain techniques classically used in cardiotoxic studies to confirm the cardiac injury that occurred in WKY rats after long-term EPM exposure. The use of different staining techniques allowed us to more fully define the extent, severity, and character of the cardiac injury and the possible role of mast cell degranulation. Based on this finding, it could be postulated that the lesions in WKY rats developed over time as a result of long-term EPM exposure, since fibrosis, myocyte degeneration, and active inflammation were all apparent within the same foci.

It is noteworthy that cardiac lesions were detected only after 16-week episodic exposures to EPM of 10 mg/m^3, 6 h/day, 1 day/week, but not after 4-week exposures (1 day/week for 4 weeks or 4 consecutive days), suggesting that long-term exposure was necessary for detectable histological changes. Although histologic evidence of cardiac injury was not discerned at shorter durations or lower exposure concentrations, biochemical and molecular changes are likely. In addition, it is possible that high-concentration exposures might have resulted in injury that is detectable in a short time. Based on the present observation, several hypotheses can be proposed about the time course of injury, the dose response, the mechanism, and the possible causative constituent responsible for cardiotoxicty of inhaled EPM. These possibilities are currently being investigated by our laboratory to identify and evaluate potential mechanisms of EPM-induced cardiac injury in Wistar Kyoto rats.

The observation of cardiac lesions resulting from EPM exposure is significant, because PM air pollution has been consistently linked to poor cardiac health and increased cardiac-related deaths (reviewed in Dockery, 2001; Morris, 2001; Peters *et al.*, 2001; Schwartz, 2001; U.S. EPA, 1996). These and other recent epidemiological studies, together with overt public concern, were responsible for EPA's new, more stringent National Ambient Air Quality Standard for PM (2.5 micrometer, 60 microgram/m^3, 24-h average, and 15 microgram/m^3 annual average) imposed in 1997. This decision was based primarily on consistent epidemiological associations; however, providing causality has been difficult, because animal studies using ambient PM exposure conditions have often led to none or minimal increases in cardiopulmonary morbidity or mortality. Moreover, the use of higher-than-ambient concentrations of combustion and ambient-derived PM has been criticized as being irrelevant. Therefore, the search for detectable pulmonary and cardiovascular effects at lower levels of PM in healthy and susceptible animal species, especially cardiopulmonary-compromised models, has been continued (Campen *et al.*, 2001; Kodavanti *et al.*, 1999, 2000b, 2002a,b; Wellenius *et al.*, 2002). Susceptible animal models are particularly useful, because epidemiological studies show that PM exerts its greatest impact on this population (Peters *et al.*, 2001; Pope, 2001).

The EPM concentration used in this study (10 mg/m^3, 6 h/day, 1 day/week, 16 weeks), while actually high, may be "translated" over the entire period of 16 weeks to 357 microgram/m^3 continuous exposure. Based on the differences in the morphology and physiology of the respiratory tract of rats and humans, it is argued that for a given PM sample of respirable size, humans may require lower concentration than rats to achieve the same deposition dose (Vincent *et al.*, 1997). Thus, higher concentrations of PM may be needed to achieve an effect in the rat, whereas humans may experience the same effect at lower ambient PM levels. However, these assumptions have not been experimentally proven for all types of particles. Particle deposition can vary with size, composition, and physical characteristics. A protracted exposure of this degree is not likely to be encountered, but the episodic nature of the single weekly exposure allowed us to test the potential for direct cardiac injury/pathology in the context of an ambient-like PM to assess a range of susceptible animal models. With this scenario, we were able to detect significant focal myocardial degeneration, fibrosis, and inflammation in WKY rats. Thus, these findings are consistent with recent epidemiological associations of increases in cardiac morbidity following exposure to ambient PM in an industrialized urban environment (Dockery, 2001; Peters *et al.*, 2001). Furthermore, the WKY rat may prove to be a useful animal model with which to investigate the mechanism by which PM exposure results in cardiac lesions.

We have at present no direct mechanistic data. One of the possible explanations for these findings is that EPM exposure stimulated systemic endothelin release and microvascular thrombosis resulting in myocardial injury (Vincent *et al.*, 2001). No significant elevation of fibrinogen or changes in white blood cells were noted in rats exposed to EPM (Kodavanti *et al.*, 2002b), suggesting that these processes may not be

significant in WKY rat cardiac disease induced by EPM. Of greater interest might be the possible direct role of zinc on cardiac tissues. Among water-leachable bioavailable metals, zinc is the only predominant metal in this EPM. Bioavailable metals are easily translocated to the circulation and may impact the heart. Moreover, occupational exposure to zinc has been blamed for causation of myocardial effects associated with pulmonary and systemic inflammation, collectively referred to as "zinc-fume fever" (Cire, 1983). The possibility of zinc being one of the causative constituents of this EPM for cardiac injury is currently being investigated.

Heavy metals have previously been shown to induce *in vivo* vascular endothelial growth factor (VEGF) expression in the heart (Levy *et al.*, 1995). While zinc has been shown to activate MAP kinases via activation of epidermal growth factor (EGF) receptors and contributes to the inflammatory response in the lung (Huang *et al.*, 2002), leachable zinc may possibly reach the coronary arteries and similarly induce activation of EGF and VEGF receptors. This activation through tyrosine kinase phosphorylation and MAP kinase cell signaling may cause increased inflammatory cytokine gene expressions (Fournier *et al.*, 1999; Huang *et al.*, 2002) in the heart, which in turn stimulate a cascade of inflammatory events. The presence of active inflammatory foci within the myocardium of EPM-exposed WKY rats was most likely reflective of the activation of cytokine genes.

In summary, this investigation demonstrated for the first time that ambient-like EPM can cause histologically discernible myocardial injury, inflammation, and degeneration in WKY rats, following long-term episodic inhalation at a concentration that does not cause significant lung injury.

ACKNOWLEDGMENTS

We thank Donald Doerfler for statistical evaluation and JoAnne Johnson for editorial assistance. We also thank William P. Watkinson, James Samet, MaryJane Selgrade, and Linda Birnbaum of the U.S. Environmental Protection Agency for their critical review of the manuscript.

REFERENCES

Adamson, I. Y. R., Prieditis, H., Hedgecock, C., and Vincent, R. (2000). Zinc is the toxic factor in the lung response to an atmospheric particulate sample. *Toxicol. Appl. Pharmacol*. **166,** 111–119.

Balachandran, S., Meena, B. R., and Khillare, P. S. (2000). Particle size distribution and its elemental composition in the ambient air of Delhi. *Environ. Int*. **26,** 49–54.

Billingham, M. E. (1991). Role of endomyocardial biopsy in diagnosis and treatment of heart disease. In *Cardiovascular Pathology* (M. D. Silver, Jr., Ed.), pp. 1465–1486. Churchill Livingston, New York.

Campen, M. J., Nolan, J. P., Schladweiler, M. C. J., Kodavanti, U. P., Evansky, P. A., Costa, D. L., and Watkinson, W. P. (2001). Cardiovascular and thermoregulatory effects of inhaled PM-associated transition metals: A potential interaction between nickel and vanadium sulfate. *Toxicol. Sci.* **64,** 243–252.

Cire, L. (1983). Cardiac symptomatology in acute zinc oxide intoxication (zinc fume fever). *Herz Kreislauf* **15,** 358–360.

Dockery, D. W. (2001). Epidemiological evidence of cardiovascular effects of particulate air pollution. *Environ. Health Perspect*. **109,** 483–486.

Dreher, K. L., Jaskot, R. H., Lehmann, J. R., Richards, J. H., McGee, J. K., Ghio, A. J., and Costa, D. L. (1997). Soluble transition metals mediate residual oil fly ash-induced acute lung injury. *J. Toxicol. Environ. Health* **50,** 285–305.

Dye, J. A., Lehmann, J. R., McGee, J. K., Winsett, D. W., Ledbetter, A. D., Everitt, J. I., Ghio, A. J., and Costa, D. L. (2001). Acute pulmonary toxicity of particulate matter filter extracts in rats: Coherence with epidemiological studies in Utah Valley residents. *Environ. Health Perspect*. **109,** 395–403.

Fournier, E., Blaikie, P., Rosnet, O., Margolis, B., Birnbaum, D., and Borg, J. P. (1999). Role of tyrosine residues and protein interaction domains of SHC adaptor in VEGF receptor 3 signaling. *Oncogene* **18,** 507–514.

Frampton, M. W. (2001). Systemic and cardiovascular effects of airway injury and inflammation: Ultrafine particle exposure in humans. *Environ. Health Perspect*. **109,** 529–532.

Harrison, R. M., and Yin, J. (2000). Particulate matter in the atmosphere: Which particle properties are important for its effects on health? *Sci. Total Environ.* **249,** 85–101.

Herman, E. H., Zhang, J., Chadwick, D. P., and Ferrans, V. J. (1996). Age dependence of the cardiac lesions induced by monoxidil in the rat. *Toxicology* **110,** 71–83.

Herman, E. H., Zhang, J., Chadwick, D. P., and Ferrans, V. J. (2000). Comparison of the protective effects of amifostine and dexrazoxane against the toxicity of doxorubicin in spontaneously hypertensive rats. *Cancer Chemother. Pharmacol.* **45,** 329–334.

Huang, Y. C., Wu, W., Ghio, A. J., Carter, J. D., Silbajoris, R., Devlin, R. B., and Samet, J. M. (2002). Activation of EGF receptors mediates pulmonary vasoconstriction induced by residual oil fly ash. *Exp. Lung Res*. **28,** 19–38.

Kodavanti, U. P., Hauser, R., Christiani, D. C., Meng, Z. H., McGee, J., Ledbetter, A., Richards, J., and Costa, D. L. (1998). Pulmonary responses to oil fly ash particles in the rat differ by virtue of their specific soluble metals. *Toxicol. Sci*. **43,** 204–212.

Kodavanti, U. P., Jackson, M. C., Ledbetter, A. D., Richards, J. H., Gardner, S. Y., Watkinson, W. P., Campen, M. J., and Costa D. L. (1999). Lung injury from intratracheal and inhalation exposures to residual oil fly ash in a rat model of monocrotaline-induced pulmonary hypertension. *J. Toxicol. Environ. Health* **57,** 101–121.

Kodavanti, U. P., Jaskot, R. H., Costa, D. L., and Dreher, K. L. (1997). Pulmonary proinflammatory gene induction following acute exposure to residual oil fly ash: Roles of particle-associated metals. *Inh. Toxicol*. **9,** 679–701.

Kodavanti, U. P., Mebane, R., Ledbetter, A. D., Krantz, T., McGee, J., Jackson, M. C., Walsh, L., Hilliard, H., Chen, B. Y., Richards, J., and Costa, D. L. (2000a). Variable pulmonary responses from exposure to concentrated ambient air particles in a rat model of bronchitis. *Toxicol. Sci.* **54,** 441–451.

Kodavanti, U. P., Schladweiler, M. C. J., Ledbetter, A. D., Hauser, R., Christiani, D. C., McGee, J., Richards, J. H., and Costa, D. L. (2002a). Temporal association between lung injury and plasma fibrinogen increase in cardiovascular-compromised rats following particulate matter exposure. *J. Toxicol. Environ. Health,* **65,** 1545–1569.

Kodavanti, U. P., Schladweiler, M. C. J., Ledbetter, A. D., Hauser, R., Christiani, D. C., Samet, J. M., McGee, J., Richards, J. H., and Costa, D. L. (2002b). Pulmonary and systemic effects of zinc-containing emission particles in three rat strains: Multiple exposure scenarios. *Toxicol. Sci.* **70,** 73–85.

Kodavanti, U. P., Schladweiler, M. C. J., Ledbetter, A. D., Watkinson, W. P., Campen, M. J., Winsett, D. W., Richards, J. H., Crissman, K. M., Hatch, G. E., and Costa, D. L. (2000b). The spontaneously hypertensive rat as a model of human cardiovascular disease: Evidence of exacerbated cardio-

pulmonary injury and oxidative stress from inhaled emission particulate matter. *Toxicol. Appl. Pharmacol*. **164,** 250–263.

Kuribayashi, T. (1987). Spontaneously occurring hypertrophic cardiomyopathy in the rat: I. Pathologic features. *Jpn. Circ. J.* **51,** 573–588.

Ledbetter, A. D., Killough, P., and Hudson, G. F. (1998). A low-sample-consumption dry-particulate aerosol generator for use in nose-only inhalation exposures. *Inh. Toxicol.* **10,** 239–251.

Leenen, F. H., and Yuan, B. (1998). Dietary sodium-induced cardiac remodeling in spontaneously hypertensive rat versus Wistar Kyoto rat. *J. Hypertens.* **16,** 885–892.

Levy, A. P., Levy, N. S., Loscalzo, J., Calderone, A., Takahashi, N., Yeo, K. T., Koren, G., Colucci, W. S., and Goldberg, M. A. (1995). Regulation of vascular endothelial growth factor in cardiac myocytes. *Circ. Res.* **76,** 758–766.

Morris, R. D. (2001). Airborne particulates and hospital admissions for cardiovascular disease: A quantitative review of the evidence. *Environ. Health Perspect.* **109,** 495–500.

Moyer, C. F., Kodavanti, U. P., Hasman, J. K., Costa, D. L., and Nyska, A. (2002). Systemic vascular disease in male B6C3F1 mice exposed to particulate matter by inhalation: Studies conducted by the National Toxicology Program. *Toxicol. Pathol*. **30,** 1–7.

Peters, A., Dockery, D. W., Muller, J. E., and Mittleman, M. A. (2001). Increased particulate air pollution and the triggering of myocardial infarction. *Circulation* **103,** 2810–2815.

Pope, C. A., III. (2001). Epidemiology of fine particulate air pollution and human health: Biologic mechanisms and who's at risk? *Environ. Health Perspect.* **108,** 713–722.

Schladweiler, M. C. J., Ledbetter, A. D., Walsh, L., Lappi, E., Hilliard, H., Gilmour, M. I., Costa, D. L., Norwood, J., Crissman, K., Hatch, G. E., Richards, J. R., and Kodavanti, U. P. (2002). Correlated pulmonary and systemic effects in spontaneously hypertensive rats following acute exposures to concentrated ambient particles. *Toxicol. Sci.* **66**(Suppl.), 361 (Abstract).

Schwartz, J. (2001). Air pollution and blood markers of cardiovascular risk. *Environ. Health Perspect.* **109,** 405–409.

U.S. EPA (1996). *Air Quality Criteria for Particulate Matter*, Vols. I–III. Office of Research and Development, U. S. Environmental Protection Agency, Washington, DC.

Vincent, R., Bjarnason, S. G., Adamson, I. Y. R., Hedgecock, C., Kumarathasan, P., Guenette, J., Potvin, M., Goegan, P., and Bouthillier, L. (1997). Acute pulmonary toxicity of urban particulate matter and ozone. *Am. J. Pathol.* **151,** 1563–1570.

Vincent, R., Kumarathasan, P., Goegan, P., Bjarnason, S. G., Guenette, J., Berube, D., Adamson, I. Y. R., Desjardins, S., Burnett, R. T., Miller, F. J., and Battistini, B. (2001). Inhalation toxicology of urban ambient particulate matter: Acute cardiovascular effects in rats. *HEI Research Report* **104,** 1–62.

Watkinson, W. P., Campen, M. J., and Costa, D. L. (1998). Cardiac arrhythmia induction after exposure to residual oil fly ash particles in a rat model of pulmonary hypertension. *Toxicol. Sci*. **41,** 209–216.

Wellenius, G. A., Saldiva, P. H., Batalha, J. R., Krishna Murthy, G. G., Coull, B. A., Verrier, R. L., and Godleski, J. J. 2002. Electrocardiographic changes during exposure to residual oil fly ash (ROFA) particles in a rat model of myocardial infarction. *Toxicol. Sci*. **66,** 327–335.