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Differential Pulmonary Retention of Diesel Exhaust Particles in Wistar Kyoto and Spontaneously Hypertensive Rats

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Spontaneously hypertensive (SH) and normotensive Wistar Kyoto (WKY) rats have been used for understanding the mechanisms of variations in susceptibility to airborne pollutants. We examined the lung burden of diesel exhaust particles (DEP) following inhalation of diesel engine exhaust (DEE) in both strains. The kinetics of clearance was also examined after single intratracheal (IT) instillation of DEP. Lungs were analyzed for DEP elemental carbon (EC) after exposure to DEE (0, 500, or 2000 μg/m^3 4 h/day, 5 days/week × 4 weeks). SH rats had 16% less DEP-EC at 500 and 32% less at 2000 μg/m^3 in the lungs, despite having 50% higher than the average minute volume. No strain-related differences were noted in number of alveolar macrophages or their average DEP load as evident from examining cells in bronchoalveolar lavage fluid (BALF). The kinetics of DEP clearance from lungs of male WKY and SH rats was studied following a single instillation at 0.0 or 8.33 mg/kg of DEP standard reference material (SRM 2975) from the National Institute of Standards Technology. SH rats cleared 60% DEP over 112 days while minimal clearance occurred from the lungs of WKY. The pattern of DEP-induced inflammatory response assessed by BALF analysis was similar in both strains, although the overall protein leak was slightly greater in SH rats. A time-dependent accumulation of DEP occurred in tracheal lymph nodes of both strains (SH > WKY). Thus, SH rats may clear DEP more efficiently from their lungs than normotensive WKY rats, with a small contribution of more effective lymphatic drainage.

Key Words: **diesel exhaust particles; lung clearance; Wistar Kyoto rats; spontaneously hypertensive rats; hypertension; lymph nodes; lymphatic drainage.**

Exposure to different types of airborne particulate matter (PM) is associated with exacerbation of a variety of pulmonary diseases like asthma, bronchitis, obstructive pulmonary disease, bacterial infections, and lung cancer (Atkinson *et al*., 1999; Gilmour *et al.* 2007; Ma and Ma, 2002; Pope *et al*., 2002; Saxena *et al*., 2003). Furthermore, the pulmonary and cardiovascular effects of PM are exacerbated in people with underlying cardiovascular conditions such as hypertension (Pope *et al*., 2004; Sunyer *et al*., 2000; O'Neill *et al*., 2005). In order to study the mechanisms responsible for greater susceptibility to the effects of airborne PM in individuals with underlying cardiopulmonary diseases, animal models of human hypertension are used (Kodavanti *et al*., 1998; 2000). Increased deposition of inhaled PM in the lung or abnormal clearance might partly explain why potential health effects might be exacerbated in individuals with chronic diseases (Brown *et al*., 2002; Phalen *et al*., 2008). PM deposition pattern and retention differences in animal models of lung diseases have been studied (Sweeney *et al*., 1995; Tryka *et al*., 1985; Valberg *et al*., 1985; Zeltner *et al*., 1991), however, the pulmonary deposition patterns of inhaled diesel exhaust particles (DEP) have not been well studied in individuals with underlying cardiovascular disease or animal models.

Normotensive parent Wistar Kyoto (WKY) strain and spontaneously hypertensive (SH) rats have been the most extensively used to study altered biological responses associated with hypertension and underlying cardiovascular disease. For the past several years these rats have also been used as a model of human cardiovascular disease to examine mechanisms of exacerbation of cardiopulmonary health effects of PM (Gottipolu *et al*., 2009; Hwang *et al*., 2008; Kodavanti *et al*., 2000; Reed *et al*., 2008; Wallenborn *et al*., 2007; Yu *et al*., 2008). In general, the pulmonary health effects of oil combustion PM, ambient PM, and tobacco smoke have been exacerbated in SH relative to WKY rats. WKY rats did not show acute cardiac physiological effects of combustion oil fly ash PM, whereas nonconducted p-wave arrhythmias were increased in SH rats (Farraj *et al*., 2009). However, we have recently shown that although the pulmonary effects of diesel

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engine exhaust (DEE) were similar in WKY and SH rats, effects on cardiac global gene expression were manifested only in WKY rats (Gottipolu *et al*., 2009). It is not clear as to how this lack of cardiac gene expression change in SH may ultimately affect already hypertensive cardiac phenotype. There appears to be PM-specific susceptibility differences between SH and WKY rats. In order to better understand the mechanisms responsible for differential health effects of DEE and combustion PM in SH, it is important to correlate the extent of biological effects of exposure to PM with the burden of particle mass in the lung. It has not been yet demonstrated if the lungs of SH retain more particles following PM delivery or if impaired clearance may be responsible for their enhanced susceptibility relative to WKY rats.

DEPs contribute to near-road PM mass, and have been recently associated with increased pulmonary and cardiovascular impairments in near-road residents (Brunekreef *et al*., 2009). Cardiovascular health effects have also been noted in several experimental studies employing atherosclerosis-prone mice (Lund *et al*., 2009) and healthy rats (Gottipolu *et al*., 2009) exposed to DEE. Information on the contribution of DEP retention and clearance on differential susceptibilities between WKY and SH rats will provide further insights into the host factors that may contribute to variations in susceptibility to cardiovascular and pulmonary health effects of DEP.

We recently developed a new method to quantitatively estimate the uptake of DEP by cultured lung epithelial cells and AMs (Saxena *et al*., 2008). In the present study, we utilized this method to estimate the accumulation of DEP from the lungs of WKY and SH rats exposed to whole DEE (gas plus particulate components), and to track the clearance of bulk collected DEP from lungs after IT instillation. Our results indicate that the SH rats accumulated significantly lower amounts of DEP in their lungs and cleared the particles more efficiently than WKY. We also found that the accumulation of DEP in tracheal lymph nodes was relatively faster in SH rats, suggesting that a better lymphatic drainage could be a small contributing factor to a faster clearance from lungs of SH rats.

MATERIALS AND METHODS

Animals

Healthy male WKY and SH rats, 12–14 weeks old, were purchased from Charles River Laboratories, Inc. (Raleigh, NC). Rats were acclimatized for at least 1 week in an Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility (21 ± 1°C, 50 ± 5% relative humidity, 12-h light-dark cycle) prior to the experimental period. Use of animals in this study was preapproved by the National Health and Environmental Effects Research Laboratory, U.S. EPA Animal Care and Use Committee. For inhalation study, animals were single housed in ventilated cages and for IT instillation study animals were housed in pairs in polycarbonate cages with beta chips bedding. All animals received standard Purina rat chow (Brentwood, MO) and water *ad libitum*.

DEE Generation and Inhalation Exposure

DEE generated by operating a 30-kW (40 hp) four-cylinder indirect injection Deutz diesel engine (BF4M1008) and readily available road-taxed diesel fuel has been recently described (Gottipolu *et al*., 2009). A small portion of the whole DEE was routed to a dilution system and passed through two-stage air dilution and was then routed to Hinner exposure chambers. Three Hinner exposure chambers were used (0, 500, and 2000 μg/m^3) in parallel. Chamber temperature (22°C) and relative humidity (55–60%) were monitored continuously. Integrated 4-h filter samples (14.1 l/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. Organic/elemental carbon (OC/EC) ratio of the airborne particles remained 3.0 ± 0.03 as periodically determined using a thermal/optical carbon analyzer (Sunset Laboratory, Inc., model 107, Tigard, OR). Particle geometric mass median aerodynamic diameter in inhalation study was measured to be below 225 nm at both concentrations (Gottipolu *et al*., 2009). It should be noted that the inhalation exposure to DEE contained gas as well as particulate components (Gottipolu *et al*., 2009), unlike the intratrachacheal instillation study where bulk DEP was used. The concentrations of carbon monoxide, sulfur dioxide, nitrogen oxide, and nitrogen dioxide were monitored along with PM mass and number concentration during inhalation exposure and the data are published recently (Gottipolu *et al*., 2009). DEE exposures were conducted for 4 h/day, 5 days/week for 4 consecutive weeks (Gottipolu *et al*., 2009). Animals were periodically monitored for changes in breathing parameters using a barometric whole body plethysmography system (Buxco Electronics, Inc., Sharon, CT) to obtain data on pulmonary ventilation as described previously (Gottipolu *et al*., 2009; Kodavanti *et al*., 2005).

IT Instillation of DEP

The DEP sample used for IT instillation contained 60% mass concentration of EC (Singh *et al*., 2004), making it an appropriate sample for quantification by tissue EC content. DEP sample (Standard Reference Material 2975, average particle diameter 120 nm, surface area 108 m^2/g from the National Institute of Standards and Technology, Gaithersburg, MD) was mixed with saline at 8.33 mg/ml concentration, vortex mixed, and probe sonicated for 10 s three times. The saline DEP suspensions were carefully mixed prior to each instillation. Normal saline (n = 5/strain determined at 2-day postinstillation) or DEP preparation was IT instilled (n = 5/group/time point) at a volume of 1 ml/kg as described previously (Kodavanti *et al*., 2002). Because no EC was expected to be present in tissues of control rats only one (group of) rats per strain was analyzed.

Necropsy and Bronchoalveolar Lavage Fluid Analysis

Inhalation study. One day following final exposure, rats were weighed and anesthetized with an overdose of sodium pentobarbital (50–100 mg/kg, ip). Bronchoalveolar lavage was performed and cytospin slides were prepared (Gottipolu *et al*., 2009). In order to gain a possible insight into a relative difference in alveolar macrophage (AM) phagocytosis ability, AM in cell differential slides were visually estimated and categorized as having a low, medium, or high DEP load. At least 100 macrophages on each cell differential slides were scored qualitatively. From this study, the right caudal lung lobes were isolated, frozen in liquid nitrogen, and stored at −80°C until analysis (n = 5/group). The procedure for bronchoalveolar lavage, cell differential slide preparation, and the data on inflammatory cells are recently published (Gottipolu *et al*., 2009).

IT studies. At 4-h, 2-day, 7-day, 14-day, 28-day, 56-day, and 112-day postinstillation, rats were anesthetized as indicated before and exsanguinated via abdominal aorta to remove blood. The right caudal lung lobe and tracheal lymph nodes were removed and frozen for analysis of EC. The trachea was cannulated and the left lung was lavaged using a volume of $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (pH 7.4) equal to 35 ml/kg body weight (left lung representing 40% of the total lung weight). Three in-and-out washes were performed with the same buffer aliquot. Aliquots of bronchoalveolar lavage fluid (BALF) were centrifuged (Shandon 3 Cytospin, Shandon, Pittsburgh, PA) to prepare cell differential slides. Slides were dried at room temperature and stained with Leukostat (Fisher Scientific Co., Pittsburgh, PA) for differential

cell counting. The number of AM and neutrophils was counted under light microscopy. The remaining BALF was centrifuged at 1500 × g to remove cells, and the supernatant fluid was analyzed for markers of lung injury. Aliquots of BALF were used for the estimations of total protein, and activities of lactate dehydrogenase (LDH), n-acetylglucosaminidase (NAG), and γ-glutamyl transferase (GGT) as described previously (Gottipolu *et al*., 2009).

Tissue EC Analysis to Determine DEP Burden

A technique developed by us (Saxena *et al*., 2008) for estimating EC in cells as a marker of DEP was used with some modifications for tissue analysis. Tissues were weighed and homogenized in 5 ml of Solvable (a tissue dissolving reagent from Perkin Elmer, Waltham, MA) using a Polytron homogenizer. Homogenate was incubated for 24 h at 60°C to allow complete dissolution of the tissue, and centrifuged at 80,000 × g for 30 min in a Beckman ultracentrifuge to pellet the insoluble DEP. The pellet was resuspended in 1 ml of Solvable and incubated for 24 h at 60°C to further dissolve any residual tissue component. Tracheal lymph nodes required no homogenization and could directly be dissolved in 1 ml of Solvable at 60°C. The DEP pellet obtained by ultracentrifugation was washed thrice with normal saline, suspended in 50 μl of normal saline, and deposited on a quartz filter punch (1 cm × 1.5 cm). Filters were dried overnight under vacuum at 50°C and analyzed using a carbon analyzer. The OC and EC in the DEP pellets were measured using thermal-optical-transmittance (Sunset Laboratories; Forest Grove, OR) and the National Institute for Occupational Safety and Health Method 5040 for estimating carbon in diesel PM (NIOSH Method 5040, 1998). Details of the method have been published elsewhere (Birch and Cary, 1996; Chow *et al*., 1993). Briefly, vacuum-dried DEP samples on quartz filters were heated stepwise to ~850°C in a temperature-programmable oven under helium (He phase). Organic matter volatilized under these conditions was oxidized to CO_2 in the presence of a MnO_2 catalyst, and then reduced to methane using a Ni metal hydride catalyst. The methane generated was detected and quantified by a flame ionization detector (FID), and the OC in the sample was computed from the quantity of methane. To analyze EC contents, after a brief cooling respite, the samples were again heated stepwise to ~900°C in a helium-oxygen environment whereby the nonvolatile EC was oxidized to CO_2. This CO_2 was then converted to methane and the EC quantified as before (Saxena *et al*., 2008).

Statistical Analysis

EC content was expressed per given tissue mass or total burden. For the IT study, all controls were sampled at 48-h postinstillation. The data were analyzed by a two-way ANOVA with exposure time and strain as factors, using commercial software (SigmaStat Software, Inc., version 3.5; Point Richmond, CA). Pair-wise group comparisons were made using the Holm-Sidak method (SigmaStat Software, Inc.). The data for breathing parameters and DEP content in the lymph nodes were analyzed using unpaired Student's *t*-test. Significance was ascribed to test results at $p < 0.05$. For statistical evaluation, BALF injury and inflammation markers data for all saline-exposed rats were treated as 0-h time point and were compared with the data obtained for each time point thereafter for DEP-exposed rats.

RESULTS

Rat Strain Differences in Breathing Parameters

Detailed analysis of breathing parameters and influence of DEE inhalation in this study has been recently published (Gottipolu *et al*., 2009). These data demonstrated no DEE effects on breathing parameters during 4 weeks inhalation in either rat strains. Summary of these published data on tidal and minute volume is given in Figure 1. Although tidal volumes were not different at baseline between WKY and SH rats

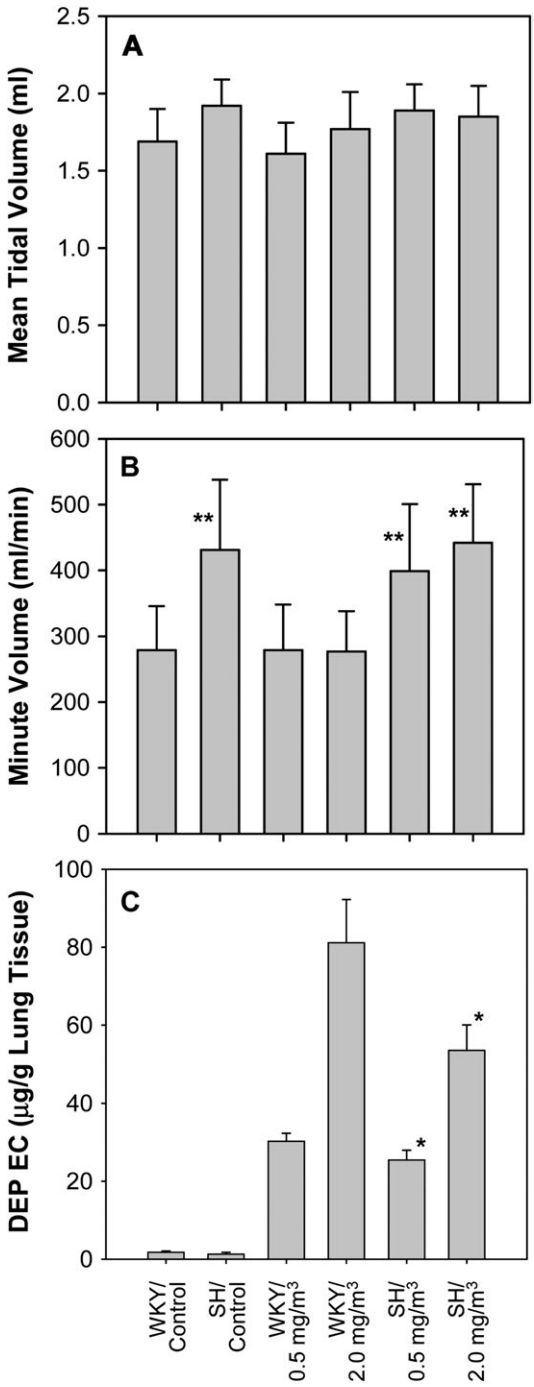

FIG. 1. Lung tidal volume (A), minute volume (B), and accumulation of DEP (C) in the lungs of WKY and SH rats exposed to whole DEE. WKY and SH rats were exposed for 4 weeks to DEE (4 h/day, 5 days/week) at 0.0, 0.5 (500 μg), or 2.0 mg (2000 μg)/m^3. (A) The tidal volume of respiration in air-control and DEE-exposed rats. Measurements were made between 7.30 to 8.30 A.M. by whole body plethysmography using the Buxco system. Panel B shows the lung minute volumes in control and DEE-exposed rats. Values represent the mean ± SD of six rats made once before the DEE exposure and on every alternate day after the initiation of DEE exposure. (C) The average accumulation of EC (DEP-EC) per gram of lung tissue. Values represent means ± SD of five rats in each group. *$p < 0.05$, **$p < 0.001$ for comparison between WKY and SH rats.

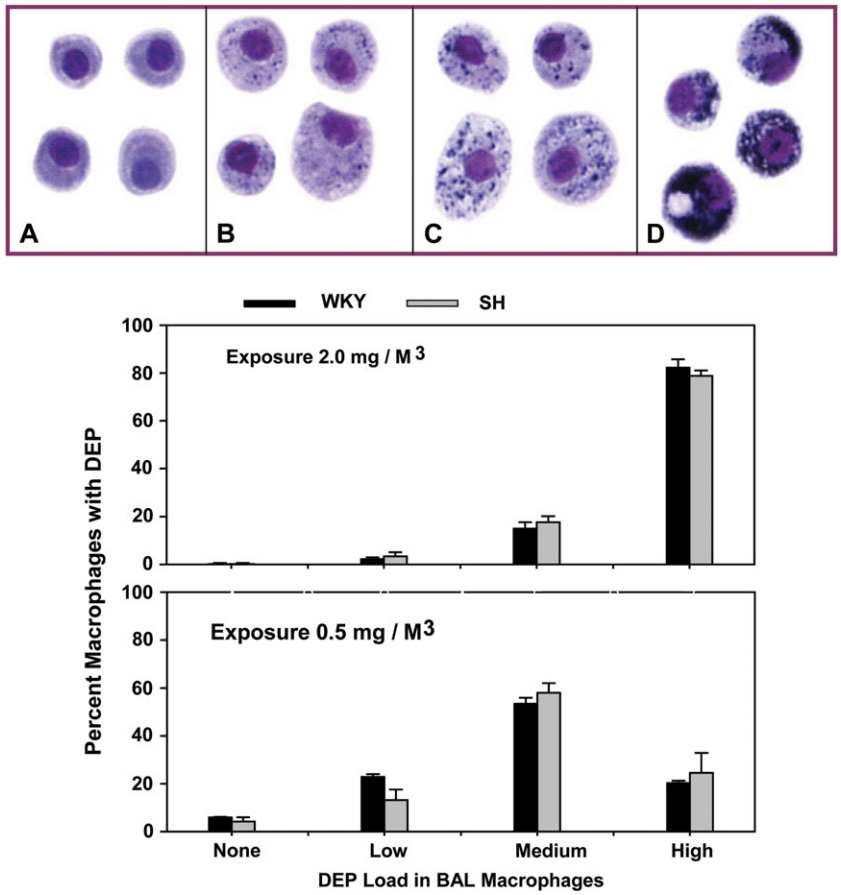

FIG. 2. Uptake of DEP by the AM of WKY and SH rats as determined from BALF analysis following 4-week inhalation exposure to DEE. Cytospin preparations of cells in BALF were stained and examined for DEP in AM. Top panel shows representative AM containing no (A), low (B), medium (C), and high (D) amounts of intracellular DEP. In each of the cytospin slide; at least 100 AM were scored for intercellular DEP. Results indicate the percentages of AM containing different levels of intracellular DEP from WKY (black-filled bars) and SH (gray-filled bars) after exposure to high 2.0 mg/m^3 (2000 μg/m^3, upper histogram) or low 0.5 mg/m^3 (500 μg/m^3, lower histogram) levels of DEE. Each value represents mean ± SD of five rats.

(Fig. 1A), because of the higher breathing frequency in SH rats (Gottipolu *et al.*, 2009), the minute volume was significantly greater when compared with WKY rats (Fig. 1B). This strain difference indicated that the SH rats inhaled more PM-containing air than WKY rats. In order to see if greater respiratory intake in SH rats resulted in higher deposition/retention of DEP in lungs, a quantitative estimation of accumulation of EC was carried out.

Accumulation of EC in Lungs of WKY and SH Rats Exposed to DEE via Inhalation

The results in Figure 1C show that the background level of EC (< 2 μg/g lung tissue) could be detected in both strains exposed to air and that exposure to DEE resulted in significant accumulation of EC in lungs that was dose dependent. The highest accumulation of 81.21 ± 11.05 μg EC/g lung tissue was observed in the WKY rats exposed to 2000 μg/m^3 of DEE for 4 weeks, and DEP-EC levels in SH were 16% ($p < 0.05$) and 34% ($p < 0.01$) lower than the WKY rats at 500 and 2000 μg/m^3 exposure levels, respectively. This could suggest that even though the exposure to DEE was likely greater for SH rats due to greater respiratory minute volume, the actual lung burden of DEP over this time period was significantly lower.

Estimation of AM DEP Load (Inhalation Study)

Total cell numbers in BALF did not change significantly following DEE exposure although trends of increase were noted which accounted for by increases in neutrophilic inflammation in both strains (Gottipolu *et al.*, 2009). The absolute number of AM in BALF of DEE-exposed WKY and SH rats was not significantly different from air-exposed rats (Gottipolu *et al.*, 2009). Engulfment of DEP by AM was assessed by grading the AM for the presence of intercellular DEP (Fig. 2, upper panel). Results in Figure 2 (lower panel) show that DEP load was significantly greater in AM of both WKY and SH rats exposed to higher dose of DEE. Although the DEP burden of AM in both strains was concentration dependent, there appeared to be no apparent strain difference in

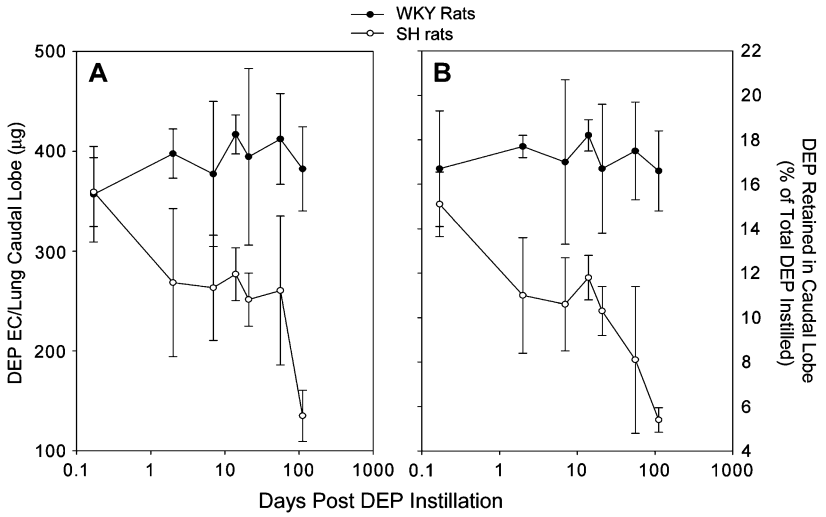

FIG. 3. Kinetics of clearance of DEP from the lungs of WKY and SH rats following IT instillation of DEP. DEP preparations were intratracheally instilled in WKY and SH rats (0.0 or 8.33 mg/kg body weight). At various time points after DEP instillation, caudal lung lobes were dissolved in Solvable as described in methods. DEP that remained insoluble were isolated by centrifugation and analyzed for the amounts of EC (DEP-EC) as a measure of DEP. Left panel (A) shows the results as amounts of DEP per caudal lung lobe and the right panel (B) shows the same data as the amounts of DEP retained as percentage of the DEP instilled in each rat. All values represent mean ± SD of data from five rats except for controls for which five animals in each strain were analyzed for DEP for only one time point of 2 days as the values of this analysis was expected near zero.

the amount of phagocytosed DEP suggesting that the ability of AM to phagocytize DEP may not be responsible for the observed differences in pulmonary DEP burden.

Lung Retention of Intratracheally Instilled DEP in WKY and SH Rats

The lower accumulation of DEP in lungs of SH rats relative to WKY following 4-week inhalation exposure could result from less deposition and/or more efficient clearance in SH lungs. In order to examine this possibility, a standard DEP (with 60% mass being EC, Singh *et al*., 2004) preparation (8.33 mg/kg body weight) was instilled intratracheally in age-matched WKY and SH rats having body weights of 272.8 ± 19.5 g and 292.9 ± 15.6 g (mean ± SD), respectively. The results in Figure 3A show that 4 h after the instillation of DEP the amounts of EC present per caudal right lung lobe were comparable for WKY (357.0 ± 47.9 µg) and SH (359.3 ± 34.6 µg) rats. These values fell with time in the SH rats and the level of EC in caudal lobes was 62% lower (135.1 ± 25.7 µg) at 112 days after the instillation of DEP. In the WKY rats, however, no time-dependent decline in lung EC levels was observed.

Because the body weights of the rats used in the DEP lung burden study differed to some extent (range 241–318 g), the absolute amount of instilled DEP varied. To avoid the variations in results arising due to variation in amount of DEP instilled in individual rats, the data were normalized as % actual DEP instilled. The results in Figure 3B show that even when the residual DEP was computed as percentage of the actual amounts of DEP instilled, the kinetics of DEP lung burden remained essentially similar to what was noted in Figure 3A. An analysis of variance indicated that the time-dependent decline in the lung load of EC in SH rats was highly significant as compared with the more or less static levels of EC in WKY lungs ($p < 0.001$).

Figure 4 shows time kinetics data on the analysis of BALF for several parameters of lung injury including total protein, and activities of LDH, NAG, and GGT. The results show that following DEP instillation, the peak of injury response occurred by day 2 or 7 in both strains of rats and the indicators of injury tended to return to normal thereafter by 21 days. Consistent with our previous studies (Kodavanti *et al*., 2002; Wallenborn *et al*., 2007), protein levels in BALF were higher at baseline in SH than in WKY, and the effect caused by DEP instillation was slightly greater although not significant in SH, despite their lower retention of DEP in the lung. DEP instillation resulted in marked increase in total cells in BALF which peaked by day 7 and then returned to control by day 21 (Fig. 5). This increase in total cells can be fully accounted for by increases in BALF neutrophils (Fig. 5). No increase in lavageable AM was noted at any time point in either strain. The degree of neutrophilic inflammation, as determined from analysis of BALF, in the lung was similar between SH and WKY rats following DEP instillation (Fig. 5).

DEP Accumulation in Bronchus-Associated Lymph Nodes of Rats

The faster clearance of DEP through the lymphatic drainage of lungs of SH than WKY rats could be a contributory factor in low pulmonary retention. Because the lymphatic drainage of the lung occurs via tracheobronchial lymph nodes

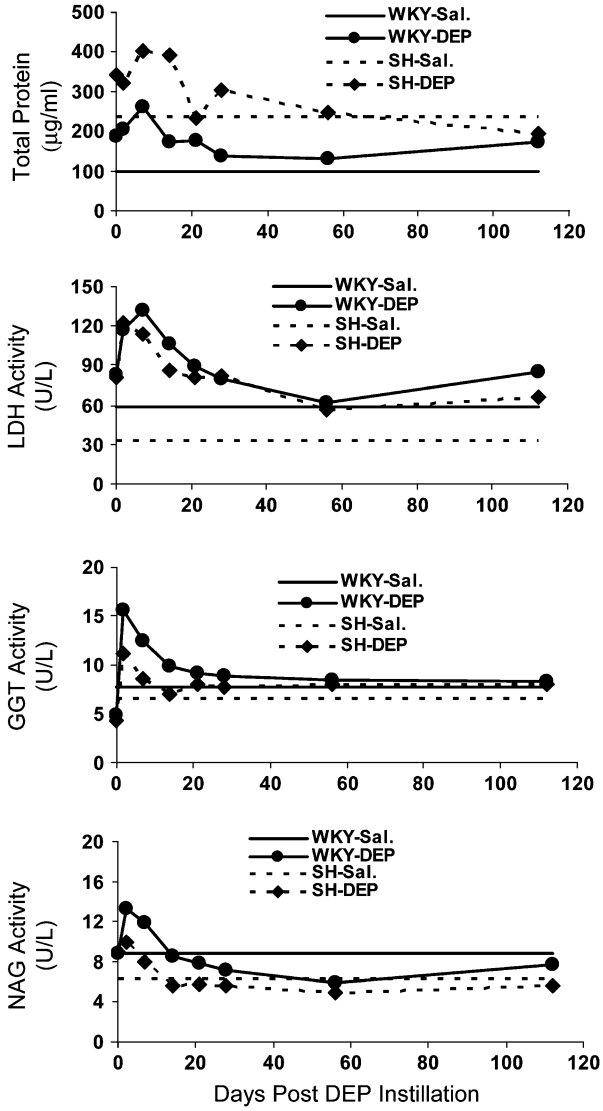

FIG. 4. Kinetics of pulmonary injury biomarkers determined by analysis of BALF following a single IT DEP instillation in WKY and SH rats. Saline or DEP preparation was intratracheally instilled in WKY and SH rats (8.33 mg/kg body weight). At various time points after DEP instillation, total protein, LDH activity, GGT activity, and NAG activity were assessed in BALF. All values represent mean ± SD of data from BALF preparations from five rats. Note that the control values determined at 2-day postinstillation in each strain are indicated by corresponding lines. Strain differences are significant ($p \leq 0.05$) for all parameters (protein, LDG, GGT, NAG) when combining data for all time points, however, not at individual group level or time. There are significant exposure effects when data for both strains are combined: protein, $p \leq 0.05$ at 1 week; LDH, $p \leq 0.05$ at 4 h, 2 days, 7 days, 2 weeks, 3 weeks, 4 weeks, and 16 weeks; GGT, $p \leq 0.05$ at 4 h, 2 days, 1 week; NAG, $p \leq 0.05$ at 2 days and 8 weeks.

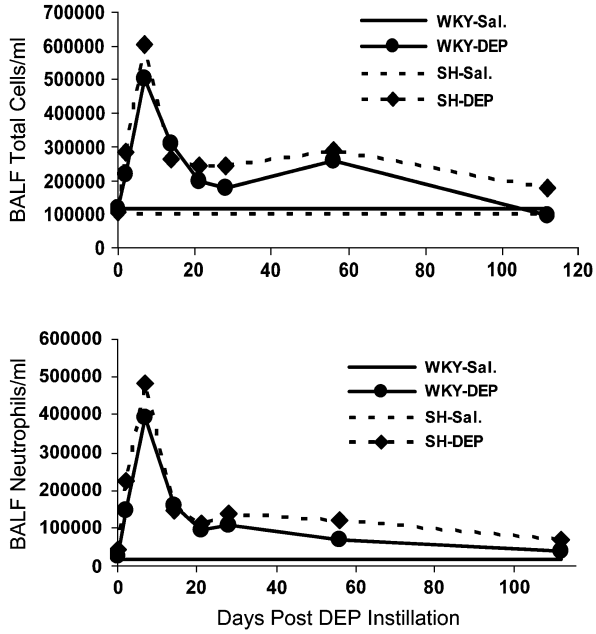

FIG. 5. Kinetics of total cells and neutrophil influx determined by analysis of BALF following a single IT DEP instillation in WKY and SH rats. DEP preparation was intratracheally instilled in WKY and SH rats (0.0 or 8.33 mg/kg body weight). At various time points after DEP instillation, inflammatory cells were assessed in BALF. All values represent mean ± SD ($n = 5$). Note that the control values determined at 2 days in each strain are indicated by corresponding lines. As evident in the figure the changes in total cell numbers are accounted by influx of neutrophils. No changes were noted in number of BALF AM at any time in either strain. Strain differences are significant ($p \leq 0.05$) for both total cells and neutrophils when combining data for all time points, however, not at individual group level or time. There are significant exposure effects ($p \leq 0.05$) for 2-day to 8-week times, regardless of strain.

(bronchus-associated), the kinetics of accumulation of DEP in these lymph nodes was examined. Results in Figure 6A show that the weights of tracheobronchial lymph nodes were significantly greater in SH rats as compared with WKY rats at 2, 21, and 112 days post-DEP instillation. Whole lymph nodes were dissolved in Solvable and amounts of insoluble DEP residue were quantitatively assessed for EC content. Results in Figure 6B show that there was a progressive accumulation in lymph nodes of both strains of rats exposed to DEP. At the earlier time points (2 and 21 days) the DEP accumulation in SH lymph nodes was more than double that of the accumulation in WKY lymph nodes ($p < 0.05$). At the 112-day time point, the greatest accumulation of DEP was seen in lymph nodes of both WKY and SH rats, but no interstrain difference was noted at this time point. These results suggest that the clearance of DEP through lymphatic drainage may be more efficient in SH rats, at least until the accumulation of DEP reaches a certain degree of saturation.

DISCUSSION

SH rats have been extensively used as a model of human cardiovascular disease to study the mechanisms by which air pollution increases morbidity and mortality (Hwang *et al.*, 2008; Kodavanti *et al.*, 2000, 2002; Reed *et al.*, 2008;

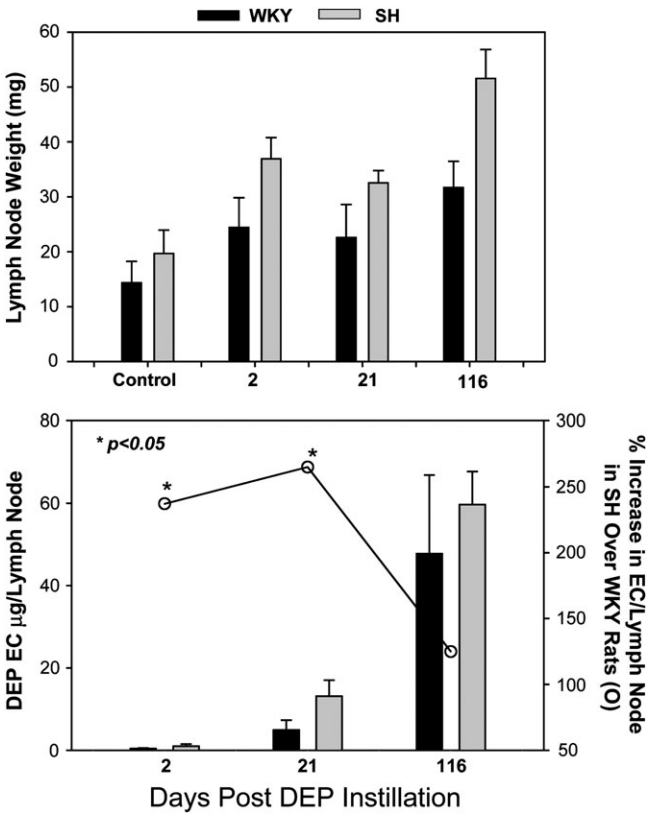

FIG. 6. Time-dependent accumulation of DEP-EC in the tracheobronchial lymph nodes of WKY and SH rats instilled intratracheally with DEP. At various time points after DEP instillation tracheobronchial lymph nodes were removed and DEP-EC contents were analyzed. Upper panel shows the mean weights of lymph nodes removed from DEP-exposed WKY (black-filled bars) and SH (gray-open bars) rats. Lower panel shows the values of DEP-EC accumulated per lymph node. Line graph in the lower panel represents the percent increase in DEP accumulation in SH rat lymph nodes as compared with WKY rats. All values represent mean ± SD of data from five rats.

Wallenborn *et al.*, 2007; Yu *et al.*, 2008). In addition to physiological alterations, differences in inhaled particle retention, deposition and clearance could also result in variation in the biological response between healthy and cardiopulmonary compromised individuals. Particle deposition in the airways has been reported to increase in people with chronic obstructive lung disease (Brown *et al.*, 2002) and could explain in part why these individuals might be more adversely affected during air pollution episodes. SH rats have been shown to exhibit greater susceptibility to combustion-derived metal-rich PM-induced lung injury and vascular effects (Hwang *et al.*, 2008; Kodavanti *et al.*, 2000, 2002; Wallenborn *et al.*, 2007; Yu *et al.*, 2008) but not DEE (Gottipolu *et al.*, 2009). The purpose of this study was to measure the deposition and retention of inhaled DEE in lungs of SH and WKY rats and to compare the temporality of lung burden following single IT instillation of bulk DEP. The results showed that despite having higher minute volumes, the SH rats accumulated lower amounts of DEP (as determined by EC content of tissues) in the lungs following inhalation exposure than the WKY rats. The DEE inhalation exposure resulted in a dose-dependent increase in lung injury markers and neutrophils in the BALF. Although lung injury markers appeared slightly more severe in SH compared with WKY, there were no strain differences in the net injury or inflammatory response following DEE inhalation as has been recently reported (Gottipolu *et al.*, 2009). Evaluation of DEP lung clearance kinetics following single IT instillation bolus confirmed that SH lungs cleared DEP more readily and rapidly than the WKY rats, and this was associated with greater accumulation of particles in the tracheobronchial (bronchus-associated) lymph nodes during earlier time points. We conclude that although SH rats exhibit similar degree of pulmonary injury from DEP exposure, they eliminate particles from the respiratory tract more readily than WKY. It remains to be studied if other types of PM, such as ambient or combustion-derived, may be eliminated as efficiently as DEP by SH lungs.

One of the physiological factors which can play a significant role in deposition of physicochemically uniform inhaled particles in the lung is the breathing mechanics, including minute volume. The SH rats had a 50% greater average minute volumes compared with the WKY rats, which was not due to rapid shallow breathing because the lung tidal volumes were comparable in the two strains. Knowing the total duration of exposure during inhalation study, the average minute volume and the concentration of DEP in the DEE, the total theoretical amounts of particles deposited during 80-h episodic exposure to DEE was calculated to be 670 and 2660 µg DEP for the WKY rats exposed to the low and high DEE, whereas the corresponding calculations for SH rats would yield 958 and 4243 µg, at low and high concentrations, respectively. The actual determination of EC due to DEP accumulation in lungs (DEP-EC), however, indicated that the SH rats retained significantly lower amounts of DEP-EC in lungs over a 4-week exposure period suggesting that the differences in respiratory mechanics between WKY and SH rats did not contribute to the lower lung DEP burden and that the clearance mechanisms must have been more efficient in SH than in WKY rats.

Mucociliary clearance of deposited particles within first 2–3 days following instillation perhaps plays a significant role in subsequent particle retention in the lung (Van der Schans, 2007). Transport of particles deposited in airways to the pharynx can primarily be influenced by mucociliary mechanism in more peripheral airways while by airflow in central large airways (Van der Schans, 2007). Although the clearance by mucociliary escalator can be very different between IT instillation of one large bolus versus inhalation over a long period of time, significant portion of poorly soluble particles, such as DEP may clear via this route. Consistently we noted a rapid decline in intratracheally instilled DEP from caudal lung lobe within first two days in SH rats. Underlying pulmonary fibrosis and exercise has been shown to increase particle clearance from lungs (Tryka *et al.*, 1985; Zeltner *et al.*,

1991). It is not clear why there was very little if any decline between 4 h and 2 days following DEP instillation in WKY rats.

DEP-loaded AM could move to the airways for removal through the mucociliary escalator (Stöber and McClellan, 1997). Contributions mucociliary clearance of DEP- or DEP-loaded AM were not considered in this paper because of the technical issues associated with the sensitivity of the assay and the likely confounding of exogenous carbon with DEP measurement in the gut. There were no significant difference in the numbers of AMs in BALF of WKY and SH rats exposed to DE. Moreover, the spectrum of DEP loading in AM was similar in the two strains at a given exposure concentration, suggesting that the differences in retention were not likely due to AM phagocytic capacity. It is also likely that macrophage ingestion of PM and transport via the mucociliary escalator or clearance through the lymphatic system may be influenced by the site (alveolar or tracheobronchial) where inhaled or instilled particles are deposited within the lung.

To further study the mechanism of DEP clearance from lungs of WKY and SH rats, equal amounts (per kg body weight) of a standard DEP suspension was instilled into the lungs and the kinetics of its clearance followed for a period of 112 days. The results showed 60% clearance over 112 days in SH but virtually no clearance in WKY. Although the macrophage DEP burden appeared similar following DEE inhalation, a time-dependent accumulation of DEP-EC was noted in the lymph nodes which was significantly greater in SH rats following 2 and 21 days post exposure (approximately 250% higher). At the later time point, however, the accumulation was high and comparable in the two strains. These results suggest that the lymphatic clearance of DEP from lungs may be more efficient in SH rats, at least until the time lymph nodes become relatively saturated with DEP, and this may partially be responsible for less retention of DEP from SH lungs. Greater presence of residual oil fly ash particles was noted in small lymphatic foci adjacent to airways of SH than in WKY rats (Kodavanti *et al*., 2002).

It should be noted that the highest lymph node load of DEP measured in these experiments was approximately 50 μg EC and accounted for only 2% of the amount of DEP instilled (8.33 mg/kg body weight, range 2140–2650 μg per rat). An insignificant decrease in pulmonary lung burden in WKY over 112 days despite accumulation of DEP in lymph nodes is reflective of only a minor contribution of this clearance mechanism. Even though there are other regional lymph nodes that drain lungs (Takahashi and Patrick, 1987), it is difficult to conceive that clearance of 60% of DEP from SH lungs occurred by transportation to lymph nodes per se, unless further translocation of particles out of lymph nodes is assumed. Particle loaded cells may exit lymph nodes through efferent lymphatic ducts and eventually find their way to blood through thoracic duct. To support this, transportation of carbon nanoparticles administered intraperitoneally through the mesenteric lymph nodes and thoracic duct has been demonstrated in rats (Maincent *et al*., 1992). From the blood the particles may have been sequestered by Kupffer cells in liver and macrophages elsewhere. To study these possibilities requires further investigation.

Although we have demonstrated lower retention of DEP in lungs of SH rats and have suggested more efficient lymphatic drainage as one of the factors that may be responsible for it, the reason why this would occur in these rats is not clear. The increased minute volume would suggest that the SH rats hyperventilate and this may improve propelling the contents of afferent lymphatics towards lymph nodes. High blood pressure in SH rats may also result in increased blood flow through lungs which could improve clearance of DEP from the lungs. High blood flow has been linked with better mucociliary clearance of particles in sheep (Wagner and Foster, 1996). The other factor that could influence clearance is the underlying increased protein leakage at baseline in SH when compared with WKY rats (Kodavanti *et al*., 2000, 2002; Wallenborn *et al*., 2007). We have also noted significant protein leakage and concentration-dependent neutrophilic inflammation in the lungs of both rat strains following 1-month DEE exposure with overall protein leakage being greater in SH rats (Gottipolu *et al*., 2009). Significant protein leakage was also noted in the first week after a large bolus instillation of DEP in both SH and WKY rats, however, this was largely revered by 1 week whereas the decrease in lung DEP burden actually occurred more readily after 1 week in SH rats, suggesting that DEP-induced vascular leakage might not play a major role in leakage of DEP from lung.

An important issue which is not addressed in this study is how different delivery methods (inhalation, instillation) might influence the distribution of DEP deposited in the lungs and subsequent clearance. Although IT instillation may not mimic the distribution of DEP resulting from inhalation, this methodology allows one to control the particle dose and thus, actual lung deposition can be calculated for subsequent measurement of retention. Our data from 4-week inhalation study show that the strain differences in DEP retention are clear despite the different exposure protocols used. Our study will not allow the direct comparison of lung retention of DEP between the inhalation and IT instillation protocols, as episodic inhalation exposure occurred four weeks but one bolus instillation dose of DEP was delivered in the IT study. The types of DEP used were also different between these exposure protocols and the chemical make-up of each DEP may influence how it is retained in the lungs of WKY and SH rats. The IT instillation dose might be large enough to overwhelm the clearance mechanism and lead to an overload situation which might not occur with inhalation over a short period of 4 weeks, making it difficult to correlate DEP retention using instillation versus inhalation. It is clear; however, that exposure of rats to DEP via instillation resulted in greater pulmonary injury and inflammation than what was observed at

the end of 4-week DEE inhalation. Thus, more controlled experiments will be needed to address the relativity of DEP clearance through both routes of exposure.

The pulmonary and cardiophysiological effects of oil combustion PM exposure are known to be exacerbated in hypertension. WKY and SH rats have been extensively used to understand the greater susceptibility to PM-induced cardiopulmonary effects associated with hypertension (Elder *et al*. 2004; Farraj *et al*., 2009; Gilmour *et al*. 2004; Kodavanti *et al*. 2002, 2005; Kooter *et al*. 2005; Wichers *et al*., 2006). These studies have demonstrated that pulmonary injury and cardiovascular effects of ambient and oil combustion-derived PM are generally exacerbated in SH when compared with healthy WKY rats. However, our recent study show that DEE inhalation produced no cardiac gene expression changes in SH although WKY rats were greatly affected. Thus, it is likely that susceptibility differences between healthy and hypertensive rats may be greatly influenced by the type of PM materials used and the response being analyzed. It is not clear if other types of PM, such as oil combustion, may be cleared faster by SH lungs relative to WKY despite those being more toxic to the lung. The strain differences in PM retention will need to be considered and further examined in considering susceptibility differences underlying cardiovascular and pulmonary diseases.

We demonstrate that in spite of similar or slightly greater degree of pulmonary injury noted in DEE and DEP-exposed SH relative to WKY rats, the pulmonary-deposited DEP particles clear faster and more efficiently from lungs of SH rats. Clearance to lymphatic drainage may be a small contributor to this rapid clearance of DEP from SH lungs.

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