Pulmonary Exposure to Diesel Exhaust Particles Enhances Coagulatory Disturbance with Endothelial Damage and Systemic Inflammation Related to Lung Inflammation

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Pulmonary exposure to diesel exhaust particles (DEP) enhances lung inflammation related to bacterial endotoxin (lipopolysaccharide [LPS]) in mice. Severe lung inflammation can reportedly induce coagulatory abnormalities and systemic inflammation. This study examined the effects of components of DEP on lung inflammation, pulmonary permeability, coagulatory changes, systemic inflammatory response, and lung-to-systemic translocation of LPS in a murine model of lung inflammation. ICR mice were divided into six experimental groups that intratracheally received vehicle, LPS (2.5 mg/kg), organic chemicals in DEP (DEP-OC; 4 mg/kg) extracted with dicloromethane), residual carbonaceous nuclei of DEP (washed DEP: 4 mg/kg), DEP-OC + LPS, or washed DEP + LPS. Both DEP components exacerbated lung inflammation, vascular permeability, and the increased fibrinogen and E-selectin levels induced by LPS. With overall trends, the exacerbation was more prominent with washed DEP than with DEP-OC. Washed DEP + LPS significantly decreased activated protein C and antithrombin-III and elevated circulatory levels of interleukin (IL)-6, keratinocyte chemoattractant (KC), and LPS as compared with LPS alone, whereas DEP-OC + LPS elevated IL-6, KC, and LPS without significance. These results show that DEP components, especially washed DEP, amplify the effects if LPS on the respiratory system and suggest that they contribute to the adverse health effects of particulate air pollution on the sensitive populations with predisposing vascular and/or pulmonary diseases, including ischemic vascular diseases and respiratory infection. Exp Biol Med 231:1626–1632, 2006

Key words: DEP; components; lung inflammation; LPS; endothelial damage

Introduction

Exposure to ambient particulate matter (PM), especially to PM with a diameter of <2.5 μm (PM2.5), is associated with daily mortality and hospital admissions related to respiratory diseases such as bronchilitis, pneumonia, and asthma (1). Also, PM are reportedly associated with mortality and morbidity from cardiovascular causes (1). It is hypothesized that PM induce pulmonary inflammation with a systemic release of cytokines, which may influence cardiovascular endpoints (2). Also, there is an epidemiological correlation among air pollution, elevated circulatory fibrinogen levels, and the risk of cardiovascular events (3).

In urban areas, diesel engines are the major source of PM2.5 (1). Diesel engine–derived diesel exhaust particles (DEP) have been experimentally correlated with a variety of respiratory disorders, such as lung cancer, pulmonary fibrosis, chronic alveolitis (4), pulmonary edema (5), and allergic airway inflammation (6). We have previously shown that DEP (7) and their components (8) enhance lung inflammation related to bacterial endotoxin (lipopolysaccharide [LPS]). Cellular profile of bronchoalveolar lavage (BAL) fluid and lung histology have shown that the enhancing effects are stronger with carbonaceous nuclei of

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DEP after extraction (washed DEP) than with the organic chemicals in DEP extracted with dichloromethane (CH_2Cl_2) (DEP-OC), which is concomitant with the lung expression of proinflammatory proteins, including interleukin (IL)-1β, macrophage inflammatory protein (MIP)-1α, macrophage chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC) (8).

DEP can reportedly induce an inflammatory response not only in the airways but also in the circulatory blood partly through enhanced prothrombotic activity *in vivo* (9). On the other hand, LPS causes endothelial damage, resulting in multiple organ dysfunction syndrome frequently accompanied by consumptive hemostatic changes, which often leads to disseminated intravascular coagulation, including the activation of coagulation and fibrinolysis (10, 11). These previous studies raise the possibility that the coexistence of DEP and LPS synergistically aggravates endothelial damage and consequent coagulopathy. Also, it can be imagined that severe endothelial damage allows large molecules such as LPS to pass into the circulation. Indeed, lung-to-systemic translocation of LPS has been evidenced under large tidal volume ventilation *in vivo* (12).

In the current study, we elucidated the effects of pulmonary exposure to DEP components on endothelial damage, coagulatory disturbance, systemic inflammatory response, and LPS translocation from the lung-to-systemic circulation after LPS exposure to the murine respiratory system.

Materials and Methods

Animals. Male ICR mice 6 to 7 weeks of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea) and given water *ad libitum*. Mice were housed in an animal facility that was maintained at 24°–26°C with 55%–75% humidity and a 12:12-hr light:dark cycle, as previously described (6).

Preparation of Particle Samples. A 4JB1-type, light-duty, four-cylinder, 2.74-L Isuzu diesel engine (Isuzu Automobile Co., Tokyo, Japan) under computer control was connected to a dynamometer (Meiden-sya, Tokyo, Japan). The details on the condition of engine and the collection of DEP were previously described (13).

Preparation of DEP-OC and Washed DEP. DEP were extracted with CH_2Cl_2 (8). Briefly, DEP were suspended in CH_2Cl_2 and sonicated for 5 mins (UD-201; Tomy Seiko, Tokyo, Japan). The suspension was centrifuged at 200 *g* for 20 mins. The supernatants were transferred to another tube, and the residue was washed with CH_2Cl_2. This manipulation was repeated three times. The residual particles of DEP were prepared as washed DEP. The extracts were combined, evaporated, dissolved in 100% dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan), and prepared as DEP-OC.

Preparation of LPS. LPS (*Escherichia coli* O55: B5) were obtained from Difco Laboratories (Detroit, MI). The LPS was extracted by the Boivin trichloracetic acid procedure.

Study Protocol. Mice were divided into six groups. The vehicle group received phosphate-buffered saline at pH 7.4 (GIBCO BRL, Life Technology, Grand Island, NY) containing 0.025% Tween 80 (Nacalai Tesque, Kyoto, Japan) and 0.25% DMSO. The washed DEP group or the DEP-OC group received 4 mg/kg of washed DEP or DEP-OC, respectively. The LPS group received 2.5 mg/kg of LPS. The washed DEP + LPS and DEP-OC + LPS groups received respective additions of LPS to each component before experimentation. In each group, vehicle, DEP components, LPS, or DEP components + LPS were dissolved in 0.1-ml aliquots and inoculated by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Hoechst, Japan, Tokyo, Japan). Twenty-four hours after the intratracheal administration, all mice were sacrificed and examined. The studies reported were carried out in accordance with the Guide for the Care and Use of Laboratory animals as adopted and promulgated by the National Institutes of Health. All animal studies were approved by the Institutional Review Board.

Histological Evaluation. After exsanguination, the lungs were fixed by intratracheal instillation of 10% neutral phosphate-buffered formalin at a pressure of 20 cm H_2O for at least 72 hrs. Slices 2 to 3 mm thick of all pulmonary lobes were embedded in paraffin. Sections of 3-μm thickness were stained with hematoxylin and eosin (H&E). Neutrophil infiltration was expressed as the number of neutrophils per high-power field by counting the number of over 30 fields at a magnification of ×400 in each slide ($n = 5$ in each group). Histologic sections were evaluated in a blind fashion.

Pulmonary Vascular Permeability. In a separate series of experiments, the bilateral lungs were weighed immediately after exsanguination and dried in an oven at 95°C for 48 hrs. Thereafter, lung water content was estimated by calculating the ratio of wet lung weight to dry lung weight (mg)/body weight (g) (Ref. 5: $n = 10$ in each group). In another experiment, protein concentrations in BAL fluid were estimated by previous method by Bradford (Ref. 14: $n = 8$ in each group).

Assays for Circulatory Fibrinogen, Activated Protein C (APC), Antithrombin (AT)-III, von Willebrand Factor (vWF), IL-1β, IL-6, KC, E-Selectin, and LPS Level. After deep anesthesia, the chest and abdominal walls were opened, and blood was retrieved by cardiac puncture. Blood samples were collected from the right ventricle and moved into 3.8% sodium citrate in a ratio of 10:1 and centrifuged at 2,500 *g* for 10 mins. Citrate plasma fibrinogen, APC, AT-III, and vWF ($n = 7$–10 in each group) were determined using commercial kits (Diagnostica Stago, Roche, Tokyo, Japan) on STA Compact (Diagnostica Stago, Roche) as described previously (15). Serum samples ($n = 10$ in each group) were analyzed by enzyme-linked immunosorbent assays for IL-1β (Endogen, Cambridge, MA), IL-6

(Biosource, Nivelles, Belgium), KC, and E-selectin (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions. Values obtained at 550 nm were subtracted from values obtained at 450 nm and converted to pg/ml using values obtained from standard curves generated with varying concentrations of recombinant IL-1β, IL-6, KC, and E-selectin with limits of detection of 3, 3, 2, and 3.8 pg/ml, respectively. The LPS level was determined by automated kinetic assay using an LPS-specific limulus amebocyte lysate reagent (Endospecy ES-50M; Seikagaku Corp., Tokyo, Japan) combined with an incubator installed microplate reader (Wellreader SK603; Seikagaku Corp.) as described previously (16). The increase in OD by released p-nitroaniline was continuously measured at 405 nm against a reference of 492 nm. LPS level was automatically calculated from a calibration curve with standard LPS preparation. No interference with the assay has been observed with sufficient recovery of nearly 100% by the addition-recovery test.

Statistical Analysis. LPS concentration is expressed as mean ± SD. Other data except for LPS concentrations are reported as mean ± SEM. Differences between groups in LPS concentrations were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney *U* test using StatView version 4.0 (Abacus Concepts, Inc., Berkeley, CA). Differences in other data were examined for statistical significance using analysis of variance and Bonferroni's test. Significance was assigned to *P* values smaller than 0.05.

Results

Effects of DEP Components on Histological Changes in the Lung. To determine the effects of DEP components on lung histology, we first evaluated lung specimens stained with H&E 24 hrs after intratracheal instillation (Fig. 1A). No pathological changes were observed in lung specimens in the vehicle group. Infiltration of inflammatory cells, including neutrophils, was slightly seen in the DEP components groups and moderately seen in the LPS group. In the washed DEP + LPS group, markedly exaggerated neutrophil sequestration and pulmonary edema were observed. On the other hand, the DEP-OC + LPS group revealed moderate neutrophilic inflammation.

We performed morphometric analysis to quantitate the number of neutrophils in the lung 24 hrs after LPS challenge (Fig. 1B). The number of neutrophils in the lung was significantly greater in the LPS group or the DEP components + LPS groups than in the vehicle group ($P < 0.01$). The number was further increased in the DEP components + LPS group ($P < 0.01$; washed DEP + LPS group vs. the LPS group).

Effects of DEP Components on Pulmonary Permeability during Lung Inflammation. To estimate pulmonary permeability, we next evaluated the lung water content and protein level in the BAL fluid 24 hrs after intratracheal instillation (Table 1). Lung water content was significantly greater in the washed DEP, the LPS, and the DEP components + LPS groups than in the vehicle group ($P < 0.01$). The value was significantly greater in the washed DEP + LPS group than in the LPS group ($P < 0.01$). The protein level in the BAL fluid was also significantly increased in the washed DEP group ($P < 0.01$) and DEP-OC + LPS groups compared to the vehicle group and was further increased in the washed DEP + LPS group ($P < 0.01$ vs. LPS group). The value was also significantly greater in the washed DEP + LPS group than in the LPS group ($P < 0.01$).

Effects of DEP Components on Coagulatory Changes during Lung Inflammation. To elucidate the effects of DEP components on coagulatory changes during lung inflammation, we next evaluated coagulatory parameters 24 hrs after the intratracheal challenge (Table 2). Fibrinogen levels were significantly higher in the washed DEP, the LPS, and the DEP components + LPS groups than in the vehicle group (Table 2; $P < 0.01$) with values further increased in the DEP components + LPS groups ($P < 0.01$ vs. the LPS group). APC was significantly smaller in the washed DEP, the LPS, and the DEP components + LPS groups than in the vehicle group ($P < 0.01$). The level was further reduced in the washed DEP + LPS group ($P < 0.05$ vs. the LPS group). AT-III values were also significantly less in the LPS and DEP-OC + LPS groups compared to the vehicle group ($P < 0.01$) and were further reduced in the washed DEP + LPS group ($P < 0.05$ vs. the LPS group). Finally, the level of vWF was significantly greater in the washed DEP + LPS group than in the vehicle group ($P < 0.05$).

Effects of DEP Components on Systemic Inflammation and Translocation of LPS during Lung Inflammation. To investigate the impact of DEP components on systemic inflammation and translocation of LPS during lung inflammation, we next estimated circulatory levels of IL-1β, IL-6, KC, E-selectin, and LPS 24 hrs after intratracheal challenge (Table 3). IL-1β levels were greater in the LPS and the DEP components + LPS groups than in the vehicle group ($P < 0.01$). The LPS group showed elevation of IL-6 level as compared with the vehicle group. IL-6 levels were also increased in the washed DEP + LPS group compared to both the vehicle and the LPS groups ($P < 0.01$). In addition, KC levels were significantly increased in the LPS ($P < 0.05$), the DEP-OC + LPS ($P < 0.05$), and the washed DEP + LPS ($P < 0.01$) groups compared to the vehicle group, and the values were further increased in the washed DEP + LPS group ($P < 0.01$ compared to LPS alone). E-selectin levels were significantly greater in the LPS and the DEP components + LPS groups ($P < 0.01$ vs. the vehicle group) and were further increased in the DEP-OC + LPS group ($P < 0.05$ vs. the LPS group) and in the washed DEP + LPS group ($P < 0.01$ vs. the LPS group) (Table 3). The LPS concentration was greater in the LPS and the DEP components + LPS groups than in the vehicle group ($P < 0.01$). The concentration was further

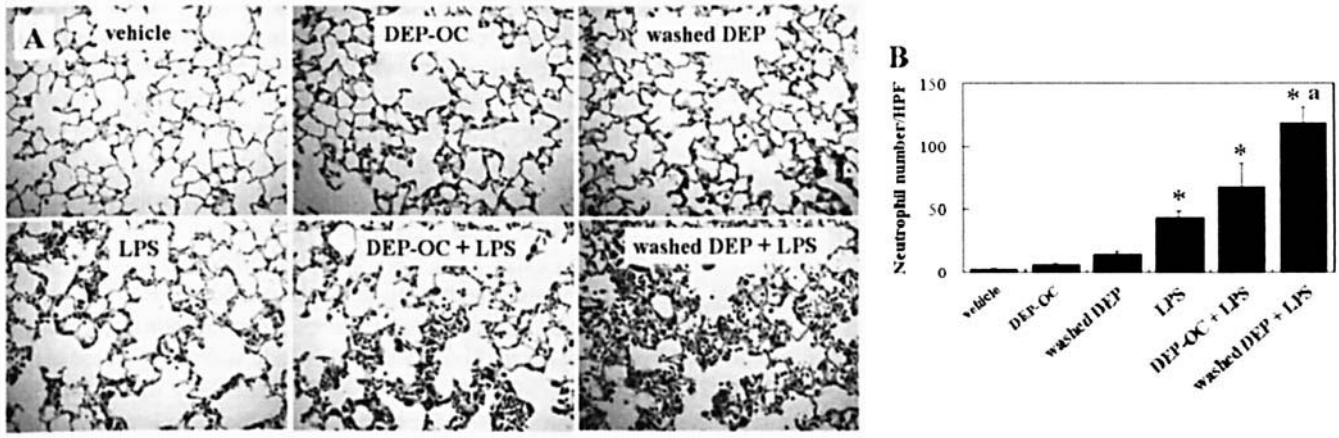

Figure 1. Effects of carbonaceous nuclei of diesel exhaust particles (DEP) after extraction (washed DEP) and organic chemicals in DEP extracted with dichloromethane (DEP-OC) on histological changes in the lung after intratracheal instillation of LPS (2.5 mg/kg). (A) Representative histopathological findings. Lungs from mice of the vehicle group, the DEP-OC group, the washed DEP group, the LPS group, the DEP-OC + LPS group, or the washed DEP + LPS group were obtained 24 hrs after the intratracheal instillation, and lung specimens were stained with hematoxylin and eosin (H&E: $n = 5$ in each group). Original magnification: ×400. (B) Quantitative analysis for neutrophil sequestration into the lung. The analysis was evaluated using lung specimens stained with H&E. Neutrophil infiltration was expressed as the number of neutrophils per high-power field by counting the number over 30 fields at a magnification of ×400 in each slide. Differences between groups were analyzed by analysis of variance and Bonferroni's test. * $P < 0.01$ versus vehicle, [a] $P < 0.01$ versus LPS. Values are the mean ± SEM in each group.

increased in the washed DEP + LPS group ($P < 0.05$ vs. LPS).

Discussion

In our recent study using the same protocol as the present one, DEP components were observed to enhance the infiltration of neutrophils into the BAL fluid in the presence of LPS (8). Impressively, washed DEP synergistically exacerbated histological changes in the lung and worsened pulmonary edema in the presence of LPS. Combined treatment with washed DEP and LPS also increased the expression of proinflammatory molecules, such as IL-1β, MIP-1α, MCP-1, and KC, in the lung compared with LPS treated alone. In the present study, we have reconfirmed that DEP components aggravate lung inflammation induced by intratracheal instillation of LPS (Fig. 1). In addition, we have demonstrated that both DEP components exacerbate vascular permeability (Table 1) and the increased fibrinogen (Table 2) and E-selectin (Table 3) levels induced by LPS. With overall trends, the exacerbation is more prominent with washed DEP rather than with DEP-OC. Finally, washed DEP + LPS significantly decreased APC and AT-III (Table 2) and elevated circulatory levels of IL-6, KC, and LPS as compared with LPS alone (Table 3).

PM exposure results in adverse health effects not only on respiratory systems but also on circulation (1–3). Indeed, inflammatory respiratory diseases and vascular diseases are highly sensitive to DEP exposure (1, 6–8). In the present study, we reconfirm that DEP components, especially washed DEP, enhance LPS-induced neutrophil inflammation (Fig. 1B). Furthermore, recent epidemiological analysis has reported that respiratory infection can act as a risk factor in the development of cardiovascular diseases, including ischemic heart diseases (17). Nonetheless, there is a shortage of molecular and/or experimental evidence to clarify the role of DEP in susceptibility to coagulatory disturbance and systemic inflammation during infectious inflammation. In the present study, we have focused on whether the effects of DEP components on lung inflammation can spread to coagulatory changes and systemic inflammation.

Lung inflammation induced by intratracheal inoculation of LPS has reportedly elevated fibrinogen level in rats (18). However, fibrinogen levels have not been examined in the other species. Furthermore, the previous report did not examine other proinflammatory mediators, such as cytokines and chemokines, in the systemic circulation (18). In the present murine model, pulmonary exposure to LPS induced significantly higher levels of circulatory fibrinogen (Table 2) and IL-1β (Table 3) than vehicle exposure. IL-1β is an important proinflammatory initiator in mammalian innate immunity (19). This study represents the first demonstration that lung inflammation related to LPS induces a systemic inflammation characterized by enhanced levels of circulating proinflammatory cytokines *in vivo*.

Intratracheal instillation of DEP leads to a rapid activation of the thrombohemostatic system, including platelet activation, suggesting that DEP can increase the risk of ischemic vascular diseases (9). On the other hand, epidemiological data have demonstrated the close correlation between high fibrinogen levels and the risk of ischemic cardiovascular diseases (20). However, the impacts of pulmonary exposure to DEP and/or their components on thrombohemostatic disturbance and circulatory inflammatory response during lung inflammation have never been experimentally examined. In the present study, the increased circulatory fibrinogen caused by LPS alone was further

Table 1. Effects of Carbonaceous Nuclei of Diesel Exhaust Particles (DEP) After Extraction (Washed DEP) and Organic Chemicals in DEP Extracted with Dichloromethane (DEP-OC) on Pulmonary Permeability After Intratracheal Instillation of Lipopolysaccharide (LPS: 2.5 mg/kg)[a]

Group	Lung water content (lung wet weight:dry weight [mg]/body weight [g])	Protein level in BAL fluid (mg/ml)
Vehicle	4.03 ± 0.07	1.57 ± 0.09
DEP-OC	4.15 ± 0.06	2.12 ± 0.13
Washed DEP	5.65 ± 0.65**	3.26 ± 0.38**
LPS	6.18 ± 0.16**	2.37 ± 0.07
DEP-OC + LPS	6.51 ± 0.28**	2.88 ± 0.15*
Washed DEP + LPS	8.72 ± 0.45**[b]	6.90 ± 0.74**[b]

[a] The bilateral lungs of mice were weighed 24 hrs after the instillation and dried in an oven at 95°C for 48 hrs. Lung water content was estimated by calculating the ratio of wet lung weight to dry lung weight (mg)/body weight (g) ($n = 10$ in each group). In another experiment, protein concentrations in bronchoalveolar lavage (BAL) fluid were estimated ($n = 7$ in each group). Differences between groups were analyzed by analysis of variance and Bonferroni's test. *$P < 0.05$ versus vehicle; **$P < 0.01$ versus vehicle.
[b] $P < 0.01$ versus LPS. Values are the mean ± SEM in each group.

significantly enhanced by its combination with the DEP components (Table 2). Furthermore, anticoagulatory factors such as APC and AT-III were lower in the DEP components + LPS groups, especially in the washed DEP + LPS group, than in the LPS group (Table 2). These findings suggest that pulmonary exposure to DEP, especially to washed DEP, activates coagulatory changes during lung inflammation. Alternatively, on the basis of the present results and the previous reports, the effects of pulmonary exposure to PM or DEP on cardiovascular events might be explained, at least partly, *via* the activated thrombohemostatic system, including fibrinogen hyperproduction.

DEP reportedly damages vascular endothelial cells *in vitro* (21). However, *in vivo* studies to examine the effects of DEP on endothelial damage in the lungs have not been performed. APC suppresses E-selectin–mediated inflammatory cell adhesion to endothelial cells (22, 23). E-selectin is an important vascular adhesion molecule in the early phase of inflammatory reactions induced by LPS (24). On the other hand, vWF is a proper marker of endothelial damage (25). In the present study, washed DEP + LPS significantly decreased APC (Table 2) and significantly elevated the levels of E-selectin (Table 3) as compared with LPS alone. In addition, vWF was significantly higher in the washed DEP + LPS group than in the vehicle group (Table 2). Furthermore, pulmonary permeability of both water and protein was exaggerated in the DEP components + LPS groups, especially in the washed DEP + LPS group, as compared with the vehicle group or the LPS group (Table 1). These findings support the adverse effects of DEP on endothelilal damage *in vivo*.

Systemic levels of IL-6 and IL-8 play important roles in initiating ischemic vascular diseases (26). In the present study, washed DEP + LPS resulted in substantial increases in circulatory levels of IL-6 and KC compared to LPS alone (Table 3). In mice, KC is the functional homologue of IL-8 in human. These reports and the present results suggest that DEP, especially washed DEP, may be involved in the increased morbidity of ischemic cardiovascular diseases, at least partly through the elevation of circulatory IL-6 and KC.

Exaggeration of lung-to-systemic translocation of LPS correlates with high mortality in rabbit under conventional artificial ventilation (12). However, studies elucidating the direct correlation between lung-to-systemic translocation of LPS, thrombohemostatic changes, endothelial damage, and systemic inflammation have not been done. Previous work by others has shown that the alveolar space is a tight compartment with very little exchange of materials to the systemic circulation (27). In the present study, however, we found that the washed DEP + LPS group showed significantly higher plasma LPS concentrations than the LPS group alone (Table 3). The high LPS concentration was consequently paralleled by the enhanced serum concentrations of fibrinogen, vWF, IL-1β, IL-6, KC, and E-selectin in overall trends. Our results

Table 2. Effects of Carbonaceous Nuclei of Diesel Exhaust Particles (DEP) After Extraction (Washed DEP) and Organic Chemicals in DEP Extracted with Dichloromethane (DEP-OC) on Coagulatory Changes After Intratracheal Instillation of Lipopolysaccharide (LPS: 2.5 mg/kg)[a]

Group	Fibrinogen (mg/dl)	APC (%)	AT III (%)	vWF (%)
Vehicle	335.6 ± 10.9	4.75 ± 0.16	120.9 ± 2.3	81.10 ± 4.49
DEP-OC	376.1 ± 14.2	4.50 ± 0.19	119.1 ± 2.2	77.30 ± 4.26
Washed DEP	488.3 ± 37.1**	3.00 ± 0.19**	112.9 ± 3.9	91.67 ± 5.40
LPS	624.1 ± 38.8**	3.25 ± 0.16**	106.9 ± 2.2**	93.64 ± 4.50
DEP-OC + LPS	742.4 ± 22.2**[b]	3.00 ± 0.19**	103.3 ± 3.0**	85.40 ± 4.39
Washed DEP + LPS	857.1 ± 20.5**[b]	2.57 ± 0.43**[c]	97.4 ± 2.4**[c]	94.70 ± 4.26*

[a] Blood samples were collected from mice 24 hrs after the intratracheal administration, and plasma fibrinogen, activated protein C (APC), antithrombin (AT)-III, and von Willebrand factor (vWF) ($n = 7$–10 in each group) were evaluated on STA Compact (Diagnostica Stago, Roche, Tokyo, Japan). Values are the mean ± SEM in each group. Differences between groups were analyzed by analysis of variance and Bonferroni's test. *$P < 0.05$ versus vehicle; **$P < 0.01$ versus vehicle.
[b] $P < 0.01$ versus LPS.
[c] $P < 0.05$ versus LPS.

Table 3. Effects of Carbonaceous Nuclei of Diesel Exhaust Particles (DEP) After Extraction (Washed DEP) and Organic Chemicals in DEP Extracted with Dichloromethane (DEP-OC) on Systemic Inflammation and the Translocation of Lipopolysaccharide (LPS) from the Lung-to-Systemic Circulation After Intratracheal Instillation of LPS (2.5 mg/kg)[a]

Group	IL-1β (pg/ml)	IL-6 (pg/ml)	KC (pg/ml)	E-selectin (ng/ml)	LPS concentration (pg/ml)
Vehicle	0 ± 0	20.00 ± 0.65	43.01 ± 12.30	21.07 ± 1.36	2.8 ± 5.2
DEP-OC	24.31 ± 13.00	19.07 ± 0.36	12.77 ± 6.91	16.93 ± 0.80	2.8 ± 2.4
Washed DEP	19.53 ± 19.53	21.45 ± 1.66	12.13 ± 9.87	23.80 ± 1.63	2.7 ± 2.1
LPS	86.62 ± 26.91**	32.67 ± 2.43	311.4 ± 49.74*	48.23 ± 1.97**	3590.6 ± 7560.9**
DEP-OC + LPS	97.39 ± 32.78**	76.65 ± 24.98	553.4 ± 179.9*	54.14 ± 3.00**[a]	7482.6 ± 16504.4**
Washed DEP + LPS	121.9 ± 16.77**	277.3 ± 98.44**[b]	1289.5 ± 375.2**[b]	59.61 ± 2.59**[b]	33145.6 ± 49217.3**[c]

[a] Blood samples were collected from mice 24 hrs after the intratracheal administration. Serum levels for interleukin (IL)-1β, IL-6, keratinocyte chemoattractant (KC), and E-selectin ($n = 10$ in each group) were evaluated by enzyme-linked immunosorbent assays, and plasma LPS concentration was evaluated by automated kinetic assay ($n = 15$–18 in each group). Values are the mean ± SD for LPS concentration and the mean ± SEM for the other value in each group. Differences between groups in LPS concentration were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney *U* test using StatView version 4.0. Differences in other data were examined for statistical significance using analysis of variance and Bonferroni's test. *$P < 0.05$ versus vehicle; **$P < 0.01$ versus vehicle.
[b] $P < 0.01$ versus LPS.
[c] $P < 0.05$ versus LPS.

indicate that washed DEP can enhance LPS translocation from the lung to circulation and may result in the accelerated systemic inflammatory response as well as endothelial damage and thrombohemostatic disturbance.

Pulmonary vascular permeability was also greater in the washed DEP group and the LPS group than in the vehicle group and further enhanced in the washed DEP + LPS group compared to the LPS group in the present study. Furthermore, the presence of red blood cells in the BAL fluid was occasionally observed in the LPS and the DEP-OC + LPS groups, whereas it was frequently seen in the washed DEP + LPS group (Ref. 8 and unpublished observation). Therefore, it might be hypothesized that endothelial–epithelial damage induced by washed DEP and/or LPS and additional damage by infiltrated effector leukocytes allows large molecules such as LPS to pass into the circulation, resulting in synergistic effects on systemic inflammation and coagulopathy. On the other hand, exposure to environmental particles reportedly generates local and systemic oxidative stress, which in turn induces/enhances inflammation and blood coagulation (28). Further, Nemmar and colleagues have demonstrated that nanoparticles instilled intratracheally rapidly diffuse from the lung into the systemic circulation *in vivo* (29). Therefore, it is also possible that intratracheally instilled particulate compounds of DEP (washed DEP in the present study) enter the circulation by themselves and contribute to high susceptibility against LPS-elicited systemic inflammation and coagulatory disturbance.

Our previous study demonstrated that washed DEP, rather than DEP-OC, predominantly contributed to the aggravation of the LPS-related lung inflammation (8). Interestingly, in that study the aggravated lung inflammation parallels the enhanced circulatory levels of fibrinogen, IL-6, and KC and decreased APC found in the present study. Therefore, we can consider washed DEP, rather than DEP-OC, as the prominent contributors to DEP toxicity also in the present study.

In conclusion, pulmonary exposure to DEP components aggravates coagulatory disturbance, including endothelial damage, systemic inflammation, and endotoxin translocation related to lung inflammation. The effects of DEP on the thrombohemostatic and inflammatory process may play a vital role in the adverse health effects of PM on the sensitive populations who have had cardiovascular and respiratory diseases, including ischemic cardiovascular diseases and respiratory infection.

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