Impairment of Endothelium-Dependent Relaxation by Diesel Exhaust Particles in Rat Thoracic Aorta

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ABSTRACT—Nitric oxide released from vascular endothelium plays important regulatory roles in cardiovascular and pulmonary systems. Epidemiological studies suggest that diesel exhaust particles (DEP) seem to be one of the causative factors responsible for the recent increase in pulmonary diseases. To clarify the pathogenic mechanism, the effects of DEP on vascular endothelial functions were investigated in terms of endothelium-dependent relaxation. Ring preparations of rat thoracic aorta were preincubated for 10 min with a DEP suspension (1, 10, 100 μg/ml) at 37℃ in organ baths and relaxed with cumulative additions of acetylcholine following precontraction with phenylephrine (10^{-6} M). The relaxation was attenuated by DEP-exposure in a concentration-dependent manner. An addition of superoxide dismutase (SOD) completely abolished the inhibitory effect of DEP at lower concentrations, but only partially at the higher concentration. DEP (10 μg/ml) neither affected the contractile response to phenylephrine in intact aortic rings nor the endothelium-independent relaxation by sodium nitroprusside in denuded rings, while DEP (100 μg/ml) significantly attenuated both responses. These results suggest that 1) inhaled DEP causes pulmonary inflammation by inhibiting the endothelial formation and/or the effect of nitric oxide and 2) SOD reduces the adverse effects.

Keywords: Diesel exhaust particle, Endothelium-dependent relaxation, Thoracic aorta (rat), Superoxide, Acetylcholine

Several epidemiological studies indicate that patients with pulmonary diseases such as asthma and chronic bronchitis are increasing in Japan, especially in children living in urban areas. Diesel exhaust contains 2 to 20 times more nitrogen oxides and 30 to 100 times more particles than gasoline exhaust. Moreover, it has been recently reported that diesel exhaust particles (DEP) suspended in phosphate buffer generate superoxide anion radicals ($O_2^{\cdot -}$) and hydroxyl radicals ($^{\cdot}OH$) on incubation at 37℃. Sagai et al. suggested that these active oxygen radicals would cause endothelial cell damage leading to pulmonary edema (1). It was reported that reactive oxygens ($O_2^{\cdot -}$, $^{\cdot}OH$ and H_2O_2) were causes of pulmonary injury (2) and that $O_2^{\cdot -}$ produced from dihydroxyfumarate induced pulmonary endothelial cell damage (3). The earliest stages of histologically observable lung injury occurred in the pulmonary capillary bed (4) and the endothelial cells in capillaries (5). Edward reports that injury of the pulmonary endothelial cells is the pathogenesis of acute lung injury (6). These observations imply that the endothelium plays important protective roles in the development of pulmonary injury.

Since the alveolar capillary endothelium lies close to the alveolar epithelium (7), inhaled DEP may easily interact with the endothelium, blood cells and blood components by releasing reactive oxygens and some soluble materials. The endothelium releases numerous biologically important materials, one of which is endothelium-derived relaxing factor (EDRF): nitric oxide (8) or nitrosothiol (9-11). EDRF released from vascular endothelial cells plays an important role in cardiovascular and pulmonary systems, being involved in vasodilation (12), inhibition of platelet aggregation (13-15), leukocyte adhesion (16-18) and vascular albumin leakage (19, 20).

Although DEP is thought to be a causative factor responsible for the recent increase in patients with pulmonary diseases, neither the cause-and-result relationship nor the pathogenic mechanism has been demonstrated.

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This study was untaken to investigate the effects of DEP on endothelial functions through changes in endothelium-dependent relaxation in rat thoracic aortic ring preparations.

MATERIALS AND METHODS

Chemicals

Recombinant human superoxide dismutase (SOD) was supplied by Asahi Chemical Industry (Tokyo). Acetylcholine chloride, cytochrome c (Type III from horse heart), L-phenylephrine hydrochloride and sodium nitroprusside were purchased from Sigma (St. Louis, MO, USA).

Collection of diesel exhaust particles and preparation of the suspension

DEP were collected as described previously (1). Briefly, a light duty four cylinder diesel engine (2,740 ml) was connected to a dynamometer and operated under the load of 6 kg/ml torque using standard diesel fuel with 2,000 rpm speed. DEP were collected on glass fiber filters (203 mm × 254 mm) in a constant-volume sampler system and kept at −35℃ in an air tight bottle until needed. DEP were sonicated for 2 min (30 sec × 4, 50 W) by a sonicator (Ohtake, Tokyo) in phosphate-buffered saline (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4 and 1.5 mM KH_2PO_4) containing 0.05% Tween 80 while cooling on ice. The DEP suspension was diluted with Tyrode's solution (158.3 mM NaCl, 4 mM KCl, 10 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 2 mM $CaCl_2$, 1.05 mM $MgCl_2$ and 5.6 mM glucose) aerated with 95% O_2 : 5% CO_2 (pH 7.4). The final Tween 80 concentration was less than 0.0005%. When indicated, DEP were washed by centrifuging the DEP suspension three times in Tyrode's solution for 5 min at 1,500 × *g*.

Measurement of superoxide from DEP

Superoxide released from DEP during the incubation was measured according to the method described by McCord and Fridovich (21). The DEP suspension (980 μl in phosphate-buffered saline) was preincubated for 90 sec at 37℃ and then incubated for 60 min with cytochrome c (10 μl of 4 mM cytochrome c in phosphate-buffered saline, final concentration of 40 μM) or vehicle only in the presence and absence of SOD (10 μl of 2,000 U/ml SOD in Krebs buffer, final concentration of 20 U/ml). Absorbances at 540 and 550 nm were measured at the end of the experiment by a spectrophotometer (U-2000; Hitachi, Tokyo). The amount of reduced cytochrome c was calculated from the difference of the absorbances at 540 and 550 nm by using a reduced-oxidized molar extinction coefficient of $19.1 \times 10^3\ cm^{-1}$. Formation of superoxide was calculated by subtracting the amount of reduced cytochrome c without SOD from that with SOD.

Measurement of vascular contraction

Male Sprague-Dawley rats at 8−10 weeks of age were anesthetized with ether and killed by exsanguination from the abdominal aorta. The thoracic aorta was rapidly excised and cleaned of adherent fat and connective tissue. The aorta was cut into 2-mm ring segments and mounted in an organ bath filled with 5 ml of Tyrode's solution maintained at 37℃ and aerated with 95% O_2 : 5% CO_2 (pH 7.4) for measurement of isometric force. The preparations were equilibrated for 90 min at a resting tension of 0.5 g and then for another 30 min at a tension of 2 g. The ring was preincubated with DEP suspension for 10 min at 37℃ and then contracted with phenylephrine (10^{-6} M). When the contraction reached a plateau (in approx. 10 min), acetylcholine (ACh, $10^{-8}-10^{-5}$ M) was cumulatively added. The relaxation was expressed as a percentage of the maximum phenylephrine (10^{-6} M) response. When indicated, SOD was added just before the addition of DEP. For the measurement of endothelium-independent relaxation, the endothelium was removed by gently rubbing the preparation with the side of a needle.

Data analysis

Results are expressed as means±S.E. for the number of rings indicated. For statistical evaluation, the data were analyzed by repeated two-way analysis of variance.

RESULTS

Generation of superoxide anion ($O_2^{\cdot -}$) by DEP-incubation

$O_2^{\cdot -}$ released from DEP during incubation was measured by cytochrome c reduction (Fig. 1). The DEP suspension (1, 10 and 100 μg/ml) was incubated for 1 hr with cytochrome c at 37℃, and $O_2^{\cdot -}$ generation was calculated by the difference between cytochrome c levels in the absence and the presence of SOD (Fig. 1a). As Fig. 1b shows, increasing the SOD-concentration to more than 20 U/ml did not affect the result. Cytochrome c reduction by DEP was inhibited by approx. 50% in the presence of 20 U/ml of SOD, suggesting that approx. 50% of the cytochrome c reduction by DEP was independent of $O_2^{\cdot -}$. $O_2^{\cdot -}$-production from DEP (1, 10 and 100 μg/ml) was 0.29, 0.73 and 4.51 nmol/hr, respectively, in this system.

The effect of DEP on ACh-induced relaxation

Figure 2 shows the effect of DEP on ACh-induced relaxation in the ring preparation of rat thoracic aorta. The ring preparation was precontracted with a submaximal concentration of phenylephrine (10^{-6} M) that induced a contraction 80% of the maximum and relaxed with cu-

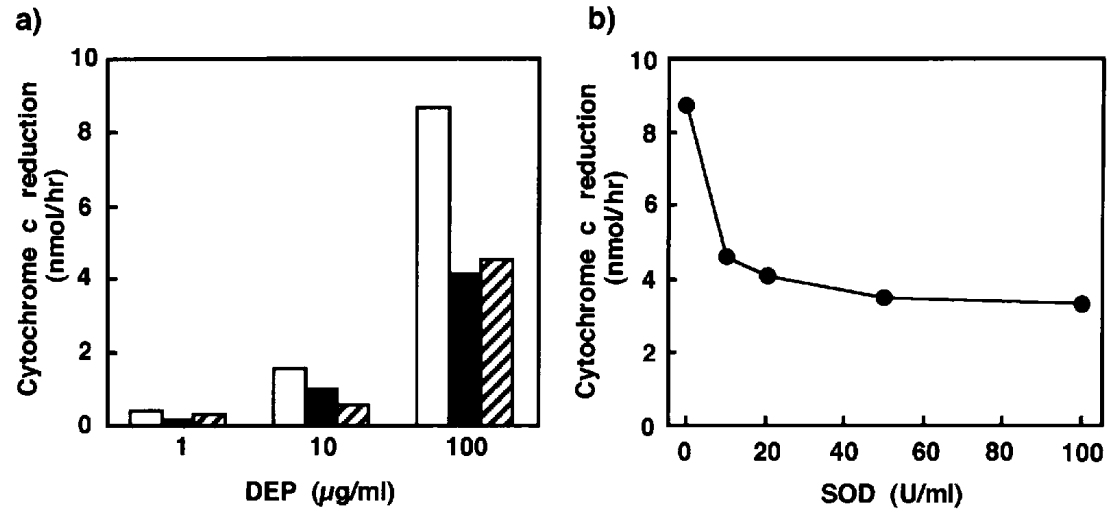

Fig. 1. Cytochrome c reduction by diesel exhaust particles (DEP) and the effect of superoxide dismutase (SOD). a) Cytochrome c reduction by varying concentrations of DEP. DEP suspension (1 – 100 μg/ml in phosphate-buffered saline containing 0.05% Tween 80) was incubated for 60 min with 40 μM cytochrome c in the presence (■) and the absence (□) of SOD (20 U/ml) at 37℃. Cytochrome c reduction was measured by the difference of the absorbances at 540 nm and 550 nm. Superoxide production (▨) was calculated by subtracting values with SOD from those without SOD. Each bar indicates the mean of triplicate determination. b) Cytochrome c reduction by DEP (100 μg/ml) as a function of SOD concentration. DEP suspension (100 μg/ml) was incubated with varying concentrations of SOD as above. Each point indicates mean of duplicate determination.

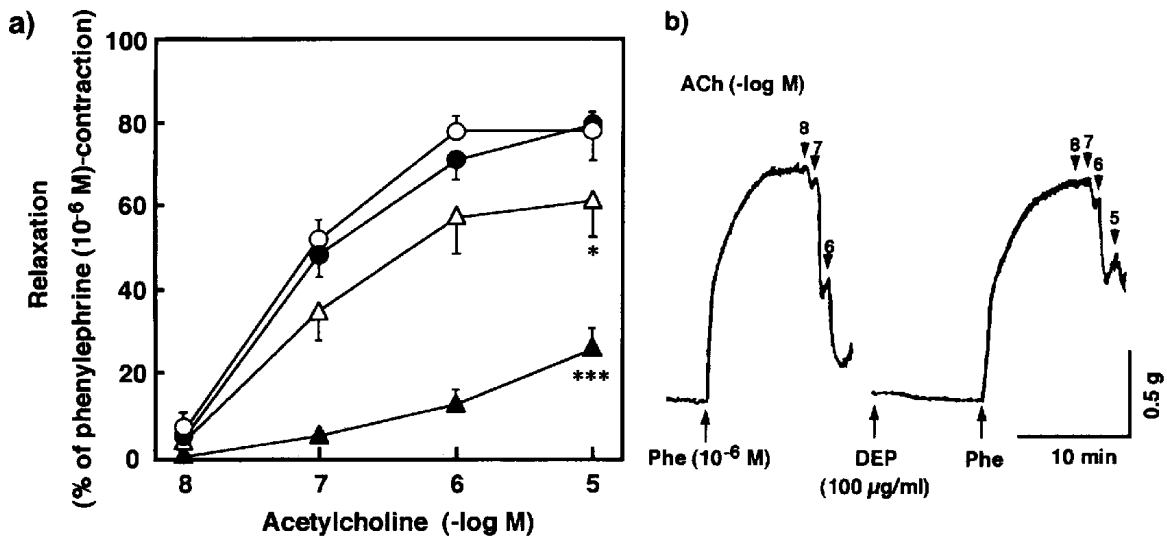

Fig. 2. Impairment of acetylcholine (ACh)-induced endothelium dependent relaxation by exposure to diesel exhaust particles (DEP) in rat thoracic aorta. The ring preparation of rat thoracic aorta was mounted in an organ chamber filled with Tyrode's solution. Following a 90-min equilibration, the resting tension was adjusted to 2 g. They were preincubated for 10 min with (●, 1 μg; △, 10 μg; ▲, 100 μg DEP/ml) or without DEP (○). After precontraction with phenylephrine (Phe, 10^{-6} M), ACh (10^{-8} – 10^{-5} M) was added cumulatively. a) ACh-induced endothelium-dependent relaxation. Relaxation was expressed as a percentage of the Phe (10^{-6} M) contraction. Each point and vertical bar indicate the mean ± S.E. (○: n=16, ●: n=8, △: n=10, ▲: n=10). Significance: *$P<0.05$, ***$P<0.001$, by ANOVA vs without DEP. b) Typical tracings.

mulative additions of ACh (10^{-8} - 10^{-5} M). ACh induced dose-dependent relaxation in the endothelium-intact ring, but not in the endothelium-denuded preparation (data not shown). Preincubation (37℃, 10 min) of aortic rings with DEP suspension did not influence ACh-induced relaxation at the concentration of 1 μg/ml, whereas DEP at concentrations of 10 and 100 μg/ml significantly inhibited the relaxation. ACh (10^{-6} M)-induced relaxation was reduced by 28% (DEP 10 μg/ml) and by 85% (DEP 100 μg/ml).

Effects of SOD on attenuation of ACh-induced relaxation by DEP

SOD reportedly prolongs the half life of EDRF (22, 23) by scavenging $O_2^{\cdot -}$. Therefore, effects of SOD were examined to see whether the attenuation of endothelium-dependent relaxation by DEP is mediated by $O_2^{\cdot -}$ production from DEP. An addition of SOD (20 U/ml) prior to DEP-preincubation completely abolished the inhibitory effect of DEP at a concentration of 10 μg/ml (Fig. 3a), while SOD blocked it partially (30-40%) at a DEP concentration of 100 μg/ml (Fig. 3b).

Effects of DEP-washing

DEP was washed in Tyrode's solution by centrifugation to examine whether inhibitory factors on endothelium-dependent relaxation were removable by washing. Formation of reduced cytochrome c from washed-DEP was detected only slightly; DEP (10 μg/ml) reduced cytochrome c (1.73 nmol/hr) before washing, while the DEP after washing reduced cytochrome c (0.001 nmol/hr). Likewise, cytochrome reduction (nmol/hr) by DEP (100 μg/ml) was 8.61 before washing and 0.004 after washing. Inhibitory effects of DEP and washed DEP on ACh-induced endothelium-dependent relaxation was compared in Fig. 4. At a low concentration (10 μg/ml) of DEP (Fig. 4a), attenuation of endothelium-dependent relaxation by DEP was not observed after washing. At a higher concentration of DEP (100 μg/ml), the inhibition was only partly reversed by washing (Fig. 4b).

Effects of DEP on phenylephrine-induced contraction

To determine whether the attenuation of ACh-induced relaxation results only from the impairment of the endothelium or together with that of smooth muscle, phenylephrine-induced contraction was examined in aortic rings treated with various concentrations of DEP for 10 min (Table 1). Treatment of aortic rings with DEP (1 and 10 μg/ml) did not significantly affect the contractile response to phenylephrine (10^{-6} M), but DEP (100 μg/ml) reduced it by approx. 23%. An addition of SOD (20 U/ml) significantly reversed the inhibitory effect of DEP (100 μg/ml) by approx. 15%. The effects of SOD was also significant at three tested concentrations of DEP

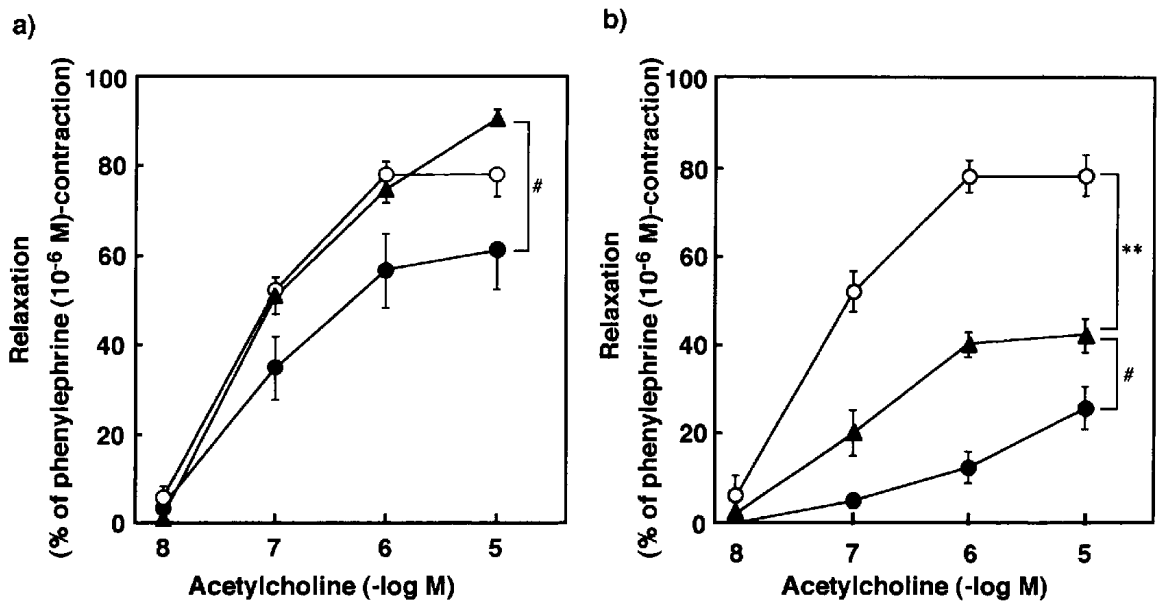

Fig. 3. Effects of superoxide dismutase (SOD) on the impairment of endothelium dependent relaxation by diesel exhaust particles (DEP). The ring preparation of rat thoracic aorta mounted in an organ chamber, as described in the legend to Fig. 2, was preincubated for 10 min with DEP (a: 10 μg/ml, b: 100 μg/ml) and without it. SOD (20 U/ml) was added 20 sec prior to the addition of DEP. Acetylcholine-induced relaxation was measured as described in the legend to Fig. 2. ●: DEP 10 μg/ml in panel a, 100 μg/ml in panel b, ▲: DEP and SOD (20 U/ml), ○: without DEP and SOD. Each point and vertical bar indicate a mean±S.E. (○: n=16, ▲: n=8, ●: n=10). Significance: **$P<0.01$ SOD vs without DEP, #$P<0.05$ DEP vs SOD by ANOVA.

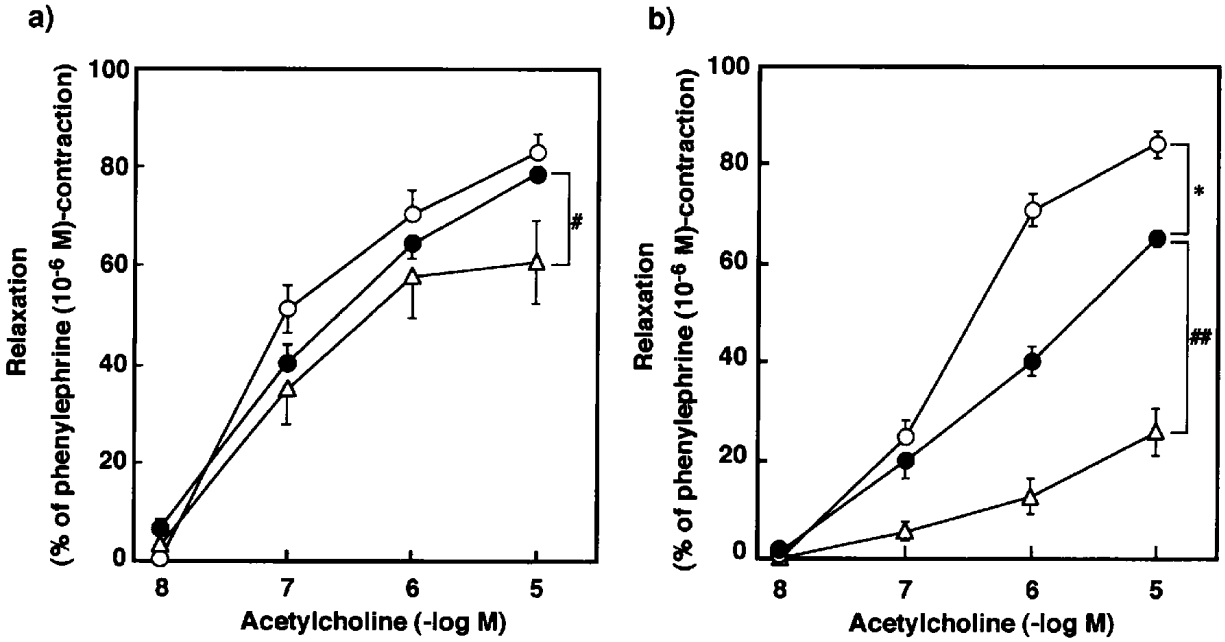

Fig. 4. Comparative effects of diesel exhaust particles (DEP) and washed DEP on acetylcholine (ACh)-induced endothelium dependent relaxation in rat thoracic aorta. The ring preparation of rat thoracic aorta was preincubated for 10 min with DEP, 10 μg/ml in panel a, 100 μg/ml in panel b or without it. DEP were washed 3 times in Tyrode's solution by centrifugation (1,500 × *g*, 5 min). ACh-induced endothelium-dependent relaxation was measured as described in the legend to Fig. 2. Each point and vertical bar indicate a mean ± S.E. △: DEP (n = 6), ●: washed DEP (n = 6), ○: without DEP (n = 10). Significance: $^{*}P < 0.05$ washed DEP vs without DEP; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ washed DEP vs DEP by ANOVA.

by ANOVA.

The effect of DEP on endothelium-independent relaxation

The effect of DEP on endothelium-independent relaxation was examined (Fig. 5). Endothelium-denuded rings were contracted with phenylephrine (10^{-6} M) and relaxed with cumulative additions of sodium nitroprusside which activates guanylate cyclase in smooth muscle cells by releasing nitric oxide (24). Although DEP (10 μg/ml) did not affect the relaxation, DEP (100 μg/ml) slightly but significantly inhibited sodium nitroprusside-induced relaxation.

DISCUSSION

Furchgott and Zawadzki first demonstrated that the endothelium releases EDRF (12), which was later identified

Table 1. Effects of diesel exhaust particles (DEP) on phenylephrine-induced contraction in rat aortic rings

DEP (μg/ml)	% contraction [as % of phenylephrine contraction (10^{-6} M)]	
	without SOD	with SOD (20 U/ml)
	mean ± S.E. (n = 22)	
1	94.1 ± 3.01	97.2 ± 1.12
10	91.3 ± 2.82	95.6 ± 3.21
100	77.3 ± 2.12	92.2 ± 0.98

The ring preparation of rat thoracic aorta was mounted in an organ bath. Rings were exposed to the DEP suspension in Tyrode's solution for 10 min in the presence or the absence of superoxide dismutase (SOD) at 20 U/ml, and then phenylephrine (10^{-6} M) was added to induce contraction. The tension developed by phenylephrine in the absence of DEP was 1.10 ± 0.10 g without SOD and 0.95 ± 0.15 g with SOD. Significance: $P < 0.05$ with SOD vs without at 3 concentrations of DEP by ANOVA.

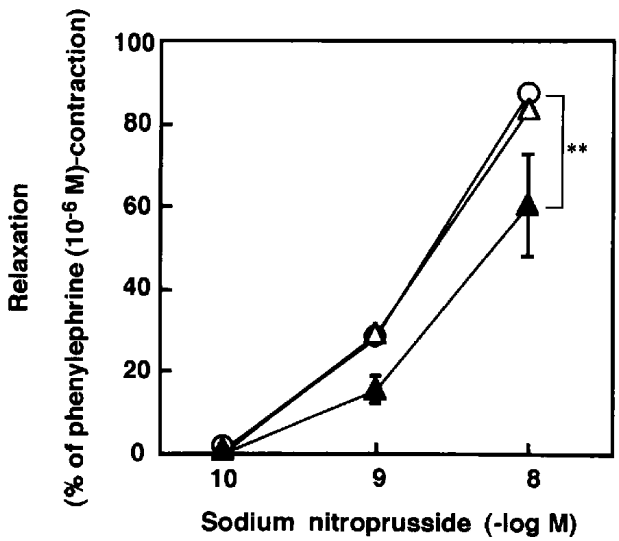

Fig. 5. Effect of diesel exhaust particles (DEP) on sodium nitroprusside-induced relaxation in endothelium-denuded aortic ring. Endothelium denuded ring preparations of rat thoracic aorta were preincubated for 10 min with DEP (△: 10 μg, ▲: 100 μg/ml) or without it (○). Sodium nitroprusside-induced relaxation was measured in phenylephrine (10^{-6} M)-precontracted rings. Relaxation was expressed as a percentage of the maximum phenylephrine (10^{-6} M) contraction. Each point and vertical bar indicate a mean ± S.E. (n = 6). Significance: **P < 0.01 by ANOVA, DEP (100 μg/ml) vs without DEP.

as nitric oxide by Palmer et al. (8). It is now known that nitric oxide is generated from the terminal guanido nitrogen atom of L-arginine by nitric oxide synthase, as demonstrated in various cells, and plays physiologically and pathophysiologically important roles in the cardiovascular system, immune system and central nervous system (25). Endogenous nitric oxide is present in exhaled air from animals and humans (26). Inhaled nitric oxide gas reverses pulmonary hypertension (27), reverses pulmonary vasoconstriction (28), improves lung function in affected patients and increases ventilation-perfusion ratios (29). In addition, nitric oxide inhibits platelet aggregation (13–15), platelet (30, 31) and leukocyte (16–18) adhesion on endothelial cells and vascular albumin leakage (19, 20). Thus, nitric oxide seems to contribute greatly to maintaining physiological functions in the lung.

In our study, rat thoracic aortic rings were directly exposed to DEP suspension at concentrations of 10 and 100 μg/ml. Ten-minute exposure of aortic ring to DEP at both concentrations greatly attenuated ACh-induced endothelium-dependent relaxation in aorta precontracted with phenylephrine, and the inhibition of ACh-induced relaxation by DEP (10 μg/ml) was completely abolished by an addition of SOD. Nitric oxide released from the endothelium is assumed to be a main factor responsible for endothelium-dependent relaxation (8), and it is rapidly converted to nitrite and nitrate in oxygenated solution (32). Since nitric oxide is known to be destroyed by $O_2^{\cdot -}$, but not by other oxygen derived radicals (22), $O_2^{\cdot -}$ produced from DEP seems to be the causative factor at the lower concentrations of DEP. Nitric oxide reacts with $O_2^{\cdot -}$ to produce peroxynitrite (33, 34), leading to endothelial cell damage (35). Attenuation of endothelium-dependent relaxation by DEP might, therefore, result from inactivation of EDRF, and/or from inhibition of nitric oxide synthase in endothelial cells by oxygen radicals produced during DEP-incubation. Since DEP (less than 10 μg/ml) neither affected phenylephrine contraction nor endothelium-independent relaxation by sodium nitroprusside that releases nitric oxide and activates guanylate cyclase in smooth muscle cells (24), DEP seem not to interfere with smooth muscle functions at these concentration levels.

Attenuation of endothelium-dependent relaxation by DEP (100 μg/ml) was only partially reversed even in the presence of sufficient amounts of SOD. DEP (100 μg/ml) also significantly inhibited phenylephrine-induced contraction in intact rings and reduced endothelium-independent relaxation by sodium nitroprusside in denuded rings. At higher concentrations, therefore, DEP impair functions both in endothelial cells and smooth muscle cells not only by $O_2^{\cdot -}$ but also by some other factors.

The alveolar capillary endothelium lies close to the alveolar epithelium (7), and inhaled DEP may easily interact with the vascular endothelium and blood by releasing reactive oxygens and some soluble materials. Japanese environmental quality standards limit the amount of suspended particulate matter (SPM) of less than 10 μm to 0.1 mg/m^3 in air. DEP with an average size of 0.4 μm makes up 20–40% of the SPM depending on the season. If an individual inhales 10 m^3 of air with 0.1 mg SPM/m^3 in a day, the amount of SPM accumulated in the lung accounts for 1 mg. When the rat weighing 380 g inhales 215 ml air/min (36), the rat lung would take up approx. 30 μg SPM in a day. Thus approx. 30 ml blood in a rat is estimated to exposed to 30 μg SPM by one day inhalation. In this context, DEP at 1–10 μg/ml would be more practical concentrations than DEP at 100 μg/ml. It is therefore concluded that 1) inhaled DEP may induce pulmonary inflammation through an impairment of the endothelial functions and/or the effect of nitric oxide and 2) SOD effectively reduces the adverse effects.

Acknowledgments

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