Motorcycle Exhaust Particulates Enhance Vasoconstriction in Organ Culture of Rat Aortas and Involve Reactive Oxygen Species

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The effects of motorcycle exhaust particulate on vasoconstriction were determined using rat thoracic aortas under organ culture conditions treated with organic extracts of motorcycle exhaust particulate from a two-stroke engine. The motorcycle exhaust particulate extract (MEPE) induced a concentration-dependent enhancement of vasoconstriction elicited by phenylephrine in the organ cultures of both intact and endothelium-denuded aortas for 18 h. Nifedipine (an L-type Ca\(^{2+}\) channel blocker), manganese acetate (an inorganic Ca\(^{2+}\) channel blocker), and staurosporine (a nonselective protein kinase C inhibitor), but not the selective protein kinase C inhibitor chelerythrine, inhibited the enhancement of vasoconstriction by MEPE. Staurosporine has also been reported as a myosin light chain kinase (MLCK) inhibitor, so we tested whether the MLCK pathway was involved in the effect of MEPE. The results showed that ML-9 (a selective MLCK inhibitor) could inhibit the enhancement of vasoconstriction by MEPE. The phosphorylation of a 20-kDa myosin light chain in a primary culture of rat vascular smooth muscle cells was also enhanced by MEPE. Moreover, we also examined the role of reactive oxygen species (ROS) in the stimulatory effect of MEPE on vasoconstriction. The antioxidant N-acetylcysteine significantly inhibited the enhancement of vasoconstriction by MEPE. A time-dependent increase in ROS production by MEPE was also detected in primary cultures of vascular smooth muscle cells. These results indicate that MEPE induces a marked enhancement of vasoconstriction in aortas under organ culture conditions and imply that a ROS–Ca\(^{2+}\)–MLCK pathway may be involved in this MEPE-induced response.

Key Words: motorcycle exhaust particulate extract; aorta; vascular smooth muscle cell; myosin light chain kinase; reactive oxygen species.

Air pollution has become a more and more serious problem in many modern cities. Motorcycle and car exhausts are the major sources of air pollution in many congested cities in Taiwan, and such heavy motorcycle density has created a major environmental burden for the urban areas (Hricko, 1994). According to a report published by the Ministry of Transportation and Communication in Taiwan, the number of registered motorcycles in 2002 was 11,983,757 and the motorcycle density was 331.51/km\(^2\), which was about twice as much as the density of cars. The use of motorcycles, especially those with a two-stroke engine, introduced about 16,000 and 15,000 tons of total suspended particle and particulate matter of 10 \(\mu\)m, respectively, per year in Taiwan (Environmental Protection Agency, R.O.C., 2000). The wide use of motorcycles in transportation provides a significant opportunity for environmental exposure to these emissions, especially in commuting hours. Jemma et al. (1995) have indicated that there were more than 100 C1–C16 hydrocarbons in the exhaust of a two-stroke engine, and the levels of xylene, toluene, and benzene in the motorcycle engine exhaust were higher than the respective levels in car engine exhaust. A study has also shown that the organic extracts of motorcycle exhaust particulates (MEPE) contained high concentrations of polycyclic aromatic hydrocarbons (PAH) (Jeng and Peng, 1997), similar to those reported from diesel engine emissions. Diesel exhaust particulates (DEP) contain more than 450 different organic compounds, including xenobiotics such as PAH and redox-active quinines (Casellas et al., 1995; Li et al., 1996). DEP or DEP extracts have been shown to affect epithelial, endothelial, eosinophil, B-cell, and macrophage function (Bai et al., 2001; Nel et al., 1998; Terada et al., 1999). Nevertheless, unlike automobile exhaust, there are few studies on the impact of motorcycle exhaust on the environment and their biological effects. Previous studies have shown that MEPE were mutagenic (Zhou and Ye, 1997) and genotoxic \textit{in vitro} (Kuo et al., 1998) and neurotoxic \textit{in vivo} (Liu et al., 2002). It has also been shown that MEPE is capable of inducing several metabolic enzymes \textit{in vitro} and \textit{in vivo} (Ueng et al., 1998; 2000). In addition, an \textit{in vitro} study has shown that the endothelium-dependent relaxation in rat aorta was impaired 10 min after the treatment of MEPE (Cheng et al., 1999). However, the effect of longer...
exposure of MEPE on the vasoconstriction is still unknown. There are also no studies that have been done to examine the direct effect of MEPE on the vascular smooth muscle cells.

The major regulatory mechanism of smooth muscle contraction and relaxation is phosphorylation and dephosphorylation of the 20-kDa myosin light chain (MLC_{20}) (Allen et al., 1994). MLC_{20} is phosphorylated by the Ca^{2+}-calmodulin (CaM)–activated myosin light chain kinase (MLCK) and dephosphorylated by the Ca^{2+}-independent myosin light chain phosphatase (MLCP). Thus, a rise in cytosolic Ca^{2+} concentration ([Ca^{2+}]_{i}) produces smooth muscle contraction by binding to calmodulin, the activation of MLCK, and the subsequent phosphorylation of MLC_{20} (Fukata et al., 2001; Wilson et al., 2002). The Ca^{2+} influx plays a major role in maintaining the sustained [Ca^{2+}]_{i} elevation during contractions. The L-type Ca^{2+} channel is considered to be a major Ca^{2+} influx pathway and is responsible for vascular smooth muscle contractility (Karaki et al., 1997; Triggle et al., 1998). Furthermore, reactive oxygen species (ROS) may also participate in the regulation of vascular tone (Suzuki and Ford, 1999). It has been indicated that ROS generated by different compounds or systems could induce vasoconstriction in various arteries such as rat aortas (Peters et al., 2000; Shen et al., 2000), porcine coronary arteries (Grover et al., 1999), rabbit carotid arteries (Heinle, 1984), rat and porcine pulmonary arteries (Jin et al., 1997; Sanderud et al., 1991), canine basilar arteries (Tosaka et al., 2002), and human umbilical arteries (Okatani et al., 1997). These studies provide evidence that H_{2}O_{2} or other ROS may be the vasoactive mediators in arteries by acting as second messengers. A recent report further suggested that ROS generated by mitochondria appear to function as second messengers during hypoxia and act to trigger the Ca^{2+}-signaling process responsible for the contraction of pulmonary arterial myocytes during hypoxia (Waypa et al., 2002). Therefore, the present study was performed to investigate the effect of MEPE on the contractile response in isolated rat aortas, to examine whether the Ca^{2+}–MLCK pathway is involved, and to determine the role of ROS in the MEPE-induced response.

MATERIALS AND METHODS

Preparation of motorcycle exhausts particulates extract. MEPE was prepared according to the method described by Ueng et al. (1998). In brief, motorcycle exhaust particulates were collected from the 0.5-mq quartz fiber filters of a Yamaha motorcycle with a 50-cm³ two-stroke engine using 95% octane unleaded gasoline. The sampling apparatus consisted of, in sequence, a 30-cm long by 2.2-cm diameter stainless dilution tube, a filter holder, and a vacuum pump. The engine was running at 150 rpm on an empty load, and the pump was set at a flow rate of 20 l/min to collect the motorcycle exhaust particulates for 30 min twice daily. The extraction of motorcycle exhaust particulates was carried out in the dark for 24 h using a Soxhlet extraction method (solvent system: dichloromethane:hexane [1:1]) (Escobal et al., 1997). The crude MEPE was vacuum-evaporated to dryness and kept desiccated at –20°C.

Aorta culture. Male Wistar rats (200–250 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University, Taipei, Taiwan. The Animal Research Committee of National Taiwan University, College of Medicine, conducted the study in accordance with the guidelines for the care and use of laboratory animals. The rats were anesthetized with sodium pentobarbital, and the thoracic aorta was removed, cleaned of fat and adventitia, and cut into ring segments of 4–5 mm length with parallel rings. When necessary, the endothelium was carefully removed by gentle rubbing of the luminal surface with a cotton-tipped applicator. These isolated aortic rings were cultured in an organ culture petri dish (Falcon) with sterile Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum and 1% antibiotic solution at 37°C. After 18 h, the rings were prepared for organ bath study. MEPE were dissolved in dimethylsulfoxide oxide (DMSO) and added to the medium. The concentration of DMSO in the medium was less than 0.1%.

Organ bath study. The vasoconstriction of aorta rings was measured by the method described by Liu et al. (1999). The aorta rings were suspended between two hooks connected to a transducer (Grass FT.03) for the measurement of isometric force. The rings were suspended in 10-m1 organ baths containing oxygenated (95% O_{2} + 5% CO_{2}) and warmed (37°C) Krebs solution containing (composition in mM) NaCl 118.3, KCl 4.7, CaCl_{2}, 2.5, KH_{2}PO_{4}, 1.2, MgSO_{4}, 1.2, NaHCO_{3}, 25.0, and glucose 11.1. The pH of the Krebs solution was 7.2–7.4. The basal tension was set at 1.0 g. The rings were allowed to equilibrate for 1 h before a concentration–response curve to phenylephrine (0.003–10 μM) was obtained. The tension was recorded by an isometric transducer (Grass FT.03) on a Biopac’s MP 100 data acquisition system with analytic software (AcqKnowledge, Version 3.0, Biopac Systems Inc., Ste E Goleta, CA), the outputs of which were written on an HP deskjet 500C printer. In some experiments, inhibitors were added simultaneously with the MEPE treatment. The absence of endothelium was confirmed by lack of a response to acetylcholine.

Primary vascular smooth muscle cell culture. Vascular smooth muscle cells (VSMCs) were obtained from the thoracic aortas of Wistar rats by the method described by Bierman et al. (1974). In brief, male rats (200–250 g) were sacrificed and the thoracic aortas were removed, cleaned of fat and adventitia, cut into small strips, and then incubated in 1 mg/ml collagenase and 0.125 mg/ml elastase at 37°C for 60 min. The cells were seeded into 10-mm diameter dishes and maintained in 10 ml of DMEM containing 10% fetal bovine serum at 37°C. The cells were used between the third and sixth passages. The cells exhibited characteristics of VSMCs. The cells were grown to 60 to 80% confluence, at which time they were rendered quiescent by the DMEM medium containing 0.1% bovine serum albumin (BSA) and maintained for 48 h before experimentation.

Measurement of MLC_{20} phosphorylation. The extent of MLC phosphorylation in VSMCs was determined using the urea-glycerol gel electrophoresis technique (Persechini et al., 1986), followed by immunoblot detection with a specific mouse monoclonal anti-MLC antibody (Sigma). In brief, the VSMCs were transferred to a denaturation solution containing 10% trichloroacetic acid in acetone and 10-mM dithiothreitol pre-chilled at –80°C. The VSMCs were then washed three times extensively and stored in acetone containing 10-mM dithiothreitol at –80°C. After the VSMCs were dried to remove the acetone, they were extracted in the sample buffer (8-M urea, Tris [hydroxy-methyl] aminomethane 20 mM, glycine 23 mM, 0.004% bromophenol blue and dithiothreitol 10 mM) at room temperature for 2 h. The supernatant was subjected to electrophoresis on 10% polyacrylamide gel containing 40% glycerol, followed by transfer onto a nitrocellulose membrane. The 20-kDa MLC, both unphosphorylated and phosphorylated, was detected by a specific antibody (1:3000) and a horse radish peroxidase-conjugated secondary antibody (1:7500). An immune complex was detected using an enhanced chemiluminescence technique (Amersham, Buckinghamshire, UK).

Detection of intracellular ROS. Intracellular ROS generation was monitored by flow cytometry using a peroxide-sensitive fluorescent probe (2′,7′-
dichlorofluorescin diacetate (DCFH-DA), Molecular Probes, Eugene, OR) as described in Lund-Johansen and Oluweus (1992). In brief, the experiments were performed under dim light. Subconfluent and serum-deprived VSMCs were loaded with 10 μM of DCFH-DA for 30 min after the treatment of MEPE and then chilled on ice and washed with cold PBS. The washed cells were detached from the culture plates by trypsin digestion. The fluorescent intensities for samples of 10,000 cells each was analyzed by flow cytometry with the use of a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

**Statistics.** The values given are as mean ± SEM. The significance of difference from the respective controls for each experimental test condition was assessed by using one-way analysis of variance followed by Dunnett’s test for each paired experiment. Patterns of vasoconstriction were analyzed by two-way ANOVA with repeated measurements. P values < 0.05 were regarded as indicating significant differences.

**RESULTS**

**MEPE Enhance Phenylephrine-Elicited Vasoconstriction in Isolated Aortic Rings**

In intact endothelium aortic rings, phenylephrine caused a concentration-dependent increase in contraction (Fig. 1). Incubation with MEPE (1–100 μg/ml) for 18 h enhanced the maximal contractile response elicited by phenylephrine in a concentration-dependent manner (Fig. 1A). In endothelium-denuded rat aortic rings, phenylephrine also triggered a dose-dependent increase in contractions. Treatment with MEPE (10 μg/ml) for 18 h caused an increase in contractions (Fig. 1B). These results indicate that the stimulatory effect of MEPE on aortic contractile response may be endothelium-independent. MEPE by itself did not affect the basal tension of the aorta in these experimental conditions.

To test the role of Ca\(^{2+}\) in the stimulatory effect of MEPE, the Ca\(^{2+}\) channel blockers nifedipine and manganese acetate were used. Co-incubation with nifedipine (10 μM) or manganese acetate (100 μM) reversed the MEPE-induced changes in response to phenylephrine (Fig. 2). These results indicate that the stimulatory effect of MEPE on the aorta was dependent on Ca\(^{2+}\) influx.

It has been shown that Ca\(^{2+}\) may sensitize the contractile apparatus of smooth muscle by activating protein kinase C (PKC) (Morgan and Leinweber, 1998), so we further investigated whether PKC was involved in the MEPE-induced response. As shown in Figure 3, co-incubation with staurosporine (1–2 nM), a nonselective PKC inhibitor, abolished the enhancement of vasoconstriction triggered by MEPE, but chelerythrine (1 μM), a selective PKC inhibitor, failed to affect the MEPE-induced response.

**Role of MLCK in MEPE-Induced Response**

Smooth muscle contraction is activated by phosphorylation of the 20-kDa myosin light chain (MLC\(_{20}\)) by Ca\(^{2+}\)/calmodulin–dependent MLCK (Fukata et al., 2001; Wilson et al., 2002). It has been reported that staurosporine acted as an MLCK inhibitor at IC\(_{50}\) 1.3 nM (Tamaoki et al., 1986). Therefore, we next investigated the effect of MEPE on MLCK activation. In the presence of ML-9 (5 μM), a selective MLCK inhibitor, MEPE failed to enhance the phenylephrine-elicted vasoconstriction in organ cultures of rat aortas (Fig. 4A). To further identify the change of MLCK activation by MEPE, we observed the 20-kDa myosin light chain (MLC\(_{20}\)) phosphorylation state in primary vascular smooth muscle cells (VSMCs) using the method of urea/glycerol PAGE. In response to MEPE stimulation, a rapid increase in the level of both monophosphorylated and diphosphorylated forms of MLC\(_{20}\) was ob-
served within 5–10 min, and in the presence of ML-9, the phosphorylation was decreased to the control level (Fig. 4B). Taken together, these results demonstrated that MEPE-enhanced vasoconstriction is MLCK-dependent.

Role of ROS in MEPE-Induced Response

It has been reported that MEPE was capable of inducing the oxidative stress in many cell systems (Kuo et al., 1998). Therefore, we were interested in seeing if ROS is also participated in MEPE-enhanced vasoconstriction. Co-incubation with antioxidant N-acetyl-L-cysteine (NAC, 0.5, and 2 mM) significantly inhibited MEPE-enhanced vasoconstriction in organ cultures of rat aortas (Fig. 5). No significant difference in the contractile pattern was found between the control group and NAC alone (data not shown). Next, the induction of ROS production by MEPE in VSMCs was measured with the fluorescent dye DCFH-DA. We found that MEPE induced the generation of DCF-sensitive ROS in VSMCs in a dose-dependent manner (Fig. 6A). MEPE (10 μg/ml) induced an increase of DCF-sensitive ROS generation as early as 30 min, gradually increased up to 3 h, and declined to a basal level around at 12 h (Fig. 6B).

DISCUSSION

The present study demonstrated that MEPE enhanced the vasoconstriction response to phenylephrine in organ cultures of both endothelium-intact and -denuded aortic rings. It suggested that the stimulatory effect of MEPE might not be endothelium-dependent, but instead might be related to vascular smooth muscle. Previous studies have shown that the acetylcholine-induced endothelium-dependent vasorelaxation in rat aorta rings was impaired 10 min after the exposure of both MEPE (Cheng and Kang, 1999) and DEP (Ikeda et al., 1995). Bai et al. (2001) have reported that DEP extract could induce the cytotoxic effects in human pulmonary artery endothelial cells in vitro (Bai et al., 2001). Nevertheless, Cheng and Kang (1999) have also found that the phenylephrine-elicted endothelium-independent vasoconstriction could be reduced 10 min after the treatment of MEPE. However, in the present study, a longer exposure (18 h) of MEPE in organ cultures of aortas...
enhanced the vasoconstriction elicited by phenylephrine (endothelium-independent). These results indicate that the organic constituents of particulate emissions can be distributed into both the endothelium and the vascular smooth muscles. The responses elicited by MEPE on the vascular smooth muscles seem to be different according to the time of exposure. The significant concentrations producing the enhancement of phenylephrine-elicited vasoconstriction by MEPE in our data (dosage range: 1–100 μg/ml; EC50 is about 10 μg/ml) are lower than the MEPE-induced vasorelaxation in the previous report (dosage range: 10–1000 μg/ml; EC50 is about 50 μg/ml; Cheng and Kang, 1999).

The primary signal for smooth muscle contraction is an increase in sarcoplasmic free Ca²⁺ concentration [Ca²⁺]i. This triggers activation of Ca²⁺-dependent myosin light chain kinase. In smooth muscle, an L-type Ca²⁺ channel is considered to be a major Ca²⁺ influx pathway (Karaki et al., 1997). The L-type Ca²⁺ channel is responsible for normal myocardial contractility and vascular smooth muscle contractility (Triggle 1998). In the present study, we found that manganese and L-type Ca²⁺ channel blocker nifedipine significantly suppressed MEPE-enhanced vasoconstriction in organ cultures of rat aortas. Therefore, the mechanism of MEPE-induced enhancement of vasoconstriction may be related to the alteration in the transmembranous influx of Ca²⁺. However, although inhibiting a physiological mediator may block the response, this alone does not prove that the test agent is operating through that particulate physiological mediator; it just proves that the mediator is required for the response. Therefore, the direct correlation between the increase of Ca²⁺ influx and vasoconstriction in the presence of MEPE needs further investigation. An increase in [Ca²⁺], saturates the four Ca²⁺-binding sites of CaM, which then binds to and activates actin-bound MLCK. The activated the MLCK phosphorylates myosin light chain, and then phosphorylated myosin interacted with actin to induce the contractile response (Karaki et al., 1997; Wilson et al., 2002). Moreover, it has also been suggested that PKC could increase Ca²⁺ sensitivity through the inhibition of MLC phosphatase in smooth muscles (Karaki et al., 1997; Morgan and Leinweber, 1998). Our present study showed that staurosporine, a nonselective PKC inhibitor, could inhibit the MEPE-enhanced vasoconstriction; however, chelerythrine, a potent and selective PKC inhibitor (Herbert et al., 1990), failed to abolish the stimulatory effect of MEPE. These results imply

FIG. 4. Effect of MLCK inhibitor on MEPE-enhanced vasoconstriction and MLC, phosphorylation. (A) Aortic rings were incubated with either vehicle (closed circle, control), MEPE (10 μg/ml, open circle), or MLCK inhibitor ML-9 (5 μM, closed triangle) plus MEPE for 18 h, and then PE was added cumulatively to induce the contractile response. Each point represents mean ± SEM from four to six separate experiments. *P < 0.05 as compared with control. #P < 0.05 as compared with MEPE alone. (B) An immunoblot detection of unphosphorylated and phosphorylated MLC. ML-9 (10 μM) was applied 30 min before and during the addition of MEPE in quiescent vascular smooth muscle cells. The cell extracts were subjected to urea/glycerol-PAGE, and immunoblotted with antibody to MLC. MLC: unphosphorylated band; MLC-P: monophosphorylated band; MLC-P2: diphosphorylated band. This is a representative of three separate experiments.

FIG. 5. Effect of antioxidant on MEPE-enhanced vasoconstriction. The aortic rings were incubated with vehicle (closed circle, control), MEPE (10 μg/ml, open circle), or antioxidant N-acetyl-L-cysteine (NAC, 0.5 mM, closed triangle; 2 mM, open triangle) plus MEPE (10 μg/ml) for 18 h, and then PE was added cumulatively to induce the contractile response. Each point represents mean ± SEM from four to six separate experiments. *P < 0.05 as compared with control. #P < 0.05 as compared with MEPE alone.
that a PKC pathway is not involved in the enhancement of vasoconstriction by MEPE. It has been reported that staurosporine could also act as an MLCK inhibitor at IC50 1.3 nM (Tamaoki et al., 1986). Our further results showed that both nonselective MLCK inhibitor staurosporine and selective MLCK inhibitor ML-9 suppress the MEPE-enhanced constriction in rat aortas. We also identified that MEPE was capable of inducing the phosphorylation of MLC20 in cultured vascular smooth muscle cells, which could be blocked in the presence of ML-9. Therefore, these findings indicate that a Ca2+/CaM/MLCK-dependent pathway is involved in the MEPE-enhanced vasoconstriction in aortas.

Increasing evidence suggests that reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are produced by a variety of cell types and may modulate physiological and pathophysiological processes (Schnackenberg, 2002). Recent studies have shown that ROS may function as a second messenger in the activation of transcription factors, gene expression, cell growth, chemotaxis, and apoptosis (Irani, 2000). These ROS also have direct vasocontractile effects on several vessels, such as rat aortas (Peters et al., 2000; Shen et al., 2000; Yang et al., 1998). Some studies have identified that H2O2 was capable of evoking vasoconstriction through a Ca2+-dependent mechanism in rat aortas (Sotnikova, 1998; Yang et al., 1998). A recent report further showed that catalase prevents elevation of [Ca2+], induced by alcohol in cultured canine cerebral vascular smooth muscle cells, which may indicate a possible relationship to alcohol-induced strokes and brain pathology (Li et al., 2003). Waypa et al. (2002) have also suggested that ROS generated by mitochondria appear to function as second messengers during hypoxia and act to trigger the Ca2+-signaling process responsible for the contraction of pulmonary arterial myocytes during hypoxia. It has been demonstrated that DEPs might stimulate ROS generation in macrophage-like cell lines RAW 264.7 and bronchial epithelial cells (Hiura et al., 1999; Li et al., 2002). Indeed, MEPE have also been reported to stimulate ROS generation in various cell systems, including hepatic, pulmonary, and macrophage-like cell lines (Kuo et al., 1998; Lee et al., 2002). In the present study, we found that antioxidant NAC significantly inhibited MEPE-enhanced vasoconstriction in rat aortas, and MEPE could stimulate ROS generation in a time-dependent manner in cultured vascular smooth muscle cells. Therefore, these findings suggest that ROS induced by MEPE may contribute to Ca2+ influx and MLCK activation and subsequently enhance vasoconstriction.

There are more than 100 C1–C16 hydrocarbons including PAHs in motorcycle emissions (Jemma et al., 1995; Ueng et al., 2000). It has been reported that short-term treatment of some PAHs could induce vasorelaxation of rat aortas in an endothelium-dependent manner (Kang and Cheng, 1997). Therefore, there is the need for more work on the chemical characterization of an MEPE sample followed by subsequent bioactivity testing of the primary chemical constituent groups in the future.

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