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Genotoxicity of Motorcycle Exhaust Particles *In Vivo* and *In Vitro*

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We studied the genotoxic potency of motorcycle exhaust particles (MEP) by using a bacterial reversion assay and chromosome aberration and micronucleus tests. In the bacterial reversion assay (Ames test), MEP concentration-dependently increased TA98, TA100, and TA102 revertants in the presence of metabolic-activating enzymes. In the chromosome aberration test, MEP concentration-dependently increased abnormal structural chromosomes in CHO-K1 cells both with and without S9. Pretreatment with antioxidants (α-tocopherol, ascorbate, catalase, and NAC) showed varying degrees of inhibitory effect on the MEP-induced mutagenic effect and chromosome structural abnormalities. In the *in vivo* micronucleus test, MEP dose-dependently induced micronucleus formation in peripheral red blood cells after 24 and 48 h of treatment. The increase of micronucleated reticulocytes induced by MEP was inhibited by pretreatment with α-tocopherol and ascorbate. The fluorescence intensity of DCFH-DA–loaded CHO-K1 cells was increased upon the addition of MEP. Our data suggest that MEP can induce genotoxicity through a reactive oxygen species–(ROS-) dependent pathway, which can be augmented by metabolic activation. Alpha-tocopherol, ascorbate, catalase, and NAC can inhibit MEP-induced genotoxicity, indicating that ROS might be involved in this effect.

Key Words: **motorcycle exhaust particles; Ames test; reactive oxygen species; chromosome aberration; micronucleus; genotoxicity.**

Recent prospective cohort studies have shown that long-term exposures to particulate air pollution might be associated with increases in the rates of morbidity and mortality from respiratory and cardiovascular diseases in the general population (Abbey *et al*., 1995; Dockery *et al*., 1993; Pope *et al*., 1995). In addition to systemic toxicity, the possible genotoxicity of small particulate matter has been investigated in recent years. On metabolism, activation, or accumulation, pollutants can become extremely toxic to vital organs, and this is often related to a strong genotoxic effect (Baulig *et al*., 2003; Harris *et al*., 1978). Airborne particles have been shown to induce chromosome aberrations (Seemayer *et al*., 1994), sister chromatic exchanges (Hornberg *et al*., 1996), the formation of DNA adduct (Gallagher *et al*., 1990), tumorigenicity (Heussen *et al*., 1996), and embryotoxicity (Matsumoto and Kashimoto, 1986). In some industrial cities, the exhaust of diesel vehicles is considered a major source of air pollution that is ''probably carcinogenic'' (Aoki *et al*., 2001; Baeza Squiban *et al*., 1999). Diesel exhaust particles (DEP) are composed of carbon nuclei, absorbed organic compounds, and trace heavy metals including iron and copper (Schuetzle and Lewtas, 1986; Schuetzle *et al*., 1981). The absorbed organic compounds consist of some highly mutagenic chemicals including polycyclic aromatic hydrocarbons (PAHs) and nitroaromatic hydrocarbons (Grimmer *et al*., 1987; Lewtas, 1988) and were shown to cause pulmonary tumors (Mauderly *et al*., 1987).

In Taiwan, motorcycles are widely used; more than 11 million motorcycles were registered in 2000 (Ministry of Transportation and Communications, 2000). The use of motorcycles, especially those with two-stroke engines, introduce about 16,000 and 15,000 tons of total suspended particles and particulate matter of 10 μm (PM10), respectively, per year in Taiwan (Environment Protection Agency, 2000). However, the impact of motorcycle exhaust on the environment and its biological effects are relatively unknown. Motorcycle exhaust particles (MEP) have been found to impair endothelium-dependent relaxation in the rat aorta (Cheng and Kang, 1999) with short-term treatment (10 min), enhance vasoconstriction in rat smooth muscle cells with long-term treatment (18 h; Tzeng *et al*., 2003), induce apoptosis in macrophages (Lee and Kang, 2002), and affect metabolic enzyme activities in rat tissues (Ueng *et al*., 1998). Also, particles collected from scooter or motorcycle exhaust were shown to cause a certain degree of DNA damage (Kuo *et al*., 1998; Zhou and Ye, 1997), similar to that reported with DEP (Casellas *et al*., 1995; Li *et al*., 1996).

Although both DEP and MEP come from vehicles' combustion, there might be some difference between the chemical components in the exhausts due to the different engine structures and the fuel used. For example, many motorcycles use two-stroke engines, which require the mixing of lubricant with the fuel. It has been shown that the addition of lubricant will lead to the incomplete combustion and mass exhaust of submicrometer carbonaceous particles (Lewtas, 1983). Chemical analysis studies demonstrated that MEP contained higher levels of benzene, xylene, and toluene than did gasoline and diesel engine exhausts (Chan *et al*., 1993; Jemma *et al*., 1995). Although both DEP and

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MEP contain PAHs, their compositions are different; the major PAH components found in DEP are phenanthrenes (Barfknecht *et al*., 1982). Therefore, it is possible that DEP and MEP might exert different biological effects.

The aim of this study was to contribute to a better understanding of the genotoxic effect of MEP and the possible involvement of reactive oxygen species (ROS) in the induction of genotoxicity using the short-term *in vitro* mutagenicity bioassay (Ames Salmonella assay), the chromosome aberration test with CHO mammalian cells, and the *in vivo* rodent micronucleus test. We found that MEP contains chemicals that can induce genotoxicity both *in vitro* and *in vivo* and that ROS are involved in this pathway.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS), penicillin/streptomycin, Dulbecco's modified Eagle medium (DMEM), F-12K medium, and trypsin were obtained from Gibco BRL (Grand Island, NY). Ascorbate, D (+)-glucose, and sodium chloride were obtained from Merck (Darmstadt, Germany). Acridine orange, *N*-acetyl-L-cysteine (NAC), catalase, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (β-NADP), α-tocopherol, colcemid, 3-methylchlolanthrene, and the positive control chemicals for the Ames test, 9-aminoacridine, 4-nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2AA), streptozotocin (STZ), benzo(a)pyrene (BaP), and mitomycin c, were obtained from Sigma Chemical Co. (St. Louis, MO).

For the micronucleus assay in mice, MEP agents were dissolved in corn oil and mitomycin c was dissolved in PBS to give solutions with different concentrations of the tested compound for intraperitoneal (ip) injections at 10 μl/g body weight of mice. For the *in vitro* Ames/Salmonella test, the compounds were dissolved in DMSO.

Collection and preparation of MEP. MEP were collected on a 0.5-μM quartz fiber filter from a 50-cm^3 Yamaha two-stroke engine using 95% octane unleaded gasoline. The sampling apparatus consisted of, in sequence, a 40-(long) × 2.2-cm (diameter) stainless still 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 particles for 1 h, four times daily. The filter with particulate matter was left to dry and repeatedly extracted with methanol, four times under sonication. The washed particles and organic components absorbed on the filter were pooled, and the methanol was removed in a vacuum evaporator. The final residues, the MEP and methanol extract, were collected and kept desiccated at −20°C. Approximately 32.7 μg of final residue (MEP) could be derived from 1 l of motorcycle exhaust. In our extraction, methanol was used to wash and bring down the particles from the filter. After removing the methanol, the final MEP residue should contain the chemicals as well as the particles absorbed on the filter. MEP were then dissolved in DMSO or corn oil before being added to the culture medium or intratracheally instilled solution.

Experimental animals. For the Ames test, Wistar rats (200 g) were used for preparation of the liver microsomal (S9) fraction. For the micronucleus test, male ICR mice, aged 8 to 9 weeks and weighing 30–40 g, were employed. All animals were purchased from the Animal Center of the College of Medicine, National Taiwan University. The animals were allowed at least a 1-week acclimation period in their animal quarters with air conditioning and a 12-h light/dark cycle. All animal treatments were approved by the Institution Animal Care and Use Committee (IACUC) of the College of Medicine, National Taiwan University, which follows the Animal Welfare Protection Act of the Department of Agriculture, Executive Yuan, Taiwan.

S9 fraction preparation. Rat liver S9 used for metabolic activation was prepared as described previously (Maron and Ames, 1983; Matsuoka *et al*., 1979). To obtain the liver microsomal fraction, each of the Wistar rats was injected ip with 3-methylcholanthrene (30 mg/kg body weight) every day, and 4 days later the rats were killed by cervical dislocation. The livers were homogenated, diluted 1:4 with 0.15 M KCl, and centrifuged for 10 min at 9000 × g. The supernatant was pulled and diluted (giving a protein concentration of 30 mg/ml), frozen in small aliquots, and stored at −70 to −80°C until use. The final preparation of the metabolizing system (S9 mixture) was made in accordance with the protocol of Ames *et al*. (1975). The composition and final concentrations of the S9 mix used for the CHO-K1 cell chromosome aberration test were as follows: glucose-6-phosphate, 4.4 mM; NADP, 0.84 mM; KCl, 30 mM; $NaHCO_3$, 0.032%; and S9 fraction, 10%.

Ames Salmonella/microsome test. The method we used followed the recommendations of Maron and Ames (1983) and Organization for Economic Cooperation and Development (OECD) guidelines (1997). The *Salmonella typhimurium* bacteria and histidine auxotrophic strains TA98, TA100, and TA102 were obtained from MOLTOX (Molecular Toxicology, Annapolis, MD) and grown for 14 h at 35 ± 2°C with continuous shaking. Bacteria were grown to a density of 1 to 2 × 10^9 cells/ml with OD600 absorbance of 0.2–0.3. Top agar, containing 2 ml of heated agar, 0.1 ml of test chemical, 0.1 ml of bacteria, and 0.5 ml of S9 solution, was mixed up and added to three different minimal glucose agar plates. All plates were incubated at 37°C for 48 h, and the number of bacteria colonies was determined. The entire experiment was replicated again on a different day with a total of six plates for each concentration of MEP with and without S9. S9 liver cell extracts contain enzymes that may activate the potential mutagen. Each tester strain was routinely checked to confirm its features for optimal response to known mutagenic chemicals as follows: 4-NQO (0.5 μg/plate), mitomycin c (0.5 μg/plate), and 2AA (5 μg/plate). BaP (10 μM) and STZ (0.5 mM) were used as ROS-dependent positive controls with and without S9, respectively. A test compound was judged to be mutagenic in the plate test if it produced, in at least one concentration and one strain, a response equal to twice (or more) of the control incidence with a dose-response relationship considered to be positive (De Serres and Shelby, 1979; Suter *et al*., 2002). The only exception was strain TA102, which has a relatively high spontaneous revertant number, where an increase by a factor of 1.5 above the control level was taken as an indication of a mutagenic effect.

Chromosomal aberrations. Chinese hamster ovary epithelial cells (CHO-K1, ATCC: CCL-61) were plated into 6-cm dishes at 5 × 10^5 cells/plate for the 24-h treatment group. After overnight incubation, cells were treated with DMSO (solvent), mitomycin c (1 μg/ml), BaP pyrene (5 μg/ml), and various concentrations of MEP for 3 h with or without S9. Then, 3 h after the end of the treatment time, 0.1 μg/ml colcemid was administered, and metaphase chromosomes were prepared as described (Tsutsui *et al*., 1983). After trypsinization, cells were treated with 0.9% sodium citrate at 37°C for 10 min, fixed in Carnoy's solution (methanol:acetic acid, 3:1), and spread on glass slides by the air-drying method. Specimens were stained with a 3% Giemsa solution in 0.07 M phosphate buffer (pH 6.8) for 30 min. For determination of both chromosome aberrations, 100 metaphases per experimental group were scored. Structural chromosome aberrations observed in each experimental group were classified into seven types as follows: chromosome-type gap (G), chromosome-type break (B), chromosome-type ring (R), chromosome-type dicentric (D), chromatid-type gap (g), chromatid-type break (b), and chromatid-type exchange (e). Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid. If there was displacement, it was recorded as a break.

Micronucleus assay. The micronucleus assay from peripheral blood cells was performed as previously described (Hayashi *et al*., 1990). The number of micronucleated cells was counted in 1000 reticulocytes (RETs) per animal. Slides were analyzed using a fluorescent microscope with a combination of a blue excitation (e.g., 488 nm) and a yellow-to-orange barrier filter (e.g., 515 nm long pass), with a ×100 objective for RETs.

Analysis of ROS production by flow cytometry. Intracellular ROS generation was measured by a flow cytometer with an oxidation-sensitive DCFH-DA fluoroprobe (Rothe and Valet, 1990). DCFH-DA (2′,7′-dichlorofluorescin diacetate) is a nonfluorescent compound that is freely taken up into cells and

hydrolyzed by esterase by removing the DA group. This nonfluorescent molecule (DCFH) is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. When DCFH-DA was deacetylated and oxidized to form the fluorescent compound, fluorescence intensity was increased in a concentration-dependent manner. We stained 2×10^6 CHO-K1 cells with 20 μg/ml DCFH-DA for 30 min at 37°C in the dark. Cells were then collected after PBS washing for fluorescence measurement. The level of intracellular ROS was determined with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. For each treatment, 10,000 cells were counted and the experiment was performed in triplicate.

Statistical analysis. Data are expressed as the mean ± SEM for the number of experiments indicated. Statistical analysis of the data was performed by Student's *t*-test, and $p < 0.05$ was considered significantly different. The values of IC_{50} were calculated and obtained from five regression lines; each regression line was constructed of at least five points. The values of inhibition of these points ranged from 20 to 80%.

RESULTS

Mutagenicity of MEP

The mutagenicity of MEP was examined by the Ames method (Maron and Ames, 1983). The assay was carried out *in vitro* using three histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, and TA102) with and without a metabolic-activating enzyme (S9). Each strain of *Salmonella* was treated with MEP at 1, 10, 100, 1000, and 2000 μg/plate, respectively. Our data showed that MEP did not increase colony formation in strains TA98, TA100, and TA102 without the S9 mix (Table 1) at concentrations of up to 2 mg/plate. On the other hand, in the presence of S9, the number of revertants in MEP-treated plates was concentration-dependently increased in TA98, TA100, and TA102 strains (Table 1). Pretreatment with antioxidants, including α-tocopherol (3.5 μM/plate), ascorbate (100 μM/plate), NAC (1 mM/plate), and catalase (1000 U/plate), showed different potencies, but all attenuated the colony formation induced by MEP (1000 μg/plate) in TA98, TA100, and TA102 strains with the S9 mix (Table 2). In particular, α-tocopherol significantly exerted attenuation of MEP-induced colony formation, even up to 30–40% inhibition of these testing strains. Two well-known, ROS-dependent mutagens, STZ and BaP, were used as positive controls (Table 1). The effects of antioxidants on these positive compounds have been widely reported. STZ can induce ROS-dependent mutagenesis without metabolic activation (Bedoya *et al*., 1996; Nukatsuka *et al*., 1988; Takasu *et al*., 1991); BaP is a promutagen (Kim and Wells, 1996; Wells *et al*., 1997; Winn and Wells, 1997).

TABLE 1

Revertants in Three Strains of *Salmonella typhimurium* Treated with Different Concentrations of MEP in the Absence and Presence of a Metabolic-Activating Enzyme (S9)

Without S9								
	His^+/plate (−S9)							
				MEP (μg/plate)				
Strain	Negative control	Positive control	STZ (0.5 mM)	1	10	100	1000	2000
TA98	20 ± 2	251 ± 34[a]	98 ± 10[a]	20 ± 1	21 ± 2	24 ± 2	31 ± 6	33 ± 5
TA100	79 ± 1	852 ± 59[a]	3989 ± 252[a]	76 ± 5	86 ± 0	101 ± 8	79 ± 7	81 ± 17
TA102	150 ± 7	1264 ± 96[a]	805 ± 60[a]	167 ± 5	179 ± 16	231 ± 6	175 ± 1	172 ± 5
With S9								
	His^+/plate (+S9)							
				MEP (μg/plate)				
Strain	Negative control	Positive control	BaP (10 μM)	1	10	100	1000	2000
TA98	28 ± 2	3802 ± 129[a]	684 ± 50[a]	37 ± 6	54 ± 13	250 ± 53[b]	387 ± 42[a]	407 ± 73[a]
TA100	92 ± 6	3473 ± 385[a]	976 ± 42[a]	92 ± 1	107 ± 13	287 ± 13[c]	291 ± 52[b]	459 ± 105[b]
TA102	250 ± 12	1699 ± 207[a]	857 ± 61[a]	248 ± 14	283 ± 22	310 ± 22	478 ± 89[c]	637 ± 68[b]

Note. Values are presented as the mean ± SE ($n \geq 6$). The negative control consisted of 2 μl DMSO/plate. The positive ontrol in the −S9 plate consisted of TA98, 2 μg/plate 4-nitro-o-phenylenediamine; TA100 and TA102, 0.5 μg/plate mitomycin C. The positive control in the +S9 plate consisted of 5 μg/plate 2-aminoanthrene. The ROS-dependent positive controls were streptozotocin (STZ, 0.5 mM without S9) and benzo(a)pyrene (BaP, 10 μM with S9).

[a] $p < 0.005$ versus negative control.

[b] $p < 0.01$ versus DMSO.

[c] $p < 0.05$ versus DMSO.

TABLE 2
The Effect of Antioxidants on the Revertants in *Salmonella typhimurium* TA98, TA100, and TA102 Treated with MEP in the Presence of Metabolic-Activating Enzymes

	His$^+$/plate (+S9)		
Treatment group	TA98	TA100	TA102
Negative control	22 ± 5	81 ± 2	249 ± 15
BaP (10 μM)	684 ± 50[a]	976 ± 42[a]	857 ± 61[a]
MEP (1000 μg/ml)	397 ± 35[a]	317 ± 64[a]	444 ± 67[a]
+ 1 mM NAC	285 ± 47[a,b] (28.2)	245 ± 22[a] (22.7)	329 ± 31[a] (25.9)
+ 100 μM ascorbate	256 ± 55[a,b] (35.5)	221 ± 23[a] (30.3)	289 ± 39[a,b] (34.9)
+ 3.5 μM α-tocopherol	244 ± 61[a,b] (38.6)	164 ± 12[a,b] (48.3)	293 ± 25[a,b] (34.0)
+ 1000 U catalase	280 ± 63[a] (29.5)	239 ± 14[a] (24.6)	339 ± 47[a] (23.6)

Note. Values are presented as the mean ± SE ($n \geq 6$). The negative control consisted of 2 μl DMSO/plate. The ROS-dependent positive control was 10 μM BaP. The percentage of inhibition (% In) = 100 − [(number of revertants per plate in the presence of antioxidant)/(number of revertants per plate in the absence of antioxidant) × 100].

[a] $p < 0.05$ versus negative control.

[b] $p < 0.05$ versus MEP (1000 μg/ml).

Induction of Chromosome Aberrations by MEP in CHO Cells

The *in vitro* effect of MEP on chromosomes was studied with CHO-K1 cells, and the results of CHO-K1 cell chromosome analysis are given in Table 3. The incidence of CHO-K1 cells with structural chromosomal aberrations significantly increased in cells treated with BaP (5 μg/ml; 13.1 ± 2.2%, $p < 0.001$; Table 3) and mitomycin c (1 μg/ml; 9.8 ± 2.2%, $p < 0.001$; Table 3) with and without S9, respectively. Both in the absence and presence of S9, MEP (0.5, 5, and 50 μg/ml) concentration-dependently increased structural chromosomal aberrations at 3 h of treatment compared to the solvent control (0.1% DMSO, Table 3). We also found that MEP- (5 and 50 μg/ml) induced chromosomal aberration was potentiated up to 2-fold by the addition of S9 (Table 3). Pretreatment with the antioxidants α-tocopherol (3.5 μM), ascorbate (100 μM), catalase (1000 U/ml), and NAC (1 mM) yielded different potencies, but all significantly attenuated the increase in aberrant cells induced by MEP (50 μg/ml) in the S9-treated group, though not in the group without S9 (Table 4).

In Vivo *Induction of Micronuclei by MEP*

Results of the micronucleus assay of ICR mice treated with different doses of MEP and the positive control are shown in Table 5. Mice injected (ip) with mitomycin C (1 mg/kg body weight) showed a significant increase in the frequency of RETs at 24 (12.2 ± 1.4) and 48 h (36.5 ± 1.6) of treatment compared to control (1.2 ± 0.2 and 1.4 ± 0.5, respectively). Intratracheal instillation with various doses of MEP (160, 200, and 240 mg/kg body weight) in mice significantly increased the formation of RETs at both 24 and 48 h, with a return to basal level at 72 h after treatment (Table 5). Cotreatment with antioxidants α-tocopherol (75 mg/kg body weight) and ascorbate (1 g/kg body weight) inhibited the MEP- (200 mg/kg body weight) induced increase of RETs formation only at 48 h (Table 5).

MEP Induced ROS Formation in CHO-K1 Cells

ROS generation induced by MEP was examined using a DCFH-DA fluorescence probe. The fluorescence intensity of oxidative DCF in reaction with various concentrations of MEP was determined in CHO-K1 cells. We found that MEP increased the fluorescence intensity as ROS level in a concentration-dependent manner (Fig. 1). MEP- (50 μg/ml) induced fluorescence intensity was potentiated up to 2-fold compared to control.

DISCUSSION

Large numbers of people in the world continue to be exposed to pollutant mixtures containing known or suspected carcinogens. Epidemiologic studies over the last 50 years suggest rather consistently that general ambient air pollution, mainly due to the incomplete combustion of fossil fuels, may be responsible for increased rates of lung cancer (Cohen and Pope, 1995). These substances are present as components of complex mixtures, which may include carbon-based particles that absorb organic compounds, oxidants such as ozone, and sulfuric acid in aerosol form (Cass *et al*., 1984; Dockery *et al*., 1993). In Taiwan, the combustion of fossil fuels for power generation and transportation (especially motorcycles), also the primary source of many organic and inorganic compounds, oxidants, and acids, contributes heavily to particulate air pollution. In this study, we found MEP extract was mutagenic in both *in vitro* and *in vivo* experimental assays.

TABLE 3
Chromosome Aberrations of CHO-K1 Cells Treated with MEP

Without S9

		Number of aberrations/100 cells						
Treatment group	Aberrant cells (%)	G	B	D	R	G	b	e
Solvent control (DMSO)	3.1 ± 0.1	1.6 ± 0.3	0	0	0	0	1.4 ± 0.3	0
MMC (1 μg/ml)	9.8 ± 2.2[a]	0	5.3 ± 0.3	0.3 ± 0.3	0	0.2 ± 0.2	3.4 ± 1.8	0
MEP (μg/ml)								
0.5	6.0 ± 2.2	2.7 ± 1.5	0.5 ± 0.3	0.2 ± 0.2	0.2 ± 0.2	0.8 ± 0.8	1.5 ± 1.3	0.2 ± 0.2
5	6.2 ± 1.9[b]	1.3 ± 0.9	0.2 ± 0.2	0.2 ± 0.2	0	3.7 ± 1.9	0.8 ± 0.4	0
50	10.0 ± 2.0[a]	1.3 ± 1.3	0.7 ± 0.3	1.0 ± 0.5	2.0 ± 1.0	2.3 ± 2.3	2.0 ± 0.5	0

With S9

		Number of aberrations/100 cells						
Treatment group	Aberrant cells (%)	G	B	D	R	g	b	e
Solvent control (DMSO)	3.4 ± 1.0	1.6 ± 0.6	0	0	0.1 ± 0.1	1.6 ± 0.8	0.2 ± 0.2	0
BaP (5 μg/ml)	13.1 ± 2.2[a]	3.8 ± 1.3	0.5 ± 0.2	0.1 ± 0.1	0.8 ± 0.4	4.0 ± 1.8	0.5 ± 0.3	1.0 ± 0.2
MEP (μg/ml)								
0.5	7.0 ± 2.9[c]	1.5 ± 0.7	1.2 ± 1.2	0.2 ± 0.2	0.5 ± 0.5	3.0 ± 1.5	0.3 ± 0.3	0.3 ± 0.3
5	13.3 ± 2.0[a]	3.2 ± 1.6	1.3 ± 1.3	1.7 ± 1.4	0.8 ± 0.8	3.8 ± 2.0	1.0 ± 0.6	1.5 ± 1.5
50	19.8 ± 3.5[a]	3.8 ± 2.0	3.0 ± 2.5	2.0 ± 2.0	2.0 ± 1.0	5.7 ± 3.0	1.0 ± 0.6	2.3 ± 1.9

Note. Values are presented as the mean ± SE ($n = 3$). MEP was dissolved in DMSO, and the solvent control did not exceed 0.1%. Mitomycin C (MMC) was the positive control in the medium without S9, and BaP was the positive control in the medium with S9. G, chromosome gap; B, chromosome break; D, dicentric; R, ring; g, chromatid gap; b, chromatid break; e, exchange.

[a] $p < 0.001$ versus DMSO.

[b] $p < 0.01$.

[c] $p < 0.05$.

Over the years, the Ames test has been used worldwide as an initial screening tool to determine the mutagenic potential of new chemicals and drugs, because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained (McCann *et al*., 1975; Sugimura *et al*., 1976; Zeiger *et al*., 1990). Our data showed that MEP contains chemicals that are mutagenic to *Salmonella* strains TA98, TA100, and TA102 upon metabolic activation. These results indicated that the active forms of MEP are their metabolites. Some carcinogenic chemicals, such as aromatic amines and PAHs, are biologically inactive unless they are metabolized to their active forms. Several mutagens, such as PAHs, nitrofluorene, and various aromatic nitroso derivatives of amine carcinogens (Isono and Yourno, 1974), have been found in DEP (Li *et al*., 2000; Schuetzle *et al*., 1981) and MEP (Ueng *et al*., 2000). It is interesting to note that while MEP (this study) and gasoline engine exhaust particulate extracts (Crebelli *et al*., 1991) showed a promutagenic effect, scooter exhaust contained chemicals that were directly acting mutagens (Zhou and Ye, 1997).

We do not know, at this point, the chemical compounds that are responsible for the mutagenicity seen in our study. Many reports in recent years have focused on the toxic effect of the extracts of DEP. They have been shown to generate intracellular ROS, leading to a variety of cellular responses (Hiura *et al*., 2000; Ma and Ma, 2002; Yang *et al*., 2001). The organic component of DEP has also been shown to generate ROS that produce 8-hydroxydeoxyguanosine (8-OHdG) in cell culture (Tsurudome *et al*., 1999). Our data showed the mutagenic effect on TA102, providing proof of the involvement of ROS. Strain TA102 was developed containing AT base pairs at the *his*G428 mutant site, and the mutation is reverted by mutagens that cause oxidative damage (Myriam *et al*., 2000; Niittykoski *et al*., 1995). The involvement of ROS in MEP-induced mutation was further evidenced by the alleviation of mutation with the use of antioxidants including α-tocopherol, ascorbate catalase, and NAC. Alpha-tocopherol and ascorbate have similar effects in preventing MEP-induced increases of revertants in TA98 (38.6% inhibition with S9) and TA100 (48.3% inhibition with S9), as did ascorbate in TA102 (34.9% inhibition with S9). Although the antioxidants can attenuate the mutagenic effect, however, the inhibition is not complete. This might suggest that there are other chemicals that are involved in the mutagenic effect but independent of ROS.

DNA breaks and the formation of clastogens could be detected by *in vitro* CHO cells in the chromosome aberration test and *in vivo* micronucleus assay. In the chromosome aberration test, MEP concentration-dependently increased the number of abnormal structural chromosomes in the absence of metabolic activation ($10.0 \pm 2.0, p < 0.001$ at 50 μg/ml) in CHO cells. However,

TABLE 4
Chromosome Aberrations of MEP-Treated CHO-K1 Cells in the Presence of Antioxidants

Treatment group	Aberrant cells (%)	
	3 h without S9	3 h with S9
Solvent control (DMSO)	3.3 ± 0.3	4.4 ± 0.7
Positive control	10.7 ± 0.9[a]	16.3 ± 1.2[a]
MEP (50 μg/ml)	10.0 ± 2.0[a]	20.3 ± 1.5[a]
+ α-tocopherol (3.5 μM)	7.0 ± 1[a]	8.5 ± 0.5[a,b]
+ ascorbate (100 μM)	9.0 ± 0[a]	9.0 ± 1.5[a,b]
+ catalase (1000 U/ml)	8.0 ± 1[a]	14.3 ± 1.7[a,b]
+ NAC (1 mM)	9.5 ± 0.5[a]	10.7 ± 2.0[a,b]

Note. Values were presented as the mean ± SE ($n = 3$). MEP was dissolved in DMSO, and the solvent control did not exceed 0.1%. MMC was the positive control in the medium without S9, and BaP was the positive control in the medium with S9. G, chromosome gap; B, chromosome break; D, dicentric; R, ring; g, chromatid gap; b, chromatid break; e, exchange.

[a] $p < 0.05$ versus control.

[b] $p < 0.05$ versus 50 μg/ml MEP.

TABLE 5
Micronucleus Formation in Peripheral Blood Cells of Mice Treated with MEP and the Effects of Antioxidants *In Vivo*

Treatment group	Time (h)		
	24	48	72
Control	1.2 ± 0.2	1.4 ± 0.5	1.0 ± 0.2
MMC (1 mg/kg)	12.2 ± 1.4[a]	36.5 ± 1.6[a]	9.3 ± 1.2[a]
MEP (160 mg/kg)	1.8 ± 0.5	2.7 ± 0.8[b]	2.0 ± 0
MEP (200 mg/kg)	2.8 ± 0.6[b]	4.8 ± 0.5[a]	2.2 ± 0.7
MEP (240 mg/kg)	5.0 ± 0.3[a]	5.9 ± 0.8[a]	2.4 ± 0.8

[a] $p < 0.005$ versus the control.

[b] $p < 0.01$ versus the control.

[c] $p < 0.05$ versus the control.

Treatment group	Time (h)		
	24	48	72
Control	1.2 ± 0.2	1.4 ± 0.5	1.0 ± 0.2
MEP (200 mg/kg)	2.7 ± 0.5[d]	5.6 ± 0.4[d]	2.1 ± 0.5
MEP (200 mg/kg) + ascorbate (1 g/kg)	2.0 ± 0.8	2.0 ± 0.2[e]	1.5 ± 0.5
MEP (200 mg/kg) + α-tocopherol (75 mg/kg)	3.1 ± 1.5[d]	2.6 ± 0.6[d,e]	1.7 ± 0.1

Note. Values are presented as the mean ± SE ($n \geq 3$). The control consisted of 1% Tween 80 in DMSO:PBS (1:9). Ascorbate (1 g/kg in water) and α-tocopherol (75 mg/kg in corn oil) were used for pretreatment by an ip injection for 30 min.

[a] $p < 0.05$ versus the control.

[b] $p < 0.05$ versus MEP (200 mg/kg).

the addition of S9 greatly enhanced the effect of the MEP-induced aberrations (19.8 ± 3.5, $p < 0.001$ at 50 μg/ml; Table 3). These data suggested that MEP contains chemicals that can induce chromosome aberrations both with and without metabolic activation in CHO cells. Previously, Kuo *et al*. (1998) also showed that MEP induced genotoxicity in a ROS-dependent manner by using cell-junction communication as an experimental model. Pretreatment with antioxidants selectively attenuated MEP-induced aberration of cells in the S9-treated group (Table 4). This result indicated that MEP might contain chemicals that exert a mutagenic effect independent of ROS and bioactivation in CHO cells. The mechanistic detail still requires further investigation.

The clastogenic effects of MEP were also detected with the *in vivo* micronucleus test in mice peripheral red blood cells. Micronuclei scoring is based on the observation that displaced chromatin, resulting from chromosome loss or breakage, may fail to be incorporated into daughter nuclei as a cell divides. The resulting micronucleus is found in the cytoplasm. During erythropoiesis, an erythroblast expels its main nucleus to become a reticulocyte (RET), while the micronuclei remain in the cytoplasm. The newly formed RET is then released from the bone marrow into the circulating bloodstream, where it develops into a normochromatic erythrocyte (NCE). Elevations in the frequency of micronuclei are indicative of genotoxic activity (Hayashi *et al*., 2000). MEP dose-dependently increased the micronucleus formation at 24 and 48 h after treatment, and this effect was slightly reversed to basal level at 72 h. Obviously, the *in vivo* study showed that the effect is reversible or can be repaired after 72 h. Intraperitoneal injection with ascorbate (1 g/kg in water) and α-tocopherol (75 mg/kg in corn oil), 30 min before MEP (200 mg/kg) treatment, greatly reduced the micronucleus formation at 48 h in the treated group.

Micronuclei can arise from acetric fragments induced by a substance causing chromosomal breakage (clastogens) as well as aneuploidy (aneugens). However, with the conventional micronucleus test, it is impossible to distinguish between these two events. We cannot rule out the possibility that MEP might be an aneugen in this study. The reason the antioxidants showed the most obvious protecting effect at 48 h might be because the micronucleus showed the most dramatic change at 48 h. It is also possible that the antioxidants only have a partial effect, as seen with the Ames and chromosome tests. The basal micronucleus observed at all time points, which was not inhibited by the antioxidants, might be caused by some chemicals in MEP that damage the chromosome independently of ROS formation.

There are some possible sources that can account for the origination of ROS generation. They include the following:

1. Among the > 100 chemicals that are present in MEP (Chan *et al*., 1993; Zhou and Ye, 1997), such as PAH, nitroderivatives of PAH, and oxygenated PAH derivatives (ketones, quinines, and diones) are candidate chemicals that may contribute to ROS generation (Alsberg *et al*., 1985; Anderson *et al*., 1998; Li *et al*., 1996; Schuetzle *et al*., 1981).

2. Quinone is reduced to semiquinone radicals by microsomally localized cytochrome P450 reductases (Chesis

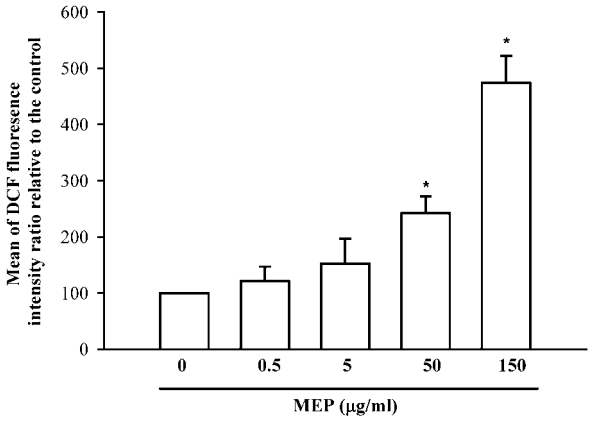

FIG. 1. ROS generation induced by MEP in CHO-K1 cells. Cells (2×10^6) were incubated with various concentrations of MEP (0.5, 5, 50, and 150 μg/ml) for 3 h with 20 μM DCFH-DA at 37°C for 30 min. Then cells were collected after PBS washing for fluorescence measurement. Data with error bars are the mean ± SE from three independent experiments. DMSO was used as the solvent control. $*p < 0.05$ compared to the control.

et al., 1984). These semiquinones group together, thereby initiating a futile redox cycle (Chesis *et al*., 1984; Monks *et al*., 1992).

3. PAH may contribute to further ROS production during cytochrome P4501A1-dependent transformation.

4. In addition to the contribution of organic chemical compounds, that of metal ions, such as Fe^{2+}, may relate to ROS generation.

5. Mitochondria have been implicated in the induction of apoptosis by a growing list of pro-oxidative chemicals, including redox-cycling quinines and PAH (Segura-Aguilar *et al*., 1998; Yamaguchi *et al*., 1996; Zoratti and Szabo, 1995).

ROS generation has been linked to the mutagenic effects of DEP chemicals (Ichinose *et al*., 1997). Although these MEP-associated chemicals need to be identified, we know that MEP contain PAHs, which have potentially harmful effects on humans. The PAH components can react with nitrous oxides in the air, such as NO_2 and NO, and form nitro-PAH. Recent studies demonstrated that nitro- and dinitropyrene play a minor role in air particulate mutagenicity (Crebelli *et al*., 1991).

Our study provides evidence of the potentially genotoxic effects of MEP extract both *in vitro* and *in vivo*. Although bioactivation and ROS generation were shown to play major roles in the genotoxic effects that we observed, MEP also contains directly acting chemicals and chemicals that act independently of ROS. The impact of this toxic effect of MEP on the health of people in Taiwan is worthy of emphasis and further investigation.

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