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Effects of paving asphalt fume exposure on genotoxic and mutagenic activities in the rat lung

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Abstract

Asphalt fumes are complex mixtures of aerosols and vapors containing various organic compounds, including polycyclic aromatic hydrocarbons (PAHs). Previously, we have demonstrated that inhalation exposure of rats to asphalt fumes resulted in dose-dependent induction of CYP1A1 with concomitant down-regulation of CYP2B1 and increased phase II enzyme quinone reductase activity in the rat lung. In the present study, the potential genotoxic effects of asphalt fume exposure due to altered lung microsomal enzymes were studied. Rats were exposed to air or asphalt fume generated under road paving conditions at various concentrations and sacrificed the next day. Alveolar macrophages (AM) were obtained by bronchoalveolar lavage and examined for DNA damage using the comet assay. To evaluate the systemic genotoxic effect of asphalt fume, micronuclei formation in bone marrow polychromatic erythrocytes (PCEs) was monitored. Lung S9 from various exposure groups was isolated from tissue homogenates and characterized for metabolic activity in activating 2-aminoanthracene (2-AA) and benzo[a]pyrene (BaP) mutagenicity using the Ames test with *Salmonella typhimurium* YG1024 and YG1029. This study showed that the paving asphalt fumes significantly induced DNA damage in AM, as revealed by DNA migration in the comet assay, in a dose-dependent manner, whereas the micronuclei formation in bone marrow PCEs was not detected even at a very high exposure level (1733 mg h/m^3). The conversion of 2-AA to mutagens in the Ames test required lung S9-mediated metabolic activation in a dose-dependent manner. In comparison to the controls, lung S9 from rats exposed to asphalt fume at a total exposure level of 479 ± 33 mg h/m^3 did not significantly enhance 2-AA mutagenicity with either *S. typhimurium* YG1024 or YG1029. At a higher total asphalt fume exposure level (1150±63 mg h/m^3), S9 significantly increased the mutagenicity of 2-AA as compared to the control. However, S9 from asphalt fume-exposed rats did not significantly activate the mutagenicity of BaP in the Ames test. These results show that asphalt fume exposure, which significantly altered both phases I and II metabolic enzymes in lung microsomes, is genotoxic to AM and enhances the metabolic activation of certain mutagens through altered S9 content. Published by Elsevier B.V.

Keywords: Paving asphalt fumes; DNA damage; Mutagenicity; CYP1A1; CYP2B1; Polycyclic aromatic hydrocarbons

1. Introduction

Asphalt fumes, commonly encountered by workers in roofing or road paving operations, are complex mixtures of aerosols and vapors that contain various organic compounds including, polycyclic aromatic

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hydrocarbons (PAHs) and heterocyclic compounds [1]. Many of the organic compounds found in asphalt fumes have been shown to be mutagenic and/or carcinogenic [2–4]. Epidemiological studies have indicated an association between cancer risk and exposure to asphalt fumes and, in general, a higher cancer risk for the roofers than for the pavers [5–8]. The mutagenic activity of asphalt fumes is correlated to the amount of three- to seven-ring PAHs in the fumes [2]. The higher mutagenic/carcinogenic PAH content in the roofing asphalt fumes, generated at higher temperatures than the paving asphalt fumes may account for the higher cancer risk for the roofers than for the road pavers.

The mutagenic/genotoxic effects of roofing asphalt fumes have been well documented through both in vitro and in vivo studies [4,9]. In contrast to roofers, road pavers are exposed to asphalt fumes that contain relatively low levels of PAHs. Paving fumes have been regarded as non-mutagenic [10,11]. Studies have demonstrated asphalt fume-induced genotoxic damage, determined as increase in DNA strand breaks and alkali-labile sites, were exhibited in peripheral mononuclear blood cells of roofers but not road paving workers [12,13]. A study that monitored the sister-chromatid exchange (SCE) and micronuclei (MN) in peripheral lymphocytes from road pavers in Sweden also did not show significant genotoxic effects due to the paving asphalt fume exposure [14]. However, an increase in SCE and MN formation in peripheral lymphocytes of road paving workers in Turkey was reported by Burgaz et al. [15]. Such discrepancy with regard to the potential genotoxicity of paving asphalt fumes also appeared in studies carried out in animal models. De Meo et al. [16] showed that road paving asphalt fume condensate was mutagenic and induced DNA adduct formation. Reinke et al. [17], on the other hand, reported that asphalt fume condensate collected from an asphalt storage tank was not mutagenic using Ames test and did not induce chromosomal aberrations. Using mice carrying a reporter gene for mutagenesis analysis and nose-only inhalation exposure, Micillino et al. [18] showed that the asphalt fume-exposed group was not different from the control group in mutation frequency or adduct formation.

These literature results indicate that the PAH content is an important factor in asphalt fume-induced genotoxicity, suggesting that sufficient levels of PAH to induce toxic effects may exist in these fumes under certain conditions. Many of the published studies were based on extra-pulmonary measurements. However, the direct pulmonary toxicity caused by the paving asphalt fumes and the mechanism(s) involved have not been reported extensively. Despite the low levels of PAHs, paving asphalt fumes are complex mixtures. The carcinogenic potency of asphalt fume cannot be predicted solely on the content of any single known carcinogenic PAH, e.g. benzo[a]pyrene (BaP), as the carcinogenic marker. The existence of other components in the asphalt fumes, and the potential for concomitant inhalation of particles and other chemical substances during paving operations, which may not themselves be carcinogenic, might act as co-carcinogens or inhibitors for tumor initiation and thus significantly modify PAH-induced carcinogenicity in the lung. In addition, PAH-mediated genotoxicity is not limited to the formation of active PAH metabolites. Other mechanism(s) such as reactive oxygen species (ROS)-mediated events, may also play a role in paving asphalt fume-induced toxicity.

The lung is the major target organ for airborne pollutants, including asphalt fumes. It is well known that many PAHs, including BaP, are activated by the cytochrome P450 monooxygenases (P450 or CYP) to exert their carcinogenic effects. Previously, we have shown that inhalation exposure of rats to whole asphalt fumes generated under road paving conditions resulted in a marked increase in CYP1A1, the PAH inducible isoform, and substantially decreased CYP2B1, the constitutive isoform in the lung [19]. In the bioactivation of PAHs, different P450 isoforms have been demonstrated to be structural specificity toward PAH molecules. In the case of BaP, CYP1A1 preferentially metabolizes BaP at the bay region, 7,8-positions, to form 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene that can be further transformed to ultimate carcinogenic metabolites [20]. CYP2B1, on the other hand, catalyzes oxidation at the 4,5-position. This metabolite can be easily removed in the lung suggesting a detoxification pathway for BaP [21]. Nevertheless, these isozymes may work in concert to provide a balance between the activation and detoxification pathways for PAH metabolism. The induction of CYP1A1 and down-regulation of CYP2B1 in the lung by asphalt fume exposure could favor PAH activation, leading to

increased genotoxicity. Thus, paving asphalt fumes, although containing low levels of PAH, may alter P450 isozyme distribution and enhance mutagenic and/or carcinogenic activity in the lung.

In addition to P450-mediated PAH activation, P450 enzymes are known to mediate other mechanism(s) including the production of ROS, which may also play a role in paving asphalt fume-induced toxicity. Recent studies showed that both CYP1A1 [22] and NADPH cytochrome P450 reductase [23] induced intracellular ROS generation during the metabolism of diesel exhaust particles (DEP) containing PAHs, some of which are similar to the PAHs in asphalt fumes. This oxidative stress promotes DNA damage [23] and induces apoptosis in alveolar macrophages [24]. Studies carried out in our laboratory further demonstrated that the induction of ROS by the organic extract of DEP was inhibited by CYP1A1 and nitric oxide synthase inhibitors [25], suggesting that P450-mediated PAH metabolism may generate both reactive oxygen and nitrogen species. This is of importance, as the PAH-mediated ROS generation may lead to DNA damage in host cells.

The present study was carried out to examine the genotoxic effects of paving asphalt fume exposure on the pulmonary system. Since paving asphalt fume markedly alters metabolic enzymes in the lung, we hypothesize that asphalt fume exposure may alter the metabolic activity of lung S9 in promutagen activation, and altered PAH metabolism may induce DNA damage in key host cells. These hypotheses were tested by measuring the DNA fragmentation in AM and lung tissue using the comet assay and by examining the metabolic activation of 2-aminoanthracene (2-AA) and BaP mutagenicity in the presence of lung S9 from air and asphalt fume exposure groups using Ames test with *Salmonella typhimurium* YG1024 and YG1029.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals were purchased from Sigma Chemical Co (St. Louis, MO): β-nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, L-histidine, D-biotin, Bacteriologil-cal agar, D-(+)-glucose, potassium phosphate, magnesium sulfate, citric acid monohydrate, sodium ammonium phosphate, normal melting point agarose, low melting point (LMP) agarose, benzo[a]pyrene and dimethyl sulfoxide (DMSO). Oxoid nutrient broth No.2 was obtained from Oxoid Inc. (Ogdensgury, NY). Aroclor-1254 induced rat liver S9 was obtained from Molecular Toxicology, Inc. (Boone, NC). 2-Aminoanthracene(2-AA) was purchased from Aldrich Chemical Company (Milwaukee, WI).

2.2. Asphalt fume inhalation exposure system

An inhalation exposure system, in which rats were exposed to asphalt fumes freshly generated under simulated road paving conditions, has been developed and described in a previous study from our laboratory [26]. Briefly, asphalt was pre-heated to 170 °C in an oven and transferred to a reservoir with the temperature maintained at 170 °C. The asphalt was passed through a heated pipe and onto a heated plate with temperature maintained at 150 °C at the inlet and 120 °C at the outlet. These temperatures are typical of those reported in the field during asphalt road paving. Humidified and temperature-controlled air was blown across the plate to mix with the asphalt vapor. The mixture was then transported through a heated pipe into the animal exposure chamber. Teflon filters of diameter 37 mm and pore size 0.45 μm were used for gravimetric analysis of the fume in the exposure chamber. These filters were backed up with an XAD-2 sorbent tube and a charcoal sorbent tube designed to collect medium and small molecular weight chemicals for later chemical analysis [26]. The filters were weighted immediately at the end of the sampling period and the fume concentration was determined. For the present study, rats were exposed to various concentrations of asphalt fume at 6 h per day, for 1–5 days, and sacrificed the next day. The total exposure level (TEL) was expressed as the product of fume concentration (mg/m^3) and the total exposure time (h/day × days). For S9 used in the Ames test, rats were exposed to asphalt fume at a concentration of 24.9 ± 0.9 or $38.3 \pm 2.1\ mg/m^3$, 6 h per day for 5 days, which corresponds to a total exposure level of 479 ± 33 or $1150 \pm 63\ mg\ h/m^3$, respectively. For micronuclei determination, bone marrow samples were taken from rats exposed to asphalt fume at $57.8 \pm 3.0\ mg/m^3$, 6 h per day for 5 days,

(TEL = 1733 ± 90 mg h/m^3). For comet assay, samples of AM were taken from the above mentioned exposure experiments and from rats exposed to asphalt fume at a concentration of 52.9 mg/m^3 for 1 h (TEL = 52.9 mg h/m^3) or 58.6 ± 5.6 mg/m^3 for 6 h (TEL = 353 ± 33.6 mg h/m^3).

2.3. Animal exposures

Specific pathogen-free female Sprague–Dawley rats [Hla:(SD)CVF] from Hilltop Lab Animals (Scottdale, PA), weighing approximately 200 g, were used for all experiments. The rats were kept in filtered ventilated cages on Alpha-dri virgin cellulose chips and hardwood Beta-chips as bedding and were provided HEPA-filtered air, autoclaved Prolab 3500 diet, and tap water ad lib. under controlled light cycle (12 h light/12 h darkness) and temperature (22–24 °C) conditions. The animal facilities are AAALAC-accredited, specific pathogen-free, and environmentally controlled. Rats were acclimated for 2 weeks in the exposure chambers before use and then exposed by inhalation to air or asphalt fumes. Control animals were exposed to air that was regulated at the same flow, temperature, and humidity as in the asphalt exposure experiment.

2.4. Isolation of alveolar macrophages (AM) and lung tissue

Animals were anesthetized with sodium pentobarbital (0.2 g/kg body weight) and exsanguinated by cutting the renal artery. AM were obtained by bronchoalveolar lavage (BAL) with Ca^{2+}/Mg^{2+}-free phosphate-buffered solution (PBS, 145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4). A total of 80 ml of BAL fluid was collected from each rat and centrifuged at $500 \times g$ for 10 min at 4 °C. Cellular pellets were resuspended in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5.5 mM glucose, and 1.0 mM $CaCl_2$; pH 7.4). The number of AM was determined according to their unique cell diameters, using an electronic cell counter equipped with a cell-sizing unit (Coulter Electronics, Hialeah, FL). Aliquots of 1 ml cell suspensions, adjusted to 2×10^6 AM, were added to each well of 6-well tissue culture plates and incubated in a humidified incubator (37 °C and 5% CO_2) for 1 h to allow AM attachment to the plastic plate. The non-adherent cells were then removed by rinsing the monolayers three times with the medium. The attached AM-enriched cells were then detached by trypsinization, centrifuged and resuspended to an approximate density of 5×10^6 cells/ml in PBS. AM were kept on ice for the comet assay.

Lung tissue was isolated according to the methods described by Sasaki et al. [27,28]. Briefly, after weighing, the lungs were minced, suspended at a concentration of 1 g/ml in chilled homogenizing buffer (0.075 M NaCl, 0.024 M Na_2EDTA, pH 7.5) and then homogenized gently using a Teflon-glass Potter-Elvejhem homogenizer at 500–800 rpm set in ice. To obtain nuclei, the homogenate was centrifuged at $700 \times g$ for 10 min at 4 °C and the precipitate was resuspended in chilled homogenizing buffer at 1 g lung/ml and allowed to settle for 1–2 min. Precipitated clumps were then removed.

2.5. Comet assay

The comet assay was performed under alkaline conditions based on a method described by Singh et al. [29]. Briefly, fully frosted microscope slides were pre-coated with 0.5% normal melting point agarose in water. Duplicated preparations of a gel mixture (75 μl), containing cells and 0.5% low melting point agarose (LMP) in Ca^{2+}- and Mg^{2+}-free PBS, were set on the pre-coated slides, and subsequently covered with 110 μl of LMP. The slides containing agarose-embedded cells were immersed in cold, freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% sodium sarcosinate, pH 10; 1% Triton X-100 and 10% DMSO added just before use) and kept at 4 °C for 1 h. The slides were then placed on a horizontal gel-electrophoresis tank, covered with cold alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) for 30 min, and subjected to electrophoresis at 25 V for 15 min. After electrophoresis, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) and stained with ethidium bromide. The above steps were conducted under very dim light or in the dark to prevent potential DNA damage during the assay procedure. Comets were visualized using a fluorescence microscope (Olympus, AX70) with an image capture system (SamplePCI, Compix Inc., Cranberry Township, PA). For each

sample, 50 cells were scored at 400× magnification. The length of the comet tail, which indicates DNA migration, from the digitized images was determined as the distance between edge of head and end of tail using an image analysis system (Optimas 6.51, Media Cybernetics Inc., Silver Spring, MD).

2.6. Isolation of lung S9

The S9 fractions from the lungs of control and asphalt fume-exposed rats were prepared according to the method of Maron and Ames [30] with some modification. Briefly, rats were anesthetized with sodium pentobarbital, and the heart and lung were removed and weighted. The lungs were perfused with 0.15 M ice-cold KCl to remove blood cells. The lung tissue was chopped four times with a McI1wain tissue chopper (Mickle Engineering Co., Gomshall, Surrey, UK) set at 0.5 mm slice thickness. The minced lungs were suspended in four times lung weight of 0.15 M ice-cold KCl and homogenized using a Teflon-glass Potter-Elvejhem homogenizer for 16 complete passes. The lung homogenates were centrifuged at 9000 × *g* for 20 min at 4 °C to yield the S9 fraction (the supernatant) and stored at −80 °C until use. All steps were carried out at 4 °C using sterile solution and instruments. Sterility of the lung S9 preparation was determined by plating 0.1 ml of S9 on minimal agar-containing histidine and biotin. The protein concentration of S9 was determined using a BCA protein assay kit with bovine serum albumin as the standard (Pierce, Rockford, IL).

2.7. Salmonella typhimurium/microsomal assay

S. typhimurium strain YG1024, which detects frameshift mutagens, and strain YG1029, which detects base-pair substitution mutagens, were used for the Ames test. 2-AA (0.015 μg per plate) and BaP (10 μg per plate), both chemical mutagens that require metabolic activation, were used as substrates. The *Salmonella* mutation test was carried out using a microsuspension assay [31] that was modified to provide enhanced sensitivity from the standard plate-incorporation method [32,33]. Briefly, *S. typhimurium* test strains YG1024 and YG1029 were grown in Oxoid nutrient broth No. 2 at 37 °C overnight. The bacteria were harvested by centrifugation (3000 rpm for 20 min, 4 °C) and resuspended to yield cell density of approximately 2.5–5 × 10^9/ml. The S9-dependent 2-AA mutagenicity was determined as follows. S9 reaction mixtures, which contained varying amounts of lung S9 from control or asphalt fume-exposed rats, were prepared in 100 mM sodium phosphate buffer (pH 7.4), containing 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, and 4 mM NADP. The reaction mixtures, containing final concentration of 0.015 μg 2-AA (in 10 μl DMSO), 65 μl of the S9 reaction mixture and 25 μl of concentrated bacterial solution, were placed in test tubes on a rotary shaker and incubated at 37 °C for 30 min. Aroclor-1254-induced rat liver S9 (10% (v/v) in S9 reaction mixture) was used as activating system and served as the positive control. After incubation, 2.4 ml of top agar, containing 0.05 mM each of histidine and biotin, was added to each test tube and the test tube contents were poured onto Vogel–Bonner minimal agar plates. The plates were inverted, incubated at 37 °C for 48 h for strain YG1029 or 72 h for strain YG1024, and counted. The number of histidine revertant colonies was manually counted, and the results were expressed as the number of revertant colonies per plate. The conditions of the bacterial background lawn were examined for all experiments to ensure that the reaction conditions did not lead to excessive cytotoxicity. For each assay or separate experiment, control plates were prepared from DMSO, S9 or substrate alone, and the revertant colony counts were analyzed. All assays were performed in duplicate plates, and each experiment was repeated for at least three times with S9 isolated from different animals. Spontaneous revertants were not subtracted from the number of revertants obtained for each assay.

2.8. Micronucleus assay

The nucleated cells in bone marrow were isolated from rats exposed to air or 1734 ± 90 mg h/m^3 asphalt fumes, using a cellulose column [34]. The erythrocytes collected from cellulose column were pelleted by centrifugation (600 × *g*, 10 min) and resuspended in fetal bovine serum. An aliquot of cell suspension was pelleted onto slides, using a Shandon Cytospin II at 600 rpm for 7 min. The slides were allowed to air-dry, fixed in absolute methanol, and stained with Giemsa: distilled water (1:6) for 10 min. These slides showed a strong blue tint in polychromatic erythrocytes

(PCEs). Slides were scored for micronuclei and expressed as the mean number of cells with micronuclei per 1000 PCEs, under 1000 × magnification. For each animal, the frequency of micronucleated PCEs in bone marrow was based on 10,000 PCEs scored.

2.9. Statistical analysis

For Ames test, paired Student's *t*-test was performed to compare the activating capability of 2-AA and BaP mutagenicity by lung S9 from air- and asphalt fume-exposed rats. For comet assay, comparison of DNA migration between asphalt fume-exposed and control groups was performed with one-way analysis of variance (ANOVA), followed by Student's *t*-test. The comparison of MN PCEs between control and asphalt fume-exposed animals was carried out by the trend test, whereas the comparison of PCEs between said groups was performed using the group *t*-test for multiple sample analysis. In all tests, statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of asphalt fume exposure on DNA damage in alveolar macrophages and lung tissues

The asphalt fume-induced genotoxicity, measured as DNA damage by the comet assay, was examined in AM from rats exposed to various levels (53, 353, 641 and 1150 mg h/m^3) of the paving asphalt fumes. Fig. 1 shows the fluorescence image of the electrophoretic migration of DNA from AM isolated from rats exposed to air (panel A) or 641 mg h/m^3 paving asphalt fumes (panel B). We have also examined DNA damage in lung tissues obtained from air and asphalt fume-exposed rats at the same exposure level (Fig. 1, panels C and D). These results show

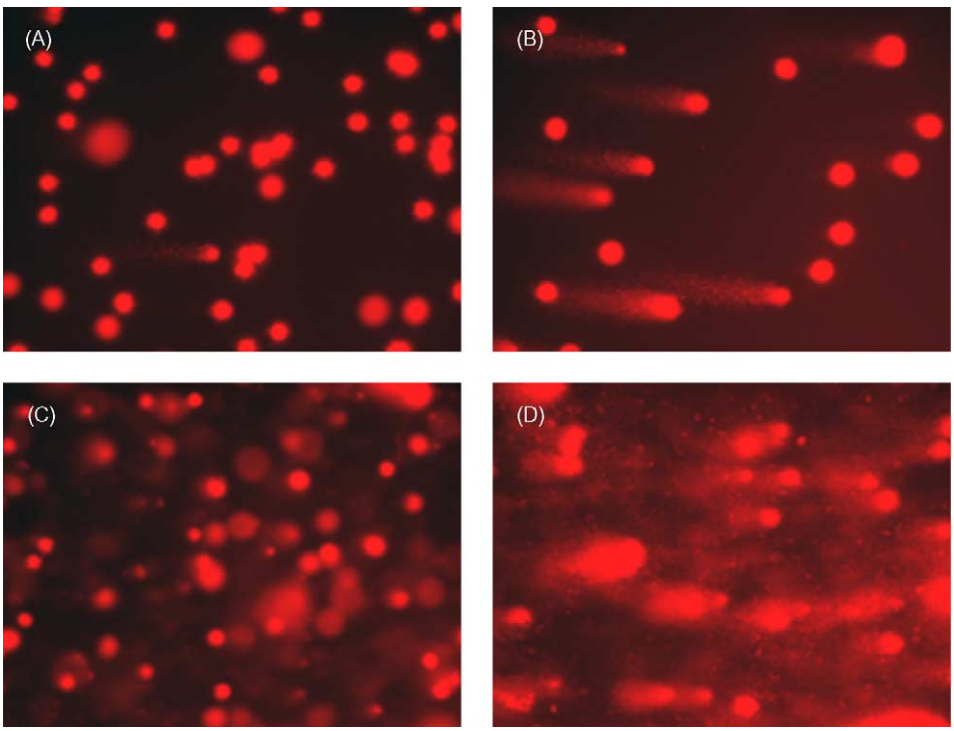

Fig. 1. Effect of asphalt fume exposure on DNA migration in AM and lung tissue using the comet assay. (A) Air-exposed AM, (B) asphalt fume (641 mg h/m^3)-exposed AM, (C) air-exposed lung tissue and (D) asphalt fume (641 mg h/m^3)-exposed lung tissue. Comets were visualized using a fluorescence microscope and quantitated with an image analysis system. Fifty cells were scored at 400× magnification for each sample.

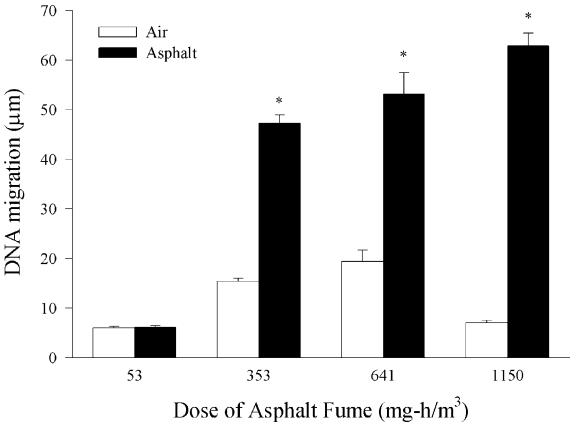

Fig. 2. Concentration-dependent effects of asphalt fume exposure on DNA migration in AM using the comet assay. AM were obtained by bronchoalveolar lavage and the length of comet tail from digitized images was determined by measuring the distance between edge of head and end of tail, and expressed as DNA migration in microns. Fifty cells were scored for each sample at 400× magnification. * indicates a significant increase from control ($P < 0.05$).

that short-term exposure to asphalt fume generated under road paving conditions significantly induced DNA damage in AM and in lung tissue. Fig. 2 shows the fluorescence imaging analysis of DNA damage in AM at varying exposure levels. The results show that asphalt fume exposure-induced DNA damage in AM is concentration dependent. Significant induction of DNA damage occurred at an asphalt fume level of 353 mg h/m^3 and higher.

In comparison to air exposure, exposure of rat to a high level of asphalt fume, 1733 mg h/m^3, did not induce micronuclei formation in polychromatic erythrocytes (PCEs) isolated from the bone marrow of exposed rats, indicating the absence of a systemic chromosomal damage (Table 1).

3.2. Effects of asphalt fume exposure on lung S9-dependent 2-AA mutagenicity

Bioactivation of 2-AA mutagenicity by S9 fractions from air- or asphalt fume-exposed rats was tested in both *S. typhimurium* strains YG1024 and YG1029. Fig. 3 shows that S9 is required for 2-AA activation, and that 2-AA mutagenicity increases with increasing concentration of lung S9 (from 0 to 200 μg per plate) in both strains. At the total exposure level of

Table 1
Effects of asphalt fume exposure on the induction of micronuclei (MN) in bone marrow polychromatic erythrocytes (PCEs) in comparison to the control

Treatment	Number of MN/1000 PCEs[a]	PCE/NCE[b] ratio
Air control	0.9 ± 0.8	1.04 ± 0.04
Asphalt fume exposure	1.6 ± 0.9	0.99 ± 0.04

Note: Values are *mean* ± *S.E.* for data from six different animals in each treatment group. Rats were exposed to air or asphalt fume at 57.8 ± 3.0 mg/m^3, 6 h per day, for five consecutive days (TEL = 1733 ± 90 mg h/m^3) and sacrificed the next day.

[a] Ten thousand PCEs were scored for each treatment group.

[b] NCE represents normal chromatic erythrocytes.

479 ± 33 mg h/m^3, S9 from asphalt fume-exposed rats did not show an increased capacity to activate 2-AA mutagenicity in either strain compared to S9 from the air control (Fig. 3). Fig. 4 shows that lung S9 isolated from rats exposed to a total fume level of 1150 ± 63 mg h/m^3 significantly increased S9-dependent activation of 2-AA mutagenicity in both test strains, when compared to the controls.

3.3. Effects of asphalt fume exposure on lung S9 induced-benzo[a]pyrene mutagenicity

Table 2 shows the measured mutations in *S. typhimurium* strains YG1024 and YG1029 using BaP as the substrate. The results of the current study show

Table 2
The effects of asphalt fume exposure on lung S9 induced-benzo-[a]pyrene mutagenicity

S9 per plate (μg)[c]	Revertants per plate[a,b]			
	YG1024		YG1029	
	Air	Asphalt	Air	Asphalt
0[d]	35 ± 2	35 ± 2	71 ± 4	71 ± 4
50	36 ± 2	41 ± 3	80 ± 5	80 ± 6
100	38 ± 1	41 ± 2	89 ± 5	86 ± 4

[a] Values are means ± S.E. from three rats, each in duplicate. Spontaneous revertants were not subtracted from the number of revertants obtained for each assay.

[b] Positive control: Aroclor 1254-treated liver S9 (10%) was used as the activating system, causing revertants for YG1024 of 382 ± 14 and for YG1029 of 406 ± 30.

[c] Lung S9 isolated from rats exposed to air or asphalt fume (1150 ± 63 mg h/m^3).

[d] Spontaneous revetants per plate.

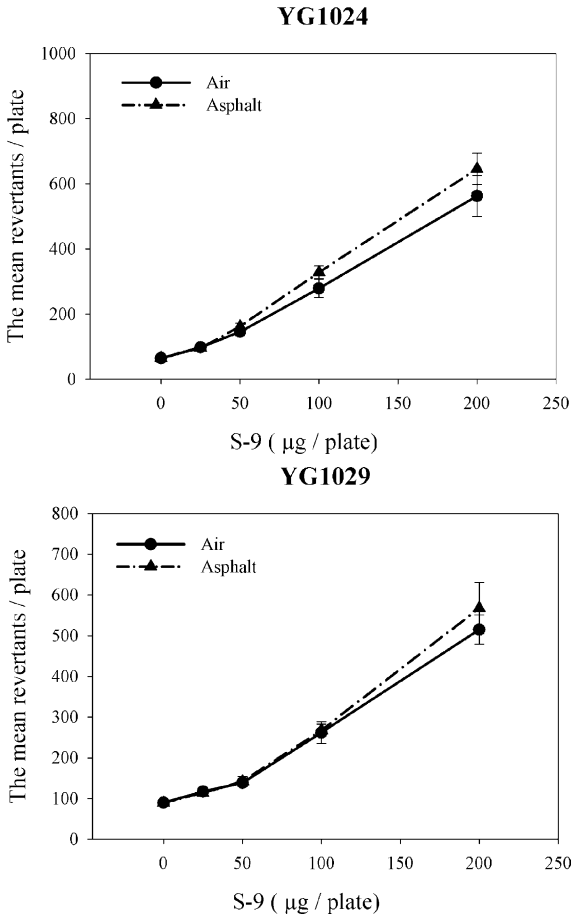

Fig. 3. Effects of asphalt fume exposure on lung S9-dependent 2-AA (0.015 μg per plate) mutagenicity in *S. typhimurium* YG1024 and YG1029. The lung S9 was isolated from air- or asphalt fume (479 mg h/m^3)-exposed rats. All the assays were performed in duplicate plates, and each experiment was repeated at least three times using different rats. Values represent means ± S.E. ($n = 3$). Spontaneous revertants (45 ± 3 per plate for YG1024, 89 ± 5 per plate for YG1029) were not subtracted from the number of revertants obtained for each assay.

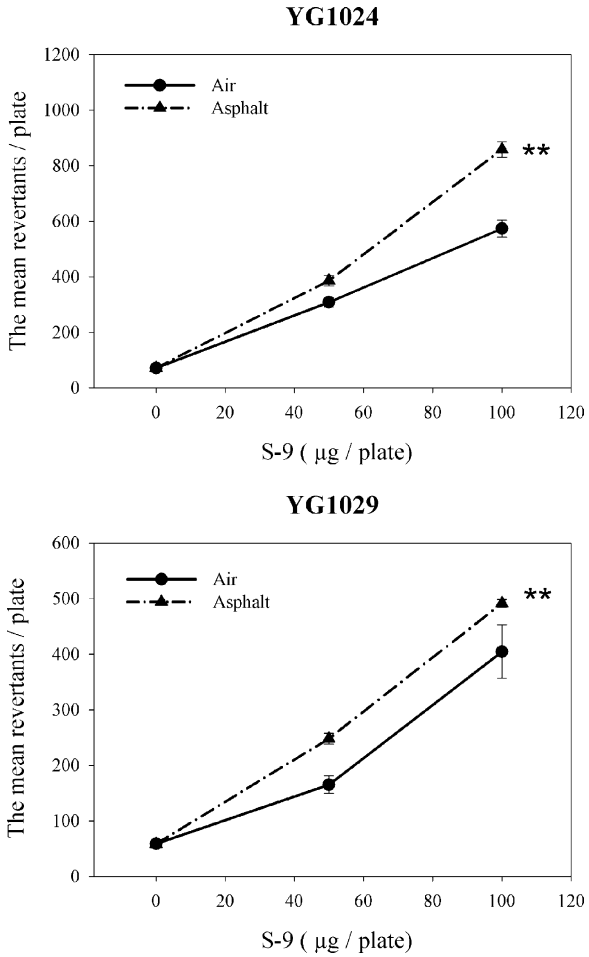

Fig. 4. Activation of 2-AA (0.015 μg per plate) by lung S9 isolated from air– or asphalt fume-exposed rats. The effects of air or asphalt fume (1150 mg h/m^3) exposure on lung S9 metabolic capability in activating 2-AA mutagenicity, using the Ames test in both *S. typhimurium* YG1024 and YG1029. Each assay was performed in duplicates and each experiment was repeated at least three times using different rats. Values represent means ± S.E. ($n = 3$). Spontaneous revertants (41 ± 2 per plate for YG1024, 80 ± 8 per plate for YG1029) were not subtracted from the number of revertants obtained for each assay. ** indicates significantly different from air controls, $P < 0.01$.

that BaP was not activated to mutagen by S9 isolated from either air- or asphalt fume-exposed rats, even thought the same S9 from asphalt fume-exposed rats was shown to induce 2-AA mutagenicity (Fig. 4).

4. Discussion

Exposure of rats by inhalation to asphalt fumes generated at paving temperature, has been shown to significantly modify xenobiotic metabolic activities in the lung, including the significant induction of CYP1A1 with concomitant down-regualtion of CYP2B1 and increased quinone reductase activity [19]. However, these studies showed that exposure of rats to asphalt fumes generated at paving temperature did not cause acute inflammation or lung injury, as

measured by neutrophil infiltration or elevation of LDH activity and protein content in acellular lavage fluid. Stimulation of respiratory burst activities of AM, measured as the release of reactive oxygen and nitrogen species was also absent, in contrast to the characteristic of activation of AM following exposure to toxic substances, such as DEP, silica, asbestos, etc. The PAH-inducible CYP1A1 is mainly responsible for the production of reactive PAH metabolites that covalently bind to DNA, leading to DNA adduct formation, mutation, and multistage carcinogenesis [35]. CYP2B1, the constitutive P450 isozyme in rat lung, has been shown to mediate epithelial type I cell stability, type II cell differentiation, and the conversion of type II to type I cell during epithelial repair [36]. In light of the importance of CYP1A1 and CYP2B1 in relation to lung stability, the alteration of CYP1A1 and 2B1 by asphalt fume exposure may be important in asphalt fume-induced pulmonary toxicity. The present study was carried out to characterize the genotoxic effects of inhalation exposure of rats to asphalt fume, generated at paving temperature under road paving conditions. The results demonstrated that asphalt fume exposure induced S9-metabolic activity dependent mutagenicity in the Ames test and induced genotoxicity in alveolar macrophage, but did not induce systemic genotoxicity, measured as micronuclei formation in bone marrow PCE.

Asphalt is heated at lower temperatures during road paving operations in comparison to roofing applications resulting in the generation of fumes containing lower levels of PAHs. Certain compounds found in asphalt fumes, such as BaP [37,38] and 3-methylcholanthrene (3-MC) [39], have been shown to increase mutation frequencies in transgenic mice carrying either the *lacI* or the *lacZ* reporter gene. However, the reports of genotoxic effects for asphalt fume exposure in road pavers are inconsistent. Several field studies have shown that asphalt fume exposure-induced genotoxicity occurred in peripheral mononuclear blood cells of roofers, but not in road pavers [37–39]. However, in one field study, paving asphalt fume was shown to enhanced SCE and MN frequencies in peripheral lymphocytes of road pavers [15]. A recent international epidemiological study of cancer hazards among workers exposed to asphalt fumes showed a small increase in lung cancer and mortality risk [40–42]. However, these studies conclude that there was no evidence of a causal link between exposure to asphalt fume and risk of cancer of the lung. In animal studies, the genotoxic effects of paving temperature asphalt fume were also inconclusive. For example, after nose-only inhalation of 100 mg/m^3, 6 h per day for 5 days, Micillino et al. [18] demonstrated that there was no significant induction of pulmonary genotoxic responses in transgenic mice with 30 days fixation time. Genevois-Charmeau et al. [43], on the other hand, were able to show the formation of DNA adducts in various tissues of BD/6 rats after exposure (nose-only) to bitumen fumes generated at 180 °C for 5 days. These discrepancies may be due partly to species difference (mice versus rats) and/or fixation time differences (30 days versus 1 day) in DNA adduct formation. Further elucidation of the genotoxic effect of paving asphalt fumes by examination of direct lung toxicity and biomarkers that are more sensitive to low-level PAH exposure is warranted.

The present study showed that exposure of rats by inhalation to asphalt fume, generated under road paving conditions and at a high total exposure level (1733 mg h/m^3), did not cause micronuclei formation in bone marrow PCEs. However, asphalt fume exposure resulted in direct pulmonary toxicity, as demonstrated by the comet assay, at much lower exposure levels. This discrepancy is probably due to measurement of genotoxic effect in the exposed organ (lung) versus systemic system (bone marrow). In addition, the comet assay has been reported to be a more sensitive, genotoxic test than micronuclei formation [44–47]. It produces fast detection of DNA damage shortly after injury and before DNA repair, without any need to wait for progression into mitosis. The results showed that short-term asphalt fume inhalation exposure induced DNA damage in AM at exposure levels of 353 mg h/m^3 and greater. However, there was no evidence of DNA damage in AM isolated from rats exposed to 53 mg h/m^3 of asphalt fume, in comparison to the control. Asphalt fume-induced DNA damage was also detected in lung tissue. These results clearly demonstrated that exposure of rats to paving asphalt fumes induced DNA damage in lung cells and this genotoxic effect in AM was asphalt fume dose-dependent. The dose dependence of DNA damage correlates with the induction of CYP1A1 by asphalt fume exposure that was carried out in

our laboratory previously [19]. Increased activity of CYP1A1 may lead to DNA damage via two pathways. One is through direct activation of PAHs into mutagenic metabolites. Genevois-Charmeau et al. [43] also demonstrated DNA adducts in various rat tissues after exposure to paving temperature bitumen fumes for 5 days. In addition, Sato et al. [48] showed that rats exposed to 6 mg/m^3 of diesel exhaust, containing similar organic compounds as those in asphalt fumes, had a 4.8-fold increase in mutant frequency over the controls and 3-fold adducts in lung DNA. They correlated the adduct formation to the increase in CYP1A1 mRNA level. This CYP1A1-derived DNA damage may result from ROS-mediated pathways. AM are one of a few cell types in the lung that express the cytochrome P450 enzymes. Recently, we have shown that the PAH containing organic extract of DEP induced ROS in AM. This ROS production was inhibited by an antioxidant, *N*-acetylcysteine, or a CYP1A1 inhibitor, α-naphthoflavone, suggesting the involvement of CYP1A1 in ROS production from AM [25]. A number of other studies support the conclusion that CYP1A1-mediated PAH metabolism can result in the generation of intracellular ROS, leading to DNA damage [22,49]. These studies shows that ROS may play a crucial role in PAH-mediated genotoxicity.

The present study shows that the asphalt fume exposure-altered xenobiotic metabolizing enzymes contained in lung S9 fraction can change the pulmonary genotoxic responses to potential mutagenic and/or carcinogenic compounds as illustrated in the Ames test. The activation of carcinogenic compounds by the P450 enzymes is highly substrate specific [50,51]. In the rat lung, studies have suggested that BaP is mainly activated by CYP1A1 [52]. The results show that lung S9 isolated from asphalt fume-exposed rats exhibited a marked increase in CYP1A1 compared to the air controls [19]. However, such S9 fractions did not activate BaP to mutagen in the Ames test. The lack of induction of BaP mutagenicity in the current study may be due to insufficient induction of CYP1A1 by asphalt fume exposure. Asphalt fume-induced CYP1A1 may not reach the activating level required to show a positive effect, as was shown using Aroclor 1254-induced liver S9. Although it did not activate BaP mutagenicity, S9 from rats exposed to asphalt fume at a level of 1150 mg h/m^3 significantly enhanced 2-AA mutagenicity. At a lower exposure level (479 mg h/m^3), the S9 did not significantly induce 2-AA mutagenicity. 2-AA is known to be preferentially metabolized by the CYP1A subfamily. It has been reported that, in addition to CYP1A1, several other P450 isozymes including CYP1A2 [53,54], CYP4B1 [55] and CYP2B1 [56], are involved in 2-AA bioactivation. Carriere et al. [56] have demonstrated that the induction of CYP2B1, by phenobarbital treatment, significantly reduced the mutagenic effects of 2-AA in the liver but not in the intestine, suggesting that the activation of 2-AA by P450 isoforms is organ-dependent. In another study, Lubet et al. [57] demonstrated that in the liver 2-AA was activated by 3-MC-induced CYP1A1, whereas CYP2B1 showed little effect on 2-AA activation. These studies did not show a clear role of CYP2B1 in 2-AA activation. To date, the specific isozymes of lung P450 involved in the activation of 2-AA to mutagenic metabolites have not been demonstrated in detail. The results from the present study show that alteration of xenobiotic metabolic enzymes in the asphalt fume-exposed lung significantly activated 2-AA to form mutagens in a dose-dependent manner.

The current study shows that lung P450 enzymes are involved in the PAH metabolism-induced genotoxicity. It should be mentioned that the actions of the P450 isozymes are complex even toward a single substrate and become increasingly so where multiple PAHs are present, such as following asphalt fume exposure. More studies are certainly needed, but the present study shows that even with a low PAH content, paving asphalt fume exposure can at least alter the metabolic enzymes that are critically involved in the activation and/or detoxification of inhaled chemicals, even if it does not play a direct role in providing toxic levels of mutagenic compounds.

In summary, the present study demonstrates that short-term inhalation of asphalt fume generated under road paving conditions is genotoxic to lung cells. Asphalt fume exposure may induce DNA damage in AM and lung tissue through PAH induction of CYP1A1 and possibly ROS-mediated pathways. In addition, the lung S9 fractions from rats exposed to paving asphalt fumes, which contain an induced level of CYP1A1 and a reduced level of CYP2B1, did not induce BaP mutagenicity but significantly enhanced the activation of 2-AA mutagenicity in the Ames test. These results

show that alteration of the P450 enzymes in the lung by asphalt fume exposure can have a profound effect on pulmonary handling of toxic chemicals.

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