DNA binding of polycyclic aromatic hydrocarbons in a human bronchial epithelial cell line treated with diesel and gasoline particulate extracts and benzo[a]pyrene

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Particulate matter of vehicle exhaust is known to contain carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAH) and is suggested to increase lung cancer risk in humans. This study examines the differences in diesel and gasoline-derived PAH binding to DNA in a human bronchial epithelial cell line (BEAS-2B). Particulate matter (PM) of gasoline exhaust was collected from passenger cars on filters and semi-volatile compounds on polyurethane foam (PUF). The soluble organic fraction (SOF) extracted from the particles was used to expose the cells and to perform PAH analysis. Gasoline extracts, benzo[a]pyrene (B[a]P) and reference materials (SRM 1650 and 1587) were used to study dose-dependent adduct formation in BEAS-2B cells. The levels of DNA adducts were in good accord with the 10 DNA adduct-forming PAH concentrations analyzed in the extracts. Gasoline extracts, SRM 1650, SRM 1587 and B[a]P formed DNA adducts dose-dependently in BEAS-2B cells. The time-dependent DNA adduct formation of 5.0 µM B[a]P was lower than that of 2.5 µM B[a]P. The results of this study indicate that reformulated and the particles used to expose the cells to diesel PM were less active compared to gasoline, respectively, when PAH–DNA adduct levels were calculated on an emission basis (adducts/mg PM/km), whereas on a particulate basis (adducts/mg PM) no difference between the diesel and gasoline extracts was observed. We conclude that the genotoxicity of diesel fuel is based on higher particulate emission rates compared to gasoline emission and although the concentration of PAH compounds was higher in diesel particulate extracts, DNA binding by the gasoline particulate-bound PAH compounds was more pronounced than that by the diesel particulate-bound PAH compounds.

Introduction

The particles present in urban air pollution are mainly derived from diesel and gasoline fueled vehicles. Exhausts are released straight into the breathing zone and small particles can easily penetrate into the lungs. Vehicle exhaust has been shown to contain many known carcinogenic compounds, such as benzo[a]pyrene (B[a]P). Chronic exposure of particles derived from diesel and gasoline engines causes several harmful health effects, including increased lung cancer risk (IARC, 1989). According to the World Health Organisation particulate matter is linked to half a million premature deaths every year. Even short-term exposures to low levels (below 100 µg/m³) of particulate matter (PM) in air are associated with adverse health effects (World Health Organisation, 2000). For this reason, the World Health Organisation cannot recommend a guideline level for short-term average PM10 value in air. Although the epidemiological evidence and the results from animal tests support human carcinogenicity, at least with respect to diesel exhaust emission, there is still no consensus about the mechanism of carcinogenicity.

Polycyclic aromatic hydrocarbons (PAH) are the most prominent among the genotoxic and carcinogenic agents present in polluted urban air. Genotoxic PAH compounds are known to react with DNA after their metabolic activation to form DNA adducts (Conney, 1982). This initiation step is thought to be relevant with respect to chemical carcinogenesis (Miller, 1970; Lawley, 1989). Furthermore, DNA adducts are useful biomarkers reflecting the internal dose and exposure to PAH and nitro-PAH derived from particles. DNA adducts have been measured in test animals (Bond et al., 1990; Gallagher et al., 1994; Savela et al., 1995; Sato et al., 2000; Aoki et al., 2001; Gerde et al., 2001) and in cultured human cells (Gallagher et al., 1993; Kuljukka-Rabb et al., 2001) after exposure to diesel particulate or urban air extracts. In human biomonitoring studies DNA adducts have been used as biomarkers of genotoxicity of traffic-related pollution, taking into account that many confounding factors, e.g. active or passive smoking and consumption of grilled and smoked food, may significantly affect the PAH–DNA adduct levels, especially when the degree of exposure is moderate or low (Hemminki et al., 1994; Phillips et al., 1995; Nielsen et al., 1996; Schoket et al., 1999; Kyrtopoulos et al., 2001).

Diesel and gasoline engines emit particles and PAH compounds due to incomplete combustion of the fuel. Particulate mass and PAH emissions from diesel engines are generally higher compared to those of gasoline engines with a catalytic converter (Mi et al., 1998, 2000). Emissions are, however, dependent on the temperature, the test vehicles and cycles used, the fuels and also the exhaust after-treatment technology. The concentrations of 14 PAH compounds analyzed in the soluble organic fraction (SOF) of direct injection gasoline engine (GDI) emissions have been shown to be 17 and 53 µg/km, when reformulated gasoline and European standard gasoline, respectively, were used (Kokko et al., 2000). In addition to the mass based particle emission, the question of particle size and number in gasoline and diesel emissions has been raised. Particle diameters <300 nm dominate the mass distribution of both gasoline and diesel emissions, but the gasoline particle diameters are on average smaller than particles found in diesel exhausts (Maricq et al., 1999; Bünger et al., 2000).

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Bronchial epithelial cells are the target of inhalable particles. Exhaust particles and particulate-bound organic compounds have been shown to induce cytokine expression and elicit a pro-inflammatory response in bronchial epithelial cells (Bayram et al., 1998; Abe et al., 2000; Bonvallot et al., 2001). Bronchial epithelial cells effectively express PAH-activating CYP1A1 and CYP1B1 when induced with B[a]P and, therefore, particle-derived PAH are metabolically activated to reactive intermediates in these cells (Mollerup et al., 2001). Phase II enzymes such as glutathione S-transferase (GST), which detoxifies PAH metabolites, are expressed in bronchial cells (Macé et al., 1994), and B[a]P induces unscheduled DNA synthesis leading to active DNA repair in these cells (Ishikawa et al., 1984; Doolittle et al., 1985). Hornberg et al. (1998) showed that airborne particles are able to damage DNA of bronchial cells and the PM$_{10}$ and PM$_{2.5}$ fractions of the airborne particles induced sister chromatid exchanges in a dose-dependent manner. Also, a strong linear correlation between B[a]P concentration and DNA adduct levels has been reported in these cells (Van Agen et al., 1997). All these findings indicate that human bronchial epithelial cell lines are suitable for in vitro studies in order to investigate chemical-induced lung carcinogenesis.

The aim of the present study was to analyze the metabolic activation and DNA binding of PAH compounds derived from diesel and gasoline particulate extracts in a human bronchial epithelial cell line (BEAS-2B). The particulate extracts were measured for 14 selected PAH, of which 10 were DNA adduct-forming compounds, and the correlation with adduct levels was determined. Further, dose-dependent metabolic activation by BEAS-2B cells to form PAH DNA-binding metabolites from gasoline extracts and Standard Reference Materials (SRM) 1650 and 1587 and time-dependent DNA adduct formation by B[a]P was studied.

Materials and methods

Chemicals and reagents

Micrococcal nuclease (from Staphylococcus aureus) was purchased from Sigma Chemical Co. (St. Louis, MO). Calf spleen phosphodiesterase was from Calbiochem (Darmstadt, Germany) and T4 polynucleotide kinase was from United States Biochemical (Cleveland, OH). [3-32P]ATP (7000 Ci/mmol) was obtained from ICN Biochemicals (Costa Mesa, CA). Polyethyleneimine (PEI)–cellulose thin layer chromatography (TLC) plates were purchased from Macherey-Nagel (Duren, Germany). Standard Reference Materials (SRM 1650a diesel particulate matter and SRM 1587 nitrated polycyclic aromatic hydrocarbons) were supplied by the National Institute of Standards and Technology (NIST) (Gaithersburg, MD) and EPA 610 PAH mixture containing 16 PAH compounds was from Ehrenstorfer (Augsburg, Germany). B[a]P was from Accud Standard (New Haven, CT). BEAS-2B cells (CRL-9609) were obtained from the American Type Culture Collection and bronchial epithelial cell growth medium (BEGM) from Cytotech ApS (Hørsholm, Denmark). All other chemicals and solvents were of analytical grade.

Emission test and particulate sampling

Exhaust particulate matter was collected from two passenger cars (Nissan Micra 1.3, 1995 model and Mitsubishi Carisma 1.8, 2000 model) using reference gasoline CEC-RF-02-99 (sulfur < 50 mg/kg, total aromatics maximum 35%). The Nissan was equipped with a multipoint port fuel injection (MPI) and the Mitsubishi with a GDI engine. Details of the particle sampling and modifications of the emission tests have been previously described (Kokko et al., 2000). Exhaust particles were collected from the Nissan at a driving speed of 120 km/h (samples G1 and G6) and from the Mitsubishi at 50 (G3 and G5) or 120 km/h (G4) through a dilution tunnel on Teflon-coated glassfiber filters (Pallflex Fiberglass T60A20, diameter 142 mm, pore size 0.3 μm). In addition to the filters, polyurethane foam (PUF) was used to collect semi-volatile compounds from three of the gasoline exhaust samples (G1, G3 and G6). Filter and PUF samples were collected during the same emission tests by positioning the PUF sampling holder downstream of the PM filters. In order to obtain sufficient particulate material for the biological tests, the emission tests were repeated several times and the extracts were combined. Diesel exhaust particles were collected from a passenger car (Toyota, indirect injection diesel engine without any oxidative catalyst) using two reformulated fuels (RD1 and RD2) and standard European fuel (EN97) during modified ECE/EUDC tests. Details of the diesel fuels and the test runs have been reported elsewhere (Kuljukka et al., 1998; Isotalo et al., 2002). The particulate samples of gasoline and diesel exhausts were extracted with dichloromethane using a Soxhlet apparatus. To be able to use the samples in the biological tests, the dichloromethane of SOF was exchanged for dimethylsulfoxide (DMSO) under nitrogen.

Determination of PAH in gasoline particulate extracts

The extracts of the particulate and PUF samples were purified for the PAH analyses using high performance liquid chromatography (HPLC). PAH concentrations in samples of gasoline particulate extract and diesel particulate extract were to the identical parallel emission tests performed for samples G6 and G6-PUF. The extracts were spiked with internal standards (d$_{10}$-pyrene, β,β-binaphthyl and indeno[1,2,3-c,d]-fluoranthene) and EPA 610 PAH mixture was used as the calibration standard. The concentrations of 14 selected PAHs in the particulates were analyzed by GC–M$(+$)S$(+$)MS using a DB-17 capillary column (Kokko et al., 2000).

Cell culture and treatment of BEAS-2B cells

BEAS-2B cells were maintained in 5 ml of BEGM medium at +37°C in a humidified atmosphere with 5% CO$_2$. The basal medium (500 ml) supplemented with 26 mg bovine pituitary extract, 2.5 mg insulin, 0.25 μg human epidermal growth factor, hydrocortisone and epinephrine, 3.25 μg tridihydroxysterone, 5 mg transferrin, 50 ng retinoic acid and 100 μM penicillin/streptomycin was changed every 2–3 days. Cell viability of BEAS-2B cells was determined by counting the number of living and dead cells using trypan blue after 48 h exposure to 2.5, 5.0, 7.5 and 10.0 μM B[a]P, 150 μg SRM 1650/ml medium and one gasoline particulate extract (200 μg/ml medium). DMSO was used as a negative control. Cell exposures were carried out twice to nearly confluent cell cultures (about 5 000 000 cells per T-25 glass bottle). Time-dependent adduct formation of 2.5 and 5.0 μM B[a]P was measured during the 48 h at several time points. Cells were exposed twice to concentrations of 100 and 475 μg/ml medium of eight gasoline and three diesel particulate extracts, respectively. BEAS-2B cells were exposed for 48 h to 15–150 μg/g SRM 1650, 1–10 μl/ml SRM 1587 and 20–400 μg/ml medium G1 and G1-PUF extracts and the dose-dependent adduct formation was detected. DMSO was used as a negative control.

DNA isolation and 32P-post-labeling of DNA adducts

DNA was isolated from the exposed and control cells. First, cells were purified twice with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris–HCl pH 8.5, 1 mM EDTA, 0.5% Tween) at 55°C for 2 h with gentle agitation. After lysis, proteinase K (0.2 mg/ml) was added and incubated at 55°C overnight until the solution was clear. RNA was digested by adding RNase A (0.2 mg/ml) and 0.1% SDS and incubating at 37°C for 1 h. After 2 min agitation, the proteins were precipitated with 1.7 M NaCl and centrifuged at 5000 r.p.m. for 15 min. Supernatant was removed to a new tube and DNA was precipitated with a double volume of absolute ethanol at −20°C for 1 h. DNA was washed twice with 70% ethanol and dissolved in distilled water. DNA concentrations were measured with an UV spectrophotometer (A$_{260}$ nm and A$_{280}$ nm). 32P DNA (4 μg) was analyzed for bulkly aromatic DNA adducts using the 32P-post-labeling method enhanced with butanol extraction (Gupta, 1993). DNA was digested to mononucleotides with micrococcal nucleases (0.04 U) and spleen phosphodiesterase (0.08 U) at 37°C for 3.5 h. The modified nucleotides were enriched by butanol extraction and DNA adducts were labeled using T4 polynucleotide kinase (4.8 U) and 40 μCi [3-32P]ATP. After two-dimensional chromatographic separation on PEI-cellulose TLC plates, DNA adducts were visualized by autoradiography and quantified based on specific activity of the [3-32P]ATP (Reddy and Randerath, 1986). Each DNA sample was analyzed three times by 32P-post-labeling assay and the average adduct levels and the standard deviations were calculated.

Results

Autoradiograms resulting from the 32P-post-labeled DNA adducts formed in BEAS-2B cells exposed to B[a]P, SRM 1650, SRM 1587, diesel particulate extract (RD2) and gasoline particulate extracts (G6, G6-PUF) are shown in Figure 1A–F. B[a]P eluted as a single spot (Figure 1A), whereas SRM 1650 and 1587 formed a diagonal radioactive zone (DRZ) of PAH and nitro-PAH adducts (Figure 1B and C). Diesel and gasoline...
particulate extracts formed less intense areas of DRZ than SRMs and three to four intense spots could be observed (Figure 1D–F). For the quantitative analysis of PAH–DNA adducts, the area of DRZ and background was excised from PEI–cellulose TLC plates and the radioactivity was determined by scintillation counting.

When analyzing DNA adducts, possible loss of adducts has been reported during the isolation and digestion of DNA to 2′-deoxyribonuclease 3′-monophosphates (Hemminki et al., 1993). In this study we lysed the cells and digested proteins with proteinase K during an overnight incubation at 55°C. This is a longer treatment at a slightly higher temperature than is usually performed in DNA isolation (1–2 h, 37–50°C), but we used it in order to digest all proteins and to have less background in the post-labeling assay. In a separate experiment we have observed that this DNA isolation procedure did not affect human lymphocyte adduct levels compared with DNA isolation using Qiagen genomic tips according to the manufacturer’s handbook (unpublished data).

Fig. 1. Autoradiograms of TLC maps of 32P-post-labeled DNA adducts enhanced with butanol extraction. Each panel shows the adduct pattern derived from B[a]P (A), SRM 1650 (B), SRM 1587 (C), RD2 diesel extract (D) and G6 and G6-PUF gasoline extracts (E and F) after exposing BEAS-2B cells for 48 h. Autoradiography was at −70°C for 3 days.
Table I. Concentrations of selected PAH compounds and B[a]P (ng/mg PM) in the gasoline and diesel extracts and the amounts of particulate matter (PM) emitted per kilometer during the test (mg/km)

<table>
<thead>
<tr>
<th>Sample</th>
<th>14 selected PAH (ng/mg PM)</th>
<th>10 DNA adduct-forming PAH (ng/mg PM)</th>
<th>B[a]P (ng/mg PM)</th>
<th>PM emitted (mg/km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gasoline extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>315.6</td>
<td>219.5</td>
<td>2.24</td>
<td>0.46</td>
</tr>
<tr>
<td>G3-PUF</td>
<td>1277</td>
<td>291.1</td>
<td>3.11</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>241.6</td>
<td>63.4</td>
<td>0.36</td>
<td>15.3</td>
</tr>
<tr>
<td>G5</td>
<td>181.0</td>
<td>141.0</td>
<td>2.80</td>
<td>1.10</td>
</tr>
<tr>
<td>G6</td>
<td>237.3</td>
<td>198.0</td>
<td>15.9</td>
<td>1.22</td>
</tr>
<tr>
<td>G6-PUF</td>
<td>4322</td>
<td>1298</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Diesel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD2</td>
<td>1015</td>
<td>486.2</td>
<td>24.5</td>
<td>42.5</td>
</tr>
<tr>
<td>RD1</td>
<td>1266</td>
<td>524.2</td>
<td>28.3</td>
<td>64.5</td>
</tr>
<tr>
<td>EN97</td>
<td>1356</td>
<td>568.4</td>
<td>20.4</td>
<td>97.0</td>
</tr>
<tr>
<td>SRM 1650</td>
<td>207.4*</td>
<td>104.0</td>
<td>1.33</td>
<td></td>
</tr>
</tbody>
</table>

G1 and G1-PUF samples were not analyzed due to the identical parallel emission test performed for sample G6 and G6-PUF.

*Certified concentrations of 13 PAHs (excluding fluorene) according to the National Institute of Standards and Technology (NIST).

PAH concentrations in the extracts

Table I shows the sum of 14 selected PAH compounds (fluorene, anthracene, phenanthrene, pyrene, fluoranthene, benzo[a]anthracene, chrysene, B[a]P, benzo[e]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene, benzo[ghi]perylen and dibenz[a,h]anthracene), of which the last 10 are DNA adduct-forming compounds (IARC, 1983; Reddy et al., 1984; King et al., 1994; Wang et al., 1995). The concentration of B[a]P in gasoline and diesel extracts and the amounts of PM emitted per kilometer, including the certified concentration of 13 PAH compounds (excluding fluorene) in SRM 1650 diesel extract according to the NIST, are also shown in Table I. The PAH concentrations in diesel extracts were analyzed earlier by Isotalo et al. (2002) and are presented here per mg PM. Concentrations of PAH compounds per mg PM were calculated for both filter and PUF samples using the PM of filter samples due to the small amount of particles obtained from PUF samples.

The highest PAH concentrations were measured in two gasoline extracts collected on PUF. Low molecular weight PAH (equal to or less than pyrene) dominated, at over 90%, PUF samples, whereas 10 DNA adduct-forming PAH compounds represented less than 30% of the 14 PAH compounds. In extracts obtained from the gasoline filters, except for sample G4 (26%), the 10 DNA adduct-forming PAH compounds constituted over 70% of the PAH compounds analyzed. The concentrations of the 14 PAH compounds in diesel extracts were 3–7 times higher than those in gasoline extracts collected on filters. Diesel particulate extracts obtained from two reformulated and standard European diesel exhaust samples contained 41–48% of 10 DNA adduct-forming PAH compounds. The amounts of B[a]P ranged from 0.4 to 16 and from 20 to 28 ng/mg in the gasoline and diesel particulate extracts, respectively. The certified concentrations of 10 DNA adduct-forming PAH compounds in SRM 1650 was about 5-fold lower and the concentration of B[a]P was nearly 20-fold lower than in SOF obtained from the reformulated and standard diesel exhaust particles. Gasoline fuel emitted particulate matter in the range 0.46–15.3 mg/km. The highest emission of 15.3 mg/km was obtained from the GDI engine (Mitsubishi) when
tested at 120 km/h (G4), while the emission of the MPI engine (Nissan) at the same speed was only 1.22 mg/km (G6). Reformulated diesel fuels emitted particulate matter 3- to 140-fold and standard reference diesel fuel 6- to 210-fold more than gasoline.

Cytotoxicity

The cytotoxicity of B[a]P, SRM 1650 and gasoline particulate extracts in BEAS-2B cells was tested after 48 h exposure. When the numbers of living and dead cells at the given concentrations of B[a]P and particulate extracts were counted, no decreases in the cell counts in comparison with the control treatment were detected (data not shown).

Time- and dose-dependent adduct formation by B[a]P

Figure 2 shows the levels of DNA adducts in BEAS-2B cells at 3, 8, 12, 24 and 48 h exposure to 2.5 and 5.0 μM B[a]P. After 3, 8 and 12 h incubations DNA binding was almost at the same level with both concentrations tested. A 4-fold increase in adduct formation for both concentrations was obtained after 24 h incubation, increasing only slightly after 48 h. The highest adduct levels were formed with 2.5 μM B[a]P after 48 h exposure, which was about 1.8-fold higher than that detected with 5.0 μM B[a]P. The dose-dependence of adduct formation after exposure to 0.5, 1.2, 2.5 and 5.0 μM B[a]P for 48 h is shown in Figure 3. A slight change in adduct levels from 0.5 to 1.2 μM B[a]P was observed, whereas the highest adduct levels were formed when BEAS-2B cells were exposed to 2.5 μM. In comparisons of the 1.2 μM dose of B[a]P with the 2.5 and 5 μM doses, 4- to 2-fold increases in DNA binding, respectively, were detected.

Adduct formation by Standard Reference Materials (SRM) and gasoline extracts

DNA adducts formed as a result of treatment of BEAS-2B cells for 48 h with two extracts of SRM were analyzed by the 32P-post-labeling assay. SRM 1650 is an extract from a heavy powered diesel engine containing complex mixtures of compounds including PAH and nitro-PAH compounds. SRM 1587 is a certified mixture of seven nitrated polycyclic hydrocarbons containing 2-nitrofluorene, 9-nitroanthracene, 3-nitrofluoranthene, 1-nitropyrene, 7-nitrobenz[a]anthracene, 6-nitrochrysene and 6-nitrobenzo[a]pyrene. Figure 4 shows the levels of...
PAH and nitro-PAH adducts formed in BEAS-2B cells after exposure to SRM 1650 (Figure 4A) and SRM 1587 (Figure 4B). Adduct levels increased only slightly when cells were exposed to SRM 1650 at concentrations from 15 to 150 µg/ml (from 7 to 11 adducts/10^8 nucleotides) and after exposure to SRM 1587 at concentrations from 1 to 10 µl/ml (from 11 to 19 adducts/10^8 nucleotides). SRM 1587 formed nearly 2-fold more DNA adducts than SRM 1650 at the concentrations tested.

PAH compounds derived from extracts of gasoline particles (G1) and the semi-volatile fraction (G1-PUF) were shown to be activated in BEAS-2B cells to DNA-binding metabolites (Figure 5). The cells were exposed for 48 h to gasoline particulate extracts at concentrations of 20, 50, 100, 200 and 400 µg PM/ml medium. The adduct levels rapidly increased from 4 and 6 to 20 adducts/10^8 nucleotides when the exposure concentration increased 5-fold, showing no increase in adduct levels thereafter. Extracts of the semi-volatile fraction (G1-PUF) formed 2-fold more adducts with the 20 and 50 µg/ml doses than the corresponding filter extract (G1), but at higher doses no differences in adduct levels were detected. After adding the PUF extract to the cell culture at a concentration of >50 µg/ml, the extract gradually started to precipitate, however, no precipitation of the filter extract was observed.

DNA adducts formed by gasoline and diesel extracts

BEAS-2B cell cultures were treated twice for 48 h with five gasoline particulate, three semi-volatile and three diesel particulate extracts to measure PAH–DNA adducts by the 32P-post-labeling assay. Table II shows adduct levels (adducts/10^8 nucleotides) which are calculated per exposure dose (37–100 µg PM/ml), per ng 10 DNA adduct-forming PAH compounds in the exposure dose and per mg PM emitted per test kilometer. Table II also includes adduct levels formed by SRM 1650 calculated per exposure dose (75 µg PM/ml) and per ng 10 DNA adduct-forming PAH. Gasoline extract G6-PUF formed the highest (291 adducts/10^8 nucleotides/mg PM) and G4 the lowest level of adducts (50 adducts/10^8 nucleotides/mg PM). PUF samples formed on average 2-fold more adducts per exposure dose than gasoline filter samples, and diesel particulate samples formed on average 2-fold less adducts than gasoline particulate samples. When adduct levels were calculated per ng 10 adduct-forming PAH compounds, gasoline sample G1 formed the highest level (1.0 adducts/10^8 nucleotides/mg PM/ng 10 PAH) and reformulated diesel sample RD1 the lowest levels of adducts (0.1 adducts/10^8 nucleotides/mg PM/ng 10 PAH). Gasoline particulate samples formed about 2.4-fold more adducts than PUF samples and 6-fold more than diesel particulate samples, when adduct levels were calculated per ng 10 adduct-forming PAH compounds in the exposure dose. DNA binding of PAH compounds in SRM 1650 was more effective than those in gasoline and diesel extracts, showing that 2- and 11-fold more adducts were formed by SRM than by gasoline and diesel particulate extracts, respectively. When the adduct levels were calculated taking into account the amount of PM emitted per test kilometer.

Table II. Levels of PAH–DNA adducts (adducts/10^8 nucleotides) in BEAS-2B cells after treatment with gasoline and diesel extracts and SRM 1650 for 48 h.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Adduct levelsa</th>
<th>(A)</th>
<th>(B)</th>
<th>(C)</th>
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<tr>
<td>Gasoline extracts</td>
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<td>G1</td>
<td>200</td>
<td>1.0</td>
<td>266</td>
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<tr>
<td>G1-PUF</td>
<td>199</td>
<td>0.15</td>
<td>264</td>
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<tr>
<td>G3</td>
<td>145</td>
<td>0.65</td>
<td>65.6</td>
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<td>G3-PUF</td>
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<td>0.51</td>
<td>68.6</td>
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<tr>
<td>G4</td>
<td>50.4</td>
<td>0.79</td>
<td>770</td>
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<tr>
<td>G5</td>
<td>82.7</td>
<td>0.59</td>
<td>91.0</td>
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<tr>
<td>G6</td>
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<td>G6-PUF</td>
<td>291</td>
<td>0.22</td>
<td>355</td>
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<td>Diesel</td>
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<tr>
<td>RD2</td>
<td>65.1</td>
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<tr>
<td>SRM 1650</td>
<td>141</td>
<td>1.4</td>
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</table>

aAdduct levels were calculated (A) per mg of particulate matter (PM), (B) per ng 10 DNA adduct-forming PAH and (C) per mg PM emitted per test kilometer.

Fig. 5. PAH–DNA adduct formation in BEAS-2B cells after exposure to gasoline particulate extracts obtained from particles (G1) and the semi-volatile fraction (G1-PUF) collected on a Teflon-coated glassfiber filter and polyurethane foam (PUF), respectively. BEAS-2B cell cultures were treated for 48 h with DMSO, as a negative control, and with doses of 20, 50, 100, 200 and 400 µg/ml particulate matter.
and CYP1B1 enzymes were expressed in BEAS-2B cells in a study, in which they showed that cytochrome P450 CYP1A1 metabolites in BEAS-2B cells compared with the mixture of autoradiograms of TLC maps between these samples were after exposure to SRM 1587 than to SRM 1650. The formation in bronchial epithelial cells is evaluated.

into consideration when the net effect of DNA adduct enzymes, but also the activities of phase II detoxifying et al., 1994), and the metabolites of B[a]P induce unscheduled DNA synthesis in human bronchial epithelium, leading to active DNA repair (Ishikawa et al., 1982; Jeffrey et al., 1990; Van Agen et al., 1990). Lee et al. (1994) showed that the mutagenicity of and DNA adduct formation by a binary mixture of 1-nitropyrene (1-NP) and B[a]P was lower than those of and 1-NP alone. In this study 126 ng pure B[a]P (0.5 µM) formed 6 times more adducts than 0.2 ng B[a]P in SRM 1650 extract (exposure dose 150 µg/ml). The same exposure dose contained other PAH compounds, of which the concentration of 10 DNA adduct-forming PAH compounds and 1-NP was 18.4 ng/ml. Due to the co-elution of several PAH–DNA adducts on TLC plates, the synergistic or antagonistic effects of DNA binding by single PAH compounds (e.g. B[a]P and 1-NP) in complex mixtures requires further study. The identification of DNA adducts by HPLC or mass spectrometric measurements have been shown to be promising (Carmichael et al., 1992; Farmer and Sweetman, 1995; Savela et al., 1995; Koganti et al., 2000).

In comparison with a human mammary carcinoma cell line (MCF-7), the highest levels of B[a]P-related DNA adducts (311 adducts/10^8 nucleotides) were formed after 24 h exposure to 5 µM B[a]P (Kuljukka-Rabb et al., 2001), whereas in BEAS-2B cells the highest adduct levels were found after 48 h exposure to 2.5 µM B[a]P (395 adducts/10^8 nucleotides). In BEAS-2B cells the metabolic activation of B[a]P was slower but more intense than in MCF-7 cells, because after 24 h exposure the adduct levels started to decrease in MCF-7 cells, but in BEAS-2B cells the adduct levels continued to increase. Also, DNA adducts formed by PAH compounds in SRM 1650 were 3- to 11-fold higher in BEAS-2B cells than in MCF-7 cells. This could be due to higher expression of PAH-metabolizing cytochrome P450 enzymes in bronchial epithelial cells or less active DNA repair in BEAS-2B than in MCF-7 cells. Moreover, the DNA adducts in this study were analyzed with butanol extraction, whereas in Kuljukka-Rabb’s study, the nuclease P1 enhancement procedure was used, which has been shown to exclude small molecular weight aromatic adducts and certain nitro-PAH–DNA adducts (King et al., 1994).

When BEAS-2B cells were exposed to several doses of gasoline extracts obtained from the filters and PFU, higher adduct formation was observed with the extract collected on the PUF at doses of 20 and 50 µg/ml. When concentrations >50 µg/ml were used, the PUF samples started to precipitate in the culture medium. The effective Soxhlet extraction could be a
possible reason for this, indicating that some as yet unknown components in the PUF were co-extracted into the SOF. Partial precipitation of the PUF extract affects the availability of PAH compounds to enter BEAS-2B cells. It remains to be determined whether the components are derived from the gasoline itself or formed during the Soxhlet extraction, however, it was independent of pH.

Semi-volatile PAH, with molecular weights equal to or lower than that of pyrene, have been shown to bind effectively to the PUF (Westerholm et al., 1991). In our study, higher concentrations of the 14 PAH and 10 DNA adduct-forming PAH compounds were measured in the gasoline extracts collected on PUF than those on filters. Gasoline particles predominantly fall into the 10–300 nm diameter range (Maricq et al., 1999). Due to the small particle size of gasoline exhaust, Teflon-coated glassfiber filters (pore size 300 nm) did not adsorb all the small particles and some of them were trapped on the PUF, situated downstream of the PM filter. This explains, in addition to the semi-volatile PAH compounds, the high concentrations of high molecular weight particulate-bound PAH compounds in the PUF extracts. Westerholm et al. (1991) studied the particulate- and semi-volatile-associated compounds in heavy duty diesel exhaust and they found 3-fold higher PAH concentrations in the PUF adsorbent than in the corresponding filter fraction. When comparing concentrations of adduct-forming PAH compounds between the filter and PUF samples of gasoline particulate extracts, the highest levels of 10 PAH compounds were detected in the G6-PUF sample, whereas B[a]P concentrations did not vary between the samples, except for gasoline filter sample G4, which contained much less B[a]P than the other gasoline samples. The low levels of carcinogenic PAH compounds in this gasoline sample in comparison with the other samples is probably due to the short (15 min) collection time of the particulate sample when compared with the 40–160 min used for the collection of the other samples. The concentrations of the 10 DNA adduct-forming PAH and B[a]P were higher in diesel samples than those of gasoline (both PUF and filter), except for sample G6-PUF. The concentrations of the 14 PAH compounds were also 3- to 7-fold higher in diesel than in gasoline extracts collected on filters. This is in accord with other studies, in which PAH compounds analyzed in diesel extracts were shown to be between 5 and 10 times higher than those found in gasoline extracts (Stenberg et al., 1983; Rantanan et al., 1996; Kokko et al., 2000). It is noteworthy that the individual PAH associated with the particulate and semi-volatile phases may vary significantly with the fuels, test cycles and collection procedures used and the individual cars and thus caution is warranted in the interpretation of the results. Standard diesel fuel emitted 34 and 56% more particles in comparison with the reformulated diesel fuels. The lowest amount of particulate emission (0.46 mg/km) was measured when a direct injection gasoline engine was tested at 50 km/h (G3). In a separate emission test under similar conditions, over 2-fold higher particulate emission was obtained from sample G5, whereas the same engine produced particulate matter at 15.3 mg/km when the velocity of the car was 120 km/h (G4). Therefore, if PAH concentrations were calculated based on the emission units (per mg PM emitted per km), all diesel fuel extracts contained higher levels of the 14 PAH compounds (43–132 μg/ km) than gasoline extracts (0.12–5.8 μg/km). Emission rates are in accord with the study by Durbin et al. (1999), in which the particulate emission rates were between 0.006 and 17.2 mg/km for 61 new gasoline vehicles and 9.6 and 1000 mg/km for 19 diesel vehicles.

Both gasoline and diesel exhaust extracts formed DNA adducts in BEAS-2B cells. The adduct levels varied greatly depending on how the results were expressed. The highest differences between the gasoline and diesel samples were obtained when adduct levels were calculated per mg PM emitted per km. Reformulated and standard diesel particulate extracts formed on average 11- and 31-fold more adducts than gasoline particulate extracts. However, when adduct levels were calculated per mg PM or per ng 10 adduct-forming PAH compounds in the exposure dose, gasoline extracts formed more adducts than diesel extracts. Therefore, gasoline particulate extracts either contained more other DNA adduct-forming compounds than those 10 analyzed here or the efficiency of PAH compounds to bind with DNA was greater in gasoline than in diesel samples. PUF samples formed fewer adducts than the corresponding filter samples when calculated per ng 10 DNA adduct-forming PAH compounds, but more when calculated per mg PM in the exposure dose. In an earlier study we showed that DNA adduct levels were higher in calf thymus DNA with diesel than gasoline extracts under oxidative (+S9) and reductive (+XO) activation conditions and that adduct levels were higher with the PUF than the filter extract when adduct levels were calculated per exposure dose or per test kilometer (Pohjola et al., 2003). It seems that PAH-activating enzymes are differently induced in BEAS-2B cells than the enzymes in rat liver S9 and, therefore, DNA adduct levels in these test systems are significantly different. Furthermore, when exposing cell cultures to vehicle extracts, the ability of the soluble organic matter to penetrate into the cells and activate DNA repair and detoxifying enzymes inside the cells affects adduct levels. Therefore, exposing human cell cultures to vehicle extracts rather than in vitro exposure of calf thymus DNA can be used to assess the genotoxicity of PAH compounds derived from vehicle exhausts.

DNA adducts calculated per mg PM correlated significantly with the sum of the 14 PAH and 10 DNA adduct-forming PAH concentrations of gasoline and diesel extracts ($R = 0.807$, $n = 9$, $P = 0.009$; $R = 0.725$, $n = 9$, $P = 0.027$), indicating that the post-labeling assay is an adequate method for analyzing PAH-induced DNA damage. Particulate matter emitted per kilometer produced large differences in DNA adduct levels between the gasoline and diesel extracts when adduct levels were calculated on an emission basis (adducts/mg PM/km). Although diesel extracts contained more particulate-associated PAH compounds than gasoline extracts, the DNA binding of particulate-bound PAH compounds was more effective in gasoline than diesel extracts. Bronchial epithelial cells effectively activated PAH compounds in diesel extracts and SRM 1650 and B[a]P to DNA-binding metabolites and thus can be used in genotoxicity studies. BEAS-2B cells also contain nitrareductive activity, leading to the formation of high levels of adducts derived from nitro-PAH compounds, shown to be highly mutagenic in diesel exhaust emission.

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