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DNA adducts and oxidative DNA damage induced by organic extracts from PM2.5 in an acellular assay

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ABSTRACT

The genotoxic activities of complex mixtures of organic extracts from the urban air particles collected in various localities of the Czech Republic, which differed in the extent and sources of air pollution, were compared. For this purpose, PM2.5 particles were collected by high volume samplers in the most polluted area of the Czech Republic – Ostrava region (localities Bartovice, Poruba and Karvina) and in the locality exhibiting a low level of air pollution – Trebon – a small town in the non-industrial region of Southern Bohemia. To prepare extractable organic matter (EOM), PM2.5 particles were extracted by dichloromethane and c-PAHs contents in the EOMs were determined. As markers of genotoxic potential, DNA adduct levels and oxidative DNA damage (8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxodG, levels) induced by EOMs in an acellular assay of calf thymus DNA coupled with ^{32}P-postlabeling (DNA adducts) and ELISA (8-oxodG) in the presence and absence of microsomal S9 fraction were employed. Twofold higher DNA adduct levels (17.20 adducts/10^8 nucleotides/m^3 vs. 8.49 adducts/10^8 nucleotides/m^3) were induced by EOM from Ostrava-Bartovice (immediate proximity of heavy industry) compared with that from Ostrava-Poruba (mostly traffic emissions). Oxidative DNA damage induced by EOM from Ostrava-Bartovice was more than fourfold higher than damage induced by EOM from Trebon (8-oxodG/10^8 dG/m^3: 0.131 vs. 0.030 for Ostrava-Bartovice vs. Trebon, respectively). Since PM2.5 particles collected in various localities differ with respect to their c-PAHs content, and c-PAHs significantly contribute to genotoxicity (DNA adduct levels), we suggest that monitoring of PM2.5 levels is not a sufficient basis to assess genotoxicity of respirable aerosols. It seems likely that the industrial emissions prevailing in Ostrava-Bartovice represent a substantially higher genotoxic risk than mostly traffic-related emissions in Ostrava-Poruba. B[a]P and c-PAH contents in EOMs are the most important factors relating to their genotoxic potential.

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1. Introduction

Epidemiological studies have demonstrated that particulate organic matter present in the ambient air is a complex mixture of chemicals such as polycyclic aromatic hydrocarbons (PAHs) that may have multiple adverse health effects including cancer (Pope et al., 2002). These adverse effects may not be accurately predicted from the effects of individual mixture components because of their significant interactions within the mixtures (White, 2002; Binkova et al., 2007). PAHs are formed during incomplete combustion processes and are found in ambient air due to industrial emissions (coke ovens, ferrous metallurgy), vehicle exhausts, domestic heating and tobacco smoke (Menzie et al., 1992). Some PAHs, especially the particulate types, are classified by the International Agency for Research on Cancer (IARC) as human carcinogens (benzo[a]pyrene, B[a]P) and several others as probably or possibly carcinogenic to humans (IARC, 2009). After metabolic activation by cytochrome P450 enzymes, B[a]P and other carcinogenic PAHs are known to bind covalently to DNA (DNA adduct formation) (Binkova and Sram, 2004). However, they can also induce oxidative lesions resulting from the production of reactive oxygen species (Penning et al., 1999; Park et al., 2006). To assess the risk associated with exposure to PAHs is a challenging task since PAHs are present in ambient air in the form of complex mixtures. The interactions of mixture components may lead to additivity of the effects, or individual components may interact leading to synergistic or antagonistic effects (Binkova et al., 2007).

Abbreviations: B[a]P, benzo[a]pyrene; B[b]F, benzo[b]fluoranthene; B[k]F, benzo[k]fluoranthene; B[a]A, benz[a]anthracene; B[ghi]P, benzo[ghi]perylene; BPDE, benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide[±]; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; CHRY, chrysene; DRZ, diagonal radioactive zone; DB[al]P, dibenzo[al]pyrene; DB[ah]A, dibenz[ah]anthracene; DCM, dichloromethane; 7,12-DMBA, 7,12-dimethylbenz[a]anthracene; EOM, extractable organic matter; HPLC, high performance liquid chromatography; I[cd]P, indeno[cd]pyrene; 8-oxoG, 8-oxoguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; PM2.5, particulate matter < 2.5 μm; RAL, relative adduct labeling; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography.

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The genotoxicity and mutagenicity of airborne particulate matter has been studied using various *in vitro* short-term assays (Claxton et al., 2004; Lewtas, 2007), including cell-free assays (Adams et al., 1996). We have recently demonstrated the possible use of cell-free assays to study DNA adducts and oxidative DNA damage by complex mixtures of organic compounds extracted from size segregated aerosols (Topinka et al., 2010; Rossner et al., 2010). In the context of a broader molecular epidemiology study dealing with the possible biological and health consequences of air pollution in the heavily polluted Ostrava region, we simultaneously analyzed bulky DNA adducts and 8-oxodG using the acellular assay with the aim of quantifying genotoxicity of particulate air pollution. In addition, we aimed to estimate the possible contribution of heavy industry (mostly in Ostrava-Bartovice) and traffic-related air pollution (mostly in Ostrava-Poruba), and assess the relative importance of bulky DNA adducts and oxidative DNA damage as basic genotoxic events induced by the mixture of organic compounds bound to respirable aerosols.

2. Materials and methods

2.1. Chemicals and biochemicals

Calf spleen phosphodiesterase was purchased from ICN Biomedicals, Inc.; micrococcal nuclease, nuclease P1 from Sigma (Deisenhofen, Germany); polyethyleneimine cellulose TLC plates (0.1 mm) from Macherey-Nagel (Düren, Germany); c-PAHs (99% pure) from Supelco, Inc; T4 polynucleotide kinase (Affymetrix, Germany); γ-^{32}P-ATP (3000 Ci/mmol, 10 μCi/μl) from Perkin Elmer. All other chemicals and solvents were of HPLC or analytical grade.

2.2. Air sampling, EOM extraction and chemical analysis

Particulate matter < 2.5 μm (PM2.5) was collected by a HiVol 3000 air sampler (model ECO-HVS3000, Ecotech, Australia) on Pallflex filters T60A20 (20 cm × 25 cm) in four localities of the Czech Republic differing in the extent and major sources of air pollution: Ostrava-Bartovice (heavily polluted industrial area), Ostrava-Poruba (high level of traffic), Karvina (industrial area) and Trebon (rural area with some houses equipped with local brown coal heating). Sampling was conducted for 24 h each day (with the exception of several days when air pollution was too high and filters were overloaded) for 30–35 days in the winter season of 2008/2009. Exposed filters were kept at −18 °C. Both the dichloromethane extractions of organic complex mixtures (EOMs) from the filters with PM2.5 samples and the chemical analyses of PAHs were performed uniformly in the laboratories of the certified company ALS Czech Republic, Prague (EN ISO CSN IEC 17025). The concentrations of eight PAHs regarded as carcinogenic PAHs (c-PAHs) according to IARC (IARC, 2009), namely, benz[a]anthracene (B[a]A), chrysene (CHRY), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), dibenzo[a,h]anthracene (DB[ah]A), benzo[g,h,i]perylene (B[ghi]P), and indeno[1,2,3-cd]pyrene (I[cd]P) were analyzed in each EOM sample. For the *in vitro* experiments, EOM samples were evaporated to dryness under a stream of nitrogen and the residue re-dissolved in dimethylsulfoxide (DMSO). The stock solution of each EOM sample contained 50 mg of EOM/ml DMSO. Samples were kept in the freezer at −80 °C until analysis.

2.3. In vitro acellular assay and DNA adduct analysis

The assay was performed as previously described (Smith et al., 1998; Binkova et al., 2007). Briefly, calf thymus DNA (1 mg/ml) was incubated with various EOM samples (100 μg EOM/ml) for 24 h at 37 °C with and without metabolic activation using the S9 fraction (1 mg protein/ml). Rat liver S9 fraction was purchased from Toxila (Pardubice, Czech Republic). B[a]P and DMSO treated calf thymus DNA samples were used as positive and negative controls, respectively. DNA was isolated by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation (Gupta, 1985), and the samples were kept at −80 °C until analysis. ^{32}P-postlabeling analysis was performed as previously described (Reddy and Randerath, 1986; Phillips and Castegnaro, 1999). Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37 °C. Nuclease P1 was used for adduct enrichment. The labeled DNA adducts were resolved by multidirectional TLC on 10 cm × 10 cm PEI-cellulose plates. The following solvent systems were used for TLC: D-1: 1 M sodium phosphate, pH 6.8; D-2: 3.8 M lithium formate, 8.5 M urea, pH 3.5; D-3: 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. Autoradiography was carried out at −80 °C for 6–24 h. The radioactivity of distinct adduct spots and diagonal radioactive zones was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (1 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously controlled for purity of the DNA. DNA adduct levels were expressed as adducts per 10^8 nucleotides. A BPDE-DNA adduct standard was run in triplicate in each postlabeling experiment in order to control for interassay variability.

2.4. DNA extraction and analysis of oxidative damage to DNA

Following incubation with the S9 fraction, as described in the previous paragraph, DNA was purified essentially as reported by Rossner et al. (2009) omitting the RNAse and proteinase K incubation steps. Briefly, samples containing CT-DNA with or without the S9 fraction were mixed with the same volume of chloroform–isoamylalcohol (24:1, v/v) and centrifuged for 5 min at 1800 × g. The upper phase was transferred to a new tube and mixed with the same volume of ice-cold absolute ethanol and a 1/10 volume of 5 M NaCl. Precipitated DNA was removed, using a pipette tip, to an Eppendorf tube, washed with 70% ethanol, vacuum dried and stored at −80 °C until further processing. To minimize artifactual DNA oxidation, all solutions were supplemented with an antioxidant, 0.1 mM deferoxamine mesylate (DFA). Before ELISA, samples were removed from the freezer, dissolved in phosphate buffered saline (PBS) at pH 7.4 containing 1 mM DFA (PBS/DFA), fragmented using a 22-G needle and incubated for 5 min at 100 °C to denature the molecules. DNA concentration was adjusted to 1 mg/ml using PBS/DFA, and samples were used for ELISA. Levels of 8-oxodG in DNA were analyzed using the competitive ELISA performed according to a modified protocol (Rossner et al., 2009). Wells of ELISA plates were coated with 5 ng of 8-oxoG conjugated with bovine serum albumin (BSA; total volume, 50 μl/well) by drying the plates overnight at 37 °C. Plates were blocked with 200 μl/well of blocking buffer (1% fetal calf serum (FCS) in PBS/Tween; PBS/Tween–0.05% Tween 20 in PBS) for 1 h at room temperature. After blocking, 50 μl of 8-oxodG standards (concentration range, 5–80 ng/ml) and DNA samples (50 μl/well containing 50 μg DNA) were added, followed by 50 μl of primary antibody (concentration 0.2 μg/ml, MOG-100P, JaICA, Japan). Plates were incubated for 1.5 h at room temperature. After incubation, 100 μl of secondary antibody conjugated with alkaline phosphatase (A4656, Sigma–Aldrich) were added and plates were incubated for another 1.5 h at room temperature. The color was developed by adding 100 μl of p-nitrophenyl phosphate substrate (1 mg/ml of 1 mol/l diethanolamine) per well and incubating the plates for 30–60 min at room temperature. Absorbance was measured with a microplate reader at 405 nm. Each sample was analyzed in triplicate. The assay was run in three independent experiments; mean 8-oxodG levels per 10^5 guanosine molecules and standard deviations were calculated and used for statistical analyses.

2.5. Statistical analysis

Pearson's correlation coefficient was used to analyze the correlation of DNA adduct levels with B[a]P and c-PAH content in EOMs extracted from size segregated aerosols collected in various localities. A comparison of 8-oxodG levels after treatment CT-DNA with and without S9 fraction was performed using Student's *T*-test.

3. Results

3.1. Air sampling and chemical analysis

PM2.5 particles were collected during the winter of 2008/2009 by high volume air samplers in four localities of the Czech Republic as described in Section 2. Threefold higher PM2.5 concentrations were found in Ostrava localities (36.7 and 25.8 μg/m^3 for Bartovice and Poruba, respectively) than in Trebon (11.4 μg/m^3), a small town in the rural area of Southern Bohemia (Table 1). Depending on the locality, EOMs represented 31–36% of the original mass of PM2.5. Although PM2.5 concentrations were comparable in Bartovice and Poruba, 3-fold higher B[a]P and c-PAHs levels were found in Bartovice (13.6 ng/m^3 and 81.6 ng/m^3, respectively), which is a residential area of Ostrava in close proximity to an industrial area (ferrous metallurgy, coke ovens, etc.), than in Poruba (4.28 ng/m^3 and 27.2 ng/m^3, respectively), which is situated at the end of a highway. This difference was further documented by daily concentrations of PM2.5, c-PAHs and B[a]P (Fig. 1) analyzed from individual filters, while DNA adducts and 8-oxodG were measured in EOMs from pooled filters. Particular high levels of c-PAHs and B[a]P in Bartovice are caused by winter temperature inversions causing high increase of the air pollution which is much higher in localities with the industrial sources of c-PAHs. This high time resolution analysis indicated the presence of extremely high peaks of daily concentrations of c-PAHs (200–600 ng/m^3) and B[a]P (30–100 ng/m^3) in Bartovice. Lower concentrations of B[a]P and c-

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