Biomarkers of exposure and effect—interpretation in human risk assessment

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Abstract The effect of exposure to carcinogenic polycyclic aromatic hydrocarbons adsorbed onto respirable air particles (PM2.5, diameter < 2.5 μm) on DNA adducts and chromosomal aberrations was repeatedly studied in Prague, Czech Republic, in groups of policemen working in the downtown area and in bus drivers. Personal exposure was evaluated using personal samplers during working shifts. DNA adducts were analyzed in lymphocytes by the 32P-postlabeling assay and chromosomal aberrations were analyzed by conventional cytogenetic analysis and fluorescent in situ hybridization (FISH). The impact of environmental pollution on DNA adducts and chromosomal aberrations was studied in a total of 950 subjects. Our results suggest that the environmental exposure of nonsmokers to concentrations higher than 1 ng benzo[a]pyrene/m3 represents a risk of DNA damage, as indicated by an increase in DNA adducts and the genomic frequency of translocations determined by FISH.

Keywords Personal monitoring · Carcinogenic polycyclic aromatic hydrocarbons · Benzo[a]pyrene · DNA adducts · Chromosomal aberrations

Introduction

Epidemiological studies have shown that prolonged exposure to particulate air pollution may be associated with an increased rate of morbidity and mortality from respiratory and cardiovascular diseases in the general population. Polycyclic aromatic hydrocarbons (PAHs) adsorbed onto respirable air particles (PM2.5, diameter < 2.5 μm) are mainly derived from incomplete combustion, including mobile sources, such as motor vehicles, and stationary sources, such as power plants, residential heating, among others. Some of these compounds exhibit carcinogenic and/or mutagenic properties (Lewtas 2007). Molecular epidemiology studies using biomarkers of exposure and early biological effects could provide invaluable information on the genotoxic effects of environmental exposure to such PAH mixtures. One promising biomarker seems to be the measurement of DNA adducts, since such measurements take into account individual differences in exposure, absorption, distribution, metabolic activation, and detoxification of PAHs in the body as well as cell turnover and the repair of DNA damage (Binkova et al. 2007a; Lewtas 2007).

Chromosomal aberrations in human peripheral lymphocytes are recognized as a valuable biomarker of effect, probably the only such biomarker that has been standardized and validated at the international level (Bonassi et al. 2008). While classic cytogenetic analysis (conventional method) is the method of choice for determining unstable types of aberrations, the fluorescent in situ hybridization technique (FISH) seems to be a rapid, sensitive, and reliable method for the detection of stable structural rearrangements that remain undiminished over time, such as translocations (Rubes et al. 1998; Verdorfer et al. 2001; Tucker et al. 2003; Sram et al. 2007a; Sigurdson et al. 2008). The FISH painting technique appears to be more sensitive than the conventional technique for detecting the genomic frequency of translocations induced by various chemical agents or irradiation (Sram et al. 2007b).

The capital city of Prague has become one of the most polluted localities in the Czech Republic, primarily due to...
traffic. Therefore, the effect of exposure to carcinogenic PAHs (c-PAHs) adsorbed onto respirable air particles on DNA adducts and chromosomal aberrations was repeatedly studied in groups of policemen working in the downtown area and in bus drivers.

Methods

The study cohort comprised 950 individuals from three studies; the subjects of the analyses were categorized into the following groups: (1) policemen in 2001 (exposed $n=53$, controls $n=52$) (Sram et al. 2007a); (2) policemen in 2004 (exposed $n=480$, 120×4) (Topinka et al. 2007); (3) bus drivers in 2005–2006 ($n=120 \times 3$; exposed I $n=50$, exposed II $n=20$, controls $n=50$) (Rossner et al. 2008). All volunteers were male city policemen working in downtown Prague and spending >8 h outdoors daily. Controls from study (1) were age- and sex-matched healthy males spending >90% of their daily time indoors and working in a suburban area. Study (3) involved 50 bus drivers working in the center of Prague (I), 20 garage men (II) and, as controls, 50 healthy administrative workers spending >90% of their daily time indoors (Table 1).

Ambient air particles (PM10, PM2.5) and c-PAHs, namely, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene (B[a]P), chrysene, dibenz[a]anthracene, and indeno[cd]pyrene, were monitored using VAPS samplers, while personal exposure was evaluated using personal samplers during working shifts. Quantitative chemical analysis of c-PAHs was performed by high-performance liquid chromatography (HPLC) with fluorimetric detection according to the U.S. Environmental Protection Agency method.

DNA adducts were analyzed in lymphocytes by the $^{32}$P-postlabeling assay, which was performed according to a standardized procedure (Fig. 1) (Binkova et al. 2007a). Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37°C; P1 nuclease was used for adduct enrichment. The labeled DNA adducts were resolved by two-directional thin layer chromatography on 10 × 10-cm PEI-cellulose plates. Three solvent systems were used for thin layer chromatography (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 72–120 h. A diagonal radioactive zone and/or distinct DNA adduct spots were excised using the same template for all samples. The $^{32}$P-radioactivity was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (0.5 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA. DNA adduct levels were expressed as adducts per $10^8$ nucleotides. A B[a]P diolepoxide-DNA adduct standard was run in triplicate in each postlabeling experiment to

### Table 1 Overview of the study data

| Biomarkers | Policemen 2001 | Policemen 2004 | Bus driver 2005 |
|------------|---------------|---------------|----------------|
|            | Exp SM | Exp NS | Con SM | Con NS | NS | Exp* NS | Exp* NS | Con NS |
| n          | 29     | 24     | 11     | 41     | 89  | 49       | 19       | 45      |
| Age (years)| 33.1±6.9 | 29.9±7.3 | 35.6±12.3 | 27.9±7.5 | 34.4±8.3 | 49.5±9.6 | 35.8±10.2 | 50.1±10.9 |
| B[a]P (ng/m³) | 2.2±2.0 | 1.4±1.0 | 0.6±0.5 | 0.9±0.6 | 1.5±1.3 | 1.3±0.7 | 3.4±4.1 | 1.7±1.0 |
| c-PAH (ng/m³) | 14.3±13.2 | 9.4±7.6 | 4.7±2.2 | 6.6±3.7 | 8.6±9.0 | 7.2±3.7 | 19.4±24.1 | 9.1±5.6 |
| Vitamin A (μmol/l) | 2.2±0.6 | 1.8±0.6 | 2.2±0.8 | 1.8±0.5 | 3.5±1.0 | 3.4±0.9 | 2.8±0.6 | 3.0±0.8 |
| Vitamin E (μmol/l) | 26.7±9.2 | 25.0±10.9 | 33.5±29.0 | 24.5±6.6 | 24.1±8.8 | 32.1±8.6 | 23.7±6.3 | 23.0±7.1 |
| Vitamin C (μmol/l) | 84.2±40.3 | 86.4±53.1 | 60.0±28.1 | 98.3±58.6 | 64.4±15.9 | 52.2±19.6 | 64.3±14.7 | 56.8±18.7 |
| Folate (μmol/l) | 18.04±11.59 | 17.51±9.90 | 13.48±7.52 | 17.48±9.04 | 25.48±18.31 | - | - | - |
| Cotinine/creatinine (ng/mg) | 1714±1563 | 21±11 | 2072±1901 | 16±11 | 16±9 | 16±11 | 13±8 | 12±9 |
| Cholesterol (mmol/l) | 4.2±1.0 | 3.3±0.9 | 4.1±1.0 | 3.6±0.7 | 3.9±1.2 | 5.9±1.1 | 5.8±1.2 | 5.5±0.9 |
| HDL cholesterol (mmol/l) | 0.8±0.2 | 0.9±0.2 | 0.9±0.3 | 1.0±0.3 | 1.0±0.3 | 1.4±0.3 | 1.5±0.2 | 1.5±0.2 |
| LDL cholesterol (mmol/l) | 2.7±0.8 | 2.2±0.7 | 2.6±0.9 | 2.2±0.6 | 2.2±0.8 | 3.5±0.8 | 3.3±0.8 | 3.2±0.7 |
| Triglycerides (mmol/l) | 2.2±1.9 | 1.3±0.9 | 2.1±1.5 | 1.1±0.7 | 1.4±1.0 | 2.0±1.1 | 2.0±1.4 | 2.1±1.7 |

Exp SM, Exposed smokers; Exp NS, exposed nonsmokers; Con SM, control smokers; Con NS, control nonsmokers; B[a]P, benzo[a]pyrene; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; HDL, high-density lipoprotein; LDL, low-density lipoprotein

*Bus drivers

*Garage men
control for interassay variability and to normalize the calculated DNA adduct levels. The data presented here are average values from three independent experiments (variability of the total and “like-B[a]P”-DNA adduct levels for each: less than ±20%).

Chromosomal aberrations were analyzed by conventional cytogenetic analysis and by FISH whole chromosome painting for chromosomes no. 1 and no. 4 (Fig. 2) (Sram et al. 2007b).

For the conventional cytogenetic analysis (CCA), a suspension from the last fixation step was dropped onto slides, and the slides were air-dried and stained with 5% Giemsa solution (pH 6.8). The slides from each culture of peripheral blood lymphocytes were numbered randomly and scored “blind” in numerical order. One hundred well-spread metaphases with 46±1 centromeres were examined per donor. The following were evaluated as chromosomal aberrations: chromatid and chromosome breaks, and chromatid and chromosome exchanges.

The FISH analysis was performed using commercial WCP (whole chromosome painting) probes differing in color (Cambio, Cambridge, UK) for chromosomes no. 1 (biotinylated) and no. 4 [fluorescein isothiocyanate (FITC)-labeled] according to the manufacturer’s chromosome painting protocol (Rubes et al. 1998). One thousand metaphases were examined for each subject under a fluorescent microscope equipped with a triple-band pass filter for visualization of 4′,6-diamidino-2-phenylindole (blue), FITC (green), and cyanine (red) signals.

Aberrant cells were classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) (Tucker et al. 1995) and recorded by ISIS 4.4.16 software (MetaSystem GmbH, Frankfurt, Germany) as translocations, reciprocal translocations, dicentric chromosomes, acentric fragments, and insertions. Other analyzed parameters were the percentage of aberrant cells (% AB.C.), aberrations per 1000 cells (AB/1000), and the number of color junctions. The genomic frequencies (F_G) of stable chromosomal exchanges were calculated according to Lucas and Sachs (Lucas and Sachs 1993) using the equation: \( F_G = \frac{F_{tg}}{2.05} \left[ f_t(1 - f_t) + f_g(1 - f_g) - f_{tg} \right] \). \( F_{tg} \) is the translocation frequency measured by FISH after two-color painting, and \( f_t \) and \( f_g \) are the fractions of the genome painted red and green, respectively. Additional biomarkers included cotinine in urine determined by radioimmunoassay, plasma levels of vitamins A, E, and C by HPLC, folic acid by enzyme-linked immunosorbenent assay (ELISA), and cholesterol and triglycerides using commercial kits; polymorphisms of metabolic genotypes [GSTM1, GSTP1, GSTT1, EPHX1, cytochrome 450 1A1-MspI (CYP1A1-MspI)] and DNA repair genotypes (XRCC1 and XPD) were determined by PCR-based restriction fragment length polymorphism (RFLP) assays.

Statistical analysis

Statistica (StatSoft, Tulsa, IK) multiple regression and SAS (SAS Institute, Cary, NC) logistic regression were used to model the association between DNA-adduct levels and variables in terms of exposure, potential modifiers of effects, and confounders. Generally, a level of \( p = 0.10 \)
was used for including a variable into the multivariate model (PIN) and a level of \( p=0.15 \) was used for removing a variable (POUT).

Bivariate and multivariate logistic regression were performed to identify the impact of the monitored markers on the level of genetic damage. For logistic regression estimates, variables were transformed into a three level scale using tertiles or into a two level scale using medians.

**Results**

Based on the personal monitoring data, during their working shifts the policemen were exposed to significantly higher concentrations of both c-PAHs and B[a]P than were the controls (median): 9.7 vs. 5.8 ng/m\(^3\) (\( p<0.01 \)) and 1.6 vs 0.8 ng/m\(^3\) (\( p<0.01 \)), respectively. The level of “like” B[a]P-derived DNA adducts was higher in the exposed group than in the controls (0.122±0.036 vs. 0.099±0.035 adducts/10\(^8\) nucleotides, \( p=0.003 \)). The results of multivariate regression analysis showed smoking, vitamin C levels, and polymorphisms of the XPD repair gene in exon 23 and the GSTM1 gene to be significant predictors for total DNA adduct levels. Exposure to ambient air pollution, smoking, and polymorphisms of the XPD repair gene in exon 6 were significant predictors for B[a]P-“like” DNA adducts (Binkova et al. 2007a). Using the FISH technique and probes for chromosomes no. 1 and no. 4, the genomic frequency of translocations were significantly higher in the exposed group than in the controls (1.07±1.18 vs. 1.07±0.81, \( p<0.01 \)) and 1.6 vs 0.94, respectively, and the frequency of chromosomal aberrations determined by CCA was 1.87±1.42, 1.28±1.84, and 1.64±1.46 \% AB.C., respectively.

In another study, bus drivers were sampled in the winter of 2005, summer of 2006, and winter of 2006. Using the personal monitoring approach, the concentrations of B[a]P for the exposed group were 1.25, 0.20, and 1.04 ng/m\(^3\) during the winter of 2005, summer of 2006, and winter of 2006, respectively; for the controls, the concentrations were 1.75, 0.24, and 0.75 ng/m\(^3\), respectively, during these same periods. The total DNA adducts in the exposed group were 1.72±0.56, 1.22±0.45, and 1.62±0.59 adducts/10\(^8\) nucleotides during the winter of 2005, summer of 2006, and winter of 2006, respectively; for garage men, the total DNA adducts were 1.24±0.41, 1.27±0.48, and 1.70±0.08 adducts/10\(^8\) nucleotides during the winter of 2005, summer of 2006, and winter of 2006, respectively; in controls, the totals were 2.15±0.61, 1.18±0.36, and 1.90±0.79 adducts/10\(^8\) nucleotides during the winter of 2005, summer of 2006, and winter of 2006, respectively. In these same three periods, the mean frequencies of translocations measured by FISH (FG/100) were 1.62±1.17, 2.18±1.75, and 1.77±1.31 in the group of bus drivers, 1.20±1.24, 0.88±1.11, and 1.01±0.78 in garage men, and 1.65±149, 1.34±1.01, and 1.87±1.29 in controls, respectively.

The obtained results were confirmed in a subsequent study in which city policemen were sampled in January, March, June, and September. Using a personal monitoring approach, the concentrations of c-PAHs were 1.58 and 9.07 ng/m\(^3\) for B[a]P and c-PAHs during January, 0.39 and 3.46 ng/m\(^3\) for B[a]P and c-PAHs during March, 0.18 and 1.92 ng/m\(^3\) for B[a]P and c-PAHs during June, and 0.45 and 3.08 ng/m\(^3\) for B[a]P and c-PAHs during September. Total DNA adducts were only slightly elevated in January (2.08±1.60) compared to March (1.66±0.65), June (1.96±1.73), and September (1.77±1.77). B[a]P-like DNA adducts, however, were significantly higher in January than in the March and June sampling periods (0.26±0.14 vs. 0.19±0.12 and 0.22±0.13, respectively; \( p<0.0001 \) and \( p=0.017 \)), indicating that c-PAH exposure probably plays a crucial role in DNA adduct formation in lymphocytes (Topinka et al. 2007). In those same periods, the mean frequency of translocations measured by FISH (FG/100) was 1.32±1.07, 0.85±0.95, 0.87±0.81, and 1.08±0.94, respectively, and the frequency of chromosomal aberrations determined by CCA was 2.07±1.48, 1.84±1.28, 1.84±1.42, and 1.64±1.46 \% AB.C., respectively.

The levels of DNA adducts were significantly affected by stationary exposure to B[a]P within the last 30 days (Table 2). Data obtained for biomarkers of exposure and effect were used for a pooled analysis. Using multivariate logistic regression, we calculated the relationship between personal exposure to B[a]P and DNA adducts (DNA adducts=1.042+B[a]P x 0.077, \( p<0.001 \); Fig. 3). These results indicate that c-PAH exposure plays a crucial role in DNA adduct formation in lymphocytes. A similar relationship was observed between personal exposure to B[a]P and
the genomic frequency of translocations measured by FISH 
\( \text{FG/100} = 1.255 + B[a]P \times 0.082, \quad p < 0.05; \) Fig. 4). Figures 3 and 4 show the quasi-linear dose response impact of pollution levels from personal monitoring to DNA adducts and FISH \( \text{FG/100} \) estimated by multivariate linear regression, respectively.

**Discussion**

Studying the ambient concentrations of c-PAHs needed to induce DNA adducts and the genomic frequency of translocations measured by FISH, we observed that the impact of active smoking was more significant than the effect of air pollution. Therefore, we propose to use only nonsmokers in future studies.

The average personal environmental exposure to approximately 10 ng/m³ of c-PAHs (or 1.6 ng/m³ of \( B[a]P \), or approx. twofold higher than the c-PAHs concentrations measured by stationary monitoring—based on the according to outdoor/indoor ratio) during the winter increased DNA adduct formation and decreased repair efficiency, which may be further affected by genetic polymorphisms. This concentration of c-PAHs and its impact on increased DNA adduct formation seems to be important for re-evaluating the risk assessment of c-PAHs. PAH-DNA adduct formation represents one of the key first steps in carcinogenesis (Gammon et al. 2004). According to Phillips (2005), the use of DNA adducts as a measure of exposure, several years prior to the onset (or clinical manifestation) of disease, can help identify individuals at a higher probability of subsequently developing cancer. Another study demonstrated significant correlations between DNA adduct levels and atherosclerosis (Binkova et al. 2002). Both examples demonstrate the potential of DNA adducts as biomarkers of risk.

DNA adducts determined by the \( ^{32} \text{P}-\text{postlabeling method} \) are sensitive biomarkers of environmental exposure to c-PAHs, if a study simultaneously includes personal and stationary monitoring, information on life style, determination of cotinine, vitamin and lipid levels, and genetic polymorphisms of metabolic and DNA repair genes.
It is currently accepted that a high frequency of chromosomal aberrations in peripheral lymphocytes is predictive of an increased risk of cancer (Bonassi et al. 2008). Therefore, we may hypothesize that environmental exposure to c-PAHs that increases the genomic frequency of translocations represents a significant health risk. Surprising results were observed in the group of city policemen who were examined in January and March: the genomic frequency of translocations decreased in a similar manner as did their exposure to c-PAHs. This result reveals that these chromosomal aberrations are not so stable as originally expected. When we studied the relationship between chromosomal aberrations and DNA adducts in the same subjects using FISH, multiple regression analysis indicated that B[a]P-like DNA adducts are a significant predictor of the genomic frequency of translocations (Binkova et al. 2007a). Whole chromosome painting using the FISH technique is more sensitive than the originally used conventional cytogenetic method, which was not affected by the studied concentrations of c-PAHs.

Carcinogenic PAHs are adsorbed on fine respiratory particles (PM2.5) and probably represent the most important biologically active group of pollutants (Binkova et al. 2007b). As c-PAHs were not originally listed among those chemicals whose concentrations should be monitored, stationary monitoring of c-PAHs was started in some European countries only recently. It has been proposed by the EU that the emission limit for B[a]P concentration in European countries only recently. It has been proposed by the EU that the emission limit for B[a]P concentration in the ambient air should be 1 ng/m³/year. Our data indicate that such a concentration can still induce genetic injury. Certainly a concentration of a chemical carcinogen that is able to induce translocations should be considered as deleterious.

Chromosomal aberrations detected as translocations by FISH are a sensitive biomarker of effect and can help to evaluate the risk of occupational and environmental exposure to mutagens and carcinogens. Our FISH results provide new knowledge about the risk of c-PAHs in polluted air, which may significantly affect human health. This new knowledge should be used for risk assessment and to determine new standards for long-term environmental c-PAHs exposure.

Conclusions

The results of our studies suggest that environmental exposure to concentrations >1 ng B[a]P/m³ represent a risk of DNA damage, as indicated by an increase in DNA adducts and an increase in translocations detected by FISH. Among our study cohort, the level of DNA adducts was significantly affected by B[a]P exposure within the last 30 days.

This is the first published report of a relationship between DNA adducts (biomarker of exposure) and chromosomal aberrations as determined by FISH (biomarker of effect).

Our results also suggest that DNA adducts in the lymphocytes of subjects exposed to increased c-PAH levels are an appropriate biomarker of a biologically effective dose, directly indicating whether or not the extent of exposure to these compounds is related to an increased mutagenic and carcinogenic risk. All results indicate that molecular epidemiology studies should be done in a very complex manner, simultaneously using biomarkers of exposure, effect, and susceptibility. Air pollution, and specifically c-PAHs, induce genetic damage and may significantly affect human health.

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