Formation and persistence of DNA adducts in different target tissues of rats after multiple administration of benzo[a]pyrene

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Introduction

Human exposure to polycyclic aromatic hydrocarbons (PAHs*), which comprise a group of well-documented carcinogens, has been and continues to be of occupational and environmental concern. Although considerable occupational data have been reported, more studies are required to clearly explain the carcinogenic mechanisms of PAHs (1). Since DNA is the cellular macromolecule believed to be most closely involved in carcinogenesis, modification (adduct formation) of DNA by carcinogens appears to be directly relevant to potential tumour formation. Therefore, quantitative indicators of human exposure to these carcinogens would be extremely useful in risk assessment studies (2). In fact, the relationship between DNA adduct accumulation and carcinogen susceptibility has led to the examination of DNA adducts as molecular dosimeters for PAH exposure, especially for workers in relevant occupations (3).

Epidemiological studies have indicated that human lung could be the most sensitive and specific target organ for the carcinogenesis of PAHs (1, 2). Most PAHs are biotransformed to reactive species capable of binding to cellular DNA. It is interesting that for some PAHs there is a strong correlation between tissue and cell specificity for formation of promutagenic DNA adducts and susceptibility to carcinogenesis (2, 3). Benzo[a]pyrene (B[a]P), a known carcinogenic PAH, is widely distributed in occupational and natural environments, being found in diesel engine emissions, automobile exhaust, cigarette smoke, food and contaminated air. Thus human exposure to this chemical is widespread and it seems likely to be closely related to overall human lung cancer risk. In experimental animals the carcinogenic effects of B[a]P have been intensively studied and shown to induce in rats a wide range of tumours which depend to a certain extent on the route of administration. Overall, the respiratory tissues form the primary target organ. In a study with a single injection of B[a]P in rats Ross et al. (4) found the apparent half-life of total DNA adducts in lung to be ~22 days, which is the longest of all tissues sampled. However, the target tissues for many chemical carcinogens are not readily accessible for monitoring exposure in humans, so other tissues, such as peripheral white blood cells, have been investigated as possible surrogates. A few studies using a high single dose have been published in recent years (4–6), but the determination of acceptable times for sampling after repeated low exposure has not been comprehensively examined (7).

It was therefore decided to investigate the effects of repeated administration of a relatively low dose of B[a]P on DNA adduct removal in various tissues to assist in the rational application of this technique to human biomonitoring. The goals of this study were: (i) to compare the specific DNA adducts formed in different tissues; (ii) to evaluate the half-lives of DNA adducts in each tissue; (iii) to determine if peripheral blood mononuclear cells (PBMs) are a suitable surrogate for DNA adducts in other tissues.

Materials and methods

Chemicals and reagents

B[a]P, protease K, RNases A and T1, micrococcal nuclease and spleen phosphodiesterase (MNSP) and nuclease P1 (NP1) were purchased from Sigma Chemical Co. (St Louis, MO). T4 polynucleotide kinase (PNK) (3'-phosphatase-free) was supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN). Ficoll-Paque was supplied by Pharmacia LKB (Uppsala,
P-Postlabelling analysis of DNA adducts was performed on the basis of previous reports (3,9-11) but with some modifications as follows.

Animals and chemical treatments

Male Sprague-Dawley (SD) rats, 6 weeks old, were supplied by The University of Sydney Animal House. Rats were fed with Allied Stock Feed (Sydney, Australia) and water ad libitum. Animals were maintained in an environment of 25°C, 60-68% relative humidity and a 12 h light/dark cycle. At the time of B[a]P administration the animals weighed between 280 and 300 g and were 7 weeks old. B[a]P was dissolved in dimethyl sulphoxide (DMSO) and 10 mg/kg body wt B[a]P was administrated by i.p. injection 3 times/week for 2 weeks. The control rats received DMSO vehicle (at a volume of 1 ml/kg body wt) alone.

After the last injection rats were kept for observation times of 1, 3, 7, 14, 28 and 56 days. At each time point four rats were sacrificed and 10 ml blood was collected from the abdominal aorta. The liver, spleen and lung were excised and frozen at -80°C until DNA isolation. The Ficoll-Paque procedure was used to isolate PBMNs (4).

DNA isolation

PBMNs or 0.2 g tissue were homogenized in 2 ml 50 mM Tris–HCl, 10 mM EDTA buffer, pH 8.0, to prepare the RNA and protein-free DNA as described previously (2,8). Some modifications were made as follows. The crude nuclei from tissues were centrifuged at 1851 × g for 10 min, then the nuclei were incubated with RNases A (100–150 µg/ml) and T1 (25–50 U/ml) in the presence of 50 mM Tris–HCl, 10 mM EDTA buffer, pH 8.0, at 37°C for 30 min. After adding 1% SDS the samples were incubated with proteinase K (250–500 µg/ml) for another 30 min at 37°C. Following phenol and chloroform/isoamyl alcohol extraction, DNA was precipitated in absolute ethanol. The DNA pellet was washed twice with 70% ethanol and air dried. The concentration and purity of DNA were measured by spectrophotometer using the A260 value (1 A260 U = 50 µg DNA), A260/A280 ratio (0.40–0.45) and A260/A320 ratio (1.80–1.85). Tissues from individual animals were analysed separately.

32P-Postlabelling analysis of DNA adducts

32P-Postlabelling analysis of DNA adducts was performed on the basis of previous reports (3,9-11) but with some modifications as follows.

MNSP digestion. Aliquots of 20 µg isolated DNA were dissolved in 20 µL 1× MNSP buffer (20 mM sodium succinate, 10 mM CaCl2, pH 6.0) followed by addition of 5 µL MNSP enzyme (1 µg/µL each enzyme). After 3 h incubation at 37°C 10 µL digested solution were taken for further assay and the remainder of the aliquot was kept on ice for total nucleotides labelling.

NP1 enrichment. Five microlitres of NP1 (0.5 µg/µL) and 1.65 µL 1 mM ZnCl2 (final concentration, 0.1 mM) were added to 10 µL of the above solution (containing 8 µg DNA). After 2 h incubation at 37°C the sample was dried using a Dynavac freeze dryer (Sydney, Australia).

PNK reaction. The dried residue was redissolved in 10 µL double distilled water to get a final DNA concentration of 8 µg/10 µL. Half of this (containing 4 µg DNA) was used for the PNK reaction. After adding 5 µL ‘hot mix’ [consisting of 3 µL (33P)ATP (30 µCi), 0.3 µL PNK (3 U), 1.0 µL 10× kinase buffer and 0.7 µL dual distilled water] incubation was carried out at 22°C for 1 h.

Mapping of adducts. Aliquots of 8 µL PNK reaction mixture were spotted onto TLC plates which had been prewashed with double distilled water. The TLC plates were developed in different solvent systems, named D1, D3, D4 and D5 as detailed in Figure 1. Chromatography was carried out on 10×12 cm PEI-cellulose plates.

MNSP chromatography and measurement. DNA adducts and total nucleotides on the TLC plates were visualized using a Bio-Imaging Analyser with BAS 1000 workstation and quantified by MacBas version 1.0 software from Fuji Photo Film Co (Tokyo, Japan). Relative adduct labelling values were calculated via the total nucleotides and then converted to amol/µg DNA adducts (3,9).
Fig. 2. $^{32}$P-Postlabelling chromatograms of DNA adducts in spleen tissue induced by B[a]P. (a) Control and at (b) day 1, (c) day 14 and (d) day 56 after B[a]P exposure ceased. Chromatography conditions were as described in Figure 1.

Fig. 3. $^{32}$P-Postlabelling chromatograms of DNA adducts in PBMNs induced by B[a]P. (a) Control and at (b) day 1, (c) day 14 and (d) day 56 after B[a]P exposure ceased. Chromatography conditions were as described in Figure 1.
Fig. 4. 32P-Postlabelling chromatograms of DNA adducts in lung tissue induced by B[a]P. (a) Control and at (b) day 1, (c) day 14 and (d) day 56 after B[a]P exposure ceased. Chromatography conditions were as described in Figure 1.

Calculation of DNA adduct half-lives and mean residence time (MRT)
Based on the preliminary plotting of the experimental data a bi-exponential model was chosen to calculate the initial and terminal half-lives and MRT (12).

\[ C = C_1e^{-\tau_1} + C_2e^{-\tau_2} \]  
\[ \tau_{1/2} = \ln 2/\gamma \]  
\[ \text{MRT} = \frac{\text{AUMC}}{\text{AUC}} \]

where \( C \) is the concentration of DNA adducts in the tissue, \( C_1 \) and \( C_2 \) are model coefficients, \( \gamma_1 \) and \( \gamma_2 \) are exponents, \( t \) is time in days and \( \tau_{1/2} \) is the half-life. AUC is the area under the concentration-time curve and AUMC is the area under the first derivative of the concentration-time curve.

\[ \text{AUC} = C_1/\gamma_1 + C_2/\gamma_2 \]
\[ \text{AUMC} = C_1/\gamma_1^2 + C_2/\gamma_2^2 \]

Statistics
Experimental results are presented as the mean ± SE. ANOVA with post hoc Duncan’s test, Student’s \( t \)-test and linear regression analysis were used for different data analyses. The significance level was set at 0.05 for all results.

Results
Administration of vehicle (DMSO) alone to rats did not produce any detectable DNA adducts in any of the tissues examined (Figures 1a, 2a, 3a and 4a). A dose of 10 mg/kg B[a]P (i.p.) resulted in the formation of several detectable DNA adducts in liver, spleen, PBMs and lung during the entire post-exposure period. The patterns of adducts in each tissue were similar at different time points (Figures 1b–d, 2b–d, 3b–d and 4b–d). Lung showed the highest DNA adduct levels in all tissues tested (Figure 5).

Maximum levels of DNA adducts appeared at day 1 post-exposure for all tissues (Figure 5). Comparison of the removal of DNA adducts from different tissues showed liver to have the most rapid reduction. At day 14 the remaining DNA adducts in lung, spleen, PBMs and lung were still >50% of the levels at day 1, but the total adducts in liver had been reduced to ∼33% (Table I). The removal of total DNA adducts was found to be liver > spleen > PBMs > lung. The half-lives and the MRTs for total DNA adducts in lung, PBMs, spleen and liver tissues are shown in Table II, calculated from mean adduct levels measured between 1 and 56 days. It is clear that all data are well described using a bi-exponential equation.
Persistence of benzo[a]pyrene-DNA adducts

Table I. Per cent of total DNA adducts* remaining in each tissue over the entire post-exposure period for rats (n = 4) administered 10 mg/kg B[a]P

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Day(s)} & \text{Liver} & \text{Spleen} & \text{PBMMNs} & \text{Lung} \\
\hline
1 & 100 & 100 & 100 & 100 \\
3 & 67.5 & 94.9 & 94.4 & 95.9 \\
7 & 54.8 & 76.1 & 60.7 & 63.4 \\
14 & 33.8 & 66.4 & 57.4 & 60.7 \\
28 & 33.7 & 35.7 & 36.1 & 47.6 \\
56 & 14.2 & 29.3 & 32.4 & 41.0 \\
\hline
\end{array}
\]

*DNA adducts at each time point/DNA adducts day 1) \times 100%.

Table II. Half-lives of total B[a]P-induced DNA adducts and MRTs in rat PBMMNs, lung, spleen and liver

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Tissue} & \text{Initial half-life (days)} & \text{Terminal half-life (days)} & \text{MRT (days)} \\
\hline
\text{PBMMNs} & 5.4 & 127.0 & 172.4 \\
\text{Lung} & 3.2 & 105.5 & 148.1 \\
\text{Spleen} & 9.5 & 80.1 & 99.6 \\
\text{Liver} & 1.5 & 30.8 & 41.8 \\
\hline
\end{array}
\]

Table III. Per cent of DNA adduct 1 over total DNA adducts in tissues of rats (n = 4) at different sampling times post-B[a]P exposure

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Day(s)} & \text{Liver} & \text{Spleen} & \text{PBMMNs} & \text{Lung} \\
\hline
1 & 42.4 & 94.2 & 95.1 & 39.5 \\
3 & 66.7 & 88.4 & 96.3 & 45.2 \\
7 & 72.6 & 92.1 & 95.3 & 37.5 \\
14 & 74.7 & 92.3 & 96.8 & 38.2 \\
28 & 79.1 & 95.1 & 94.7 & 30.9 \\
56 & 71.9 & 96.7 & 97.5 & 32.4 \\
\hline
\end{array}
\]

Table IV. Per cent of DNA adduct 4 over total DNA adducts in tissues of rats (n = 4) at different sampling times post-B[a]P exposure

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Day(s)} & \text{Liver} & \text{Lung} \\
\hline
1 & 52.6 & 13.7 \\
3 & 30.8 & 13.6 \\
7 & 26.8 & 4.2 \\
14 & 24.4 & 7.3 \\
28 & 70.2 & 20.5 \\
56 & 28.1 & 18.4 \\
\hline
\end{array}
\]

Table V. Per cent of DNA adduct 5 over total DNA adducts and adduct 5 remaining in lung tissue of rats (n = 4) at different sampling times post-B[a]P exposure

\[
\begin{array}{|c|c|c|}
\hline
\text{Day(s)} & \text{Adduct 5/total adducts (%)} & \text{Per cent remaininga} \\
\hline
1 & 43.7 & 100 \\
3 & 40.8 & 89.4 \\
7 & 47.8 & 69.4 \\
14 & 49.6 & 68.9 \\
28 & 39.6 & 43.2 \\
56 & 38.4 & 36.1 \\
\hline
\end{array}
\]

*aDNA adduct at each time point/DNA adducts day 1) \times 100%.

Fig. 6. Adducts 1, 4 and 5 in different tissues from rats administrated B[a]P. Each bar represents the mean ± SE, n = 4.

The liver tissue has the shortest initial and terminal half-lives (1.5 and 30.8 days) and the shortest MRT (41.8 days) of DNA adducts after repeated low level exposure to B[a]P. In contrast, the initial and terminal half-lives (5.4 and 127.0 days) and MRT values (172.4 days) were the longest in PBMMNs.

A major individual adduct, designated adduct 1, was detected in all tissues and showed a gradual disappearance (Figure 6). Over the observation period adduct 1 was the major DNA adduct in spleen and PBMMNs, at ~90% or even higher. However, in lung adduct 1 showed a relatively lower proportion at ~30-45% of total DNA adducts. Overall adduct 1 seemed to be the major one in liver, although levels were <50% at days 1 and 28 (Table III). A second major adduct, at ~4-20%, designated adduct 4, was relatively lower in lung. Adduct 4 showed increases at 28 days in both liver and lung tissues (Figure 6 and Table IV). Another adduct, adduct 5, was only detected in lung tissue and comprised a major proportion of total DNA adducts (38-49%) for this organ (Table V). Adduct 5 was observed to persist over the entire post-exposure period (Figure 6). Some minor adducts (2 and 3) were found in different tissues with levels ranging from 0.5 to 5%.

After B[a]P treatment the correlation between PBMMNs and other tissues for total DNA adducts was found to be highly significant, with \( r \) values of 0.925-0.997 (Figure 7).

Discussion

The results from this study show that repeated low level exposure to B[a]P significantly increased total DNA adducts in all tissues tested, with lung having the highest level of all. At day 14 total DNA adduct levels in lung, spleen and PBMMNs were still >50% of the levels at day 1. The removal rate of total DNA adducts was found to be liver > spleen > PBMMNs.
Fig. 7. Correlation of total DNA adduct levels between PBMNs and lung, spleen or liver over the entire post-exposure period (analysed by Student’s t-test).

These findings are consistent with other previous reports, though only single high doses of B[a]P, ~10– to 25-fold higher than our dose, had been administered to the experimental animals (4,6,7).

In experimental animals the carcinogenic effects of B[a]P have been intensively studied and shown to induce a wide range of tumours which depend on the route of administration, such as lung tumours following inhalation, i.p. injection or oral administration, forestomach tumours after oral administration or skin tumours when applied topically. However, the respiratory system, especially lung, is considered as the primary target organ for B[a]P-induced carcinogenesis in rats (4,7).

Withney et al. (13) have found that B[a]P was distributed to a greater extent in lung tissue compared with other tissues sampled immediately post-dosing (lung > blood > liver > kidney > fat > fetus) in pregnant rats after exposure to higher concentrations of B[a]P (200 or 800 mg/m³) by inhalation. The total metabolites of B[a]P were also concentrated more highly in the lung tissue (lung > blood > liver > kidney > fat > fetus > fat). In another study after a single i.p. dose of B[a]P (100 mg/kg) to SD rats increases in total DNA adducts were also observed in a number of tissues, with lung DNA exhibiting consistently higher adduct levels than liver or peripheral blood lymphocytes (PBLs) (5). In a study with other PAH carcinogens, such as benzo[b]fluoranthene, the DNA adducts in liver and PBLs were present at <10 amol/µg DNA, while in lung these were 100 amol/µg DNA at 56 days after benzo[b]fluoranthene administration (5). All of the previous reports are consistent with the results obtained in the present study, in which we found that the lung tissue presents a higher level of DNA adducts than other tissues sampled (Figure 5). These current data may, at least in part, account for the findings in this study, have the longest half-life and MRT value of all tested tissues. Therefore, based on this behaviour, the DNA adducts present and was also observed in PBLs, accounting for ~10% of the total adducts, and in liver, where it made up <5% of the total. These data suggest that adduct no. 9 is possibly derived from a metabolic pathway that includes the formation of 9-hydroxy-B[a]P. Comparison of the three major adducts (nos 4, 7 and 9) in the study by Ross et al. (4) with the DNA adducts (1, 4 and 5) found in the present study shows similarities. As lung is the most commonly recognized target organ for B[a]P, the role and importance of (our) DNA adduct 5 in lung tumour induction should be further investigated in experimental animals and humans.

With regard to the relationship between PAH–DNA adduct levels in peripheral blood samples and other tissues, Nesnow et al. (7) reported correlation values (r²) of 0.78 for lung and 0.75 for liver respectively. Our data showed an even stronger relationship for total DNA adduct levels between lung and PBMNs during the entire post-exposure period. Such a close relationship supports the use of DNA adducts in PBMNs as surrogates for target organ DNA adducts after B[a]P exposure. As PBMNs are readily accessible cells in humans the continued development of human biomonitoring techniques examining DNA adducts in PBMNs exposed to PAHs in the workplace is warranted. However, it should be noted here that the relationship between DNA adducts in PBMNs and lung is not necessarily highly correlated for all PAHs. For instance, Ross et al. (2) found a decreased correlation of DNA adducts in these two tissues after benzo[b]fluoranthene treatment (r² = 0.67) in the experimental animals.

At day 14 after exposure ceased DNA adducts in liver tissue were ~34% of their level at day 1. In other tissues the adduct levels were still at least 50% of the initial levels. At day 56 DNA adducts were still 41% of day 1 levels in lung tissue and 34% in PBMNs. These data indicate that samples taken days or weeks after exposure will still yield DNA adducts at detectable levels. Furthermore, a half-life for DNA adducts in lung tissue has been calculated in our study as 3.2 and 105.5 days for initial and terminal half-lives respectively. Some differences in the half-lives in the PBMNs, spleen and liver tissue were also observed. However, both initial and terminal half-lives for DNA adducts in liver tissue of 1.5 and 30.8 days under our experimental conditions are the shortest of all tissues examined. The MRT value in liver tissue is ~41.8 days. These results indicate that when applying this technique in human biomonitoring studies there would be some flexibility in the sampling time for the exposed workers. It is very interesting that PBMNs, an easily accessible surrogate chosen in this study, have the longest half-life and MRT value of all tested tissues. Therefore, based on this behaviour, the DNA adducts in PBMNs will be much more convenient to use for the estimation of B[a]P in lung, which is the primary target organ of B[a]P. It should be noted here that the results for half-lives in this study are based on a bi-exponential model, which is the best model for describing our data. Therefore, our figures differ from Ross et al. (4) and Nesnow et al. (7), who used linear regression for half-life calculations. Nevertheless, when
linear regression is applied to our data the results obtained are similar to the previous publications (4,7), giving 25.3 days for lung, 19.7 days for PBMs, 17.4 days for spleen and 9.0 days for liver.

In conclusion, the results from our study indicate that B[a]P induced a significant increase in DNA adduct levels in all tissues tested within the first few days after in vivo treatment. The DNA adducts were still readily detectable at 56 days after exposure had ceased. Based on the high correlation values, it is apparent that total PBMs adducts reflect total lung tissue adduct levels. Thus the results support the use of PBMs as surrogates for estimation of B[a]P exposure in lung, the primary target organ for carcinogenesis. However, the significance of some specific individual adducts, such as adduct 5, which only occurs in lung, requires further investigation.

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