Hemoglobin adducts of benzo[a]pyrene diolepoxide in newspaper vendors: association with traffic exhaust

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Benzo[a]pyrene diol epoxide adducts with hemoglobin (Hb) were measured to detect human exposure to environmental benzo[a]pyrene from traffic exhaust. Benzo[a]pyrene tetrahydroxetrols (BPTs) released from Hb after acid hydrolysis were quantitated by gas chromatography–mass spectrometry after immunoaffinity chromatography. Fifty three newspaper vendors were enrolled. The median adduct concentration was 0.3 fmol BPTs/mg Hb in high density traffic-exposed vendors and ≤0.1 fmol BPTs/mg Hb in those exposed to low density traffic; the difference was not significant (P = 0.09). Among non-smokers, adducts were detectable in 60% of high exposure subjects (median 0.3 fmol BPTs/mg Hb) and in 28% of those with low exposure (median ≤0.1 fmol/mg Hb). This difference was significant (P = 0.02). In low exposure smokers the median of adducts was 0.26 fmol BPTs/mg Hb, while in low exposure nonsmokers it was ≤0.1 fmol BPTs/mg Hb (P = 0.08, not significant). Adduct concentration was no different for low and high density traffic-exposed smokers (P = 0.82). The data indicate a significant difference in adduct concentration related to traffic exhaust exposure among non-smokers.

Introduction

Benzo[a]pyrene (B[a]P) is a ubiquitous pollutant encountered in the workplace, urban air and food and is a constituent of mainstream and sidestream cigarette smoke (1). It is frequently used as an indicator of exposure to polycyclic aromatic hydrocarbons (PAH), which has been associated with an increased risk of respiratory chronic and neoplastic diseases (2). B[a]P is metabolically activated to (±) r-7, r-8-dihydroxy-t-9, t-10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), the ultimate carcinogenic metabolite known to bind covalently to DNA and blood proteins (3–5).

The reaction of B[a]P with human hemoglobin (Hb) has been investigated. The presence of carboxylic esters formed by BPDE alkylation of one or more carboxylate groups in Hb has been demonstrated (6–8). These studies showed that ester adducts are the most abundant ones formed by BPDE. They are stable in native Hb, but upon disruption of the tertiary structure of the protein, they release benzo[a]pyrene tetrots (BPTs), used as a quantitative measure of exposure to B[a]P. The application of this approach in humans was first reported by Day et al. (9) and by Taghizadeh and Skipper (10), providing clear evidence of the formation of BPDE–Hb adducts in vivo.

Despite the apparent advantage of measuring BPDE–Hb adducts in molecular epidemiology studies, only a few reports exist on their detection.

The major challenge in B[a]P dosimetry arises from the fact that adduct analyses typically involve trace quantitation of BPTs. Ordinary human environmental exposures to carcinogens studied so far result in a range of adduct levels (pmol adduct/g Hb) (4).

There are numerous techniques currently available for these studies, all varying in their specificity and sensitivity (11,12). One of the most successful methods for BPT quantitation has been gas chromatography–mass spectrometry (GC-MS) (9,11,13,14). The application of GC-MS with negative ion chemical ionization (NICI) analysis appeared to give the best combination of sensitivity and selectivity (10,13,14). Successful measurement by GC-MS depends on several factors, including sample work-up and quantification procedures, the most important of which is probably the use of a good internal standard. However, to date only one method has reported the use of isotope-enriched BPTs ([14C]BPTs) for quantification by GC-MS with single ion recording (SIR) (10). Unfortunately, only two mass units molecular weight difference between analyte ([12C]BPTs) and internal standard ([14C]BPTs) can give rise to complications in MS analysis, thus preventing accurate measurement of low BPT levels.

For these reasons we synthesized a novel, fully deuterated BPT (BPT-d12), to be used in a simpler, quantitative and specific method for GC-MS analysis of BPTs released from Hb.

Most previous reports on the detection of BPDE–Hb in humans relate to the monitoring of populations exposed to high ambient concentrations of B[a]P in the workplace (15,16). The need for more data to establish the real value of BPDE–Hb adducts as biological markers for environmental PAH exposure prompted us to validate our improved method for measuring BPDE–Hb adducts in newspaper vendors in Milan, Italy, exposed to low levels of B[a]P, mainly from traffic exhaust.

Materials and methods

Chemicals and reagents

B[a]P-d12 (99% deuterated) was obtained from Cambridge Isotope Laboratories (Inneberg, Switzerland). [14C]anti-BPDE (sp. act. 48 mCi/mmol) was purchased from Chem-Science Laboratories (Lenexa, KS) and BPTs from the NCI Chemical Carcinogen Reference Standard Repository (c/o Midwest Research Institute, Kansas City, MO). N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) and iodotrimethylsilane (ITMS) were obtained from Fluka (Switzerland). 3-Methylcholantherene (3-MC) was from Sigma Chimica.

*Abbreviations: B[a]P, benzo[a]pyrene; PAH, polycyclic aromatic hydrocarbons; BPDE, (±)-r-7, r-8-dihydroxy-t-9, t-10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; Hb, hemoglobin; BPTs, benzo[a]pyrene tetrots; GC-MS, gas chromatography–mass spectrometry; NICI, negative ion chemical ionization; SIR, selected ion recording; MSTFA, N-methyl-N-trimethylsilyl trifluoroacetamide; ITMS, iodotrimethylsilyl; 3-MC, 3-methylcholantherene; NADP, nicotinamide adenine dinucleotide phosphate; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; PBS, phosphate-buffered saline TMS, trimethylsilyl; HRGC, high resolution gas chromatography; RBC, red blood cells; CV, coefficient of variation.
Isolation of BPTs from human hemoglobin

BPT-d12 biosynthesis

Formation of BPT-d12 was achieved using a hepatic microsomal fraction from 3-MC-pretreated male Sprague–Dawley rats.

Male CD rats (200 ± 10 g body wt) were purchased from Charles River (Calco, Como, Italy). Procedures involving animals and their care were conducted in conformity with the institutional guidelines, in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication no. 85-23, 1985).

Induction by 3-MC was by i.p. injection of 25 mg/kg body wt in 0.5 ml corn oil once daily for 3 days. Animals were killed 24 h after the last 3-MC injection. Microsomes were then prepared from rat liver as described (17,18).

Microsomal incubations contained 3-MC-induced microsomes at a concentration of 1 mg protein/ml and an NADPH generating system (0.5 mM NADP, 7 mM G-6-P, 20 U G-6-PDH per microsomal incubation). The reactions were started by adding 30 mM B[a]P-d12. Reaction volumes were 10 ml in phosphate-buffered saline (PBS), pH 7.4. The samples were incubated at 37°C for 2 h in the dark. Incubations were terminated by chilling in ice. The microsomal mixtures were directly loaded onto Extrelut-20 pre-packed disposable columns. The deuterated B[a]P metabolites were eluted with 40 ml ethyl acetate. The organic eluates were dried, dissolved in 45% methanol/water and analyzed by HPLC.

HPLC analyses were on a Beckman System Gold liquid chromatograph programmed solvent module equipped with a Beckman System Gold diode array detector. A C-18 Tepchips (Analytical Technology, Cernusco S/N, Italy) reverse phase column (150×4.6 mm) was used for the separation of deuterated B[a]P metabolites, employing a gradient of 45% methanol/water to 60% methanol/water over 15 min, then to 94% at 10 min at a flow rate of 1 ml/min. The detector was operated over the range 200–450 nm. Peak signals were monitored at wavelengths of 254 and 344 nm. The BPT fractions were collected, combined and their structure further confirmed by GC-MS.

Derivatization of BPTs for GC-MS

BPTs were derivatized to their trimethylsilyl (TMS) derivatives. The dried sample was first dissolved in 10 μl anhydrous tetrahydrofuran and mixed by vortexing. Then 40 μl of a derivatizing mixture of MSTFA + 0.2% ITMS was added. After incubation at 60°C for 15 min, the reaction mixture was taken to dryness under a stream of nitrogen and the residues reacted with the MSTFA/1TMS mixture and analyzed by GC-MS.

Analysis of BPTs by GC-MS

Gas chromatographic and mass spectrometric data were obtained on a Finnigan HIGH RESOLUTION (HRGC)–NICI–SIR analyses were by mass spectrometer equipped with a Finnigan Trace GC–Mass spectrometer. Gas chromatographic and mass spectrometric data were obtained on a Finnigan 140 mass spectrometer equipped with a Finnigan Trace GC–Mass spectrometer. Gas chromatographic and mass spectrometric data were obtained on a Finnigan Trace GC–Mass spectrometer equipped with a Finnigan Trace GC–Mass spectrometer.

Results

Upon C-18 HPLC diode array UV analysis, the rat microsomal incubations produced several peaks attributable to deuterated B[a]P metabolites. BPT-d12 was identified by comparison with standard unlabeled BPT HPLC retention times and UV absorption spectra and the structure was confirmed by MS analysis.

As reported in Figure 1a, the observed fragmentation pattern in the NCI spectra of TMS-BPTs showed the most intense...
peak at \( m/z \) 446, resulting from loss of the neutral fragment TMS-O-TMS from the molecular ion (M-162)\(^-\) and was consistent with previous reports (6,9). A second loss of TMS-O-TMS gave the ion at \( m/z \) 284 (relative abundance 22%). By analogy, TMS-BPT-d12 showed the most intense peaks at \( m/z \) 458 (100%) and 296 (22%) (Figure 1b). Quantitation was by monitoring the ions at \( m/z \) 446 (BPTs) and \( m/z \) 458 (BPT-d12). The ions at \( m/z \) 284 and \( m/z \) 296 were also recorded, in order to increase the specificity of the method.

The amount of biosynthesized BPT-d12 was quantitated by GC-MS and found to be \( \sim \)1% of the B[a]P concentration added to the microsomal incubation.

Figure 2 shows typical HRGC-SIR chromatograms obtained by injecting 0.25 pg (0.8 fmol) standard BPTs and 15 pg (45 fmol) BPT-d12 standard (Figure 2a), a human Hb sample containing detectable amounts of BPTs (Figure 2b) and a human Hb sample with no detectable BPTs (Figure 2c).

Calibration curves drawn with increasing amounts of BPTs (0.8–16 fmol injected) and a constant amount of internal standard BPT-d12 (45 fmol injected) showed a linear response over the range of concentrations considered, with a correlation coefficient of 0.999. The limit of sensitivity for the detection of BPTs in human Hb was \( \leq \)0.1 fmol/mg Hb.

The immunoaffinity columns used for sample purification had a capacity of 12.5 ng BPTs/0.25 ml gel/22 μg antibodies. Overall recovery was \( \sim \)30% from human Hb samples.

Reproducibility, determined by triplicate analyses of a few human Hb samples, showed a coefficient of variation (CV) generally within 10%. Accuracy, estimated by repeated injections of the same samples, gave CV \( \leq \)2%.

We performed time course experiments in vitro, in order to validate the efficiency of the acid hydrolysis in releasing BPTs from adducted Hb. Samples containing adducted BPDE-Hb were hydrolyzed as described above for 30, 60, 90 or 120 min. No significant differences in BPT release were found (data not shown), so 60 min was chosen, a hydrolysis time similar to those previously reported (10,11).

Fifty three newspaper vendors were enrolled in the study, all working in the city of Milan. Demographic characteristics, distribution of subjects and smoking habits of the volunteers are shown in Table I. On the basis of information provided by the Municipal Police Traffic Division of Milan, we classified newspaper vendors whose stand was located in heavy traffic areas, where the daily traffic flow was \( >1300 \) vehicles/h, with a flow rate in the rush hour of 1650–6800 vehicles/h, as high exposure to traffic exhaust. Low exposure newspaper vendors were those working in news-stands in low density traffic areas (<1300 vehicles/h). The flow rate of 1300 vehicles/h represents the median of all traffic flow measurements. Smokers and non-smokers were evenly distributed between the two exposure groups.

Individual BPDE-Hb adduct levels are shown in Figure 3, in which the adduct levels are expressed as fmol BPTs released/mg Hb hydrolyzed. Adduct levels showed wide interindividual variability, with a range of \( \leq \)0.1–3.3 fmol BPTs/mg Hb. The median BPT level in Hb from high exposure vendors was 0.3 fmol/mg protein, while the median for low exposure individuals was below the limit of sensitivity of the method (\( \leq \)0.1 fmol/mg Hb). The difference between the two groups was not significant, although an association of borderline significance (\( P = 0.09 \)) cannot be excluded between levels of adducts and degree of exposure.

Table II shows the levels of BPDE-Hb adducts in the high exposure and low exposure vendors ranked by their smoking habit. Adducts were detectable in 60% of high exposure non-smokers (median 0.3 fmol BPTs/mg Hb) and in 28% of low exposure ones (median \( \leq \)0.1 fmol BPTs/mg Hb). The difference was significant (\( P = 0.02 \)).

High exposure and low exposure smokers had medians of 0.27 and 0.26 fmol BPTs/mg Hb (\( P = 0.82 \)). Smoking did not affect the levels of adducts in the high exposure individuals.
Fig. 3. Distribution of BPDE-Hb adducts in low exposure newspaper vendors (LE: news-stands located in low density traffic areas) and high exposure newspaper vendors (HE: news-stands located in high density traffic areas), each consisting of smokers (triangles) and non-smokers (circles). The adduct levels are expressed as fmol BPTs released per mg protein hydrolyzed. Each sample was run in duplicate. Each data point represents the measurement average. The bold lines represent the median. The dotted line indicates our detection limit. Samples with non-detectable adducts are grouped below the dotted line. *LE/HE, \( P = 0.09 \) by Mann-Whitney U test.

Table I. Demographic characteristics, distribution of subjects and smoking habits of the study newsvendors

<table>
<thead>
<tr>
<th></th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>53 (100)</td>
</tr>
<tr>
<td>Male</td>
<td>32 (60)</td>
</tr>
<tr>
<td>Female</td>
<td>21 (40)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>20-39</td>
<td>16 (30)</td>
</tr>
<tr>
<td>40-59</td>
<td>30 (57)</td>
</tr>
<tr>
<td>60-over</td>
<td>7 (13)</td>
</tr>
<tr>
<td>Work years</td>
<td></td>
</tr>
<tr>
<td>1-9</td>
<td>19 (36)</td>
</tr>
<tr>
<td>10-19</td>
<td>10 (19)</td>
</tr>
<tr>
<td>20-29</td>
<td>19 (36)</td>
</tr>
<tr>
<td>30-over</td>
<td>5 (9)</td>
</tr>
<tr>
<td>High-traffic exposed</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>30 (57)</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>10</td>
</tr>
<tr>
<td>Low-traffic exposed</td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>23 (43)</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>9</td>
</tr>
</tbody>
</table>

(P = 1). Levels of adducts were higher in low exposure smokers (median 0.26 fmol BPTs/mg Hb) than low exposure non-smokers (median <0.1 fmol BPTs/mg Hb), although the difference was not significant (\( P = 0.08 \))

Table II. BPT in Hb (fmol/mg) from newspaper vendors in the city of Milan

<table>
<thead>
<tr>
<th></th>
<th>Low exposed (^a)</th>
<th>High exposed (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>( \leq 0.1-0.41 )</td>
<td>( \leq 0.1-3.3 )</td>
</tr>
<tr>
<td>Median</td>
<td>&lt;0.1</td>
<td>0.26</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>( \leq 0.1-1.75 )</td>
<td>( \leq 0.1-2.9 )</td>
</tr>
<tr>
<td>Median</td>
<td>0.26</td>
<td>0.27</td>
</tr>
</tbody>
</table>

\(^a\)Low exposed: newspaper vendors working in news-stands in low-traffic areas.

\(^b\)High exposed: newspaper vendors working in news-stands in heavy-traffic areas.

\(^c\)High exposed non-smokers/low exposed non-smokers: \( P = 0.02 \) (Mann-Whitney U-test). None of the other comparisons were significant.

Discussion

Although the presence of PAH and B[el]P in the air of large European cities has progressively declined in the last 20 years (20,21), accurate estimates of exposure are still needed in order to obtain a better understanding of the health hazards connected with PAH air pollution.

We used acid hydrolysis combined with immunoaffinity chromatography and HRGC-NICI-MS to quantitate BPTs released from Hb in a selected population exposed to low environmental levels of B[el]P. BPTs were released reproducibly after acid hydrolysis of the protein and were recovered clearly from up to 100 mg Hb by fast extraction on Extrelut columns.

A unique aspect of our procedure is the use of deuterated B[el]P tetrahydrortetrolys as internal standards. This was added to the human hemolysates at the hydrolysis step and, with stable isotope dilution mass spectrometry, permitted accurate quantification of the true BPT levels in the samples, overcoming the relatively low recovery.

An important problem is that unadducted BPTs in a human blood sample might be quantified as adducts. In order to rule this out we performed an experiment in which hemolysates of positive human samples were filtered through a YM-10 (10 kDa cut-off) Amicon membrane in a 50 ml Amicon stirring cell under \( N_2 \) pressure. The filtrates, containing low molecular weight material (e.g. GSH adducts, free B[el]P metabolites) were subjected to acid hydrolysis and to the subsequent steps of purification. GC-MS analysis (data not shown) showed no BPTs in the filtrates, confirming that the BPTs measured after hydrolysis of the protein derived from covalently bound BPDE adducts sensitive to acid treatment.

To date, no studies have investigated the relationship between environmental B[el]P exposure and BPDE-Hb in humans. Most attention has been focused on BPT-DNA adduct measurements in cohorts from industry or from extreme environmental conditions, where the B[el]P concentrations were >50 ng/m\(^3\) (22,23). It has been reported that BPDE-Hb adduct levels in foundry workers are correlated with airborne PAH when the workplace total PAH concentration reaches 4 \( \mu g/m^3 \) (15).

We addressed exposure to PAH in newspaper vendors as a prototype of a population chronically exposed to low environmental levels of B[el]P. In Italy, newspaper vendors have a long daily exposure to air pollution, being in the stand for at least 10–12 h, 6 days a week. Furthermore, most news-stands are kiosks, often located at crossroads in streets with heavy
traffic. Unfortunately, there is no systematic information available on PAH air pollution at all the stand addresses considered in the study, but in the city of Milan the B[α]P air concentration in high traffic areas has been estimated to be in the range 1–3 ng/m³ during sampling (24).

Since the samples were collected in summer, we can exclude any contribution of residential heating, reported to affect the air levels of PAH (25), so environmental B[α]P appears to be mainly traffic generated.

The present study indicates that a biomarker such as the BPDE–Hb adduct might be useful for studies of non-occupationally PAH-exposed people.

To our knowledge, this is the first evidence of a measurable difference in BPDE–Hb adduct levels in individuals essentially exposed to low levels of traffic-derived B[α]P, although no correlation was observed between adduct levels and number of vehicles per hour (data not shown).

The amounts of BPDE–Hb adducts we detected were at least 50 times lower than those reported for occupationally exposed subjects (15), but in the range of the levels reported for the general population (10).

The significantly higher level of BPDE–Hb adducts in non-smoker high density traffic-exposed vendors compared with the low density traffic-exposed non-smoking group suggests a relationship between exposure to traffic exhaust and levels of adducts. Similar trends were observed by Hemminki (26) in Stockholm taxi drivers and by Autrup (27) in young women not occupationally exposed to PAH. These two studies showed B[α]P-equivalent–serum albumin adduct levels to be higher in people living in the city than in suburban areas.

Smoking increases exposure to PAH and B[α]P, but data concerning the effect of cigarette smoking on BPDE–Hb adduct levels are scanty and conflicting (10,11,15). In the present study, a tendency to higher levels of adducts was noted in low exposure smokers compared with the low exposure non-smokers, suggesting that smoking could have an impact on adduct concentration.

Most of the smokers enrolled in the study were moderate smokers, with an average of 15 cigarettes/day, yielding ~300–600 ng B[α]P inhaled per day. These figures are higher than the estimated B[α]P inhaled by traffic-exposed subjects (10–30 ng B[α]P/10 m³/working day), therefore, it was unexpected to find similar BPT levels in high density traffic-exposed subjects (15), but in the range of the levels reported for the general population (10).

In conclusion, we have developed a specific and sensitive method for BPDE–Hb adduct quantification, relevant to the validation of the use of this biomarker on a general population exposed to low levels of B[α]P. There was a significant difference in adduct concentration related to traffic exhaust exposure among non-smokers, suggesting that the use of this marker in assessing exposure to low levels of B[α]P should take into consideration the interference of possible confounders.

Acknowledgements

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