Quantitation of enantiomers of \( r-7,t-8,9,c-10 \)-tetrahydroxy-7,8,9,10-tetrahydrobenzo[\( a \)]-pyrene in human urine: evidence supporting metabolic activation of benzo[\( a \)]pyrene via the bay region diol epoxide

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Benzo[\( a \)]pyrene (BaP), a potent polycyclic aromatic hydrocarbon carcinogen, is widely distributed in the human environment. All humans are exposed to BaP through the diet and contact with the general environment; cigarette smokers have higher exposure. An important pathway of BaP metabolism proceeds through formation of diol epoxides including the ‘bay region diol epoxide’ \( 7R,8S \)-dihydroxy-95,10R-epoxy-7,8,9,10-tetrahydrobenzo[\( a \)]pyrene [BaP-(7R,8S)-diol-(95,10R)-epoxide] and the ‘reverse diol epoxide’ 95,10R-dihydroxy-7R,8S-epoxy-7,8,9,10-tetrahydrobenzo[\( a \)]pyrene [BaP-(95,10R)-diol-(7R,8S)-epoxide]. The bay region diol epoxide is considered a major ultimate carcinogen of BaP based on studies in cell culture and laboratory animals, but the available data in humans are less convincing. The bay region diol epoxide and the reverse diol epoxide react with \( H_2O \) to produce enantiomeric BaP-tetraols that are excreted in the urine. We used chiral stationary-phase high-performance liquid chromatography and gas chromatography–negative ion chemical ionisation–tandem mass spectrometry to quantify these enantiomeric BaP-tetraols in the urine of 25 smokers and 25 non-smokers. The results demonstrated that the BaP-tetraol enantiomer representing the carcinogenic bay region diol epoxide pathway accounted for 68±6% (range 56–81%) of total BaP-tetraol in smokers and 64±6% (range 46–78%) in non-smokers. Levels of the major BaP-tetraol enantiomer decreased by 75% in smokers who quit smoking. These data provide convincing evidence in support of the bay region diol epoxide mechanism of BaP carcinogenesis in humans.

Introduction

Benzo[\( a \)]pyrene (BaP), a highly carcinogenic polycyclic aromatic hydrocarbon (PAH) and one of the most extensively investigated of all chemical carcinogens (1), is considered ‘carcinogenic to humans’ by the International Agency for Research on Cancer (2). Considerable evidence supports the role of BaP and other PAHs as important causative factors for lung cancer in cigarette smokers (3). All cigarettes generate BaP and other PAHs in their smoke as a result of incomplete combustion of tobacco (4). Although current levels of BaP in mainstream cigarette smoke are fairly low (typically ~7 ng/cigarette) (5), this carcinogen is accompanied by a plethora of other PAH of varying structures, many of which are also carcinogenic (6). All non-smokers are also exposed to BaP through the diet and in the general environment (2). BaP is a potent carcinogen in laboratory animals inducing tumours at the site of application as well as distant sites, depending on the study design. Inhalation studies of BaP in Syrian golden hamsters resulted in the induction of upper respiratory tract and upper digestive tract tumours, and intrapulmonary installation of BaP in the rat lung caused malignant lung tumours. Many other tumour types have been induced by various routes of administration (2).

BaP requires metabolic activation to exert its carcinogenic effects and evidence exists for at least three mechanisms (2,7–9). The one discussed here involves the formation of carci-

nogenic diol epoxides, which react easily with DNA (2,7–9). A second mechanism centres on DNA damage by metaboli-

cally formed radical cations (10). A third invokes quinone metabolites of BaP resulting in direct and indirect damage to

DNA, the latter through redox cycling (11). The diol epoxide mechanism, presented in more detail in the next paragraph, results in metabolites that react at specific sites in the \( TP53 \) tumour suppressor gene (12–14). Mutations at these same sites have been found in lung tumours from smokers (15).

The metabolism of BaP by the diol epoxide pathway is summarised in Figure 1 (2,7–9,16–20). The initial step involves epoxide formation at the 7.8 or 9.10 position, catalysed by cytochromes P450, principally P450s 1A1, 1A2 and 1B1. Induction of these P450s via activation of the aryl hydrocarbon receptor is commonly observed in smokers, probably due to PAH exposure (2,4,21,22). The epoxides are converted to dihydrodiols 1 and 2 by addition of \( H_2O \), catalysed by microsomal epoxide hydrase. The initial epoxidation and the hydration are highly stereospecific. Thus, BaP-(7R,8R)-diol (1) and BaP-(9R,10R)-diol (4) are the major enantiomers formed in virtually all systems examined. These dihydrodiols are then converted to diol epoxides in a second cytochrome P450-catalysed reaction. The formation of the \( anti \)-diol epoxide (in which the epoxide ring is \( trans \)- to the benzylic hydroxyl group) is highly favoured in the case of 1, producing BaP-(7R,8S)-diol-(9S,10R)-epoxide (2). Less is known about the formation of 5. Diol epoxide 2 has come to be known as a ‘bay region diol epoxide’ because it is located near the ‘bay region’ of the tetrahydro-BaP molecule. Diol epoxide 5 has therefore been called a ‘reverse diol epoxide’. Diol epoxide 2 reacts easily with DNA to form adducts, mainly with deoxyguanosine (23). The major DNA adduct formed from 2 is identical to that formed \( in vitro \) and \( in vivo \) from the parent compound BaP in the presence of metabolic activation, providing powerful evidence in support of this mechanism of DNA damage (1,2,8). This adduct is formed at specific sites in the \( TP53 \) gene, as noted previously (13). Products indicative of formation of the reverse diol epoxide 5 were not conclusively detected in metabolism studies of race-

mic BaP-9,10-diol with rat liver microsomes, and racemic BaP-

9,10-diol was not tumorigenic in tests on mouse skin (8,24).

The synthetic racemic \( syn \)- and \( anti \)- reverse diol epoxides were
considerably less mutagenic than the bay region diol epoxides in Salmo nella typhimurium TA100 and in V79 Chinese hamster cells; however, discordant results were reported for the racemic reverse diol epoxide 5 in S. typhimurium TA98 with one study finding less mutagenicity than the racemic bay region diol epoxide 2, whereas a second study found higher mutagenicity of 5 than 2 (24,25). Based on an immense amount of data from in vitro experiments and studies in laboratory animals, the bay region diol epoxide pathway proceeding from BaP to diol epoxide 2 is convincingly more important in the metabolic activation of BaP than the reverse diol epoxide pathway via diol epoxide 5, but there is far less evidence in humans. The bay region diol epoxides and reverse diol epoxides of chrysene and dibenz[a,h]-anthracene have also been investigated in some detail (26,27).

Individual susceptibility to the carcinogetic effects of BaP logically will depend in part on the extent to which the bay region diol epoxide 2 is formed. The occurrence of this pathway in exposed humans has been examined in multiple studies by quantification of DNA and protein adducts of 2. This work is challenging because the adducts are generally formed in very low yields and the amount of DNA or protein available for analysis may be quite small. The results of these studies have been reviewed (28–31). Our approach to this problem focuses on analysis of tetrroals 3 and 6 because the major reaction of diol epoxides 2 and 5 is with DNA and protein, but rather with H2O. Thus, analysis of BaP-tetroals in human urine potentially can provide a quantitative indicator of inter-individual differences in BaP metabolic activation and potentially cancer susceptibility in smokers and other exposed individuals. In a previous study, we demonstrated the feasibility of this approach for the analysis of racemic BaP-tetroal in smokers’ urine (32). However, the results of that study did not allow us to distinguish the bay region diol epoxide and reverse diol epoxide pathways. We are aware of only one study in the literature that examined these two pathways separately—our analysis of the urine of four creosote workers exposed to unusually high levels of BaP. The results showed that the bay region diol epoxide pathway to BaP-tetroal 3 comprised 78% of total BaP-tetroal (33). To our knowledge, no information is available in the literature on the relative amounts of the two pathways in smokers and non-occupationally exposed non-smokers. It was possible that the extent of bay region diol epoxide formation could differ markedly among individuals and between these two groups; such information could be critical in assessing individual cancer susceptibility, which is very important in approaches to cancer prevention. Therefore, in this study, we analysed the urine of smokers and non-smokers for BaP-tetroals 3 and 6.

Materials and methods

Study design

These studies were approved by the University of Minnesota Institutional Review Board. For the comparison of tetroal levels in smokers and non-smokers, subjects were recruited by a member of the research staff of the University from a large group of research volunteers, and spot urine samples were collected. Inclusion criteria were as follows: current smoker of at least 10 cigarettes per day for the past year, or non-smoker in good physical and mental health with no unstable medical condition as determined by medical history and investigator assessment. Smoking status was confirmed by determination of exhaled CO (35). For the quit smoking study, 24-h urine samples were obtained as previously described (36). Two baseline urine samples were collected while the subjects were still smoking. Subsequent samples analysed here were collected 7, 21 and 56 days after quitting.

Chemicals, enzymes and chromatography supplies

Racemic r-7,r-8,3,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (a 50:50 mixture of BaP-tetroals 3 and 6) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO, USA). The internal standard, racemic 10C]-BaP-tetroal, was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Purities of these standards were >99%, as determined by high-performance liquid chromatography (HPLC) analysis. A Vydac 210TP3515 (150 x 3.0 mm, 5 μm) C18 HPLC column was obtained from Grace Davison (Deerfield, IL, USA). β-Glucuronidase and arylsulphatase (from Helix pomatia) were obtained from Roche Diagnostics Corp. (Indianapolis, IN, USA). Strata-X polymeric SPE cartridges (100 mg/3 ml, #8B-S100-EBJ) were procured from Waters. High-temperature deactivated fused silica (0.25 mm inside diameter, #160-2845-10) was obtained from Agilent Technologies.

Analysis of BaP-tetroal enantiomers 3 and 6 in urine

This was performed using a modification of a previously described method (32,33). For the analysis of the enantiomers in the urine of smokers and non-smokers, a 30-ml urine sample was required. This 30-ml sample was initially divided into ten 3-ml aliquots, each of which was processed identically in the β-glucuronidase hydrolysis and first (Strata-X) solid-phase extraction described below. The eluents from this solid-phase extraction were divided into five aliquots for the second (Oasis MAX) solid-phase extraction. The eluents from that step were combined and processed by HPLC as a single sample. Due to limitations in sample availability in the quit smoking study, 3-ml urine samples were used and each was processed as described below. Because of the smaller sample size, only BaP-tetroal 3 was quantifiable in that study.

To a 15-ml polypropylene centrifuge tube containing the 3-ml aliquot of urine was added 60 μl of [10C]-racemic BaP-tetroal in 20 μl of isopropanol and 0.450 ml of 2.5 M NaOAc buffer, pH 5. The tube was vortexed, 60 μl of β-glucuronidase was added, and the mixture was incubated with shaking for 20 h at 37°C. The sample was then added to a preconditioned (3 ml of CH3OH

Fig. 1. Metabolism of BaP to enantiomeric tetroals 3 and 6 via bay region diol epoxide 2 and reverse diol epoxide 5.
followed by 3 ml of H₂O Strata-X solid-phase extraction cartridge. The cartridge was washed sequentially with 3 ml of 50% CH₃OH, 0.25 ml of 50% CH₃OH containing 2% NH₄OH, and then BaP-tetraol was eluted with 2 ml of 90% CH₃OH in H₂O. The analyte fraction was collected in a 4-ml silanised vial, and the solvents were removed on a Speedvac. The sample was dissolved in 750 μl of 30% CH₃OH in H₂O with sonication and loaded onto a preconditioned (3 ml of CH₃OH followed by 3 ml of H₂O) Oasis MAX cartridge (30 mg). The cartridge was washed with 0.5 ml of 2% formic acid in 50% CH₃OH, 0.5 ml of 50% CH₃OH, 0.5 ml of 2% NH₄OH in 50% CH₃OH, 0.5 ml of 50% CH₃OH, then 0.25 ml of 50% CH₃OH to eliminate residual NH₄OH, and then BaP-tetraol was eluted with 0.75 ml of 90% CH₃OH in H₂O. The analyte fraction was collected in a 1-ml silanised vial, and the solvents were removed on a Speedvac. The residue was dissolved in 100 μl of CH₃OH with sonication and vortexing, transferred to an insert vial, dried on a Speedvac and dissolved in 50 μl of 30% CH₃OH in H₂O. The sample, along with 15 μl of wash CH₃OH containing 2 μl/ml of benzene and toluene as UV markers, was injected onto the C18 HPLC column, which was held at 23°C, and eluted with a linear gradient program from 30 to 51% CH₃OH in H₂O for 20 min at 0.4 ml/min with detection by UV (254 nm). The two UV markers were eluted at 12 and 22 min and BaP-tetraol eluted at 20 min. BaP-tetraol was collected during a 3-min window in a 1-ml silanised vial and dried on a Speedvac. The residue was dissolved in 100 μl of CH₃OH with sonication, transferred to an insert vial, concentrated to dryness and dissolved in 50 μl of isopropanol. The BaP-tetraol enantiomers 3 and 6 were separated on a Pirkle chiral HPLC column as previously described (33), silylated and analysed by gas chromatography–negative ion chemical ionisation–tandem mass spectrometry (GC–NICI–MS/MS) as previously described (32) using a high-temperature deactivated fused silica MS transfer line.

### Statistical analysis

The non-parametric Mann–Whitney U-test was used to compare levels of BaP-tetraol enantiomers (BaP-tetraol 3 vs. BaP-tetraol 6), total BaP-tetraol (smokers vs. non-smokers) and BaP-tetraol 3 in smokers (before vs. after quitting smoking).

### Results

The analytical method used in this study was essentially identical to the validated methods previously described (33,37) except that the boronic acid solid-phase extraction step was replaced by Oasis MAX solid-phase extraction and HPLC purification steps. The boronic acid step was deleted because in our hands the commercially available cartridges were no longer able to retain PAH-tetraols with cis-hydroxyl groups. The method used in this study resulted in enrichment of BaP-tetraols 3 and 6, which were identified and separated on a Pirkle column as previously described (33). The separation, illustrated in Figure 2, was complete and similar to that reported previously. Overall recovery of internal standard averaged 40%.

The separated tetrads were silylated and analysed by GC–NICI–MS/MS, which produced clean chromatograms with readily quantifiable peaks, as illustrated in Figure 3A and C, while the internal standard traces are shown in Figure 3B and D. A peak eluting just prior to silylated BaP-tetraol 3 in Figure 3A was identified as the tetra-trimethylsilyl derivative of r-8,10,11-trihydroxy-8,9,10,11-tetrahydronbenzo[k]-fluoranthene by comparison of its retention time and mass spectrometric fragmentation intensities to those of a synthetic standard; all other isomeric tetrads (derived from BaP, benzo(e)-pyrene, benzo(b)fluoranthene and benzo(j)fluoranthene) were eliminated as the origin of this peak based on consideration of their behaviour on boronate columns, or retention times, or mass spectrometric fragmentation of their silylated derivatives (see supplementary Material, available at Mutagenesis Online).

Urine samples were obtained from 25 cigarette smokers (age 38.8±8.8 years [mean ± SD]; 10 males; cigarettes per day, 21.4±5.5 [mean ± SD]) and 25 non-smokers (age 40.0±11.0 years; 7 males; see supplementary Table S1, available at Mutagenesis Online). The results of the enantiomer analysis are presented in Figure 4 and supplementary Table S1, available at Mutagenesis Online. BaP-tetraol 3, representing...
the carcinogenic bay region diol epoxide pathway, accounted for 68±6% (mean ± SD) of total BaP-tetraol in smokers (range 56–81%) and 64±6% (range 46–78%) in non-smokers, both significantly greater than levels of BaP-tetraol 6 (P < 2×10−3⁰). Levels of total BaP-tetraol averaged 0.20±0.13 (mean ± SD) fmol/ml urine (range 0.03–0.61 fmol/ml) in smokers and 0.08±0.07 fmol/ml urine (range 0.009–0.25 fmol/ml) in non-smokers, a significant difference (P < 0.0004).

The results of the quit smoking study are presented in Figure 5 and supplementary Table S2, available at Mutagenesis Online. Only the major enantiomer, BaP-tetraol 3, was quantified because the available urine samples were only one-tenth the size of those used in the smoker vs. non-smoker study. Levels of BaP-tetraol 3 averaged 253±120 fmol/24h at baseline and 62±61 fmol/24h after 56 days of abstaining from smoking, a 75% decrease, which was significant (P = 0.004).

Discussion

Our results demonstrate for the first time that the carcinogenic bay region diol epoxide of BaP, structure 2 of Figure 1, is the major precursor to BaP-tetraol in the urine of non-occupationally exposed smokers and non-smokers. The pathway via 2 accounted for 64–68% of BaP-tetraol in the urine of these 50 subjects with similar relative percentages in smokers and non-smokers. These results support the role of bay region diol epoxide 2 in carcinogenesis by BaP in humans. We are aware of only one previous report of this type, which was our study of four creosote workers exposed to relatively high levels of BaP; 78% of BaP-tetraol was formed via bay region diol epoxide 2 (33). The critical features of the method used in this study and the previous one were the baseline separation of the BaP-tetraol enantiomers 3 and 6 on a Pirkle chiral HPLC column, and the highly sensitive and specific GC–NICI–MS/MS quantitation of the separated tetraols.

An extensive and convincing body of evidence has clearly established bay region diol epoxide 2 as the principal metabolite of BaP involved in DNA adduct formation. The major adduct—10-(deoxyguanosin-N2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (N2-BPDE-dG)—has been detected in cells and tissues of laboratory animals treated with BaP and in various in vitro systems including human cells exposed to BaP (2,19,38–40). This overwhelming body of data leaves no doubt about the significant role of bay region diol epoxide 2 in DNA adduct formation and carcinogenicity of BaP. Data on DNA adduct formation in exposed humans also support the role of diol epoxide 2, but not nearly as convincingly. Among the various methods employed for DNA adduct detection in tissues or cells of exposed humans, only HPLC-fluorescence and mass spectrometry have the requisite sensitivity and specificity to identify N2-BPDE-dG (30). HPLC-fluorescence studies of DNA adducts of bay region diol epoxide 2 are quite numerous and have provided convincing evidence for acid catalysed release of BaP-tetraol (as a racemic mixture of 3 and 6) from N2-BPDE-dG and possibly from other related DNA adducts; this released BaP-tetraol is then quantified (41–44). This method as currently employed does not distinguish between adducts formed from the bay region diol epoxide 2 vs. the reverse diol epoxide 5, as both types of adducts would produce the detected BaP-tetraol. Liquid chromatography–tandem mass spectrometry has been used to quantify N2-BPDE-dG specifically, but this adduct was detected in only 1 out of 26 human lung DNA samples (39). Similarly, data on specific detection of protein adducts of diol epoxide 2 remain sparse and inconclusive (29). Our results, taking advantage of the fact that the major reaction of diol epoxide 2 is with H2O to produce tetraol 3, strongly support the common occurrence of the carcinogenic bay region diol epoxide pathway of BaP metabolism in humans.

There is scant evidence in the literature for the formation of reverse diol epoxide 5. One study of the metabolism of BaP-(9R,10R)-diol (4) with rat liver microsomes or a reconstituted monooxygenase system reported predominant formation of a phenolic metabolite(s) tentatively identified as either 1- and/or 3-hydroxy-BaP-(9R,10R)-diol, with some evidence for minor amounts of reverse diol epoxides, possibly including 5 (24). Our previous work demonstrated that racemic reverse diol epoxide 5 was a better substrate for glutathione-S-transferase conjugation than racemic diol epoxide 2 (45). We are not aware of any other studies in the literature that have identified products of the reverse diol epoxide pathway of BaP metabolism. The results
presented in this study, in which all subjects had some BaP-tetraol 6, varying from 32–36% of total BaP-tetraol, in their urine, appear to be the first data establishing the formation of the reverse diol epoxide 5 of BaP in any system. An alternative pathway to BaP-tetraol 6 could proceed via formation of a diol epoxide from BaP-(7S,8S)-dioi, the minor stereoisomer formed from BaP [e.g. the enantiomer of BaP-(7R,8R)-dioi(1)]. Consistently, however, literature reports indicate that BaP-(7S,8S)-dioi is metabolised mainly to the corresponding syn-diol epoxide, which is not a precursor to 6 (8,16,18,40). It is possible that the detection of BaP-tetraol 6 in our study reflects a microbiologically catalysed pathway in the human colon, followed by reabsorption and excretion in the urine. This would not have been observed under the conditions of most previous studies.

Levels of BaP-tetraol (3 + 6) were about twice as great in smokers (0.20 fmol/ml) compared with non-smokers (0.08 fmol/ml), which is consistent with the results of our previous study of racemic BaP-tetraol in urine (32). However, in that study, overall levels were ~3 times higher in both smokers (0.64 fmol/ml) and non-smokers (0.26 fmol/ml) than reported in this study. As the amount of smoking (19.3 ± 7.8 cigarettes per day in the earlier study vs. 21.4 in this study), the methods used and the quality of the data were similar and excellent in both studies, it is likely that the current set of subjects had lower exposure to BaP per cigarette and through their diet. The 75% decrease in levels of BaP-tetraol 3 upon smoking cessation is also roughly consistent with the differences in BaP-tetraol levels between smokers and non-smokers. In a previous analysis of urinary 1-hydroxypyrene levels in subjects who quit smoking (from the same study as reported here), we found a 50% decrease (36). The lack of complete disappearance of urinary PAH metabolites upon cessation of smoking is clearly due to alternate sources of exposure to BaP or pyrene, typical PAH, which are ubiquitous in the diet and the general environment.

The amounts of BaP-tetraol in the urine of the smokers in this study, averaging 0.20 fmol/ml urine, were low. BaP-tetraol levels were only ~1/25,000 as great as the typical amounts of phenanthrene tetraol observed in smokers’ urine; for non-smokers, this ratio was ~1/20,000 (46,47). There are at least three reasons for these relatively low amounts. First, levels of BaP in cigarette smoke are quite low, typically ~7 ng per cigarette, and levels of BaP—a five ring PAH—in both cigarette smoke and the general environment are lower than those of phenanthrene, a three-ring PAH (5,47). Second, and perhaps more important, most BaP metabolites are excreted in the faeces in contrast to phenanthrene metabolite excretion, which is mainly via urine (48–50). In one study in which [14C]BaP was administered to rats, the percent of dose recovered in the faeces after 168 h averaged 85%, while only 1–3% was excreted in the urine (51). Third, BaP-tetraol and phenanthrene tetraol are relatively minor metabolites of BaP and phenanthrene, resulting from three independent enzymatic steps plus hydrolysis and conjugation. In the case of phenanthrene, this pathway represents 0.5–15% of the dose, depending on the individual studied (52). We note, however, that formation of tetraol metabolites can be rapid; in our previous study of [D$_{48}$]phenanthrene metabolism in smokers, tetraol levels were maximal 30 min after inhalation (53).

In summary, we report in this study convincing evidence supporting the bay region diol epoxide pathway of BaP metabolic activation in humans—both smokers and non-smokers. These results support the designation of BaP as ‘carcinogenic to humans’ and serve to emphasise the importance of decreased human exposure to this common combustion product.

**Supplementary data**

**Supplementary Material and Tables S1 and S2** are available at *Mutagenesis* Online.

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