ABSTRACT:

Many studies have examined the relationship between polymorphisms in glutathione S-transferase genes and cancer in people exposed to polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene (BaP), but the results to date have been modest. Missing from these studies has been an exploration of the formation of the appropriate glutathione conjugates in humans. We incubated human hepatocytes from 10 donors with racemic anti-BaP-7,8-diol-9,10-epoxide (BPDE), believed to be a major ultimate carcinogen of BaP, or with the noncarcinogenic reverse diol epoxide, racemic anti-BaP-9,10-diol-7,8-epoxide (rev-BPDE). Incubations were carried out for 12 or 24 h. We used high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring at m/z 464 → m/z 317 to analyze the incubation mixtures for the mercapturic acid products that would result from glutathione conjugation. The standard mercapturic acids were synthesized by reaction of BPDE or rev-BPDE with N-acetylcysteine. We obtained convincing evidence in human hepatocytes for mercapturic acid formation from rev-BPDE in all 10 samples, in amounts up to 17 pmol/ml. However, we could detect mercapturic acids from BPDE in only 1 of 10 samples (0.05 pmol/ml).

Taken together with our similar previous results of analyses of phenanthrene metabolites in human hepatocytes and human urine, the results of this study indicate that conjugation of BPDE with glutathione is a minor pathway in humans, indicating that glutathione S-transferase genotyping is not an effective method for assessing risk of PAH-induced cancer in humans, at least with respect to the diol epoxide pathway of PAH carcinogenesis.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are well established environmental carcinogens that may play a significant role in the causation of skin and lung cancer in occupationally exposed humans and lung cancer in smokers (International Agency for Research on Cancer, 1983, 1984, 1985; Dipple et al., 1984; Luch, 2005; Straif et al., 2005). The prototypic PAH is benzo[a]pyrene (BaP), one of the most extensively studied of all carcinogens, and considered carcinogenic to humans by the International Agency for Research on Cancer (Straif et al., 1983, 1984, 1985; Dipple et al., 1984). BPDE reacts easily with DNA to form 9,10-epoxide (BPDE), illustrated in Fig. 1A (Conney, 1982; Cooper et al., 1989). 9,10-epoxide of benzo[a]pyrene in

Preferential Glutathione Conjugation of a Reverse Diol Epoxide Compared with a Bay Region Diol Epoxide of Benzo[a]pyrene in Human Hepatocytes

Received April 28, 2010; accepted June 14, 2010

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbons; BaP, benzo[a]pyrene; BPDE, anti-BaP-7,8-diol-9,10-epoxide; GST, glutathione S-transferase; Phe, phenanthrene; Phe-1,2-D-3,4-E, anti-Phe-1,2-diol-3,4-epoxide; Phe-3,4-D-1,2-E, anti-Phe-3,4-diol-1,2-epoxide; Phe-1,2-D-4-NAC, 4-(N-acetylcycteineyl)-1,2,3-trihydroxy-1,2,3,4-tetrahydrophenanthrene; Phe-3,4-D-1-NAC, 1-(N-acetylcysteineyl)-2,3,4-trihydroxy-1,2,3,4-tetrahydrophenanthrene rev-BPDE, anti-BaP-9,10-diol-7,8-epoxide; Phe-9-hydroxy-10-NAC, 10-(N-acetylcysteineyl)-9-hydroxy-9,10-dihydrophenanthrene; NAC, N-acetylcysteine; rev-BPDE-7-NAC, 7-(N-acetylcysteineyl)-8,9,10-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; BPDE-10-NAC, 10-(N-acetylcysteineyl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; CDNB, 1-chloro-2,4-dinitrobenzene; LC-ESI-MS/MS-SRM, liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring.
bay region, a feature closely associated with PAH carcinogenicity; however, Phe is not generally considered to be carcinogenic. Human exposure to Phe is thousands of times higher than to BaP, thus facilitating analysis of its urinary metabolites. We analyzed human urine for the mercapturic acid 4-N-acetylcysteinyl)-2,3,4-trihydroxy-1,2,3,4-tetrahydrophenanthrene (Phe-3,4-D-1-NAC) (Fig. 1A). Consistent with the results of our analyses previously (Upadhyaya et al., 2006). Racemic BPDE and rev-BPDE were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). N-Acetylcytisteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO).

7-(N-Acetylcysteineyl)-8,9,10-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (rev-BPDE-7-NAC) was prepared essentially as described previously (Hecht et al., 2008). In brief, a solution of racemic rev-BPDE (2.4 mg, 0.008 mmol) in 2.0 ml of dry tetrahydrofuran was added to 2 ml of NAC (3.0 M) (adjusted to pH 10.0). The mixture was stirred for 2 h, and the isomers were separated by high-performance liquid chromatography (HPLC) and collected using a 250 × 10 mm, 10µ, C18 Vydac 210TP column (Separations Group, Hesperia, CA) eluted at 3 ml/min with 30 mM NH4HCO3 (60%) and 40% CH3OH, overall yield, 1.6 mg, 45.3%. Isomer 1 (670 m/z) pyrene (rev-BPDE-7-NAC) was prepared as described previously (Upadhyaya et al., 2006). The mercapturic acid metabolites illustrated are produced by normal metabolic processing of initially formed GSH conjugates by successive action of γ-glutamyltranspeptidase, cysteinylglycine dipeptidase, and cysteine S-conjugate N-acetyltransferase.

Materials and Methods

Chemicals. 10-(N-Acetylcysteineyl)-9-hydroxy-9,10-dihydrophenanthrene (Phe-9-hydroxy-10-NAC) was prepared as described previously (Upadhyaya et al., 2006). Racemic BPDE and rev-BPDE were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). N-Acetylcytisteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO).

**Fig. 1.** Overview of the question posed in this study (A) and our previous results in studies (B) of GSH conjugation of Phe diol epoxides (Hecht et al., 2008, 2009). The mercapturic acid metabolites illustrated are produced by normal metabolic processing of initially formed GSH conjugates by successive action of γ-glutamyltranspeptidase, cysteinylglycine dipeptidase, and cysteine S-conjugate N-acetyltransferase.
(CH10), 54.3 (CHNH), 49.3 (CH7), 31.8 (CH5), 21.8 (COCH3). Isomer 2 (1.0 mg), retention time 34.56 min; UV \( \lambda_{max} \) 201, 248, 328, 345 nm; MS (negative ion ESI) m/z 464 [M - H] - (100); MS/MS of m/z 464; m/z 446 (100), 317 (51); 1H NMR (D2O) 68.21 (s, 1H, H11), 8.13 (m, 1H, H6), 7.87 - 7.79 (m, 3H, H5, H3, H5), 7.69 (dd, J = 7.2, 7.2 Hz, H2), 7.60 (m, 3H, H4, and H12), 5.51 (b, J = 3.6 Hz, 1H, H10), 4.43 (m, 1H, H9), 4.34 (m, 2H, H7 and H8), 4.27 (m, 1H, CHNH), 2.95 (dd, J = 4.8, 13.2 Hz, 1H, SCH2a), 2.60 (m, 1H, SCH2b), 1.81 (s, 3H, CH3); 13C NMR (D2O) \( \delta \) 127.6, 127.1, 126.7, 125.9, 125.0 (CH1, CH2, CH3, CH4, CH5, CH12), 123.7 (CH11), 123.8 (CH6), 73.8 (CH9), 70.9 (CH8), 71.1 (CH10), 71.0 (CH7), 54.8 (CHNH), 35.0 (SCH2), 22.0 (COCH3). Isomer 2 (622 mg), retention time 31.25 min; UV \( \lambda_{max} \) 201, 248, 328, 345 nm; m/z 464 [M - H] - (100); MS/MS of m/z 464; m/z 446 (100), 335 (58); 317 (53.5); 283 (13.6); 1H NMR (D2O) \( \delta \) 7.78 (s, 1H, H11), 7.73 (s, 1H, H6), 7.48 - 6.96 (m, 6H, H1, H2, H3, H4, H5, and H12), 4.98 (d, J = 8.4 Hz, 1H, H10), 4.73 (m, 1H, H7), 4.57 (m, 1H, H9), 4.51 (m, 2H, H8, and CHNH), 3.29 (m, 1H, SCH2a), 2.80 (m, 1H, SCH2b), 1.76 (s, 3H, CH3); 13C NMR (D2O) \( \delta \) 128.0, 127.8, 127.0, 126.4, 125.5, 125.5 (CH1, CH2, CH3, CH4, CH5, CH12), 126.0 (CH11), 123.3 (CH6), 73.0 (CH9), 69.8 (CH8), 68.7 (CH10), 54.6 (CHNH), 49.2 (CH7), 32.6 (SCH2), 22.0 (COCH3).

**Hepatocyte Incubations**. Primary human hepatocytes were purchased from CellzDirect (St. Louis, MO). In brief, freshly isolated hepatocytes were plated onto 12-well plates (7 x 10^5 cells/well) and overlaid with Matrigel 24 to 48 h after attachment. Cells were shifted overnight on cold preservation media, and, upon receipt, media were replaced with serum-free Williams' E media (without phenol red) and supplements as described previously (Hecht et al., 2009). Cells were allowed to recover from shipping for 10 h at 37°C in an atmosphere containing 5% CO2. Before incubation with substrate, the media were exchanged with 2 ml of fresh media per well. Hepatocytes (approximately 0.12 mg of protein per well) were incubated with BPDE or rev-BPDE, dissolved in 20 μl of dimethyl sulfoxide, for a final substrate concentration of 10 μM, except as noted in Table 1. Aliquots (0.4 ml) of media were removed 12 h after addition of substrate. At 24 h after addition, the remaining media were collected. Samples were stored at -20°C until analysis. Cell viability was assessed at 24 h by trypan blue exclusion staining. Expression of GSTM1 and GSTA1 and measurement of GST activity by conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) were carried out as described previously (Hecht et al., 2009).

**Analysis of Mercapturic Acids**. The internal standard Phc-9-hydroxy-10-NAC was added to a 0.2 ml of aliquots of incubation medium, and the mixture was applied to a Strata-X polymeric sorbent cartridge (33 μm, 30 mg/1 ml; Phenomenex, Torrance, CA) that had been activated with 1 ml of CH3OH and 1 ml of H2O. The cartridge was washed with 1 ml of H2O and the analyte was eluted with 1 ml of 90% CH3OH. The eluant was collected in a 2-mI vial, and the sorbent was removed on a SpeedVac. The residue was taken up in 250 μl of CH3OH and 5 μl of NH4OAc (4%), transferred to an insert vial, and concentrated to dryness. The residue was dissolved in 20 μl of aqueous NH4OAc (1%), and 8 μl was analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC-ESI-MS/MS-SRM) on a Thermo Finnigan TSQ Quantum Discovery MAX instrument (Thermo Fisher Scientific, Waltham, MA) interfaced with an Agilent 1100 Capillary HPLC System (Agilent Technologies, Palo Alto, CA). The HPLC was equipped with a 5 μm, 150 x 0.5 mm Zorbax SB-C18 column (Agilent Technologies, Santa Clara, CA) and a Kudaker disposable precolumn filter (Phenomenex). The column was eluted at 10 μl/min with 30 mM

**TABLE 1**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Bm</th>
<th>Gender</th>
<th>Age</th>
<th>Race</th>
<th>Body Mass Index</th>
<th>BPDE 7-μM</th>
<th>GSTA1 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1006</td>
<td>F</td>
<td>M</td>
<td>69</td>
<td>31</td>
<td>24</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1072</td>
<td>M</td>
<td>F</td>
<td>66</td>
<td>28</td>
<td>28</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1083</td>
<td>M</td>
<td>F</td>
<td>63</td>
<td>35</td>
<td>28</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1085</td>
<td>M</td>
<td>F</td>
<td>67</td>
<td>27</td>
<td>22</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1091</td>
<td>M</td>
<td>M</td>
<td>47</td>
<td>27</td>
<td>22</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>65.2 ± 41.8</td>
<td>54 ± 11</td>
<td>27 ± 4</td>
<td>21 ± 2</td>
<td>24 ± 1</td>
<td>0.84 ± 0.35</td>
<td>1.00 ± 0.05</td>
</tr>
</tbody>
</table>

*BMI, body mass index; F, female; ND, not detected; M, male.*

*The internal standard Phc-9-hydroxy-10-NAC was added to a 0.2 ml of aliquots of incubation medium, and the mixture was applied to a Strata-X polymeric sorbent cartridge (33 μm, 30 mg/1 ml; Phenomenex, Torrance, CA) that had been activated with 1 ml of CH3OH and 1 ml of H2O. The cartridge was washed with 1 ml of H2O and the analyte was eluted with 1 ml of 90% CH3OH. The eluant was collected in a 2-mI vial, and the sorbent was removed on a SpeedVac. The residue was taken up in 250 μl of CH3OH and 5 μl of NH4OAc (4%), transferred to an insert vial, and concentrated to dryness. The residue was dissolved in 20 μl of aqueous NH4OAc (1%), and 8 μl was analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC-ESI-MS/MS-SRM) on a Thermo Finnigan TSQ Quantum Discovery MAX instrument (Thermo Fisher Scientific, Waltham, MA) interfaced with an Agilent 1100 Capillary HPLC System (Agilent Technologies, Palo Alto, CA). The HPLC was equipped with a 5 μm, 150 x 0.5 mm Zorbax SB-C18 column (Agilent Technologies, Santa Clara, CA) and a Kudaker disposable precolumn filter (Phenomenex). The column was eluted at 10 μl/min with 30 mM
**NH₄HCO₃ (60%) in CH₃OH.** The analysis was carried out essentially as described previously (Hecht et al., 2009), except that the transition m/z 464 → m/z 317, negative ion mode, [M - 1]⁻ → [M - 1]⁻ - (CH₃CH(CO₂)NHCOCH₃ + H₂O)⁻, was monitored for rev-BPDE-7-NAC or BPDE-10-NAC, and m/z 356 → m/z 209 was monitored for Phe-9-hydroxy-10-NAC.

**Results**

Each reaction of racemic BPDE or rev-BPDE with NAC produced a mixture of two isomers, separable by HPLC. An example of the reaction of rev-BPDE with NAC is illustrated in Fig. 2A. Each isomer had a UV spectrum, ¹H and ¹³C-NMR spectra, and MS consistent with the expected products, BPDE-10-NAC and rev-BPDE-7-NAC (Fig. 1A). Analogous to previous work (Hecht et al., 2008), the first and second isomers probably resulted from cis and trans addition of NAC to the diol epoxides, but further characterization was not pursued.

Demographic data for the hepatocyte donors are summarized in Table 1. Each sample was positive for expression of GSTA1, and four were positive for GSTM1 expression. CDNB activity averaged 65.2 ± 41.8 nmol conjugate/(min • mg) protein. These data are consistent with previous results (Hecht et al., 2009).

A chromatogram obtained upon LC-ESI-MS/MS-SRM analysis of a sample in which rev-BPDE was incubated for 24 h with human hepatocytes is illustrated in Fig. 2C, along with the internal standard for the analysis (Fig. 2B). A clear peak corresponding to the second rev-BPDE-7-NAC isomer was observed. All samples in which rev-BPDE was added to human hepatocytes gave similar chromatograms, although the amounts of the two peaks varied. The results are summarized in Table 1. Mean levels of isomers 1 and 2 of rev-BPDE-7-NAC, respectively, in the nine samples incubated with 10 µM rev-BPDE were 0.017 ± 0.023 and 0.30 ± 0.61.

**Fig. 2.** Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis of products formed in the reaction of racemic rev-BPDE with NAC (A); internal standard Phe-9-hydroxy-10-NAC for the hepatocyte analysis (B); and medium from hepatocytes incubated with 10 µM rev-BPDE for 24 h (C).
pmol/ml in the 12-h incubations and 0.18 ± 0.12 and 3.59 ± 5.95 pmol/ml in the 24-h incubations. BPDE-10-NAC (isomer 2) was observed in only one of the 10 samples incubated with BPDE at levels of 0.015 (12 h) and 0.035 (24 h) pmol/ml.

Discussion

The results reported here demonstrate that the reverse diol epoxide rev-BPDE undergoes GSH conjugation in human hepatocytes, but the carcinogenic bay region diol epoxide, BPDE, does not (except for relatively small amounts in one sample). These results are completely consistent with our previous study of GSH conjugation of Pho diol epoxides in human hepatocytes, summarized in Fig. 1B. The reverse diol epoxide, Phe-3,4-D-1,2-E, was a far better substrate for GSH conjugation, as determined by analysis of Phe-3,4-D-1-NAC in the medium, than was the bay region diol epoxide Phe-1,2-D-3,4-E, for which only minimal conjugation to Phe-1,2-D-4-NAC was observed. Reverse diol epoxides such as rev-BPDE and their diol precursors generally have little or no carcinogenic or mutagenic activity in contrast to bay region diol epoxides (Conney, 1982; Glatt et al., 1993). Consequently, these results have substantial implications for molecular epidemiologic studies that have examined, using genotyping strategies, the relationship between GSTM1 null or GSTP1 low activity variants and cancer in people exposed to PAH. We find little or no evidence to support the assumption, inherent in many of these studies, that carcinogenic bay region diol epoxides of PAH are detoxified by GSH conjugation in unadulterated human cells.

Robertson and coworkers (Robertson et al., 1986) were apparently the first to study conjugation of BPDE and other PAH diol epoxides by human enzymes. This was followed by a series of studies using purified human GSTs (Robertson et al., 1986), cellular subfractions with GST activity (Jernström et al., 1989), recombinant GSTs and their variants (Jernström et al., 1996; Sundberg et al., 1997, 1998), and Chinese hamster V79 cells overexpressing human GSTs (Seidel et al., 1998; Sundberg et al., 2001, 2002). These studies demonstrated that bay region and fjord region diol epoxides can be converted to GSH conjugates by GST M1, GST P1, and GST A1, and that such conjugation was in most cases protective against DNA damage and mutagenicity. However, in one study using unadulterated MCF-7 human breast carcinoma cells incubated with BPDE, no activity was found (Romert et al., 1989). Another notable study found that only approximately 1 to 2% of the rate expected for GSH conjugation of BPDE was actually observed in cells, which indicated the importance of competing reactions of BPDE (Sundberg et al., 2002; Townsend and coworkers (Fields et al., 1998; Kushman et al., 2007a,b)) also studied GSH conjugation of BPDE in cells overexpressing human GSTs and found protection against BPDE-DNA binding and mutagenicity. Singh and coworkers (Hu et al., 1999; Srivastava et al., 1999) obtained similar results and made the interesting observation that the GSH conjugate of BPDE inhibits its own formation. We are not aware of any studies in the literature that report the GSH conjugation of BPDE in unadulterated human cells or in humans.

The studies discussed above present a comprehensive and authoritative examination of PAH diol epoxide conjugation catalyzed by GSTs. One conclusion of these studies is that diol epoxide stereochernistry strongly affects conjugation. Our results are consistent with this finding. In BPDE, the 10-position of the diol epoxide, where conjugation would occur, is sterically more hindered because of its presence in the bay region than the 7-position of rev-BPDE. The major difference between the earlier studies and those presented here is that they used hamster V79 cells stably expressing different human GSTs, whereas our work was performed with human hepatocytes that were not engineered to affect GST conjugation, and this difference is undoubtedly critical in explaining the divergent results. However, one aspect that is not examined in this study is the possible differences in transport or secretion of the GST conjugates of rev-BPDE and BPDE into the medium.

In summary, the results presented here do not support the hypothesis that the carcinogenic bay region diol epoxide of BaP is detoxified by GSH conjugation in human hepatocytes. Therefore, a basic assumption underlying molecular epidemiology studies of GST variants and cancer in people exposed to PAH may be incorrect.

Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota

References


Robertson IG, Guttenherg C, Mannervik B, and Jernström B (1986) Differences in stereoselectivity and catalytic efficiency of three human glutathione transferases in the conjugation of
glutathione with 7 β,8 dihydroxy-9 α,10 a-oxo-7,8,9,10-tetrahydrobenzo(a)pyrene. *Cancer Res* 46:2220–2224.

**Address correspondence to:** Stephen S. Hecht, Ph.D., Masonic Cancer Center, University of Minnesota, MMC 806, 420 Delaware St. SE, Minneapolis, MN 55455. E-mail: hecht002@umn.edu