NADPH-Supported and arachidonic acid-supported metabolism of the enantiomers of \( \text{trans-7,8-dihydrobenzo[a]pyrene-7,8-diol} \) by human liver microsomal samples

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Using a new sensitive reverse-phase HPLC assay relying on UV detection at 344 nm, the capacity of 18 human liver microsomal samples to support NADPH-dependent, cytochrome P450-mediated oxidation and arachidonic acid-dependent oxidation of the enantiomers of \( \text{trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (B[a]P-7,8-DHD)} \) was determined. The \(-\)-7\( R, R \)-enantiomer, the preferred substrate of cytochrome P450, formed 94% diolepoxide 2 (anti-isomer; 7\( R, 8S \)-dihydroxy-9\( S \),10\( R \)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene) measured as derived alcohols, and the \(+\)-7\( S, 8S \)-enantiomer formed 67% diolepoxide 1 (syn-isomer; 7\( S, 8R \)-dihydroxy-9\( R \),10\( R \)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene). Arachidonic acid-supported oxidations gave \( \sim 70\% \) diolepoxide 2 from each enantiomer. The involvement of different sets of cytochrome P450 isozymes was supported by incubations in the presence of \( \alpha \)-naphthoflavone (\( \alpha \)-NF) (50 \( \mu M \)) and correlation studies. In the absence of \( \alpha \)-NF, a positive correlation was found between the metabolism of the \(-\)-enantiomer but not the \(+\)-isomer of B[a]P-7,8-DHD and the relative content of P450IA2. In the presence of \( \alpha \)-NF, the P450IIA3/4 content correlated positively with the metabolism of both the \(+\)-enantiomer and the \(-\)-enantiomer. Gestodene (100 \( \mu M \)) inhibited the \( \alpha \)-NF-stimulated metabolism, confirming the involvement of cytochrome P450IIA3/4. No difference was found between the extent of arachidonic acid-supported, peroxyl radical-mediated metabolism of the \(+\)- and \(-\)-enantiomers of B[a]P-7,8-DHD. The metabolism was almost completely abolished by 2 \( \mu M \) butylatedhydroxyanisole and 100 \( \mu M \) nordihydroguaiaretic acid, confirming the free radical nature of the reaction.

Introduction

\( \text{Trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (B[a]P-7,8-DHD)} \) can undergo oxidative metabolism catalyzed by rodent liver microsomes to form the B[a]P-7,8-diol-9,10-epoxides (1). These exist as a pair of diastereomers in which the 7-hydroxyl group is either \( \text{cis} \) or \( \text{trans} \). There are two possible mechanisms by which the epoxidation of B[a]P-7,8-DHD can take place: oxidation may be mediated by the mixed-function oxidase system involving cytochrome P450 (3,5–7). Peroxyl radicals are generated in a variety of biochemical situations and co-oxidation of B[a]P-7,8-DHD by epoxidation at the 9,10-double bond occurs. This diolepoxide formation occurs during microsomal lipid peroxidation in liver microsomes triggered by the addition of ascorbate or NADPH in the presence of iron(III) and ADP (9), and in mouse keratinocytes (10). Enzymatic generation of peroxyl radicals occurs during the lipid hydroperoxide-dependent co-oxidations of B[a]P-7,8-DHD involving prostaglandin H synthase (8,10–15) and lipoxigenases (13,16,17). B[a]P-7,8-DHD epoxidation also occurs during hematin-catalyzed decomposition of fatty acid hydroperoxides (18,19) and bisulfite peroxidation (20) or autoxidation (21). In addition the cytochrome P450 enzymes have peroxigenase activity and can oxidize tolidine and methylaniline supported by hydroperoxides (22), can convert lipid hydroperoxides to lipid peroxidation products (23), and can reductively cleave hydroperoxides in the presence of NADPH (24).

It has been reported from animal studies that the stereoselectivity of epoxidation of B[a]P-7,8-DHD by peroxyl radicals is distinct from that exhibited by the cytochromes P450. Peroxyl radicals epoxidize \(+\)-B[a]P-7,8-DHD-mainly to the \(-\)-enantiomer of the diolepoxide 2 and the cytochromes P450 epoxidize it mainly to the \(+\)-enantiomer of the diolepoxide 1. \(-\)-B[a]P-7,8-DHD is epoxidized by both pathways to the \(+\)-enantiomer of the diolepoxide 2 (9,19,25,26).

The aim of the present study was to determine the relative contribution of the NADPH-dependent, cytochrome P450-mediated oxidation and the arachidonic acid-dependent, peroxyl radical-mediated oxidation of the \(+\)- and \(-\)-enantiomers of B[a]P-7,8-DHD in human liver microsomes. The stereoselectivities of the reactions and the effects of various additives were also examined.

Materials and methods

Chemicals

The following chemicals were purchased from the NCI Chemical Carcinogen Repository through Chemsyn Science Laboratories, Lexena, KS, USA: (\(+\), \(-\), \(+\)- and \(-\)-trans-\( \text{B[a]P-7,8-DHD} \), racemic; \( 78,8\alpha,9\alpha,10\alpha\text{-tetrahydro-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P-7,8-DHD)} \), \( 78,8\alpha,9\alpha,9\alpha,10\alpha\text{-tetrahydro-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P-7,8-DHD)} \), \( 78,8\alpha,9\alpha,9\alpha,10\alpha\text{-tetrahydro-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P-7,8-DHD)} \), \( 78,8\alpha,9\alpha,9\alpha,10\alpha\text{-tetrahydro-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P-7,8-DHD)} \)).
9-triol), 78,8a,9,10-tetrahydro-7,8,9-trihydroxybenzo[a]pyrene (B[a]P-7,8,9-triol), and B[a]P-7,8-diol-9,10-epoxide. B[a]P-7,8-diol-9,10-epoxide and trans-dihydro-4,5-dihydrobenzo[a]pyrene (B[a]P-4,5-DHD).

**Biochemicals**

NADPH was purchased from Boehringer Mannheim Corp., Sydney, Australia. Butyrylhydrazine (BHA), nordihydroguaiaretic acid (NDGA), \( \alpha \)-naphthoflavone (\( \alpha \)-NF), and phenolphthalein were obtained from the Sigma Chemical Company, St. Louis, MO, USA. SKF525A was purchased from Smith Kline and French Laboratories, Sydney, Australia. K,K',K'-nitro-1,1'-fluor-naphthol (Fluka Chemicals, Darmstadt, FRG) and HPLC-grade methanol (Mallickroteck Specialty Chemicals Co., KY, USA) were obtained from the sources listed. Gestodene (13-ethyl-17β-hydroxy-18,19-dihydro-17α-progesterone-4,15-diene-20-yn-3-one) was generously donated by Dr. H. Kuhn (University of Frankfurt, Germany).

**Liver microsomes**

**Rat liver tissue.** Control and phenobarbital-induced rat liver microsomal samples were prepared as described (27).

**Human liver tissue.** All the human liver microsomal samples except SV2 were prepared from the liver bank held in the Department of Clinical Pharmacology of the University of Sydney, Sydney, Australia. The SV2 liver tissue was obtained from patients suffering from liverfailure (subjects H12, H13, and SV2). In these cases, an apparently normal part of the tissue was taken. All other samples were removed from organ transplant donors 30-60 min after death (29). The clinical details of the subjects are shown in Table 1. The use of human liver tissue in this study has received the approval of both Hospital and University Human Ethics Committees.

**Methods**

NADPH-Supported metabolism of (+)- and (-)-[B[a]P]-7,8-DHD by human liver microsomes. Metabolic studies were carried out using 1 ml incubations in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.5 mg human liver microsomal protein in 40 μl methanol, and 50 μM for α-NF (added in 20 μl acetone). The substrate, (+)- or (-)-B[a]P-7,8-DHD (20 μmol in 20 μl acetone) was added to each. A period of further vortexing (10 s) followed by 1 h in iced water, and then NADPH in buffer (0.1 ml, 5 mM, final concentration 0.6 μM) was added. After incubation at 37°C in a shaking water bath for 30 min, the reaction was terminated by rapid cooling in ice and 1 M phosphate buffer, pH 3.5 (0.1 ml) was added. After vortexing, the incubation mixtures were allowed to stand in ice for 1 h, and worked up as described above for NADPH-supported metabolism. Blanks contained no phenobarbital acid.

**Quantitation of the B[a]P-7,8-DHD metabolites**

Preparation of standard curves. Seven standard solutions of the four [B[a]P]-7,8,9,10-tetraols and the two [B[a]P]-7,8,9-triols derived from the anti- and syn-isomers of B[a]P-7,8-diol-9,10-epoxide by hydrolysis and reduction were prepared in DMSO so that 50 μl contained 7.5, 15, 30, 60, 120, 180 and 240 ng of each compound respectively. In separate test tubes, an aliquot (50 μl) of each standard solution was added to liver microsomal protein (0.5 mg) prepared from phenobarbital-pretreated rats in 0.05 M Tris-HCl buffer, pH 7.4 (1 ml). The test tubes were vortexed for 10 s and 1 M phosphate buffer, pH 3.5 (0.1 ml) was added. After vortexing, the incubation mixtures were allowed to stand in ice for 1 h, and worked up as described above for NADPH-supported metabolism. Blanks contained no phenobarbital acid.

**Table 1. Clinical data for liver donors**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Smoking history</th>
<th>Alcohol consumption</th>
<th>Drug treatment</th>
<th>P450 (nmol/mg)</th>
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<tr>
<td>H5</td>
<td>62</td>
<td>M</td>
<td>unknown</td>
<td>unknown</td>
<td>phenytoin, desmethylamphetamine, gentamycin, rutinidline, cotrimoxazole</td>
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<td>unknown</td>
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*All liver donors were Caucasians except subject H14 who was of Asian origin.*
**Results and discussion**

**Metabolic assay methods**

Assays for B[α]P-7,8-DHD metabolism have generally relied on the use of radioactive substrates and subsequent radiochemical determination of metabolic products (3,15,17) or changes in UV absorbance due to consumption of substrate (48). The current study employed a reverse-phase HPLC of compounds derived from the diastereoisomers of B[α]P-7,8-diol-9,10-epoxide by hydrolysis and NADPH-mediated reduction and an internal standard. Products were B[α]P-7,10,8,9-tetraol, B[α]P-7,8,9,10-tetraol and B[α]P-7,9,10-tetraol from the *anti*-diolepoxide diastereoisomer, and B[α]P-7,9,10/8-tetraol, B[α]P-7,9/8,10-tetraol and B[α]P-7,9/8-tetraol from the *syn*-diolepoxide diastereoisomer. Quantitation was carried out using 1,1′-bisp-naphthol as the internal standard, and peak area ratios were determined after incubation mixtures were adjusted to pH 3.5 with phosphate buffer and allowed to stand for a sufficient period of time to guarantee complete hydrolysis. Using the *anti*-diolepoxide, which is the isomer of lesser reactivity towards hydrolysis under neutral conditions (30), experiments involving addition of the epoxide to incubation buffer at pH 7.4 containing 0.5 mg/ml rat liver microsomal protein followed by immediate extraction gave incomplete recoveries of the diolepoxide (as hydrolysis products) in the organic extracts. When solutions of the *anti*-diolepoxide in the presence of protein were adjusted from pH 7.4 to pH 3.5 by addition of phosphate buffer, and allowed to stand for periods of up to 1 h, hydrolysis increased. After 15 min the reaction was 85% complete and by 1 h total hydrolysis had occurred. Therefore a 1 h hydrolysis period at pH 3.5 was included in the assay. Problems with the analysis of these diolepoxides have been recognized by others. Thus during hematin-catalyzed oxidation of B[α]P-7,8-DHD, initiated by unsaturated fatty acid hydroperoxides, poor recovery of radioactivity was noted (18), and during methanol/water gradient HPLC of the diolepoxides themselves (3) or of prostaglandin synthase co-oxidation products 10-0-methylated B[α]P-tetraols were formed (31). The limit of detection of the assay was ~2 ng for each analyte, which under the conditions of the incubation and HPLC translates to ~1 pmol metabolites/mg protein/min.

Incubations designed to determine NADPH-supported cytochrome P450-catalyzed oxidation of B[α]P-7,8-DHD were effected in the presence of 2 μM BHA. Preliminary experiments with human liver microsomes in the absence of BHA gave high blank values (no NADPH), which made measurements of the NADPH-supported reaction unreliable. Using rat liver microsomal protein to effect the NADPH-supported oxidation of B[α]P-7,8-DHD even higher blanks were obtained, but in the presence of 1, 2, 5 and 10 μM BHA blanks were very substantially reduced. The chosen concentration of 2 μM gave minimal inhibition of the rat liver microsomal catalysed oxidation. The oxidation of B[α]P-7,8-DHD seen even in unincubated blanks in the absence of BHA is probably mediated by peroxides both present in the microsomal preparation and formed during incubations (8,23,24), in part perhaps by lipoxygenase (13,16,17,47) (see later discussion). The very substantial reduction of these blank values in the presence of a low concentration of BHA supports this proposal.
DEI and DE2, diolepoxide 1 and 2.

Specific enzyme activities are expressed as pmol metabolites/mg protein/min or pmol metabolites/nmol P450/min, determined in the presence of 0.6 mM NADPH and 2 μM BHA. Data were corrected using incubations containing either 2 μM BHA only or 2 μM BHA and 50 μM α-NF and no NADPH. Both trials were combined and expressed as a percentage of total metabolites; the proportions of DE-1 and DE-2 were normalized to 100%. Factor showing an increase in metabolism (>1.00), or inhibition of metabolism (<1.00) when incubations were conducted in the presence of 50 μM α-NF. Means of duplicate determinations. Substrate concentration was 40 μM. For all other human liver microsomal samples the substrate concentration was 20 μM.

Data collected in the Tables show the capacity of 18 samples of human liver microsomes to catalyze the NADPH-supported oxidation (Tables II and III) and arachidonic acid-supported oxidation (Tables IV and V) of the enantiomers of B[a]P-7,8-DHD to B[a]P-diolepoxides 1 and 2. The total metabolic capacity and the relative amounts of the two diolepoxides derived from the sums of their hydrolysis and reduction products are given. The proportion of this found as triols ranged from 4 to 30%. Protein-bound products from the diolepoxides could not be determined but in other work the proportion was low (~5%) (14) or moderate (>30%) (3).

**NADPH-dependent metabolism of (+)- and (−)-B[a]P-7,8-DHD**

Tables II and III display the extents of NADPH-supported metabolism of (+)- and (−)-B[a]P-7,8-DHD by human liver microsomal samples in the presence and absence of α-NF. The activity towards the (−)-enantiomer of B[a]P-7,8-DHD was about twice that seen towards the (+)-enantiomer (Figures 2B and C for sample H23) and the product ratios were characteristic for each enantiomer of the dihydrodiol. The (−)-enantiomer was metabolized to a mean 67% diolepoxide 1, while the (+)-enantiomer was converted to a mean 94% diolepoxide 2. With cytochromes P450LM2 and P450LM4 purified from rabbit liver, LM4 showed a much greater activity towards these B[a]P derivatives than LM2 (5.25), and LM4 preferentially metabolizes the (−)-isomer. In a similar fashion, the (−)-isomer was converted predominantly to diolepoxide 2. Thakker et al. (1) reported the high stereoselectivity in the formation of diolepoxide 2 relative to diolepoxide 1 from the 7R,8R-enantiomer by liver microsomes from 3-methylcholanthrene-pretreated rats and with purified cytochrome P448 (IA1). Microsomes from control and phenobarbitone-treated rats metabolized (+) and (−)-B[a]P-7,8-DHD with less stereoselectivity. These differences in stereoselectivity in the metabolism of the B[a]P-7,8-dihydrodiols reflect the differences in the proportion of various cytochrome P450s present in liver microsomes from control, MC-induced and phenobarbitone-induced rats.

In the present work, differences between individuals in the proportions of diolepoxide 1 and diolepoxide 2 derived metabolites formed from (+)- and (−)-B[a]P-7,8-DHD also probably reflect differences in the content of particular cytochrome P450s in the liver microsomes. Drug pretreatment, smoking habit and genetic factors have been shown to influence the expression of specific P450s in human liver. α-NF has been reported to be capable of stimulating some P450-mediated reactions in human liver microsomes and inhibiting others (4,6,33-36). In the present work, 50 μM α-NF increased the metabolism of (+)-B[a]P-7,8-DHD by a mean 2.27 fold (P <
0.001) (Figure 2D; Table II). This stimulating effect occurred in all samples; however, the extent of stimulation varied from 1.26-fold to 4.24-fold. The α-NF caused a mean 4.3-fold increase in the amounts of diol epoxide 2 hydrolysis products formed, while the amounts of diol epoxide 1-derived products remained virtually unchanged. (There was a mean 1.3-fold change.) This translated as a decreased proportion of diol epoxide 1 products and an increased proportion of tetraols derived from diol epoxide 2.

The effect of 50 μM α-NF on the metabolism of (+)-B[a]P-7,8-DHD was to decrease it by a mean 0.86-fold (P < 0.05) (Table III). This inhibitory effect was not observed in all samples, and six of the 18 samples showed increases in metabolism. α-NF generally increased the amounts of diol epoxide 1 metabolites formed and decreased the amounts of diol epoxide 2 metabolites formed, resulting in the changed proportions shown in Table III.

The liver samples used were those described in McManus et al. (29), and the relative P450IA2 and P450IIIA3/4 contents of these samples were available. Those results were used to determine the correlation of the relative content of these P450 isozymes with the rates of metabolism of (+)- and (-)-B[a]P-7,8-DHD. Statistically significant correlations were observed between the P450IIIA3/4 levels and the metabolism (pmol metabolites/mg protein/min) of (+)-B[a]P-7,8-DHD (r = 0.77, r² = 0.587) and (-)-B[a]P-7,8-DHD (r = 0.82, r² = 0.667) in the presence of α-NF, but not in its absence. The human P450IIIA3 IgG used for the quantitation of the P450IIIA3 content of the human liver microsomal samples used in the present work was a polyclonal antibody (29) and would therefore recognize at least P450IIA9, P450NF25 and P450NF10 in human liver microsomes.

It is therefore possible to conclude that the P450IIIA3/4 proteins play an important role in the metabolism of (+)- and (-)-B[a]P-7,8-DHD after stimulation by α-NF. This conclusion was also supported by the observation that the human liver microsomal samples obtained from patients known to be receiving dexamethasone, a synthetic glucocorticoid, as a medication (patients H5, H7 and H15) were among those that showed the highest fold increases in metabolism of (+)- and (-)-B[a]P-7,8-DHD after stimulation by α-NF. The samples H5, H7 and H15 gave 4.24-, 3.41- and 2.22-fold increases in the metabolism of the (+)- enantiomer, and 2.25-, 1.08- and 1.06-fold increases in the metabolism of the (-)- enantiomer respectively when α-NF was included in the incubation mixture. The specific activities of these three samples towards both the (+)- and (-)-enantiomers of B[a]P-7,8-DHD in the absence of α-NF were not significantly higher than the 15 other human liver microsomal samples. Since dexamethasone is an inducer of the cytochrome P450IIA enzymes (37), these results support the correlation studies which point to an involvement of the P450IIIA3/4 enzymes in the metabolism of the (+)- and (-)-enantiomers of B[a]P-7,8-DHD only after stimulation by α-NF, but not in the absence of α-NF.

In all human liver microsomes examined, P450IA2 is the dominant P450IA protein present (29). However, due to the difficulty associated with separating P450IA1 and IA2 proteins on SDS-PAGE, the presence on P450IA1 mRNA in 11 of the 18 samples obtained from patients known to be receiving dexamethasone, a synthetic glucocorticoid, as a medication (patients H5, H7 and H15) were among those that showed the highest fold increases in metabolism of (+)- and (-)-B[a]P-7,8-DHD after stimulation by α-NF. The samples H5, H7 and H15 gave 4.24-, 3.41- and 2.22-fold increases in the metabolism of the (+)- enantiomer, and 2.25-, 1.08- and 1.06-fold increases in the metabolism of the (-)- enantiomer respectively when α-NF was included in the incubation mixture. The specific activities of these three samples towards both the (+)- and (-)-enantiomers of B[a]P-7,8-DHD in the absence of α-NF were not significantly higher than the 15 other human liver microsomal samples. Since dexamethasone is an inducer of the cytochrome P450IIA enzymes (37), these results support the correlation studies which point to an involvement of the P450IIIA3/4 enzymes in the metabolism of the (+)- and (-)-enantiomers of B[a]P-7,8-DHD only after stimulation by α-NF, but not in the absence of α-NF.

In all human liver microsomes examined, P450IA2 is the dominant P450IA protein present (29). However, due to the difficulty associated with separating P450IA1 and IA2 proteins on SDS-PAGE, the presence on P450IA1 mRNA in 11 of the 23 livers tested in our laboratory (38), and the fact that the polyclonal anti-rabbit P450IA2 IgG used in these studies recognizes both P450IA proteins, the possibility of the P450IA2 band containing P450IA1 protein cannot be totally discounted. In the present study in the absence of α-NF, the metabolism (pmol metabolites formed/mg protein/min) of the (-)-enantiomer of B[a]P-7,8-DHD correlated positively with P450IA2 protein content (r = 0.72, r² = 0.518) of human liver microsomes. No
such positive correlation between 450IA2 levels and the metabolism of the (+)-enantiomer of the substrate was evident. These correlative studies suggest that in the absence of α-NF P450IA2 may play some role in the metabolism of the (−)-enantiomer of B[a]P-7,8-DHD by human liver microsomes, but not of the (+)-enantiomer.

α-NF must stimulate P450IIIA3/4 and inhibit P450IA1 and P450IA2 since the overall effect of 50 μM α-NF on the metabolism of (−)-B[a]P-7,8-DHD was to inhibit it by a mean 0.86-fold. Inhibition of P450IA1 protein activity is supported by the poor correlation of their specific P450 content with the metabolism of (−)-B[a]P-7,8-DHD in the presence of α-NF. Stimulation of P450IIIA3/4 and inhibition of P450IA1 and P450IA2 by α-NF is consistent with previous observations reported in the literature. Shimada and co-workers (6,39) have previously provided evidence that human P450nf (IIA4) shows a stimulatory response to α-NF. McManus et al. (29) observed that the ability of expressed human P450IA1 and P450IA2 to N-hydroxylate AAF (2-acetylaminofluorene) was strongly inhibited by α-NF. In addition, α-NF at low concentrations also inhibited the capacity of the P450IA1 and P450IA2 proteins to activate IQ (2-amino-3-methylimidazo[4,5-f]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) in the Ames test (29), and at high concentrations stimulated the ability of P450IIIA proteins to metabolize these compounds to mutagens (34). Shimada et al. (6) also observed strong inhibition of P450Pa (P450IA2) catalytic activities by α-NF.

The differential effects of α-NF and the correlative studies suggest that different P450 isozymes are involved in the metabolism of the (+)- and (−)-enantiomers of B[a]P-7,8-DHD.

Table V. Arachidonic acid-supported oxidation of (−)-B[a]P-7,8-DHD by human liver microsomes

<table>
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<th>Sample no.</th>
<th>Sp. act.*</th>
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<th>Fold change*</th>
<th>NDGAc</th>
<th>Fold change*</th>
<th>Phenbutac</th>
<th>Fold change*</th>
<th>Indometh</th>
<th>Fold change*</th>
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<td>H5</td>
<td>53.0 (63)b</td>
<td>7.0</td>
<td>0.13</td>
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<td>0.07</td>
<td>1.1</td>
<td>0.02</td>
<td>56.7</td>
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<tr>
<td>H6</td>
<td>52.5 (79)</td>
<td>12.5</td>
<td>0.24</td>
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<td>0.25</td>
<td>12.4</td>
<td>0.24</td>
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<tr>
<td>H7</td>
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<td>0.30</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>H8</td>
<td>64.5 (82)</td>
<td>19.6</td>
<td>0.30</td>
<td>26.1</td>
<td>0.40</td>
<td>6.9</td>
<td>0.11</td>
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<tr>
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<td>20.7 (72)</td>
<td>3.0</td>
<td>0.15</td>
<td>5.6</td>
<td>0.27</td>
<td>0.4</td>
<td>0.02</td>
<td>41.9</td>
<td>2.03</td>
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<tr>
<td>H10</td>
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<td>4.7</td>
<td>0.28</td>
<td>—</td>
<td>—</td>
<td>3.9</td>
<td>0.23</td>
<td>8.9</td>
<td>0.53</td>
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<tr>
<td>H11</td>
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<td>—</td>
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<td>—</td>
<td>17.0</td>
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<td>54.2</td>
<td>0.82</td>
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<td>0.07</td>
<td>0</td>
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<td>0.26</td>
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<td>0.26</td>
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<td>0.16</td>
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<td>H16</td>
<td>16.8 (73)</td>
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<td>0.06</td>
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<tr>
<td>H19</td>
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<td>3.3</td>
<td>0.13</td>
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<td>0.03</td>
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<tr>
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<td>0.26</td>
<td>2.1</td>
<td>0.15</td>
<td>1.1</td>
<td>0.08</td>
<td>18.0</td>
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<tr>
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<td>0.04</td>
<td>1.0</td>
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<td>153.5</td>
<td>4.92</td>
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<tr>
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<td>0.06</td>
<td>1.0</td>
<td>0.04</td>
<td>0.5</td>
<td>0.02</td>
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<td>1.0</td>
<td>4.0</td>
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<tr>
<td>Mean</td>
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<td>4.6</td>
<td>0.23</td>
<td>5.2</td>
<td>0.27</td>
<td>3.8</td>
<td>0.15</td>
<td>50.5</td>
<td>1.69</td>
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<tr>
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<td>0.44</td>
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<td>0.15</td>
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*Specific enzyme activities are expressed as pmol metabolites/mg protein/min using 100 μM arachidonic acid and a substrate concentration of 20 μM.

bAdditives were BHA (2 μM), NDGA (100 μM), phenylbutazone (100 μM) or indomethacin (100 μM).

cThese data have been corrected for endogenous oxidation in the presence of the additive and absence of added arachidonic acid.

dFold change shows the inhibition (factor < 1.0) or enhancement (factor > 1.0) of metabolism when incubations were conducted in the presence of the additive BHA, NDGA, phenylbutazone or indomethacin.

The percentage of diol epoxide 2 is formed in the uninhibited reaction is shown in parenthesis.

Cytochrome P450IA2 appears to have an affinity for the (−)-enantiomer of B[a]P-7,8-DHD but not for the (+)-enantiomer. Cytochrome P450IIIA3/4 is able to activate both enantiomers of the substrate and favours diol epoxide 2 formation from both enantiomers of the dihydrodiol after stimulation by α-NF.

The observations made in the present work do not totally agree with those of Shimada and co-workers (7). By measuring the umu gene response, they found that α-NF stimulated the activation of (±), (+)- and (−)-B[a]P-7,8-DHD in incubations with human liver microsomes and with a reconstituted system containing P450NF (P450IIIA4). Polyclonal antibodies to P450NF inhibited the microsomal umu gene response with (±), (+)- and (−)-B[a]P-7,8-DHD, suggesting that P450NF is the major enzyme in the activation of B[a]P-7,8-DHD in human liver. Correlation studies supported this conclusion. In the present work α-NF stimulated the metabolism of (−)-B[a]P-7,8-DHD and inhibited the metabolism of (−)-B[a]P-7,8-DHD. According to the correlation studies, cytochrome P450IIIA3/4 was important in the metabolism of both enantiomers of the substrate only after stimulation by α-NF. This conclusion was drawn by Shimada et al. (6), but contrary to the present findings, they found no correlation of either B[a]P-7,8-DHD isomer umu gene responses with the activities catalyzed by P450Pa (P450IA2). We examined the effect of gestodene, an ethynyl steroid with gestational properties and which was shown to be an effective inactivator of the cytochromes P450IIIA (40–43). Using three microsomal preparations (H5, H11 and H12), preincubation of the microsomes with NADPH and gestodene in experiments conducted in the absence of α-NF had only a relatively small inhibitory effect on the metabolism of (+)- and (−)-B[a]P-
7,8-DHD (~22% decrease) compared to experiments in which α-NF was added to the preincubation. Here metabolism of (-)-B[a]P-7,8-DHD was inhibited by 78.3 ± 3.0% while that of (+)-B[a]P-7,8-DHD was inhibited by 66.3 ± 9.1% (n = 3). These results further show the major role of the ΙΙΙΑ3/4 proteins in the metabolism of both enantiomers of the substrate after α-NF pretreatment, while illustrating the minor role these enzymes play when there is no activation by α-NF. The extent of the inhibition by gestodene was not dependent on the prior activation of the ΙΙΙΑ protein by α-NF since results were independent of the order of preincubation.

In animal liver microsomes, 2-diehtylaminomethyl 2,3-diphhenylvalerate (SKF525A) has been shown to be a potent inhibitor of the cytochrome P450 system (44,45). In the present work, further confirmation of the involvement of the cytochrome P450 enzymes in the NADPH-dependent metabolism of (+)- and (-)-B[a]P-7,8-DHD is provided through the significant inhibition effected by 500 μM SKF525A. In the presence of 2 μM BHA, the metabolism of the (+)-enantiomer was inhibited by a mean 35% and the metabolism of the (-)-enantiomer was inhibited by a mean 42%. Similar extents of inhibition were observed in the absence of BHA. The presence of SKF525A in the incubation mixtures generally had little effect on the proportions of diolepoxide 1- and diolepoxide 2-derived products produced (data not shown).

Arachidonic acid-supported conversion of (+)- and (-)-B[a]P-7,8-DHD to diolepoxides

Tables IV and V display the extents of arachidonic acid-supported conversion of (+)- and (-)-B[a]P-7,8-DHD by human liver microsomal samples in the presence and absence of various additives. The activity towards the (+)-isomer was about twice that seen in the NADPH-supported reaction (Table II), while that towards the (-)-B[a]P-7,8-DHD was approximately equal to the NADPH-supported oxidation (Table III). In the presence of both arachidonic acid and either BHA, NDGA or phenylbutazone, the activities were reduced to <27% of the conversion in the presence of added arachidonic acid only. The (+)-enantiomer of the substrate gave a mean 73% diolepoxide 2-derived products and the (-)-enantiomer afforded a mean 69% diolepoxide 2-derived metabolites. This stereochemistry is in agreement with that of the peroxyl radical-mediated oxidations (8–10,16–18, 21,26).

There was no significant difference between the mean extent of arachidonic acid-supported oxidation of the (+)- and (-)-enantiomers of B[a]P-7,8-DHD but there was a wide variation in activities within the human liver microsomal samples. Sample SV2, for example, had a very low activity towards both of these substrates, whereas sample H8 showed a higher than average activity. The SV2 liver sample was obtained by liver resection from a patient (a smoker) with a liver tumor, while H8 liver sample was obtained from a renal transplant donor who was also a smoker.

Confirmation of the free radical nature of the arachidonic acid-dependent metabolism of (+)- and (-)-B[a]P-7,8-DHD is provided by the substantial inhibition effected by the antioxidants BHA and NDGA. Prostaglandin synthase co-oxidations are inhibited at the hydroperoxidase step by both BHA and NDGA, but this enzyme was not thought to be involved in these samples in a peroxide radical-mediated process because 100 μM indomethacin, an inhibitor of cyclooxygenase, did not inhibit the metabolism consistently and in many samples increases occurred. The ratio of diolepoxide 2 to diolepoxide 1 is also inconsistent with a major role for prostaglandin synthase oxidation of the B[a]P-7,8-DHDs because the latter enzyme forms little or no diolepoxide 1 (11,15).

Alternatively, lipoxygenase may be responsible for this activity. Such an involvement in the arachidonic acid-dependent metabolism of (+)- and (-)-B[a]P-7,8-DHD was consistent with the substantial inhibition by 100 μM NDGA, a lipoxygenase inhibitor (46) as well as an antioxidant. This proposal is supported by observations by Nemato and Takayama (47) who suggested a role for lipoxygenase in the arachidonic acid-dependent activation of B[a]P to protein-binding species with cytosolic and microsomal fractions from rat liver and lung. The observation that phenylbutazone (100 μM) inhibited the arachidonic acid-supported metabolism of (+)- and (-)-B[a]P-7,8-DHD generally by >80% appears to discount this pathway in the present work. If lipoxygenase was involved in the metabolism of these compounds an increase in 7,8-dihydrodiol oxidation would have been seen. Reed et al. (48) found that 100 μM phenylbutazone markedly stimulated the prostaglandin H synthase-catalyzed oxidations of B[a]P-7,8-DHD, and we found that phenylbutazone (100 μM) markedly augmented the metabolism of (+)-B[a]P-7,8-DHD by soyabean lipoxygenase (data not shown). The augmentation was higher in the absence of added arachidonic acid.

It appears more likely that oxidation of the enantiomers of B[a]P-7,8-DHD occurs by a mechanism in which the peroxidase activity of cytochrome P450 is involved together with lipid peroxides or hydroperoxide present in the microsomal preparations (9,18,24). In such a mechanism phenylbutazone may act as an alternative substrate to the B[a]P-7,8-DHD and undergo preferential oxidation (48). However, a contribution from active peroxyl radicals produced by lipoxygenase via arachidonic acid cannot be completely discounted.

The ability of these microsomal preparations to convert B[a]P-7,8-DHD enantiomers to diolepoxides via hydroperoxide-mediated or oxene-mediated reactions represent significant bioactivation pathways. The formation of the (+)-enantiomer of diolepoxide 2 from B[a]P-7,8,9R,DHD is a particularly significant pathway since this 7,8-diol-9,10-epoxide is highly tumorigenic in rodents in contrast to its enantiomer and the two diolepoxide 1 enantiomers (1,4). This ultimate rodent carcinogen is virtually the only detectable product of the NADPH-supported human hepatic microsomal metabolism of (-)-B[a]P-7,8-DHD and is the major product of the pathway supported by arachidonic acid. While rodent hepatic metabolism of B[a]P is known to favour oxidation of the (-)-enantiomer of B[a]P-7,8-DHDs because the latter enzyme forms little or no diolepoxide 1 enantiomers (1,4). This ultimate rodent carcinogen is virtually the only detectable product of the NADPH-supported human hepatic microsomal metabolism of (-)-B[a]P-7,8-DHD and is the major product of the pathway supported by arachidonic acid.

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References


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