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Cytochrome b_5 and epoxide hydrolase contribute to benzo[*a*]pyrene-DNA adduct formation catalyzed by cytochrome P450 1A1 under low NADPH:P450 oxidoreductase conditions

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Keywords: benzo[*a*]pyrene; cytochrome P450 1A1; NADPH:cytochrome P450 oxidoreductase; epoxide hydrolase; cytochrome b_5

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Abbreviations: AHR, aryl hydrocarbon receptor; BaP, benzo[a]pyrene; BPDE, BaP-7,8-dihydrodiol-9,10-epoxide; CYP, cytochrome P450; dG- N^2 -BPDE, 10-(deoxyguanosin- N^2 -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene; EROD, 7-ethoxyresorufin *O*-deethylation; HPLC, high pressure liquid chromatography; HRN, Hepatic P450 Reductase Null; mEH, microsomal epoxide hydrolase; NMR, nuclear magnetic resonance; PA, phenacetin; PAH, polycyclic aromatic hydrocarbon; POR, NADPH:cytochrome P450 oxidoreductase; RAL, relative adduct labeling; RCN, Reductase Conditional Null; TLC, thin-layer chromatography; WT, wild-type.

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Abstract

In previous studies we had administered benzo[*a*]pyrene (BaP) to genetically engineered mice (HRN) which do not express NADPH:cytochrome P450 oxidoreductase (POR) in hepatocytes and observed higher DNA adduct levels in livers of these mice than in wild-type mice. To elucidate the reason for this unexpected finding we have used two different settings for *in vitro* incubations; hepatic microsomes from control and BaP-pretreated HRN mice and reconstituted systems with cytochrome P450 1A1 (CYP1A1), POR, cytochrome *b*₅, and epoxide hydrolase (mEH) in different ratios. In microsomes from BaP-pretreated mice, in which Cyp1a1 was induced, higher levels of BaP metabolites were formed, mainly of BaP-7,8-dihydrodiol. At a low POR:CYP1A1 ratio of 0.05:1 in the reconstituted system, the amounts of BaP diones and BaP-9-ol formed were essentially the same as at an equimolar ratio, but formation of BaP-3-ol was ~1.6-fold higher. Only after addition of mEH were BaP dihydrodiols found. Two BaP-DNA adducts were formed in the presence of mEH, but only one when CYP1A1 and POR were present alone. At a ratio of POR:CYP1A1 of 0.05:1, addition of cytochrome *b*₅ increased CYP1A1-mediated BaP oxidation to most of its metabolites indicating that cytochrome *b*₅ participates in the electron transfer from NADPH to CYP1A1 required for enzyme activity of this CYP. BaP-9-ol was formed even by CYP1A1 reconstituted with cytochrome *b*₅ without POR. Our results suggest that in livers of HRN mice Cyp1a1, cytochrome *b*₅ and mEH can effectively activate BaP to DNA binding species, even in the presence of very low amounts of Por.

1. Introduction

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that has been classified as human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC, 2010). BaP and other PAHs are produced mainly by incomplete combustion of organic matter and are ubiquitous in the environment, leading to measurable levels of exposure in the general population (IARC, 2010). Beside the inhalation of polluted air, the main sources of exposure are tobacco smoke and the diet (Kim et al., 2011; Labib et al., 2012; Phillips and Venitt, 2012; Sidden et al., 2012). Chronic exposure of laboratory animals to BaP has been associated with the development of cancer, primarily skin, stomach and lung (IARC, 2010).

BaP requires metabolic activation prior to reaction with DNA (Baird et al., 2005). Cytochrome P450 (CYP) enzymes, mainly CYP1A1 and 1B1, are the most important enzymes in this process (Baird et al., 2005; Hamouchene et al., 2011), in combination with microsomal epoxide hydrolase (mEH). First, CYP1A1 or 1B1 enzymes oxidize BaP to an epoxide that is then converted to a dihydrodiol by mEH (*i.e.* BaP-7,8-dihydrodiol); then further bioactivation by CYP1A1 or 1B1 leads to the ultimately reactive species BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) that can react with DNA, forming adducts preferentially at guanine residues. The 10-(deoxyguanosin-*N*²-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-*N*²-BPDE) adduct is the major product of BPDE with DNA *in vitro* and *in vivo* (Phillips, 2005). BaP is, however, oxidized also to other metabolites such as the other dihydrodiols, BaP-diones and hydroxylated metabolites (Bauer et al., 1995; Chun et al., 1996; Kim et al., 1998; Jiang et al., 2007; Zhu et al., 2008). Among them, 3-hydroxy-BaP (BaP-3-ol), formed by CYP1A1, is considered the major detoxification product of BaP oxidation, because it is known to inhibit mutagenesis and tumorigenesis (Huang et al., 1986). In mouse hepatoma Hepa1c1c7 cells, which express inducible CYP1A1, this metabolite was

the major metabolite formed after exposure to BaP (Holme et al., 2007). Even though this CYP1A1-mediated BaP oxidation product as well as most of the other BaP metabolites are detoxification products, 9-hydroxy-BaP (BaP-9-ol) is a precursor of 9-hydroxy-BaP-4,5-epoxide (Fig. 1) which can form another adduct with guanine (Schoket et al., 1989; Nesnow et al., 1993; Fang et al., 2003). Thus, the levels and activities of CYP1A1 seem to be crucial both for the initiation of BaP-mediated carcinogenesis and BaP detoxification. The expression of CYP1A1 is known to be up-regulated by the aryl hydrocarbon receptor (AHR) and BaP can bind to and activate AHR thereby enhancing its own metabolic activation (Hockley et al., 2007).

The level of BaP-DNA adducts in cells is most probably the result of a balance between their formation and their loss through either DNA repair processes, or cell turnover. Collectively, BaP genotoxicity depends on various factors: (i) metabolism of BaP by phase I enzymes (activation) to reactive DNA-binding species; (ii) detoxification of reactive BaP metabolites by both phase I and phase II enzymes (conjugation); (iii) rate of repair of BaP-DNA adducts; and (iv) BaP-induced expression of genes such as those encoding enzymes involved in activation/detoxification or in DNA damage response (Uno et al., 2004).

Recently, controversial results have been found showing a more important role of CYP1A1 in BaP detoxification *in vivo* than in its activation (Uno et al., 2004; 2006; Arlt et al., 2008; 2012). *Cyp1a1*($-/-$) mice treated with repeated oral doses of BaP (125 mg/kg body weight [bw]/day), die within ~28 days due to immune suppression, whereas wild-type (WT) mice remain healthy for at least 1 year on this regimen (Uno et al., 2004). Using the Hepatic P450 Reductase Null (HRN) and the Reductase Conditional Null (RCN) mouse models we also showed that hepatic CYP enzymes appear to be more important for detoxification of BaP *in vivo* (Arlt et al., 2008; 2012). In these mice NADPH:P450 oxidoreductase (POR), the essential electron donor to CYPs is deleted specifically in hepatocytes, resulting in a decrease

in hepatic CYP function (Henderson et al., 2003; Finn et al., 2007). We found however that the levels of dG-*N*²-BPDE adducts in livers of these mice treated with BaP were higher than in WT mice (Arlt et al., 2008; 2012).

We postulate that the reason for these unexpected findings could be found *in vitro* by analyzing the roles of the different contributing enzymes at different ratios to each other. This can be achieved in reconstituted systems where single enzymes can be added at definite amounts without interference from other constituents as in microsomal systems. These data were compared with the metabolism of BaP and its activation to BaP-DNA adducts *in vivo*. We evaluated CYP1A1-mediated BaP metabolism and its bioactivation in mouse hepatic microsomes and by recombinant CYP1A1 reconstituted with POR. Because microsomal cytochrome *b*₅ has been found to participate in transfer of electrons to CYP enzymes during the CYP catalysis or cause allosteric changes in CYP protein structures that can augment the CYP mediated reactions (Porter, 2002; Schenkman and Jansson, 2003; Guengerich, 2005; Stiborová et al., 2006; 2012; Kotrbová et al., 2011; Riddick et al., 2013), we also analyzed its effects on BaP oxidation in the reconstituted system of CYP1A1 with POR. Moreover, since mEH is essential for the formation of the dG-*N*²-BPDE adduct (Kim et al., 1998; Baird et al., 2005), its influence upon BaP-DNA adduct levels formed in a CYP1A1 reconstituted system was also elucidated. The levels of CYP1A1, POR, cytochrome *b*₅ and EH in mouse hepatic microsomes were determined in previous studies (Arlt et al., 2008; 2012). In the present work, we used a wide range of various ratios of these enzymes in the reconstituted systems to analyze their individual contribution to oxidation/activation of BaP. Among them, at least the reconstitution systems containing low concentrations of POR likely mimic the situation in the tested hepatic microsomes or *in vivo*. The formation of BaP metabolites was measured by high pressure liquid chromatography (HPLC) with UV-detection. DNA adduct formation was investigated by the thin-layer chromatography (TLC)-³²P-postlabeling method.

2. Material and Methods

2.1. Chemicals

BaP (>96%) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical purity or better.

2.2. Animal experiments

All animal experiments were carried out under license in accordance with the law, and with local ethical approval. HRN (*Por^{lox/lox}/Cre^{ALB}*) mice on a C57BL/6 background were derived as described previously (Henderson *et al.*, 2003). BaP was dissolved in corn oil at a concentration of 12.5 mg/ml. Groups of female HRN mice (3 months old, 25-30 g, *n*=3) were treated intraperitoneally with 125 mg/kg bw of BaP for five days. Control mice received corn oil only (Arlt *et al.*, 2008). Animals were killed 24 h after the last dose. Livers were removed, snap frozen and stored at -80°C until analysis.

2.3. Preparation of microsomes

Hepatic microsomes were isolated as described previously (Arlt *et al.*, 2008) and pooled for further experiments. Protein concentration in the microsomal fraction was measured using bicinchoninic acid protein assay (Wiechelman *et al.*, 1988) with bovine serum albumin as a standard.

2.4. Isolation of CYP1A1, POR, cytochrome *b*₅ and mEH

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA (Stiborová *et al.*, 2002), in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic). Rabbit liver POR was purified as described (Stiborová *et al.*, 2002). Cytochrome *b*₅ was isolated from rabbit liver microsomes by the procedure described by Roos (1996). mEH was purified from liver microsomes of rabbits pretreated with phenobarbital as described by Ariyoshi *et al.* (1994). These enzymes

were used for the CYP1A1 reconstitution experiments. As shown in our previous studies (Stiborová et al., 2002; 2004; 2006; 2012; Kotrbová et al., 2009; 2011), the enzymatic activity of rat or human CYP1A1 reconstituted with rabbit POR and cytochrome *b*₅ was essentially the same as that of the enzyme reconstituted with the rat orthologs of these enzymes.

2.5. Microsomal and enzymatic incubations

Incubation mixtures used to study BaP metabolism in mouse hepatic microsomes or by purified CYP1A1 (100 pmol) reconstituted with POR (5-150 pmol), with or without cytochrome *b*₅ (100-500 pmol), with or without mEH (20-250 pmol) contained 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1 mM NADP⁺, 10 mM D-glucose-6-phosphate, 1 U/ml D-glucose-6-phosphate dehydrogenase), 0.5 mg of microsomal protein or 100 pmol CYP1A1 in the reconstituted system, 50 µM BaP (dissolved in 5 µl dimethyl sulfoxide) in a final volume of 500 µl. The enzyme reconstitution was performed as described [(Stiborová et al., 2002; 2006; Kotrbová et al., 2011), using different molar ratios of CYP1A1 to POR and cytochrome *b*₅ and/or mEH (see Results for details). Briefly, purified CYP1A1 was reconstituted with pure POR as follows (2 µM CYP, 0.1-3 µM POR, 0.5 µg/µl CHAPS, 0.1 µg/µl liposomes [dilauroyl phosphatidylcholine, dioleoyl phosphatidylcholine, and dilauroylphosphatidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH, pH 7.4). The reaction was initiated by adding 50 µl of the NADPH-generating system. Control incubations were carried out either without enzymatic system (microsomes or the CYP1A1-reconstituted system), or without NADPH-generating system, or without BaP. After incubation (37°C, 20 min), 5 µl of 1 mM phenacetin (PA) in methanol was added as an internal standard. BaP metabolites were extracted twice with ethyl acetate (2 × 1 ml), solvent evaporated to dryness, residues dissolved in 25 µl methanol and BaP metabolites separated by HPLC.

Incubation mixtures used to analyze BaP-induced DNA adduct formation were as

described above but contained 1 mM NADPH instead of the NADPH-generating system plus 1 mg calf thymus DNA. The reaction was initiated by adding BaP. Incubations were carried out at 37°C for 90 min; BaP-DNA adduct formation was linear up to 120 min (Arlt et al., 2008). Control incubations were carried out either without CYP, without NADPH, without DNA, or without BaP. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Stiborová et al., 2002).

2.6. *CYP1A1* enzyme activity assays

The samples containing CYP1A1 reconstituted with POR (see the procedure described above) were characterized for enzyme activity using Sudan I oxidation for CYP1A1 (Stiborová et al., 2002) and 7-ethoxyresorufin *O*-deethylation (EROD) for CYP1A (Guengerich and Shimada, 1991).

2.7. HPLC analysis of BaP metabolites

HPLC analysis of BaP metabolites was performed on a Nucleosil® C18 reverse phase column, (250 × 4 mm, 5 µm; Macherey Nagel, Düren, Germany) using a Dionex system consisting of a pump P580, a UV/VIS Detector UVD 170S/340S, an ASI-100 Automated Sample Injector, a termobox COLUMN OVEN LCO 101 and an In-Line Mobile Phase Degasser Degasys DG-1210 Dionex controlled with Chromeleon™ 6.11 build 490 software. The conditions used for the chromatographic separation of BaP metabolites were as follows: 50% acetonitrile in water; (v/v), with a linear gradient to 85% acetonitrile in 35 min, then an isocratic elution with 85% acetonitrile for 5 min, a linear gradient from 85% acetonitrile to 50% acetonitrile in 5 min, followed by an isocratic elution of 50% acetonitrile for 5 min. Detection was by UV absorption at 254 nm. BaP metabolite peaks (Fig. 2A) were collected and analyzed by NMR and/or mass spectrometry. The areas under the curve at 254 nm were calculated relative to the peak area of the internal standard phenacetin and expressed as relative peak areas.

2.8. NMR spectrometry

NMR spectra (δ , ppm; J , Hz) of BaP and its metabolites M1, M4, M5 and M7 were measured on a Bruker Avance II-600 and/or Bruker Avance II-500 instruments equipped with a cryoprobe (600.1 or 500.0 MHz for ^1H and 150.9 or 125.7 MHz for ^{13}C) in hexadeuterated acetone and CDCl_3 and referenced to the solvent signals (δ 2.05 and 7.27, respectively). Due to the low amounts of metabolites it was not possible to acquire ^{13}C NMR spectra or perform heteronuclear correlation experiments.

1) BaP. ^1H NMR spectrum of BaP contains five isolated spin systems: one three-spin system of hydrogens H-1, H-2 and H-3, one four-spin system of H-7, H-8, H-9 and H-10, one isolated spin of H-6 and two two-spin systems of H-4, H-5 and of H-11, H-12. The assignment of signals in the spectrum could be done with the help of homonuclear COSY spectrum, where strong cross-peaks corresponding to three-bond couplings were observed. However, the signals of the two two-spin systems (H-4,H-5 and H-11, H-12) could not be assigned with the use of COSY spectrum only and heteronuclear correlation spectra (HSQC and HMBC) had to be used. The assignment of all ^1H signals and coupling constants of BaP and its metabolites is shown in Table 1 (for structures see Fig 2B).

2) M1. Two signals of the four-spin system of M1 were shifted upfield (to 4.5 and 5.7 ppm). These values are too low for the fully aromatic BaP skeleton. The structure of M1 was identified as *trans*-9,10-dihydro-BaP-9,10-diol (BaP-9,10-dihydrodiol) by comparison with previously reported NMR data of this compound (Platt and Oesch, 1983). The *trans* arrangement of the two hydroxy groups was supported by the inspection of the vicinal coupling constant between hydrogen atoms H-9 and H-10. The torsion angle between these two hydrogen atoms calculated using the generalized Karplus type equation (Haasnoot et al., 1980) was predicted to be 48° . This value is very close to the torsion angle observed in the molecular model of the *trans*-derivative with the two hydroxyl groups in pseudo-axial

positions. The molecular model of the *cis*-derivative predicts the torsion angle between the two hydrogen atoms to be close to -75° . We were unable to determine the absolute configuration on the new asymmetric centers (C-9 and C-10). It is possible that both enantiomers are present in metabolite M1.

3) M4 and M5. In the spectra of both compounds we observed the four-spin system of H-7, H-8, H-9 and H-10 and three two-spin systems. Two substituents are therefore attached to the BaP skeleton: one in position 6 and the second one in position 1 or 3. Furthermore, the unusually shielded H-2 proton at 6.7 ppm was characteristic of the α -proton (next to carbonyl) in phenalones (Prinzbach et al., 1967) suggesting that the structures of M4 and M5 could be BaP-1,6-dione and -3,6-dione, respectively. Because both compounds were synthesized previously and their ^1H NMR spectra was reported (Leeruff et al., 1986; Xu et al., 2009), spectra in CDCl_3 were used for comparison and the metabolites could be identified as BaP-1,6-dione (M4) and BaP-3,6-dione (M5).

4) M7. In the spectrum of M7, the three-spin system was replaced with a two-spin system suggesting that one substituent is attached to the position 1 or 3 of the BaP. The structure of M7 was confirmed to be BaP-3-ol by comparison of the chemical shifts and coupling constants with those reported previously (Xu et al., 2009).

5) M2, M3 and M6. Because the amounts of M2, M3 and M6 samples were insufficient for NMR spectroscopy, these metabolites were analyzed only by mass spectrometry only as described below.

2.8. Mass spectrometry

Mass spectra of BaP and its metabolites M2, M3 and M6 were measured on a matrix-assisted laser desorption/ionisation reflectron time-of-flight MALDITOF mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany). Positive spectra were calibrated externally using the monoisotopic $[\text{M}+\text{H}]^+$ ion of MRFA peptide 524.26 m/z and CCA matrix

peaks 190.05, 379.09 m/z . A 10 mg/ml solution of α -cyano-4-hydroxy-cinnamic acid or 2,5-dihydrobenzoic acid in 50% MeCN/0.3% acetic acid was used as MALDI matrix. A 0.5 μ l of sample dissolved in MeCN was premixed with 0.5 μ l of the matrix solution on the target and allowed to dry at ambient temperature. The MALDI-TOF positive spectra were collected in reflectron mode. Positive $[M^+]$ of BaP corresponded to this compound (m/z 252.1). The metabolites with retention times of 11.9 (M2) and 12.9 min (M3) gave a positive molecular ion each at m/z 286.1 which is indicative of BaP-dihydrodiol metabolites. The metabolite eluted at 24.6 min (M6) gave a positive molecular ion at m/z 268.1 which is indicative of a hydroxylated BaP metabolite. These results are consistent with previous studies on the metabolism of BaP by human CYP1A1 (Bauer et al., 1995; Kim et al., 1998), in which these metabolites were identified as BaP-4,5-dihydrodiol (M2), BaP-7,8-dihydrodiol (M3), and BaP-9-ol (M6).

2.9. BaP-DNA adduct detection by ^{32}P -postlabeling analysis

DNA adducts were measured using the nuclease P1 enrichment version of the TLC- ^{32}P -postlabeling method essentially as described previously (Arlt et al., 2008; Phillips and Arlt, 2007). Briefly, DNA samples (4 μ g) were digested with micrococcal nuclease (120 mU; Sigma) and calf spleen phosphodiesterase (40 mU; Calbiochem), enriched and labeled as reported. Solvent conditions for the resolution of ^{32}P -labeled adducts on polyethyleneimine-cellulose (PEI) TLC were: D1, 1.0 M sodium phosphate, pH 6; D3, 4.0 M lithium-formate, 7.0 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8. After D3 or D4 developments to the top of the plates, the plates were developed an additional 30 min with the TLC tank partially opened, to prolong the resolution time of the BaP-DNA adducts. After chromatography TLC plates were scanned using a Packard Instant Imager (Dowers Grove, IL, USA). DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Arlt et al., 2008). Results were expressed as DNA adducts/ 10^8 nucleotides. An external BPDE-

DNA standard (Phillips and Castegnaro, 1999) was employed to identify adducts in experimental samples.

3. Results

3.1. Oxidation of BaP by hepatic microsomes and CYP1A1 reconstituted with POR, cytochrome b, and mEH

BaP metabolism in the CYP1A1 reconstituted system containing POR combined with cytochrome *b*₅ and/or mEH was compared with its metabolism by hepatic microsomes isolated from control (untreated) and BaP-pretreated HRN mice. In studies using mouse hepatic microsomes, a spectrum of hydroxylated BaP metabolites, BaP-dihydrodiols as well as BaP diones (quinones) was found (Fig. 2). Seven BaP oxidation products (designated as M1-M7) were generated both by hepatic microsomes of control HRN mice and by those with induced CYP1A1 expression mediated by pretreating HRN mice with BaP. These metabolites have been reported to be formed by CYP1A1 in combination with mEH (Bauer et al., 1995; Kim et al., 1998; Baird et al., 2005). The HPLC method using an acetonitrile/water gradient as mobile phase and UV detection at 254 nm resulted in mostly base-line separation of all BaP metabolites (Fig. 2A).

The BaP metabolites formed by mouse hepatic microsomes were collected and subsequently characterized by NMR or mass spectrometry. They were identified to be BaP-9,10-dihydrodiol (M1), BaP-4,5-dihydrodiol (M2), BaP-7,8-dihydrodiol (M3), BaP-1,6-dione, (M4), BaP-3,6-dione (M5), BaP-9-ol (M6), and BaP-3-ol (M7) (see Fig. 2). Very low expression levels of mouse Por in the liver of untreated HRN mice that constitutively express Cyp1a1 (Arlt et al., 2008) resulted in the formation of very low amounts of these BaP metabolites. However, Cyp1a1 induction by BaP pretreatment of HRN mice (Arlt et al., 2008) was sufficient for the effective oxidation of BaP by hepatic microsomes, even at the very low Por concentrations in these microsomes (Fig. 3). The efficiency of these microsomes to oxidize BaP to BaP-7,8-dihydrodiol, the precursor of BPDE, was more than 34-fold higher than that of microsomes of untreated mice, while formation of other metabolites was less

pronounced. Amounts of BaP-9,10-dihydrodiol, BaP-4,5-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-9-ol and BaP-3-ol were 6.6-, 9.6-, 2-, 3.4-, 10- and 7.7-times higher, respectively, in microsomes of HRN mice exposed to BaP than in those of untreated HRN mice (Fig. 3).

To investigate the role of CYP1A1 in oxidation of BaP to individual metabolites, purified CYP1A1 was reconstituted with POR and used as the oxidation system. CYP1A1 reconstituted with POR was active with its typical substrates, Sudan I (Stiborová et al., 2002) and 7-ethoxyresorufin (Guengerich and Shimada, 1991) (data not shown). Isolated CYP1A1 reconstituted with POR oxidized BaP to only four metabolites, namely BaP-1,6-dione (M4), BaP-3,6-dione (M5), BaP-9-ol (M6), and BaP-3-ol (M7) (Fig. 4). As expected, no BaP-dihydrodiols were detected in this system because of the absence of mEH which is important for the hydration of BaP epoxides. In the enzymatic systems where mEH was reconstituted with CYP1A1 and POR, BaP-9,10-dihydrodiol (M1) and BaP-7,8-dihydrodiol (M3) were formed as expected, however the whole metabolic profile of BaP could not be quantified because some impurities in the mEH preparation interfered with BaP metabolites eluting between 22 and 35 min during HPLC (data not shown).

Interestingly, no significant differences in levels of BaP-1,6-dione, BaP-3,6-dione and BaP-9-ol were found in the systems where CYP1A1 was reconstituted with low amounts of POR (the CYP1A1:POR ratio was 1:0.05) or equimolar concentrations of these enzymes (Fig. 4). Only BaP-3-ol formation was significantly higher (1.6-fold) in the system containing equimolar CYP1A1:POR ratios. No BaP metabolites were formed when POR was omitted from the CYP1A1 reconstitution system (Fig. 4). However, when CYP1A1 was reconstituted with cytochrome *b*₅ without POR (under CYP1A1:cytochrome *b*₅ ratios of 1:1, 1:2.5 or 1:5), BaP was oxidized (Fig. 5A) to only low amounts of BaP-9-ol. Addition of cytochrome *b*₅ to the CYP1A1/POR reconstituted system led to a cytochrome *b*₅-concentration-dependent

increase in BaP oxidation (compare Fig. 4 and Fig. 5B and C); the increase was found both at 1:0.05 and 1:1 CYP1A1:POR ratios. BaP-3-ol was the major metabolite formed under both CYP1A1:POR ratios but its amount was up to 1.8-fold higher when POR was present at an equimolar ratio to CYP1A1 than at the 1:0.05 CYP1A1:POR ratio. In contrast, the amount of BaP-9-ol was always higher in the system with low POR concentrations. Like BaP-3-ol, the amount of BaP-3,6-dione formed in the system was always higher at equimolar CYP1A1/POR concentrations relative to the systems containing low POR concentrations, while overall no differences were found for BaP-1,6-dione. Collectively, all these results indicate that cytochrome *b*₅ may contribute quite substantially to the CYP1A1-mediated oxidation of BaP, even under conditions with very low quantities of POR.

*3.2. BaP-DNA adduct formation by hepatic microsomes and CYP1A1 reconstituted with POR, cytochrome *b*₅ and mEH*

In further experiments the formation of BaP-derived DNA adducts was investigated in analogous enzymatic systems.

Activation of BaP by CYP1A1 reconstituted with POR in the presence of DNA resulted in the formation of only one BaP-DNA adduct (adduct spot 1 shown in Fig. 6A). Therefore, this adduct is formed by CYP1A1-catalyzed oxidation of BaP, without the contribution of mEH. Comparison with previous ³²P-postlabeling studies (Schoket et al., 1989; Nesnow et al., 1993; Fang et al., 2001) showed that adduct spot 1 has similar chromatographic properties on PEI-cellulose TLC to a guanine adduct derived from reaction with 9-hydroxy-BaP-4,5-epoxide (see Fig. 1). In accordance with these previous results, DNA adduct 1 (Fig. 6) had more hydrophobic properties on PEI-cellulose than another BaP-derived DNA adduct (assigned adduct spot 2) formed by hepatic microsomes (compare Fig. 6D), which was identified as dG-N²-BPDE (Schoket et al., 1989; Fang et al., 2001; Arlt et al., 2008).

As shown in Figure 7A formation of BaP-DNA adduct 1 was dependent on the

CYP1A1:POR ratio and was formed even at low POR concentrations (*i.e.* a CYP1A1:POR ratio of 1:0.2). However, when BaP was incubated with DNA using a CYP1A1:POR ratio of 1:2, lower levels of this adduct were produced (Fig. 7A). Addition of increasing amounts of mEH to the CYP1A1/POR reconstituted system led to a decrease in the formation of this adduct, while BaP-DNA adduct 2 (adduct spot 2 shown in Figure 6B and 6D) was generated (Figs. 7B, 8A and 8C). No BaP-DNA adducts were detected by ^{32}P -postlabeling when NADPH, the cofactor of the NADPH-dependent CYP system, was omitted from incubations with the CYP1A1 reconstituted systems (Fig. 6C).

Addition of cytochrome b_5 to the CYP1A1 reconstituted system resulted in increased formation of both BaP-DNA adducts formed under a CYP1A1:POR ratio of 1:2, both in the absence and presence of mEH (Fig. 8). However, levels of BaP-DNA adduct 1 at a CYP1A1:POR ratio of 1:0.2 were nearly identical in the presence or absence of cytochrome b_5 when mEH was absent, whereas addition of cytochrome b_5 to this system in the presence of mEH increased formation of both BaP-DNA adducts (Figs. 8A and B). The levels of adduct 1 formed by CYP1A1, POR and cytochrome b_5 in a ratio of 1:2:3 were higher than in any other enzyme combination (Fig. 8). Among all enzyme combinations used, the highest levels of both BaP-DNA adducts were produced by the system containing CYP1A1, POR, mEH and cytochrome b_5 in a ratio of 1:2:0.5:3 (Fig. 8D) followed by the systems containing these enzymes in ratios of 1:0.2:1:3 (Fig. 8B) or 1:2:1:3 (Fig. 8D). These results showed that even under low POR concentrations, CYP1A1 in the presence of appropriate concentrations of mEH and cytochrome b_5 efficiently activates BaP to DNA binding species. These results are in line with the BaP metabolite profile in the CYP1A1 reconstituted experimental systems described above.

The DNA adduct results obtained in reconstituted enzyme systems corresponded to those found in hepatic microsomes of HRN mice. As shown in our previous study (Arlt et al.,

2008), incubation of BaP with hepatic microsomes from these mice led to DNA adducts detectable by ^{32}P -postlabeling. Using this method one major asymmetric BaP-DNA adduct spot was detectable, in which the dG- N^2 -BPDE adduct was identified by cochromatographic analysis, and DNA adduct levels increased substantially in incubations with microsomes from BaP pretreated HRN mice (Arlt et al., 2008). After closer inspection of this asymmetric BaP-DNA adduct spot and improved chromatographic separation, two major BaP-DNA adducts (still partially overlapping one another) were detectable. Better separation of these BaP-DNA adducts as well as those found in liver of HRN mice treated with BaP was achieved on PEI-cellulose TLC (Fig. 6D and 6E) by a slightly modified development in D3 and D4 directions as outlined in Material and Methods. The chromatographic properties of both adducts on TLC were similar to the two BaP-DNA adducts found in experiments utilizing CYP1A1-reconstituted systems (compare adduct spots 1 and 2 in Fig. 6B and 6D). Therefore, in the present study we re-analyzed the BaP-DNA adduct levels found in hepatic microsomal incubations from control (untreated) HRN mice and those pretreated with BaP. While only low levels of both BaP-DNA adducts were formed by hepatic microsomes from untreated HRN mice, formation of both adducts increased substantially (~50-fold) in hepatic microsomes isolated from BaP-pretreated HRN mice (Fig. 9). These results indicate that Cyp1a1 induction in combination with relative low Por expression still provides an efficient BaP activation system leading to DNA adduct formation. BaP-DNA adduct 1 was, however, hardly detectable in liver DNA from BaP-treated HRN mice (Fig. 6E) which is in full concordance with results obtained previously (Arlt et al., 2008) where dG- N^2 -BPDE (assigned BaP-DNA adduct 2 in the present study) was the major adduct identified *in vivo*.

4. Discussion

Previously we worked with two different mouse models lacking Por expression in hepatocytes and found that the levels of BaP-DNA adducts were up to 13-fold higher in livers of HRN mice treated with BaP than in those of WT mice (Arlt et al., 2008). More recently, these results were confirmed in the RCN mouse model (Arlt et al., 2012), indicating that hepatic CYP enzymes are more important for detoxification of BaP *in vivo* despite being required for its bioactivation *in vitro*. Nevertheless, hepatic microsomes from HRN mice expressing very low Por levels, and particularly those from BaP-pretreated HRN mice were capable of activating BaP to form DNA adducts *in vitro* (compare Fig. 6 in Arlt et al., 2008 and Figs. 6D and 9 in the present work), suggesting that Cyp1a1-mediated bioactivation of BaP takes place in these microsomes. In order to confirm this finding in a flexible modular experimental system we investigated the oxidative metabolism of BaP and its bioactivation to BaP-DNA adducts in a reconstituted enzyme system that allows the addition and removal of individual enzymes. Purified CYP1A1 was reconstituted with POR and other components of the microsomal enzymatic system (cytochrome *b*₅ and mEH) in various combinations and ratios were employed. For comparison mouse microsomal enzymatic systems were utilized. Among the tested reconstituted systems, at least those containing low concentrations of POR likely mimic the situation in mouse hepatic microsomes or *in vivo*. However, it should be noted that the reconstituted systems may only provide mechanistic insights and cannot be fully extrapolated to microsomes and/or to the *in vivo* situation.

Interestingly, as shown in Figures 2 and 3 the results obtained in the present study demonstrated that BaP is oxidized by hepatic microsomes of control (untreated) and BaP-pretreated HRN mice containing very low amounts of Por to hydroxylated BaP metabolites (BaP-3-ol and BaP-9-ol), BaP-dihydrodiols (BaP-4,5-dihydrodiol, BaP-7,8-dihydrodiol and BaP-9,10-dihydrodiol) as well as BaP diones (BaP-1,6-dione and BaP-3,6-dione). The same

metabolites have been formed in several previous studies utilizing enzyme systems of CYP1A1 combined with POR and mEH (Kim et al., 1998; Jiang et al., 2007; Zhu et al., 2008). We also found that BaP is activated by these microsomes to form BaP-DNA adducts (Fig. 6) which have been detected in previous studies (Nesnow et al., 1993; Schoket et al., 1989; Fang et al., 2001; Siddens et al., 2012).

As expected, mEH was essential for the formation of BaP-dihydrodiols; CYP1A1 reconstituted with POR but without mEH generated only BaP-1,6-dione, BaP-3,6-dione, BaP-3-ol, and BaP-9-ol. It is known that oxidation of various substrates by CYP1A1 or other CYPs is dependent on the POR:CYP1A1 ratio; optimal ratios of these enzymes are usually 1:1 or higher (Buters et al., 1995; Chun et al., 1996; Schwarz et al., 2001; Shimada et al., 1999; 2001; 2004; Yamazaki et al., 2001; Stiborová et al., 2002; Guengerich, 2005; Farooq and Roberts, 2010; Davydov, 2011; Kotrbová et al., 2011; Davydov et al., 2013). Interestingly, in contrast to these findings, with the exception of BaP-3-ol, formation of BaP metabolites by CYP1A1 reconstituted with POR was the same, even when the CYP1A1:POR ratio was only 1:0.05. This CYP1A1:POR ratio essentially mimics the expression level of these enzymes in the livers of HRN mice (Arlt et al., 2008), while a higher than equimolar POR:CYP1A1 ratio more reflects the situation in the livers of WT mice suggesting an effective transfer of electrons from NADPH to the CYP1A1-BaP binary complex mediated by POR. The only detoxification BaP metabolite, BaP-3-ol, was formed at higher amounts in the CYP1A1 system reconstituted with an equimolar ratio of POR. This high efficiency of BaP metabolism at ratios of CYP1A1:POR of less than 0.5 are unusual, but seems to be a specific phenomenon for oxidation of BaP by CYP1A1 rather than a general feature. For other substrates such as Sudan I (Stiborova et al., 2002) and ellipticine (Mrazova et al., 2007, Kotrbova et al., 2011) a reconstitution system of CYP1A1 with POR at a ratio of 1:1 was optimal. It seems that binding of BaP to CYP1A1 mediating formation of the high-spin state

of CYP1A1 facilitates the enzyme oligomerization (aggregation) in an artificial membrane of liposomes and such CYP1A1-CYP1A1 interactions may have evolved to optimize the functioning of the reconstitution system at limiting concentrations of POR. Indeed, there are numerous observations demonstrating a crucial effect of oligomerization of microsomal CYPs on their functional properties. One of the most prominent consequences of oligomerization is given by “persistent heterogeneity” or apparent divergence of a pool of a single CYP species into non-interconverting and functionally separate fractions (Davydov, 2011). This suggestion was supported by several investigations for substrate oxidation by various CYP enzymes in the reconstituted systems (Kaminsky and Guengerich, 1985; Backes et al., 1989; Iwase et al., 1991; Fernando et al., 2007; 2008; Davydov, 2011). The reasons for the results found in the present work seem to be even more complex, as BaP oxidation by the CYP1A1 reconstituted system was substantially stimulated by cytochrome b_5 .

The major BaP-DNA adduct formed in HRN/RCN mice and in hepatic microsomes of HRN mice was previously identified by TLC, HPLC and LC-MS/MS to be dG- N^2 -BPDE. In addition, another adduct found and assigned adduct 1 in the present study, exhibited more hydrophobic properties on ^{32}P -postlabeling TLC (see Fig. 1 in Arlt et al., 2008 and Fig. 2 in Arlt et al., 2012) and could be separated from adduct 2 (*i.e.* dG- N^2 -BPDE). Since mouse hepatic Cyp enzyme activity had been largely obliterated by the permanent (HRN model) or conditional (RCN model) deletion of *Por* in hepatocytes, the levels of BaP-DNA adduct formation in livers in HRN or RCN mice *in vivo* and in hepatic microsomes of HRN mice *ex vivo* was difficult to rationalize (Arlt et al., 2008; 2012). Previous results indicated that the process leading to DNA adducts did not involve the generation of a reactive species different from that formed in WT mice (Arlt et al., 2008; 2012). Since the structure of the dG- N^2 -BPDE adduct formed *in vivo* was identified by mass spectrometry (Arlt et al., 2008), BPDE (Fig. 1)

must be the reactive species formed both in HRN/RCN and WT mice. Moreover, enzymes different from CYPs such as cyclooxygenases were not found to be involved in BaP activation in these mouse models (Arlt et al., 2008). Indeed, formation of dG- N^2 -BPDE by hepatic microsomes of HRN mice *ex vivo*, which depended on NADPH, and was attenuated by inhibitors of CYP1A1 (α -naphthoflavone, ellipticine) and POR (α -lipoic acid), supported this conclusion (Arlt et al., 2008). Although the CYP specificity of chemical inhibitors can sometimes be problematic (Rendic and DiCarlo, 1997), these results indicated that hepatic Cyp1a1 in combination with even a very low Por expression level can be responsible for the formation of BaP-DNA adducts in hepatic microsomes of HRN mice.

Induction of Cyp1a1 in HRN mice with BaP resulted in a more than 34-fold increase in the production of BaP-7,8-dihydrodiol in *ex vivo* incubations of BaP with such microsomes relative to microsomes of untreated mice. BaP-7,8-dihydrodiol is the precursor of BDPE which subsequently can generate the dG- N^2 -BPDE adduct, explaining the formation of the dG- N^2 -BPDE adduct in the microsomes of HRN mice pretreated with BaP (Arlt et al., 2008 and present work]. In contrast, BaP-3-ol the major CYP1A1-mediated metabolite detoxicating BaP (Baird et al., 2005) only increased 7.7-fold after pretreatment of mice with BaP.

In this study two DNA adducts were generated by hepatic microsomes of HRN mice and levels of both were dependent on pretreatment of these mice with BaP, a CYP1A1 inducer. When CYP1A1 reconstituted with POR only was used for BaP activation, one BaP-DNA adduct (assigned adduct 1; Fig. 6A) was generated. After addition of mEH to the CYP1A1/POR reconstituted system, the dG- N^2 -BPDE adduct (assigned adduct 2; Fig. 6) was also formed. Again, higher amounts of both BaP-DNA adducts were formed by CYP1A1 in the presence of low POR levels (*i.e.* a ratio of CYP1A1:POR of 1:0.2) than at equimolar ratios in the reconstitution system. Moreover, cytochrome b_5 substantially stimulated BaP-DNA adduct formation. Both these BaP-DNA adducts have also been found by Schoket et al.

(1989) and Fang et al. (2001) to be generated by rat hepatic microsomes and separated as two adduct spots by TLC-³²P-postlabeling. The highly hydrophobic adduct 1 was tentatively identified by these authors as an adduct derived from 9-hydroxy-BaP-4,5-epoxide. This BaP metabolite is bound to the exocyclic amino group of a guanine residue, and the site of attachment is most likely either the 4 or the 5 position of BaP (Fang et al., 2003). Analogous to our results, the formation of adduct 1 was independent of the activity of mEH; an inhibitor of this enzyme, 1,2-epoxy-3,3,3-trichloropropane, did not influence its formation (Schoket et al., 1989). In contrast the formation of dG-*N*²-BPDE (adduct 2) was strongly inhibited by this mEH inhibitor. Nevertheless, even though a precursor of 9-hydroxy-BaP-4,5-epoxide, BaP-9-ol, was formed both in mouse hepatic microsomes and by the CYP1A1 reconstitution system, further work is required to demonstrate that adduct 1 is derived from 9-hydroxy-BaP-4,5-epoxide. The synthesis of an authentic standard should help with the structural identification.

It is well known that BaP is a strong CYP1A1 inducer in many organisms including HRN mice (Arlt et al., 2008). BaP also induced expression of Por protein (by 2.9-fold) and the activity of this enzyme as well as mEH and cytochrome *b*₅ in HRN mice (Arlt et al., 2008; 2012), thereby enhancing its own metabolic activation. Indeed, pretreatment of HRN mice with BaP led to more than ~50-fold higher DNA adduct levels *ex vivo* using hepatic microsomes (Fig. 9). Because microsomal cytochrome *b*₅ influences the CYP1A1-mediated activation of BaP *in vitro*, and is induced by BaP treatment in HRN mice (Arlt et al., 2012), this protein can modulate Cyp1a1-catalyzed activation of BaP also in this mouse model *in vivo*. Several mechanisms of cytochrome *b*₅-mediated modulation of CYP catalysis have been suggested. One of them suggests a role of cytochrome *b*₅ in the direct transfer of the second electron to the CYP enzyme, which is considered to be the rate limiting step in the catalytic cycle of the CYP monooxygenase reaction. The electron transfer from reduced cytochrome *b*₅ to CYP is faster than the input of electrons from POR. Due to this stimulation effect CYPs are

prevented from uncoupling of partially activated oxygen. Moreover, the addition of cytochrome *b*₅ enhances the stability of the oxy-CYP complex, therefore a higher amount of activated oxygen is available to form hydroxylated products. Another possible mechanism of the cytochrome *b*₅ action is the formation of a complex between cytochrome *b*₅ and CYP, which can receive two electrons from POR in a single step, one for reduction of CYP and another for that of cytochrome *b*₅. While CYP without cytochrome *b*₅ has to undergo two separate interactions with POR to complete one catalytic cycle, in the presence of cytochrome *b*₅ a single interaction of the complex of CYP and cytochrome *b*₅ with POR is sufficient; cytochrome *b*₅ provides the second electron to CYP promptly after oxygen binding. Cytochrome *b*₅ can also act as an allosteric modifier of the oxygenase (Porter, 2002; Schenkman and Jansson, 2003; Guengerich, 2005; Kotrbová et al., 2011; Stiborová et al., 2012; Riddick et al., 2013). Thus, in HRN mice cytochrome *b*₅ may not only modulate the Cyp-mediated (*i.e.* Cyp1a1) bioactivation of BaP *in vivo* if very low levels of Por are present, but it may also stimulate CYP-mediated metabolic activation of BaP because of its increased expression induced by BaP. Therefore, it would be interesting to examine the potential role of cytochrome *b*₅ in the metabolic activation of BaP *in vivo*. Recently, a mouse model has been developed, in which cytochrome *b*₅ has been deleted in all tissues [Cytochrome *b*₅ Complete Null (BCN)] (McLaughlin et al., 2010), allowing the investigation of general functions of cytochrome *b*₅ in carcinogen metabolism *in vivo*. To dissect the role of hepatic cytochrome *b*₅ *versus* hepatic Por in Cyp-mediated bioactivations a conditional liver-specific cytochrome *b*₅ knockout mouse (*Cytb*₅^{lox/lox}/*Cre*^{ALB}; HBN mouse) has been generated (Finn et al., 2008) with or without the expression of hepatic Por (Henderson et al., 2013). In the future we plan to investigate the balance between Cyp-mediated activation and detoxification of BaP *in vivo* in these potentially improved mouse models.

As shown in the present study, even low Por expression in livers of HRN mice (Por

probably expressed in non-parenchymal cells of liver), in combination with the induction of Cyp1a1, cytochrome *b*₅ and mEH by BaP, might be sufficient for an efficient oxidative activation of BaP. This feature might follow from a specific pattern of protein levels and activities of these enzymes in hepatic microsomes of HRN mice pretreated with BaP that resulted in a more than 10- and 34-fold increase in formation of metabolic precursors essential for the generation of BaP-DNA adducts; BaP-9-ol as the precursor of 9-hydroxy-BaP-4,5-epoxide potentially forming adduct 1, and BaP-7,8-dihydrodiol as the precursor of BPDE forming the dG-*N*²-BPDE adduct (adduct 2), respectively. We believe that these findings might be one of the potential reasons for the high levels of BaP-DNA adducts formed in livers of HRN mice relative to WT mice, but other factors may also contribute. Since it has been shown that BaP can increase the activity of some conjugation enzymes (*i.e.* glutathione *S*-transferase) participating in BaP detoxification (Banni et al., 2009), their expression levels increased by induction by BaP and contribution to the detoxication of BaP metabolites in the HRN model needs further investigation. In addition, the formation and rate of repair of BaP-DNA adducts and the effect of BaP on the expression of DNA repair proteins may be different in HRN and WT mice. Furthermore, the ratio of the hydrophobic DNA adduct 1 to dG-*N*²-BPDE formed by hepatic microsomes and in the CYP1A1-reconstituted systems were much higher than the ratios detected in the livers of HRN (compare Fig. 6E) and RCN mice *in vivo*. This might be caused, beside other factors, by differences in the DNA repair of the two adducts *in vivo* or due to efficient conjugation of the BaP-9-ol intermediate.

Conflict of interest

All authors have no conflict of interest.

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Table 1

¹H chemical shifts of BaP and its metabolites M1, M4, M5 and M7 in hexadeuterated acetone.

Coupling constants are in parenthesis.

	BaP	M1	M4	M5	M7
H-1	8.33 d (7.8)	8.26 ^a d (7.6)	-	8.02 d (9.7)	8.20 d (8.5)
H-2	8.04 t (7.6)	8.04 t (7.6)	6.74 d (9.8)	6.69 d (9.7)	7.68 d (8.4)
H-3	8.18 d (7.4)	8.27 ^a d (7.6)	8.03 d (9.7)	-	-
H-4	8.09 d (9.2)	8.12 ^b d (8.9)	8.19 d (7.4)	8.83 ^a d (7.7)	8.30 d (9.3)
H-5	8.01 d (9.1)	8.15 ^b d (8.9)	8.68 d (7.4)	8.71 ^a d (7.7)	8.00 d (9.5)
H-6	8.65 s	8.09 s	-	-	8.52 s
H-7	8.37 dm (8.1)	7.02 d (9.5)	8.41 dm (7.7)	8.41 dm (7.9)	8.32 s (8.1)
H-8	7.83 ddd (8.0, 6.7, 1.2)	6.38 dd (9.6, 5.3)	7.73 m	7.70 m	7.79 m
H-9	7.88 ddd (8.4, 6.7, 1.4)	4.50 dd (5.2, 1.7)	7.92 m	7.90 m	7.81 m
H-10	9.18 dm (8.5)	5.69 m	8.66 dm (8.0)	8.61 dm (8.1)	9.12 d (8.3)
H-11	9.20 d (9.2)	8.58 d (9.2)	8.60 ^a d (7.9)	8.76 d (7.7)	8.97 d (9.1)
H-12	8.43 d (9.1)	8.23 d (9.3)	8.92 ^a d (7.9)	8.12 d (7.7)	8.30 d (9.3)

^{a,b}Chemical shifts with the same letter in one column may be interchanged.

Legends to figures

Fig. 1. Pathways of biotransformation and DNA adduct formation of BaP catalyzed by CYP1A1 and mEH. The two-step activation process by CYP1A1 leading to the formation of the ultimately reactive species, 9-hydroxy-BaP-4,5-epoxide, that can react with deoxyguanosine in DNA (adduct 1) is shown on the left. The typical three-step activation process with oxidation by CYP1A1 followed by hydrolysis by mEH leads to the ultimately reactive species, BPDE, forming the dG- N^2 -BPDE adduct (adduct 2) is shown on the right.

Fig. 2. **A**, HPLC separation of the BaP metabolites generated by hepatic microsomes of HRN mice treated with BaP using Nucleosil[®] C18 reverse phase column. PA, phenacetin, M1-M7, BaP metabolites [BaP-9,10-dihydrodiol (M1), BaP-4,5-dihydrodiol (M2), BaP-7,8-dihydrodiol (M3), BaP-1,6-dione (M4), BaP-3,6-dione (M5), BaP-9-ol (M6) and BaP-3-ol (M7)]. **B**, Structures of the BaP metabolites.

Fig. 3. Relative peak areas of BaP metabolites at 254 nm formed by hepatic microsomes isolated from control and BaP-treated HRN mice. Values are averages $\pm$ SD of three parallel experiments. Comparison was performed by *t*-test analysis; **P* < 0.001, different from HRN control microsomes.

Fig. 4. Relative peak areas of BaP metabolites at 254 nm formed by rat CYP1A1 reconstituted with POR. Values are averages $\pm$ SD of three parallel experiments. ND - not detected. Comparison was performed by *t*-test analysis; **P* < 0.001, different from BaP oxidation by CYP1A1:POR in a ratio of 1:1 than by CYP1A1:POR in a ratio of 1:0.05.

Fig. 5. The effect of increasing amounts of cytochrome *b*₅ on oxidation of BaP by **A**, rat CYP1A1 without POR; **B**, CYP1A1 reconstituted with POR in a ratio of CYP1A1 to POR 1:0.05 and **C**, of 1:1. The values are averages $\pm$ SD of relative peak areas of

three parallel experiments. Comparison was performed by *t*-test analysis; **P* < 0.001, different from BaP oxidation with the CYP1A1/POR system containing cytochrome *b*₅ in a ratio of CYP1A1:cytochrome *b*₅ of 1:5 than by that with CYP1A1:cytochrome *b*₅ of 1:1 or 1:2.5.

Fig. 6. Autoradiographic profile of ³²P-labeled DNA adducts generated in calf thymus DNA by BaP after its activation with **A**, CYP1A1 reconstituted with POR (CYP1A1:POR of 1:0.2) in the presence of NADPH; **B**, CYP1A1 reconstituted with POR, mEH and cytochrome *b*₅ at ratios of 1:0.2:1:3 in the presence of NADPH; **C**, the same reconstituted system but without NADPH (control incubation); **D**, hepatic microsomes from HRN mice treated with BaP. **E**, adduct profile of DNA from liver of HRN mice treated with BaP. Analyses were performed by the nuclease P1 version of the ³²P-postlabeling assay with the TLC conditions described in Materials and Methods. Adduct 1 - derived from reaction of 9-hydroxy-BaP-4,5-epoxide with deoxyguanosine in DNA; Adduct 2 - dG-*N*²-BPDE.

Fig. 7. BaP-DNA adducts formed by CYP1A1 reconstituted with **A**, POR and **B**, with POR and mEH at a ratio of CYP to mEH of 1:5. RAL - relative adduct labeling. Values are averages ± SD of three parallel experiments. Comparison was performed by *t*-test analysis; **P* < 0.001, different from BaP-DNA adduct formation by the CYP1A1/POR system containing microsomal epoxide hydrolase (mEH) than by that without this enzyme. ND - not detected.

Fig. 8. The effect of cytochrome *b*₅ on BaP-DNA adduct formation catalyzed by CYP1A1 reconstituted with POR and mEH at different ratios of the four constituents: **A**, a ratio of CYP1A1 to POR of 1:0.2 with mEH added either without cytochrome *b*₅; **B**, with this protein at a ratio of 1:3 (CYP to cytochrome *b*₅); **C**, at a CYP1A1 to POR ratio of 1:2 with mEH either without cytochrome *b*₅; **D**, with this protein at a ratio of

1:3 (CYP to cytochrome *b*₅). ## - the samples were not analyzed. ND - not detected. RAL - relative adduct labeling. Values are averages $\pm$ SD of three parallel experiments. Comparison was performed by *t*-test analysis; **P* < 0.001, different from BaP-DNA adduct formation by the CYP1A1/POR system containing cytochrome *b*₅ than by that without this protein.

Fig. 9. BaP-DNA adducts formed by liver microsomes isolated from control and BaP treated HRN mice. RAL - relative adduct labeling. Values are averages $\pm$ SD of three parallel experiments (Adduct 1 and 2 see Fig. 6). Comparison was performed by *t*-test analysis; **P* < 0.001, different from HRN control microsomes.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9