ORIGINAL ARTICLE

Oxidation of carcinogenic benzo[a] pyrene by human and rat cytochrome P450 1A1 and its influencing by cytochrome b_5 – a comparative study

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Abstract

OBJECTIVES: Cytochrome P450 (CYP) 1A1 is the most important enzyme in both activation and detoxification of carcinogenic benzo[a]pyrene (BaP), in combination with microsomal epoxide hydrolase (mEH). To evaluate metabolism of BaP in human, identification of a suitable animal model that mimics the metabolic fate of BaP in human is of great importance. The aim of this work was to compare BaP oxidation by human CYP1A1 and CYP1A1 of one animal model, rat. Investigation of the effect of cytochrome b_5 on BaP oxidation by CYP1A1 was another target of this study.

METHODS: High performance liquid chromatography (HPLC) was employed for separation of BaP metabolites formed by enzymatic systems. Their structures were identified by mass- and NMR-spectrometry.

RESULTS: Human hepatic microsomes oxidized BaP to BaP-9,10-dihydrodiol, BaP-4,5-dihydrodiol, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione and BaP-3-ol. The same metabolites were generated by rat liver microsomes, but BaP-9-ol and a metabolite Mx, the structure of which has not been identified as yet, were also formed in these microsomes. Human CYP1A1 expressed with NADPH:CYP reductase (POR) in Supersomes™ oxidized BaP to the same metabolites as microsomes, but BaP-4,5-dihydrodiol has not been detected. Rat recombinant CYP1A1 in this SupersomesTM system oxidized BaP to BaP-9,10-dihydrodiol, a metabolite Mx, BaP-4,5-dihydrodiol, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-9-ol and BaP-3-ol. Addition of cytochrome b_5 to rat and human recombinant CYP1A1 systems led to a more than 2-fold increase in BaP oxidation.

CONCLUSION: The results show similarities between human and rat CYP1A1 in BaP oxidation and demonstrate rats as a suitable model mimicking BaP oxidation in human.

Abbreviations:

BaP	- benzo[a]pyrene
BPDE	- BaP-7,8-dihydrodiol-9,10-epoxide
COSY	- correlation spectroscopy
CYP	- cytochrome P450
dG-N^2-BPDE	- 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo-[a]pyrene
DMSO	- dimethyl sulfoxide
HMBC	- heteronuclear multiple-bond correlation spectroscopy
HPLC	- high performance liquid chromatography
HSQC	- heteronuclear single-quantum correlation spectroscopy
IARC	- International Agency for Research on Cancer
MALDI-TOF	- matrix-assisted laser desorption/ionization time-of-flight
mEH	- epoxide hydrolase
NADPH	- nicotinamidadeninedinucleotide phosphate (reduced)
NMR	- nuclear magnetic resonance
PAH	- polycyclic aromatic hydrocarbon
POR	- NADPH:cytochrome P450 reductase
r. t.	- retention time
UV	- ultraviolet

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 requires metabolic activation catalyzed by cytochrome P450 (CYP) enzymes prior to reaction with DNA (Baird *et al.* 2005). Of the CYP enzymes, CYP1A1 is one of the most important CYP enzymes in metabolic activation of BaP to species forming DNA adducts (Baird *et al.* 2005; Hamouchene *et al.* 2011), in combination with microsomal epoxide hydrolase (mEH). First, CYP1A1 oxidizes BaP to an epoxide that is then converted to a dihydrodiol by mEH (*i.e.* BaP-7,8-dihydrodiol); then further bio-activation by CYP1A1 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 the reaction of BPDE with DNA *in vitro* and *in vivo* (Fig. 1) (Phillips & Venitt 2012).

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). Even though most of these metabolites are the detoxification products, BaP-9-ol is a precursor of 9-hydroxy-BaP-4,5-epoxide, which can form another adduct with deoxyguanosine in DNA (Schoket *et al.* 1989; Nesnow *et al.* 1993; Fang *et al.* 2001). Therefore, regulation of CYP1A1-mediated oxidation of BaP leading to either metabolites forming BPDE, 9-hydroxy-BaP-4,5-epoxide or the BaP metabolites that are the detoxification products is of major importance.

In order to modulate CYP1A1-catalyzed oxidation of BaP in human, knowledge on such modulation of the CYP1A1 enzyme from suitable animal models that might mimic oxidation of BaP in human should be investigated and the results found applied to regulation of BaP oxidation by human CYP1A1. In fact, the first step of such investigations is to find which of the animal model CYP1A1 enzyme oxidizes BaP similarly to human CYP1A1. In addition, there are still not clearly explained how an electron transfer mediated by another component of the microsomal enzymatic system, NADPH:CYP reductase (POR), on CYP1A1 during BaP oxidation occurs, and whether microsomal cytochrome b_5 might influence this electron transfer. Namely, the oxygen needed for BaP oxidation is activated in the active center of CYPs by two electrons transferred from NADPH and/or NADH by means of POR and cytochrome b_5, respectively (Coon 1978). Whereas POR is an essential constituent of the electron transport chain towards CYP, the role of cytochrome b_5 is still quite enigmatic (Porter 2002; Stiborova *et al.* 2006, 2012b; Kotrbova *et al.* 2011; Koberova *et al.* 2013). Indeed, in the case of CYP1A1, the influencing of the POR-mediated electron transfer from NADPH to this CYP, which dictates a velocity of BaP oxidation, by cytochrome b_5 is not known.

Therefore, this study is focused on two major targets: (i) evaluation of a suitability of rat CYP1A1 enzymatic systems to mimic BaP oxidation by human CYP1A1, and (ii) investigation of the effect of cytochrome b_5 on oxidation of CYP1A1 by these CYP1A1 enzymes. Human and rat CYP1A1 expressed with POR in microsomes of insect cells (Supersomes™) and these enzyme

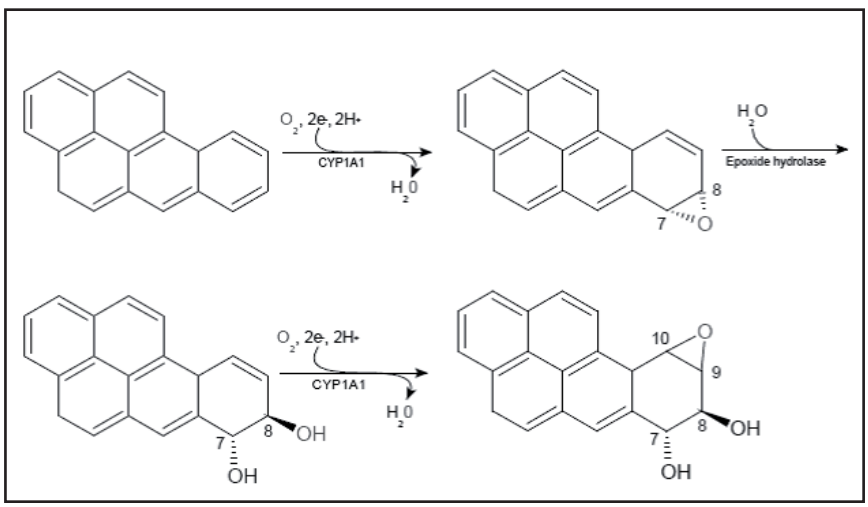

Fig. 1. Metabolic activation of benzo[a]pyrene

systems reconstituted with purified cytochrome b_5 were utilized for such a study. Human and rat hepatic microsomes containing a natural spectrum of CYPs and other enzymes located in a membrane of endoplasmic reticulum were used as positive controls.

MATERIAL AND METHODS

Chemicals and enzymes

Male human hepatic microsomes (pooled sample; cat. no. 452172), Supersomes™, microsomes isolated from insect cells transfected with a baculovirus construct containing cDNA of human or rat CYP1A1 and expressing POR were purchased from Gentest Corp. (Woburn, MI, USA). Microsomes from rat livers were isolated and characterized for CYP activities as described (Stiborova *et al.* 2011, 2012a). Cytochrome b_5 was isolated from rabbit liver microsomes by the procedure described by Roos (1996). This protein was used for the reconstitution experiments. There were no problems with CYP1A1 enzyme activity when this enzyme was reconstituted with other enzymes that were isolated from rabbit. As shown in our previous works, the enzymatic activity of rat (and/or human) CYP1A1 reconstituted with rabbit POR and cytochrome b_5 was essentially the same as that of the enzyme reconstituted with rat orthologs of these enzymes (Stiborova *et al.* 2002, 2006, 2012b; Kotrbova *et al.* 2009, 2011).

$NADP^+$, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase, dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co (St Louis, MO, USA). All these and other chemicals were reagent grade or better.

Preparation of rat microsomes and assays

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Microsomes from livers of ten male untreated Wistar rats and those of ten male rats pre-treated with Sudan I were prepared by the procedure described previously (Stiborova *et al.* 2003, 2011; 2013). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman *et al.* 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on absorption of the complex of reduced CYP with carbon monoxide. Hepatic microsomes of control (uninduced) rats and rats induced with Sudan I contained 0.6 and 1.8 nmol CYP/mg protein, respectively.

Microsomal and CYP1A1 enzymatic incubations

Rat hepatic microsomes of control (untreated) rats and rats treated with Sudan I, and human hepatic microsomes (pooled sample; cat. no. 452172), Supersomes™, microsomes isolated from insect cells transfected with a baculovirus construct containing cDNA of human or rat CYP1A1 and expressing POR were used to study BaP oxidation. Human and rat CYP1A1 expressed in these microsomes oxidized a CYP1A1-marker substrate, Sudan I (Stiborova *et al.* 2002; 2005). However, rat CYP1A1 exhibited ~2-fold lower efficiencies in Sudan I oxidation than human CYP1A1 (data not shown). Incubation mixtures used for studying BaP metabolism in human and rat hepatic microsomes or in Supersomes™ 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 nM human or rat CYP1A1 in Supersomes™ and 50 μM BaP (dissolved in 5 μl dimethyl sulfoxide, DMSO) in a final volume of 500 μl. In additional experiments, the Supersomal CYP1A1 systems were reconstituted with cytochrome b_5. The enzyme reconstitution utilizing the above systems (Supersomes™ containing human or rat CYP1A1 and expressing POR) with cytochrome b_5 were performed as described (Stiborova *et al.* 2002; 2006; 2012b). 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 systems), 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.

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, flow rate of 0.6 ml/min. Application of samples (20 μl) at ambient temperature) and HPLC were carried out at 35 °C (Moserova *et al.* 2009). Detection was by UV at 254 nm. BaP metabolite peaks (Figure 2) were collected and analyzed by NMR and/or mass spectrometry. The peak areas at 254 nm were calculated relative to the peak area of the internal standard PA, and expressed as relative peak areas.

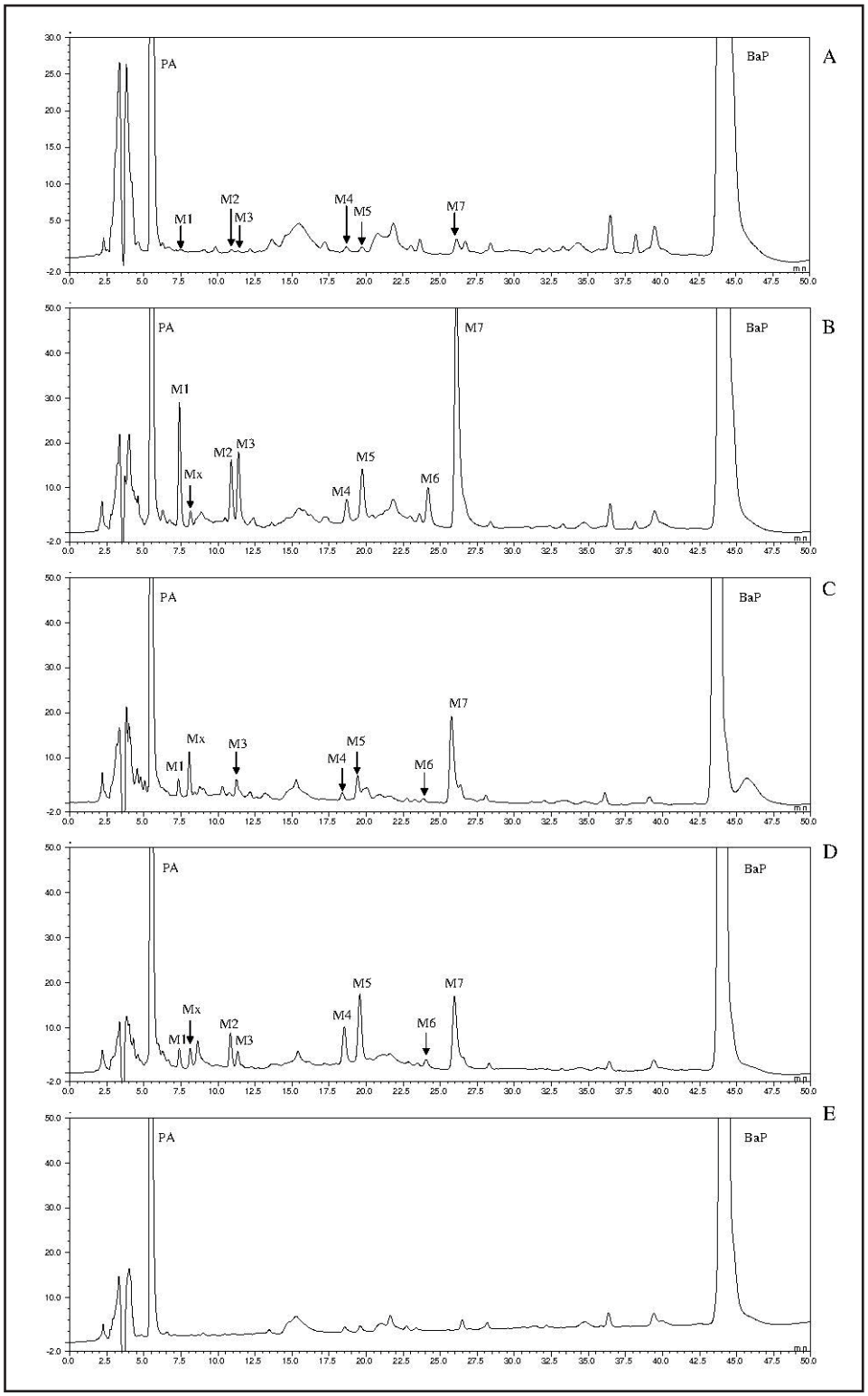

Fig. 2. High performance liquid chromatography (HPLC) of benzo[a]pyrene (BaP) metabolites formed by human hepatic microsomes (A), hepatic microsomes of rats treated with Sudan I (B), human recombinant cytochrome P450 (CYP) 1A1 (C), and rat recombinant CYP1A1 (D). (E) HPLC of control incubation mixture containing BaP and human recombinant CYP1A1, but without the NADPH (reduced nicotinamidadeninedinucleotide phosphate)-generating system. For BaP metabolites M1–M7 and Mx, see Fig. 3.

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 ^{1}H 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. ^{1}H 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 correlation spectroscopy (COSY) spectrum (data not shown), 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 [heteronuclear multiple-bond correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC)] had to be used.

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 & 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 not able 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 M4 and M5, 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 the 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 these two compounds were synthesized previously and their ^{1}H NMR spectra reported (Leeruff *et al.* 1986; Xu *et al.* 2009), spectra in $CDCl_3$ could be compared and the metabolites 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 by mass spectrometry only as described below.

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 (MALDI-TOF) mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany). Positive spectra were calibrated externally using the monoisotopic $[M+H]^+$ 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% acetonitrile/0.3% acetic acid was used as MALDI matrix. A 0.5 µl of sample dissolved in acetonitrile was premixed with 0.5 µl 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 at *m/z* 286.1 that 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).

Statistical analyses

For statistical data analysis we used Student's *t*-test. All *p*-values are two-tailed and considered significant at the 0.05 level.

RESULTS

Oxidation of BaP by rat and human hepatic microsomes

Human and rat hepatic microsomes are natural systems containing all components of a monooxygenase system located in a membrane of endoplasmic reticulum, CYPs, POR, cytochrome b_5, and its reductase NADH:cytochrome b_5 reductase, in addition to mEH. Human hepatic microsomes oxidized BaP to six metabolites that were separated by HPLC (Figure 2A). These BaP metabolites and those formed by other enzyme systems used in this work were collected and subsequently characterized by NMR and/or mass spectrometry. The BaP metabolites formed by human hepatic microsomes 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) and BaP-3-ol (M7) (see Figures 2A and 3), all corresponding to the metabolites that were formed by CYP1A1 in combination with mEH in other studies (Bauer *et al.* 1995; Kim *et al.* 1998; Baird *et al.* 2005).

Rat liver microsomes, in which CYP1A was induced by Sudan I, generated beside all these metabolites also BaP-9-ol (M6) and a metabolite Mx, the structure of which has not been identified as yet (Figures 2B and 3).

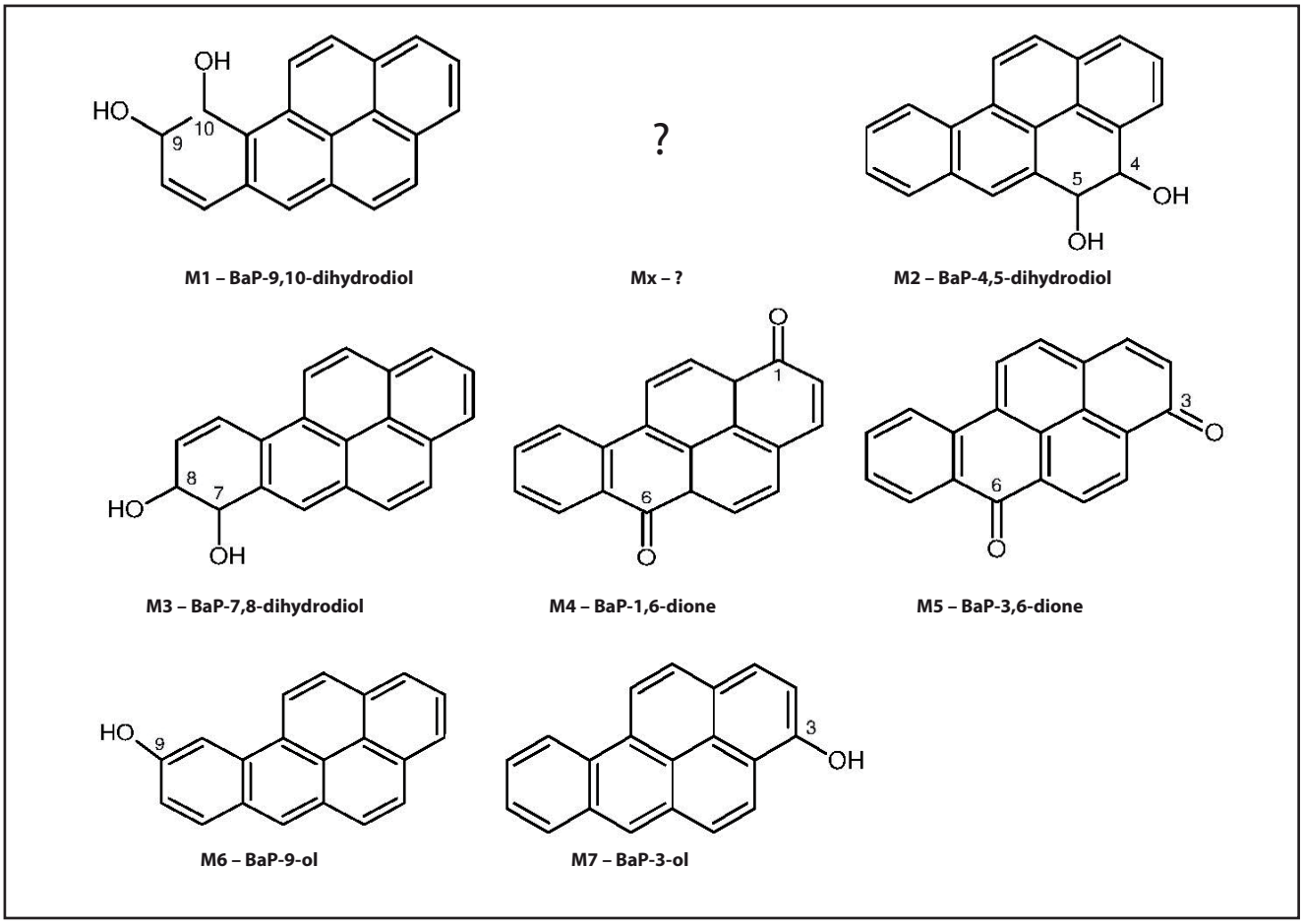

Fig. 3. Benzo[a]pyrene (BaP) metabolites formed by human and rat hepatic microsomes and human and rat cytochrome P450 1A1.

The same BaP metabolites were also formed by hepatic microsomes of control (untreated) rats, but to a more than 3.5-fold lower extent (data not shown).

The BaP-3-ol, considered as a detoxification BaP metabolite, was the major oxidation product generated both by human and rat hepatic microsomes (Figure 2B). Essentially no BaP metabolites were found when NADPH-generating system was not present in the incubation mixtures containing human and rat hepatic microsomes (data not shown).

Oxidation of BaP by human and rat CYP1A1 in Supersomes™

Human CYP1A1 expressed with POR in a microsomal system of Supersomes™ oxidized BaP to seven metabolites, BaP-9,10-dihydrodiol, a metabolite Mx, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-9-ol and BaP-3-ol (Figures 2C and 3). One of the dihydrodiols formed by human and rat hepatic microsomes, BaP-4,5-dihydrodiol (M2), has not been detected in the system containing human CYP1A1.

Rat recombinant CYP1A1 expressed with POR in Supersomes™ oxidized BaP to the analogous spectrum of metabolites as human CYP1A1, but rat CYP1A1 generated also BaP-4,5-dihydrodiol (Figure 2D). In addition, lower amounts of most BaP metabolites were formed by human than by rat CYP1A1, mainly the amount of BaP-3-ol (Figures 2 and 4). Such a lower efficacy of rat CYP1A1 corresponded to lower activity of this enzyme with a CYP1A1 marker substrate, Sudan I (data not shown).

The results found using these human and rat CYP1A1 systems indicate that BaP is metabolized not only by CYP1A1 present in these systems, but also by mEH, which is important for the hydration of BaP epoxides to produce dihydrodiols. Indeed, BaP dihydrodiols were formed in these human and rat CYP1A1 systems. Therefore, mEH is expressed in microsomes of the Supersomal system.

Essentially no BaP metabolites were found when the NADPH-generating system was deleted from the incubation mixtures containing human and rat CYP1A1 (see Figure 2E for human CYP1A1).

The effect of cytochrome b_5 on BaP oxidation by human and rat CYP1A1

Addition of cytochrome b_5 to human and rat CYP1A1 in Supersomes™ led to up to a more than 2-fold increase in BaP oxidation to its metabolites. The highest stimulation effect of cytochrome b_5 on human

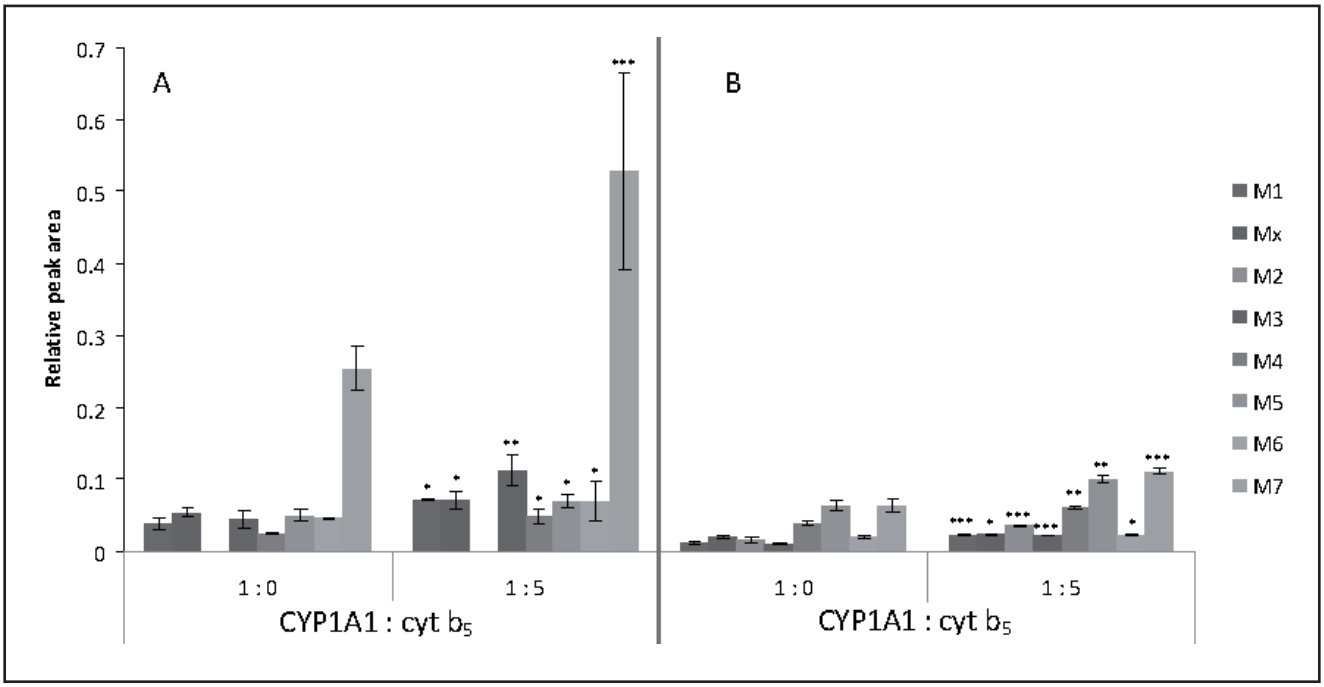

Fig. 4. Oxidation of benzo[a]pyrene (BaP) by human (A) and rat (B) cytochrome P450 (CYP) 1A1 and the effect of cytochrome b_5 (cyt b_5) on this oxidation. All values are given as means and standard deviations of triplicate incubations (n=3). Comparison was performed by *t*-test analysis; **p*<0.05, ***p*<0.01, ****p*<0.001, different from CYP1A1-mediated oxidation of BaP without cytochrome b_5. For BaP metabolites M1–M7 and Mx, see Fig. 3.

CYP1A1 has been found on formation of BaP-3-ol and BaP-7,8-dihydrodiol, followed by the effect on generation of BaP-9,10-dihydrodiol, BaP-1,6-dione, BaP-9-ol, a metabolite Mx, and BaP-3,6-dione. In the case of rat CYP1A1, the highest increase in formation of BaP-7,8,dihydrodiol, BaP-9,10-dihydrodiol and BaP-3-ol was found (Figure 4).

DISCUSSION

The results found in this work demonstrate that oxidation of BaP by human CYP1A1 is similar to that catalyzed by rat CYP1A1; the only one difference has been found. Whereas BaP-9,10-dihydrodiol, a metabolite Mx, BaP-4,5-dihydrodiol, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-9-ol and BaP-3-ol were formed both by human and rat CYP1A1, BaP-4,5-dihydrodiol was not detectable in the system containing human CYP1A1. Similar results were found in experiments where human bronchoalveolar H358 cells expressing CYP1A1 were treated with BaP; no BaP-4,5-dihydrodiol was detectable as a BaP metabolic product (Jiang *et al.* 2007). Also in the case of human and rat hepatic microsomes, where a spectrum of analogous BaP metabolites such as BaP-phenols, BaP-diones and BaP-dihydrodiols was generated, one difference in metabolic products was found; BaP-9-ol has not been detectable in human hepatic microsomes. Low levels of CYP1A1 expressed in human livers (Stiborova *et al.* 2002, 2005), resulting in low efficiencies of this subcellular system, might be responsible for no detectable amounts of BaP-9-ol.

The structure of one BaP metabolite, a metabolite Mx, that has been formed by human and rat CYP1A1 remains to be explored. Nevertheless, its chromatographic properties on HPLC indicate its polarity; it is eluted from a HPLC column with a retention time between that of BaP-9,10-dihydrodiol and BaP-4,5-dihydrodiol. All results found in this work indicate that rat CYP1A1 enzymatic systems are appropriate models that might be utilized to investigate BaP oxidation by human CYP1A1 and, therefore, to be used to evaluate risk of BaP carcinogenicity for human.

The pattern of BaP metabolites found to be formed by human and rat CYP1A1 in Supersomes™ demonstrate that not only human and rat CYP1A1, but also POR and mEH enzymes, necessary to metabolize BaP to the metabolites found, are expressed in the Supersomal microsomes. However, cytochrome b_5, which is a natural component of insect microsomes (Supersomes™), seems to be present in this system in the amounts which is not sufficient to mediate oxidation of BaP by CYP1A1 to its highest catalytic efficiencies. Namely, the addition of cytochrome b_5 to the human and rat CYP1A1 in Supersomes™ resulted in an increase in formation of BaP metabolites. This is an important finding that was shown in this study for the first time. A stimulation of CYP1A1-mediated catalysis by cytochrome b_5 has already been found in oxidation of its marker substrate Sudan I (Stiborova *et al.* 2005, 2006)

and an anticancer drug ellipticine (Kotrbova *et al.* 2011), but not in that of 7-ethoxyresorufin (Stiborova *et al.* 2005). Two mechanisms of cytochrome b_5-mediated modulation of CYP catalysis have been suggested previously: it can affect the CYP catalytic activities by donating the second electron to CYP in a CYP catalytic cycle and/or act as an allosteric modifier of the oxygenase (Yamazaki *et al.* 1997; 2001; Loughran *et al.* 2001; Porter 2002; Schenkman & Jansson 2003; Guengerich 2005; Zhang *et al.* 2005; Kotrbova *et al.* 2009, 2011; Stiborova *et al.* 2012b). The mechanism(s) underlying such allosteric effects, based on reports that apo-cytochrome b_5 can stimulate CYP catalysis, remains uncertain. It does seem clear, however, that cytochrome b_5 binding can cause conformational changes to the substrate access channel and binding pocket in the CYP enzyme (Yamazaki *et al.* 1997, 2001; Loughran *et al.* 2001; Porter 2002; Schenkman & Jansson 2003; Guengerich 2005; Zhang *et al.* 2005; Kotrbova *et al.* 2009, 2011; Stiborova *et al.* 2012b). Because addition of cytochrome b_5 changed the levels of all BaP metabolites formed by human and rat CYP1A1, the effect of this protein on an electron transfer to CYP1A1 seems to be the predominant mechanism responsible for the observed increase in oxidation of BaP. Nevertheless, the allosteric effects due to cytochrome b_5 cannot be excluded. Namely, an increase in formation of individual BaP metabolites was not the same for all these metabolites. Therefore, the mechanism responsible for the effects of cytochrome b_5 on CYP1A1-mediated oxidation of a variety of substrates needs to be explored in further investigations.

CONCLUSION

The results found in this study demonstrate that the enzymatic system of rat CYP1A1 is a suitable enzyme model that might be utilized for investigation of BaP metabolism by human CYP1A1. In addition, the results also show for the fist time that cytochrome b_5 modulate BaP metabolism catalyzed by human and rat CYP1A1, stimulating formation of all its metabolic products. All these findings demonstrate that rats seem to be a suitable animal model that might mimic BaP oxidation in human.

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