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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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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; Siddens et al., 2012). Chronic

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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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., 2001). 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)-^{32}P-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. This BaP dosage induces mutagenicity in multiple organs (Hakura et al., 1999) and is carcinogenic (Hakura et al., 1998). It is also noteworthy that the deletion of the *Por* gene in mouse hepatocytes resulted in higher expression of several Cyp proteins in female than in male mice (Henderson et al., 2003) and thus female mice were used in our previous study (Arlt et al., 2008). 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