NADH:Cytochrome b_5 Reductase and Cytochrome b_5 Can Act as Sole Electron Donors to Human Cytochrome P450 1A1-Mediated Oxidation and DNA Adduct Formation by Benzo[a]pyrene

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S *Supporting Information*

ABSTRACT: Benzo[a]pyrene (BaP) is a human carcinogen that covalently binds to DNA after activation by cytochrome P450 (P450). Here, we investigated whether NADH:cytochrome b_5 reductase (CBR) in the presence of cytochrome b_5 can act as sole electron donor to human P450 1A1 during BaP oxidation and replace the canonical NADPH:cytochrome P450 reductase (POR) system. We also studied the efficiencies of the coenzymes of these reductases, NADPH as a coenzyme of POR, and NADH as a coenzyme of CBR, to mediate BaP oxidation. Two systems containing human P450 1A1 were utilized: human recombinant P450 1A1 expressed with POR, CBR, epoxide hydrolase, and cytochrome b_5 in Supersomes and human recombinant P450 1A1 reconstituted with POR and/or with CBR and cytochrome b_5 in liposomes. BaP-9,10-dihydrodiol, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-9-ol, BaP-3-ol, a metabolite of unknown structure, and two BaP-DNA adducts were generated by the P450 1A1-Supersomes system, both in the presence of NADPH and in the presence of NADH. The major BaP-DNA adduct detected by ^{32}P-postlabeling was characterized as 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-BaP (assigned adduct 1), while the minor adduct is probably a guanine adduct derived from 9-hydroxy-BaP-4,5-epoxide (assigned adduct 2). BaP-3-ol as the major metabolite, BaP-9-ol, BaP-1,6-dione, BaP-3,6-dione, an unknown metabolite, and adduct 2 were observed in the system using P450 1A1 reconstituted with POR plus NADPH. When P450 1A1 was reconstituted with CBR and cytochrome b_5 plus NADH, BaP-3-ol was the predominant metabolite too, and an adduct 2 was also generated. Our results demonstrate that the NADH/cytochrome b_5/CBR system can act as the sole electron donor both for the first and second reduction of P450 1A1 during the oxidation of BaP *in vitro*. They suggest that NADH-dependent CBR can replace NADPH-dependent POR in the P450 1A1-catalyzed metabolism of BaP.
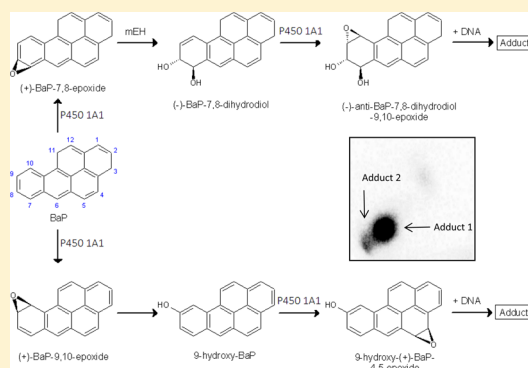
INTRODUCTION

Benzo[a]pyrene (BaP) (Figure 1) is a polycyclic aromatic hydrocarbon (PAH) that has been classified as a human carcinogen (Group 1) by the International Agency for Research on Cancer.[1] It is a pro-carcinogen requiring metabolic activation catalyzed by cytochrome P450 (P450) enzymes prior to reaction with DNA.[2] Among the P450s, P450 1A1 is the most important enzyme, in combination with microsomal epoxide hydrolase (mEH), involved in the metabolic activation of BaP to species forming DNA adducts.[2,3] First, P450 1A1 oxidizes BaP to an epoxide (i.e., BaP-7,8-epoxide) that is then converted to a dihydrodiol by mEH (i.e., BaP-7,8-dihydrodiol).

Further bioactivation by P450 1A1 leads to the ultimate reactive species, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE), that can react with DNA, forming adducts preferentially at guanine residues (Figure 1). 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*.[4−10] However, BaP is also oxidized to other metabolites such as other dihydrodiols, BaP-diones, and further hydroxylated metabolites.[2,6,11−16] Although
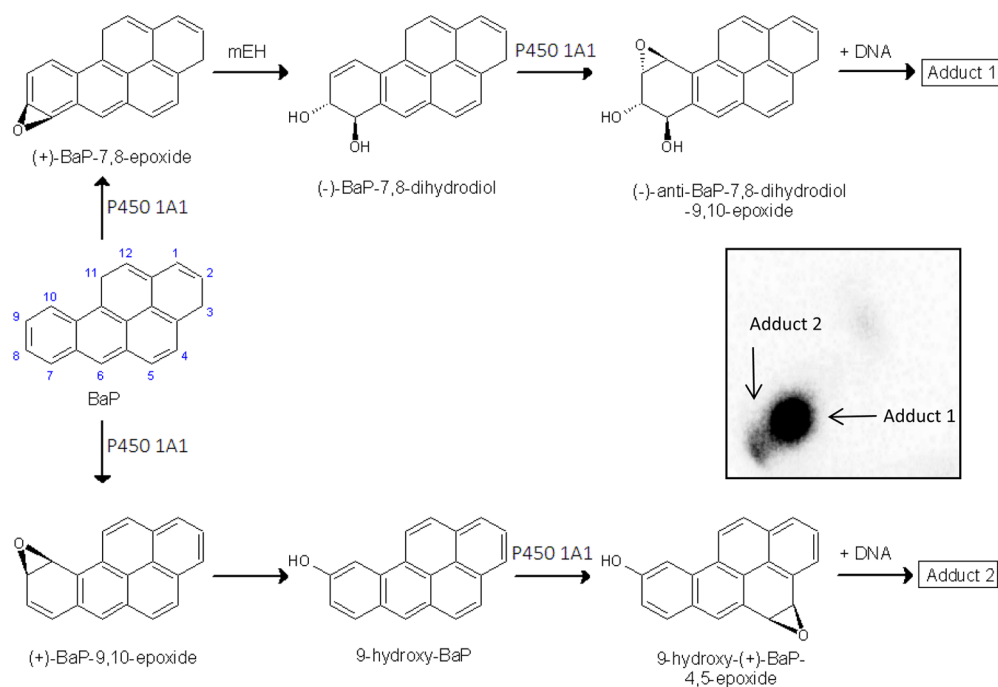
Figure 1. Proposed pathways of biotransformation and DNA adduct formation of BaP catalyzed by P450 1A1 and mEH. Upper panel: the typical three-step activation process by P450 1A1 followed by hydrolysis by mEH leads to BPDE which forms dG-N^2-BPDE (adduct 1). Lower panel: the two-step activation process by P450 1A1 leads to the formation of 9-hydroxy-BaP-4,5-epoxide that can react with deoxyguanosine in DNA (adduct 2). Insert: Autoradiographic profile of BaP-DNA adducts formed by human P450 1A1 in Supersomes with mEH in the presence of NADH and cytochrome b_5 as evaluated by TLC ^{32}P-postlabeling as described previously.[19]

most of these metabolites are detoxification products, BaP-9-ol is the precursor of 9-hydroxy-BaP-4,5-epoxide that can form another adduct with deoxyguanosine in DNA (Figure 1).[7,8,10,15,17−19]

The P450 enzymes, including P450 1A1, are components of a mixed-function oxidase (MFO) system located in the membrane of the endoplasmic reticulum (microsomes). This enzymatic system also contains other enzymes, the multidomain flavoprotein NADPH:cytochrome P450 oxidoreductase (POR) and cytochrome b_5 accompanied by its NADH:cytochrome b_5 reductase (CBR). Mammalian microsomal P450s function by catalyzing the insertion of one atom of molecular oxygen into a variety of xenobiotics, including BaP, while reducing the other atom to water, a reaction that requires two electrons.[20] The oxygen is activated in the active center of P450s by two electrons. It is generally accepted that POR with NADPH serves as donor of electrons for both reductions of P450 in the MFO reaction cycle.[20] However, the second electron may also be provided by CBR with cytochrome b_5 and NADH, but cytochrome b_5 seems to have also additional roles in the monooxygenase system.[20−28]

Although POR is considered an essential constituent of the electron transport chain toward P450,[20] its exact role in the P450-mediated reaction cycle is still not clearly established. Recently, we used two mouse models in which the expression of POR has been permanently (the Hepatic P450 Reductase Null (HRN) line) or conditionally (the Reductase Conditional Null (RCN) line) deleted in hepatocytes leading to a lack of almost all hepatic POR activity. Despite this lack of POR, the levels of the P450-mediated dG-N^2-BPDE adducts in the livers of mice of both lines exposed to BaP were higher than that in BaP-treated wild-type mice.[7,8,19] These findings suggested BaP activation in other liver cells other than hepatocytes (e.g.,

Kupffer or endothelial cells)[29] or bioactivation of BaP by non-P450 enzymes (e.g., prostaglandin H synthase and lipoxygenases),[30,31] or that combinations of these mechanisms were operative. However, these phenomena might also indicate that another reductase such as microsomal CBR can contribute to the P450-mediated BaP oxidation in these animal models. The latter possibility is supported by experiments with rat P450s indicating the involvement of cytochrome b_5 in the NADH-dependent hydroxylation of BaP in a reconstituted P450-containing system.[32,33] Studies using cytochrome b_5-knockout mouse lines, namely, HBN mice (hepatic cytochrome b_5 null) with a conditional hepatic deletion of cytochrome b_5, and HBRN mice (hepatic cytochrome b_5/P450 reductase null), in which POR and cytochrome b_5 are deleted specifically in the liver, also indicate the involvement of CBR in the P450-mediated metabolism of some other P450 substrates.[27,34,35]

We have recently demonstrated that the NADH/cytochrome b_5/CBR system is indeed able to function as the sole electron donor for both reduction steps of rat P450 1A1 during the oxidation of BaP *in vitro*.[15] Although this function of the NADH/cytochrome b_5/CBR system is valid for rat P450 1A1, its role in the reaction cycle catalyzed by the human orthologue of P450 1A1 remained to be explored. To address this question, the present study was carried out, in which we utilized enzymatic systems containing recombinant human P450 1A1 overexpressed in Supersomes or pure human recombinant P450 1A1 reconstituted with CBR and cytochrome b_5 in liposomes. Results presented here provide evidence that the NADH/cytochrome b_5/CBR system can function as the sole electron donor to human P450 1A1 during BaP oxidation *in vitro*.

■ EXPERIMENTAL PROCEDURES

Caution: *BaP is a human carcinogen and should be handled with care. Exposure to ^{32}P should be avoided, by working in a confined laboratory area, with protective clothing, plexiglass shielding, Geiger counters, and body dosimeters. Wastes must be discarded according to appropriate safety procedures.*

Chemicals and Material. BaP (CAS no. 50-32-8; purity ≥96%), NADH (as disodium salt; purity ~95%), NADPH (as tetrasodium salt; purity ~98%), dilauroylphosphatidylcholine, glutathione, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), and calf thymus DNA were purchased from Sigma-Aldrich (St. Louis, MO, USA) with ACS purity (purity meets the standards of the American Chemical Society), unless stated otherwise. P450 1A1-Supersomes, microsomes isolated from insect cells transfected with a baculovirus construct containing cDNA of human P450 1A1 and POR that are therefore overexpressed in these microsomes, were purchased from Gentest Corp. (Woburn, MI, USA). However, because they are microsomes (particles of broken endoplasmic reticulum), other enzymes (proteins) of the endoplasmic reticulum membrane [i.e., NADH:cytochrome b_5 reductase (CBR), microsomal EH (*m*EH), and cytochrome b_5] are also expressed at basal levels in these Supersomes (Gentest Corp., Woburn, MI, USA).

Preparation of Human Recombinant P450 1A1 and Rat Recombinant POR. Human recombinant P450 1A1 (EC 1.14.14.1) and rat POR (EC 1.6.2.4) were prepared by heterologous expression in *E. coli* by Jan Milichovsky and purified to apparent homogeneity (i.e., as single bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis) as described recently.[36] The specific content of human recombinant CYP1A1 was 11.5 nmol/mg protein. The specific activity of rat recombinant POR measured with cytochrome *c* as a substrate was 49.7 μmol cytochrome c/min/mg protein. Both recombinant enzymes were utilized in the reconstitution experiments.[37]

Isolation of Rat NADH:Cytochrome b_5 Reductase and Rabbit Cytochrome b_5. Cytochrome b_5 reductase (CBR) (E.C. 1.6.2.2) was isolated from rat liver microsomes by a procedure described by Perkins and Duncan.[38] The specific activity of rat CBR measured as NADH-ferricyanide reductase was 49.2 μmol ferricyanide/min/mg protein. Cytochrome b_5 was isolated from rabbit liver microsomes as described.[39] Both proteins purified to apparent homogeneity[38,39] were utilized in the reconstitution experiments.

Determination of P450 and Protein Content. The concentration of P450 was estimated according to Omura and Sato,[40] based on the absorption of the complex of reduced P450 with carbon monoxide. The P450 1A1 content was calculated using an extinction coefficient of 91 mM^{-1} cm^{-1} and estimated to be 11.5 nmol per mg protein. Protein concentrations were determined using a bicinchoninic acid assay (BCA; Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a standard.[41]

Reconstitution of Human P450 1A1 with POR or CBR in the Absence and Presence of Cytochrome b_5 in Liposomes. Reconstitution of human P450 1A1 with POR, CBR, and/or cytochrome b_5 in liposomes was carried out as described previously[19,37] with small modifications. Briefly, dilauroylphosphatidylcholine was dissolved in chloroform (20 mg/mL), and a lipid film was obtained by evaporation of chloroform by a stream of nitrogen. The lipid film was dispersed with 50 mM HEPES/KOH buffer, pH 7.4, containing 3 mM reduced glutathione and 0.1 μM CHAPS, and sonicated twice at 20 °C for 3 min. The appropriate amounts of human recombinant P450 1A1 and rat recombinant POR or human recombinant P450 1A1 and purified rat CBR (200 pmol in a ratio of 1:1), without or with cytochrome b_5 (at a ratio of P450 1A1 with reductase to cytochrome b_5 of 1:5), were added to the prepared dispersion and incubated at 20 °C for 10 min. As shown in previous studies,[19,25,37,42,43] the enzymatic activity of human P450 1A1 reconstituted with POR and cytochrome b_5 from several animal models was the same as that of the enzyme reconstituted with the human orthologues of these enzymes.

Incubations to Study the Metabolism of BaP by Human Recombinant P450 1A1 in Supersomes or by Human Recombinant P450 1A1 Reconstituted with POR or CBR in the Presence or Absence of Cytochrome b_5 in Liposomes. Incubation mixtures used to study BaP metabolism by human recombinant P450 1A1 in the presence of other components of the MFO-systems in Supersomes contained 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH or NADH, 50 μM BaP (dissolved in 5 μL DMSO), and 100 nM human recombinant P450 1A1 in a final volume of 0.5 mL. The same amount of a solvent (DMSO) was used in control incubations without BaP. With this DMSO concentration (1% as a final concentration), no inhibition of the NADPH-dependent P450-catalyzed oxidation of several substrates has been found previously.[7,8,19,25,28,44] The reaction was initiated by adding NADPH or NADH.

In separate experiments, 100 nM pure human recombinant P450 1A1 reconstituted individually with other components of the MFO-system was used instead of P450 1A1 in Supersomes. Negative control reactions lacked either P450 1A1, reductases, or BaP. After incubation (37 °C, 20 min), 5 μL of 1 mM phenacetin in methanol was added as an internal standard. BaP metabolism by the P450 1A1 systems has been shown to be linear up to 30 min of incubation.[15,16,19] BaP metabolites were extracted twice with ethyl acetate (2 × 1 mL), the solvent was evaporated to dryness, the residues were dissolved in 25 μL of methanol, and subsequently BaP metabolites were separated by HPLC as reported.[19,45] BaP metabolite peaks were analyzed by HPLC by comparison with metabolite standards whose structures were determined previously by NMR and/or mass spectrometry.[19]

Determination of BaP-DNA Adduct Formation by ^{32}P-postlabeling. Incubation mixtures used to assess DNA adduct formation by BaP activated with all enzymatic systems containing human P450 1A1 consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH or NADH, 100 nM human recombinant P450 1A1 plus other enzymes as indicated in the figures (or as described above), 0.1 mM BaP (dissolved in 7.5 μL of DMSO), and 0.5 mg of calf thymus DNA in a final volume of 0.75 mL as described previously.[19] The reaction was initiated by adding 0.1 mM BaP, and incubations were carried out at 37 °C for 60 min. BaP-DNA adduct formation has been shown to be linear up to 90 min.[7,19] Control incubations were carried out without P450 1A1, without reductases, without cytochrome b_5, without NADPH (or NADH), without DNA, or without BaP. After the incubation, DNA was isolated from the residual water phase by standard phenol/chloroform extraction. DNA adduct formation was analyzed using the nuclease P1 version of the ^{32}P-postlabeling technique.[7,19] Resolution of the adducts by thin-layer chromatography (TLC) using polyethylenimine-cellulose plates (Macherey and Nagel, Düren, Germany) was carried out as described.[7,19,46] DNA adduct levels (RAL, relative adduct labeling) were calculated as described.[47]

Statistical Analyses. Statistical analyses were carried out on the means ± standard deviations of three parallel experiments with Student's *t*-test (UNISTAT Statistics Software v6, Unistat Ltd., Highgate, London N6 5UQ, UK), and $p < 0.05$ was considered significant. Statistical association between amounts of BaP metabolites formed by oxidation of BaP by the P450 reconstituted systems containing POR or CBR and levels of BaP-DNA adduct 2 formed by the same systems were determined by the Spearman correlation coefficients using Statistical Analysis System software, version 6.12. Spearman correlation coefficients were based on a sample size of 6. All *Ps* are two-tailed and considered significant at the 0.05 level.

■ RESULTS

Oxidation of BaP by Human P450 1A1 Expressed in Supersomes and Pure Human Recombinant P450 1A1 Reconstituted with POR or CBR. In order to evaluate the role of POR and CBR in the reduction of human P450 1A1 during BaP oxidation, two enzymatic systems containing this human P450 were utilized. The first system used Supersomes containing human recombinant P450 1A1 expressed with POR,
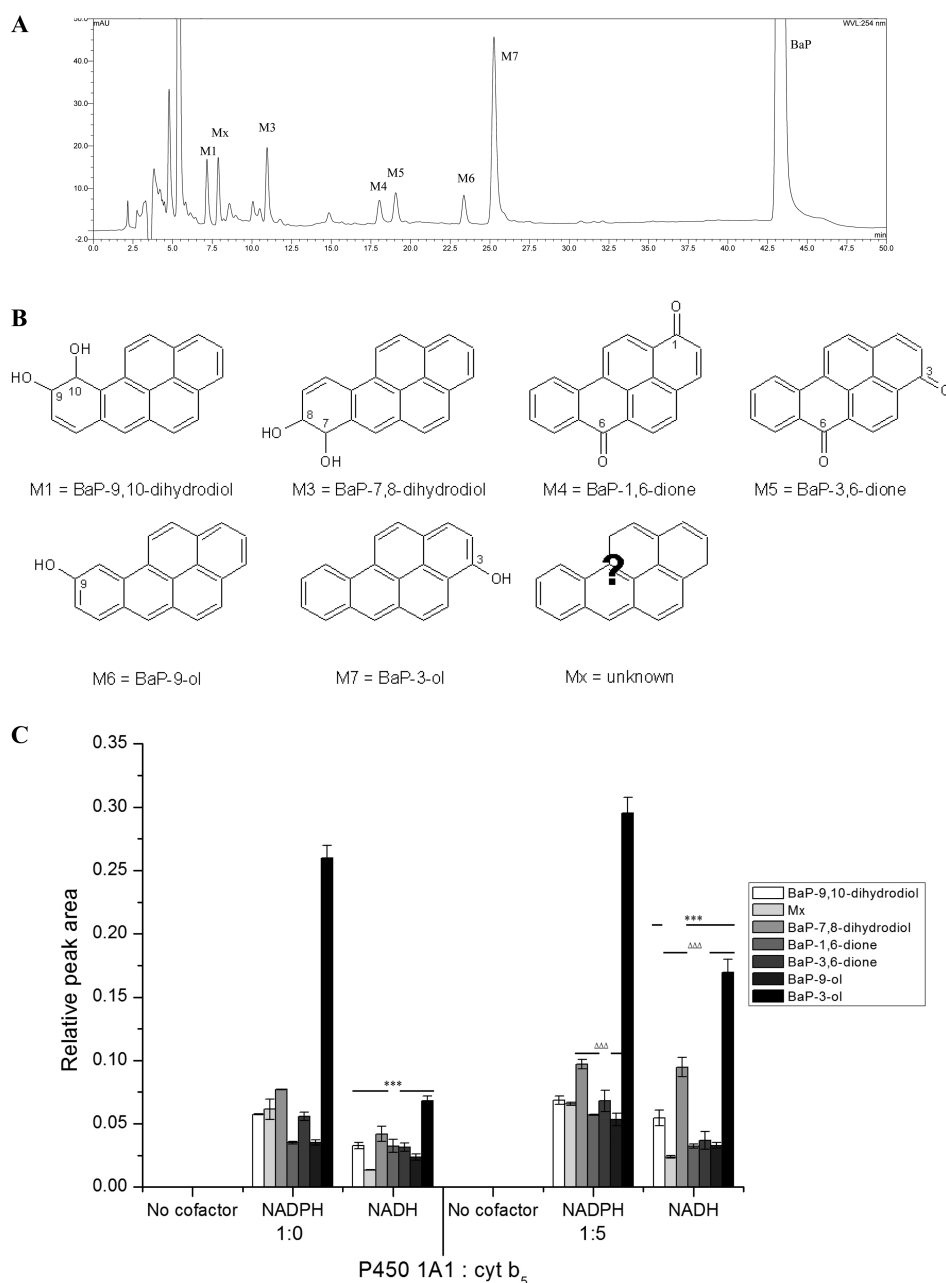
Figure 2. HPLC analysis of BaP metabolites formed by human recombinant P450 1A1 expressed in Supersomes in the presence of NADPH (A). Structures of BaP metabolites formed by human P450 1A1 (B). Amounts of BaP metabolites formed by human recombinant P450 1A1 expressed in Supersomes in the presence of either NADPH or NADH and the effect of cytochrome b_5 (cyt b_5) on this metabolism (C). Note that besides human P450 1A1 and POR overexpressed in Supersomes, this enzymatic system also contained mEH and CBR. Values represent the mean ± SD from three parallel measurements. ***$P < 0.001$ (Student's t-test), significantly different from incubations using NADPH as cofactor; $^{\triangle\triangle\triangle}P < 0.001$ (Student's t-test), significantly different from incubations without cytochrome b_5.

CBR, mEH, and/or cytochrome b_5. In the second system, pure human recombinant P450 1A1 was reconstituted in liposomes with either pure POR or CBR. The latter enzymatic system was utilized with or without cytochrome b_5 to examine the function of both reductases as electron donors to P450 1A1 during BaP metabolism with NADPH (cofactor of POR) or NADH (cofactor of CBR). The BaP metabolites formed by human P450 1A1 in these enzyme systems were analyzed by HPLC (Figure 2A).

Seven BaP metabolites were formed in Supersomes containing human P450 1A1, both in the presence of NADPH and NADH (Figure 2). They were structurally characterized previously[16,19] as BaP-9,10-dihydrodiol (M1), BaP-7,8-dihydrodiol (M3), BaP-1,6-dione (M4), BaP-3,6-dione (M5), BaP-9-ol (M6), and BaP-3-ol (M7). The structures of these BaP metabolites are shown in Figure 2B. In addition, a metabolite of unknown structure (Mx) was detected. Essentially, no BaP metabolites were found when both NADPH and NADH were omitted from the incubation mixtures containing the P450 1A1-Supersomes (Figure 2C). NADH was less effective than NADPH to act as electron donor to human P450 1A1 in Supersomes (Figure 2C). Addition of cytochrome b_5 to the incubation mixtures led to an increase in

P450 1A1-mediated BaP oxidation both in the presence of NADPH and in the presence of NADH (Figure 2C).

In the second enzymatic system, where pure human recombinant P450 1A1 was reconstituted with POR with or without cytochrome b_5 in liposomes, this P450 enzyme was also able to oxidize BaP in the presence of NADPH (Figure 3).
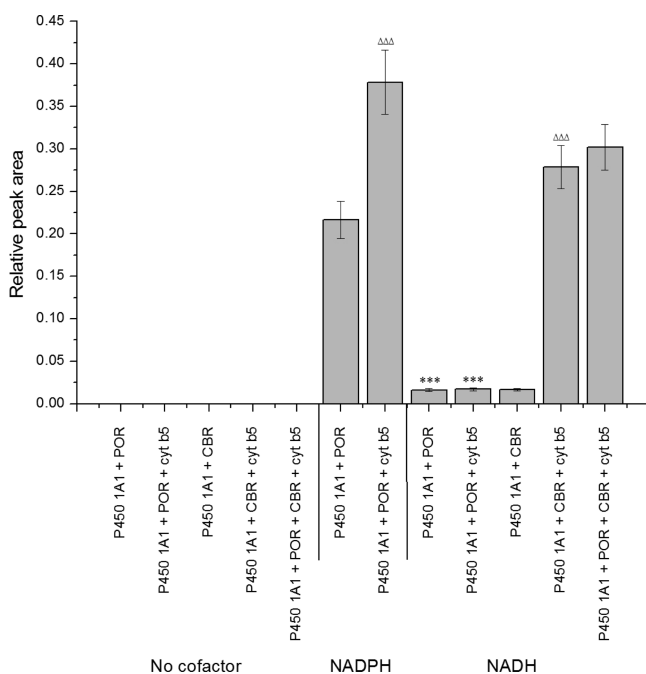

Figure 3. BaP metabolism catalyzed by human P450 1A1 reconstituted with POR or CBR with or without cytochrome b_5 (cyt b_5). Columns show the sum of individual BaP metabolites formed by P450 1A1 (BaP-3-ol, BaP-9-ol, BaP-1,6-dione, BaP-3,6-dione, and the unknown metabolite Mx) Incubations were carried out in the presence of NADPH or NADH. $^{\triangle\triangle\triangle}P < 0.001$ (Student's t-test), significantly different from incubations without cytochrome b_5; ***$P < 0.001$ (Student's t-test), significantly different from incubations with NADPH as cofactor.

Only five BaP metabolites were formed, which were BaP-3-ol (M7) as the major metabolite, BaP-9-ol (M6), BaP-1,6-dione (M4), BaP-3,6-dione (M5), and metabolite Mx (Figure S1). Because mEH was absent from the system, no dihydrodiols were formed. Addition of cytochrome b_5 to this P450 1A1 system increased the formation of all BaP metabolites, in particular BaP-3-ol (Figure 3 and Figure S1). In contrast, NADH did not lead to significant BaP oxidation by P450 1A1 reconstituted with POR (Figure 3 and Figure S1), which confirms that NADH functions as a coenzyme of POR at a very slow rate that is negligible relative to NADPH, as we showed recently with cytochrome c as a substrate for POR.[15] No BaP metabolites were found when both NADPH and NADH were omitted from the incubation mixtures containing the human recombinant P450 1A1 reconstituted with POR or CBR (Figure 3).

In the enzymatic system where human P450 1A1 was reconstituted with CBR and cytochrome b_5 in liposomes, BaP was predominantly oxidized to BaP-3-ol (M7) and to a lower extent to BaP-9-ol (M6), BaP-1,6-dione (M4), BaP-3,6-dione (M5), and a metabolite Mx (Figure S1). Cytochrome b_5 as a substrate of CBR was necessary for BaP oxidation in the system of human P450 1A1 reconstituted with CBR with NADH as

the cofactor. Without this protein, essentially no BaP oxidation was detectable by P450 1A1 reconstituted with CBR (Figure 3 and Figure S1). Addition of POR to the reconstituted system of human P450 1A1 with CBR and cytochrome b_5 did not change BaP metabolite levels (Figure 3 and Figure S1).

Formation of BaP-DNA Adducts by Human P450 1A1 Expressed in Supersomes and by Human P450 1A1 Reconstituted with POR or CBR. In further experiments, the formation of DNA adducts by BaP incubated with human P450 1A1 expressed in Supersomes and human P450 1A1 reconstituted with POR or CBR was analyzed. First, we utilized human recombinant P450 1A1 expressed in Supersomes with other enzyme components of the microsomal monooxygenase system. Up to two DNA adducts (assigned adducts 1 and 2; see insert in Figure 1) were detected by ^{32}P-postlabeling in incubations where BaP was activated with human P450 1A1 in Supersomes, in the presence of either NADPH or NADH. No such BaP adducts were found when both NADPH and NADH were omitted from the incubation mixtures containing the P450 1A1-Supersomes (Figure 4). Adduct 1 was the BaP-DNA adduct predominantly formed in this system (Figure 4). Comparison with previous ^{32}P-postlabeling analyses[10,19] showed that adduct 1 is the dG-N^2-BPDE adduct.[10,19] The other adduct, which was formed in this enzymatic system as only a minor product, if detectable at all (e.g., there was no formation of this adduct in Supersomes in the presence of NADPH), has similar chromatographic properties by TLC to a guanine adduct derived from the reaction with 9-hydroxy-BaP-4,5-epoxide (see adduct spot 2 in the insert of Figure 1 and Figure 5A). Addition of cytochrome b_5 to P450 1A1 in Supersomes increased the levels of the dG-N^2-BPDE adduct generated by human P450 1A1 in the presence of NADH (Figure 4), similar to the increase observed in BaP oxidation (Figure 2).

Pure human recombinant P450 1A1 reconstituted with POR in the presence of NADPH formed only adduct 2 (Figures 5B and 6). Because of the absence of mEH, no adduct 1 (i.e., dG-N^2-BPDE) was formed. Addition of cytochrome b_5 to this P450 1A1 system decreased the levels of adduct 2, but this decrease was not statistically significant (Figure 6). NADH was essentially ineffective to mediate the activation of BaP by P450 1A1 reconstituted with POR (Figures 5B and 6), which again indicates that NADH functions as a coenzyme of POR at a very slow rate that is negligible relative to NADPH.

Human P450 1A1 reconstituted with CBR and cytochrome b_5 also generated adduct 2. The presence of cytochrome b_5 as a substrate of CBR and NADH as its cofactor was essential for this adduct formation; NADPH, the cofactor of POR, was ineffective (Figures 5B and 6). No BaP-DNA adduct 2 was found when both NADPH and NADH were omitted from the incubation mixtures containing human recombinant P450 1A1 reconstituted with POR or CBR (Figure 6). Levels of adduct 2 formed by human P450 1A1 in the presence of the NADH/cytochrome b_5/CBR system were lower than those formed in the system of human P450 1A1 containing POR and NADPH (Figures 5 and 6). These results corresponded to lower levels of BaP-9-ol (a precursor of 9-hydroxy-BaP 4,5-epoxide generating this adduct) and other BaP metabolites formed in this system (see Figure S1). Significant correlations were found between levels of adduct 2 and BaP-9-ol ($r = 0.943$, $P < 0.001$, Spearman's correlations) or BaP-3-ol ($r = 0.943$, $P < 0.001$), whereas no such correlations were found between levels of this
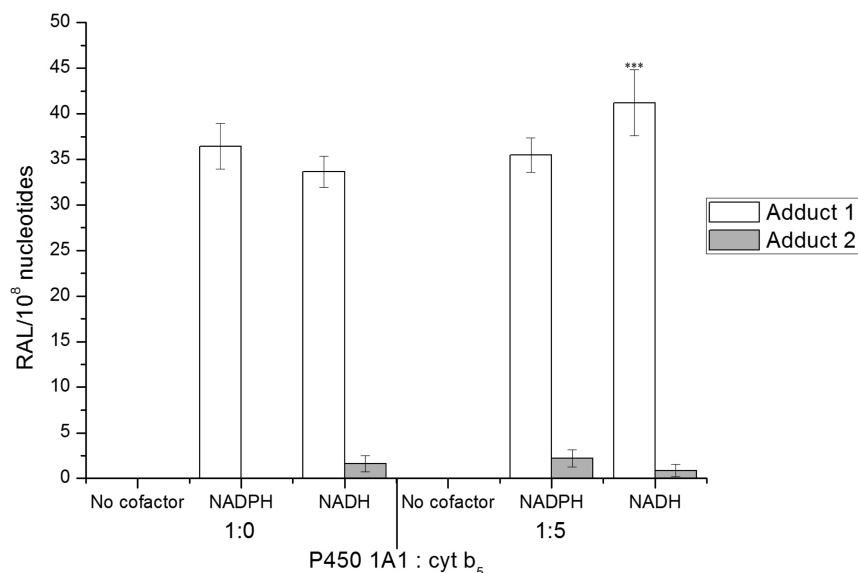
Figure 4. DNA adduct formation (RAL, relative adduct labeling) by BaP activated with human recombinant P450 1A1 in Supersomes in the presence of either NADPH or NADH and the effect of cytochrome b_5 (cyt b_5) on this reaction. Note that besides P450 1A1 and POR overexpressed in Supersomes, this enzymatic system also contained mEH and CBR. Values represent the mean total RAL ± SD (n = 3; analyses of three independent *in vitro* incubations). ***$P < 0.001$ (Student's t-test), levels of BaP-adduct 1 significantly different from incubations with NADH but without cytochrome b_5.
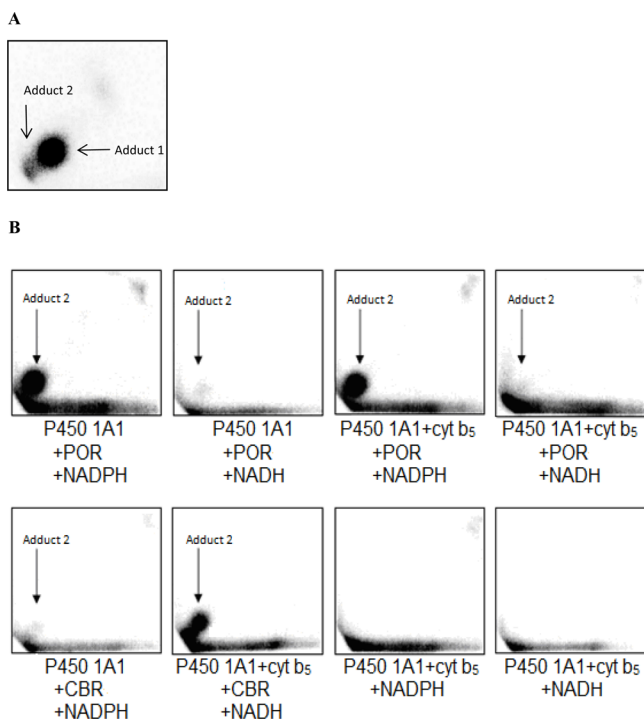

Figure 5. Autoradiographic profile of BaP-derived DNA adducts generated in DNA by BaP activated with human P450 1A1 in Supersomes with mEH in the presence of NADH and cytochrome b_5 (A); with pure human P450 1A1 reconstituted with POR in liposomes in the presence of NADPH (B); P450 1A1 with POR in the presence of NADH; P450 1A1 with POR and cytochrome b_5 in the presence of NADPH; P450 1A1 with POR and cytochrome b_5 in the presence of NADH; P450 1A1 with CBR and cytochrome b_5 in the presence of NADPH; P450 1A1 with CBR and cytochrome b_5 in the presence of NADH; P450 1A1 with cytochrome b_5 in the presence of NADPH; and P450 1A1 with cytochrome b_5 in the presence of NADH.

adduct and BaP-1,6-dione, BaP-3,6-dione or a metabolite Mx ($r < 0.5$).

■ DISCUSSION

The metabolism of BaP has been extensively studied over the past decades,[2] and various studies have examined the role of P450 enzymes, particularly P450 1A1 of several species, to metabolize this carcinogen.[2,6−8,15,16,19,28,44] However, the mechanism of the reaction cycle of BaP oxidation catalyzed by P450 1A1, particularly the roles of POR and CBR as electron donors to P450 1A1, has not yet been fully resolved.[7,8,15,19] Enigmatic results have been found in two mouse models where deletion of POR in hepatocytes did not lead to an expected decrease in BaP-DNA adduct formation in the liver *in vivo* but instead to higher BaP-DNA binding.[7,8,19] We also showed that in livers of HRN mice P450 1A1, cytochrome b_5 and mEH can effectively activate BaP to DNA binding species, even in the presence of very low amounts of POR.[19] Because this feature has biological significance, studying the role of the enzymes reducing P450 1A1 is important to better understand the mechanism(s) involved in BaP metabolism. Recently, we demonstrated that the NADH/cytochrome b_5/CBR system is able to function as the sole donor of electrons for both reduction steps of rat P450 1A1 during BaP oxidation *in vitro*.[15] This finding indicates that CBR as an NADH-dependent reductase might substitute POR in the P450 1A1-mediated BaP metabolism and might help to explain our enigmatic results in the POR-knockout mouse models.[7,8,19] However, the question whether this novel function of the NADH/cytochrome b_5/CBR system as electron donor to rat P450 1A1 in BaP metabolism represents a general feature for the P450 1A1 reaction cycle in other species including humans remained to be answered. Therefore, the primary aim of this study was to determine whether the NADH/cytochrome b_5/CBR system can be the exclusive donor of both electrons to the P450 1A1 human orthologue during BaP metabolism *in vitro*. To this end, two enzymatic systems containing this human
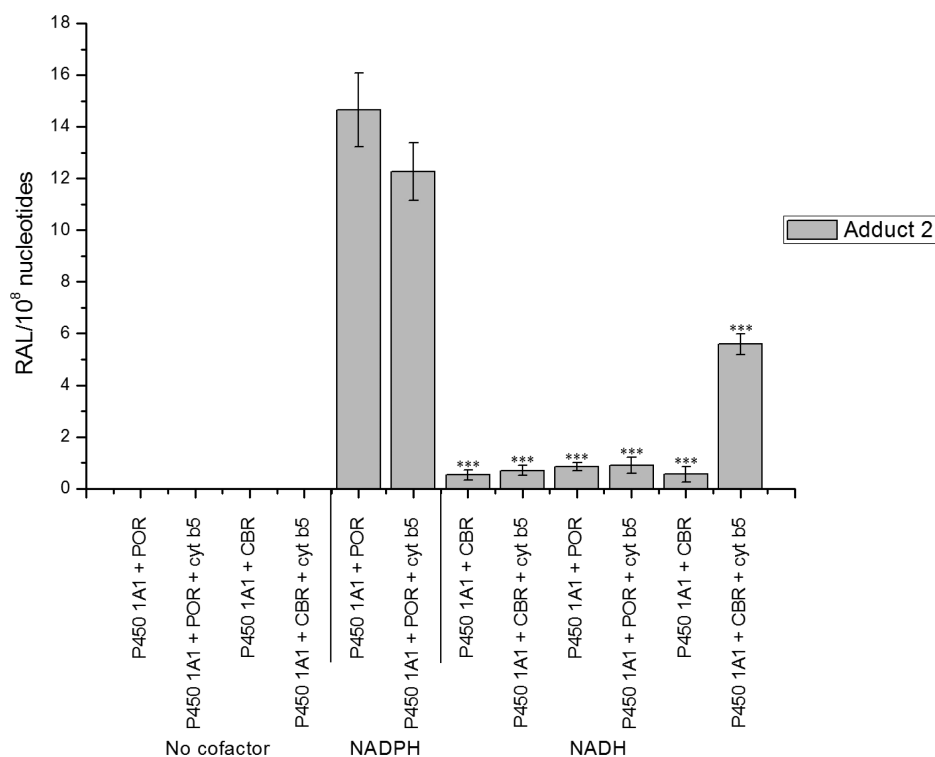
Figure 6. Effect of NADPH and/or NADH on BaP-DNA adduct formation (RAL, relative adduct labeling) catalyzed by human recombinant P450 1A1 reconstituted with POR or CBR with or without cytochrome b_5 in liposomes. Values are averages ± SD of three independent experiments. ***P < 0.001 (Student's t-test), levels of BaP-adduct 2 significantly different from incubations with human P450 1A1, POR, and NADPH.

P450 were utilized: (1) microsomes of baculovirus-infected insect cells (Supersomes) containing human recombinant P450 1A1 expressed with POR, CBR, mEH, and cytochrome b_5 and (2) pure human P450 1A1 heterologously expressed in *E. coli* reconstituted either with pure POR or CBR with cytochrome b_5 in liposomes.

Using the first system, human P450 1A1-Supersomes, we proved that NADH acts as electron donor for both reductions of the P450 1A1 human orthologue in BaP oxidation independent of NADPH and POR if CBR and cytochrome b_5 is present. This conclusion is supported by the fact that NADH functions as a poor coenzyme of POR when cytochrome *c* is used as its substrate.[15] Our results therefore confirm the novel feature of the mechanism of the catalytic cycle of human P450 1A1 during BaP oxidation previously described for rat P450 1A1. We demonstrated that the reaction cycle of BaP oxidation catalyzed by human P450 1A1 can proceed by ways that differ from the generally accepted mechanism, where the first reduction of P450 is considered to be catalyzed by POR without cytochrome b_5.[20,48−52] Considering the redox potentials of cytochrome b_5 (+20 mV)[53,54] and ferric substrate-bound P450 (−237 mV),[27,53] it is thermodynamically impossible for cytochrome b_5 to provide the first electron in the P450 catalytic cycle.[55] Given that the redox potential of oxyferrous P450 is also approximately +20 mV, it is feasible that cytochrome b_5 can supply the second electron into the catalytic cycle.[27,53,55] However, based on the redox potential of CBR determined under the anaerobic conditions (−265 mV)[56] it could provide the first electron in the P450 catalytic cycle.[27,57] Moreover, considering the effect of the given Le Chatelier's principle, the reduced P450 will rapidly bind dioxygen under aerobic conditions of the experiments. Indeed, in the present study we demonstrate that the first reduction of P450 1A1 which previously had been considered to be mediated exclusively by the NADPH/POR system can be substituted by the NADH/cytochrome b_5/CBR system. This reaction was found not only in Supersomes containing human recombinant P450 1A1 but, even more importantly, in the system of pure human P450 1A1 reconstituted with CBR and cytochrome b_5. Such NADH/cytochrome b_5/CBR-mediated activity of the human P450 1A1 systems was proven by the formation of up to seven BaP metabolites [BaP-9,10-dihydrodiol (M1), BaP-7,8-dihydrodiol (M3), BaP-1,6-dione (M4), BaP-3,6-dione (M5), BaP-9-ol (M6), BaP-3-ol (M7), and a metabolite of unknown structure (Mx)]. In addition, these systems generated two BaP-DNA adducts, the dG-N^2-BPDE adduct (adduct 1) and/or a guanine adduct derived from the reaction with 9-hydroxy-BaP-4,5-epoxide (adduct 2). The BaP metabolite profiles formed by P450 1A1 with the NADH/cytochrome b_5/CBR system were the same as those in the system where NADPH and POR were used as electron donors. BaP-4,5-dihydrodiol (M2), which was previously found to be formed by rat P450 1A1,[15,19] was not generated by the human P450 1A1 orthologue (Figure 2). This finding is in line with previous findings showing that this BaP metabolite was not formed in human bronchoalveolar H358 cells expressing P450 1A1 after BaP exposure.[13]

Interestingly, essentially no differences in the levels of dG-N^2-BPDE adducts were seen when using either NADPH or NADH as cofactors in the P450 1A1-Supersomes system (see Figure 4). However, it should be noted that the oxidation reaction of BaP to its metabolites was catalyzed less efficiently by NADH than by NADPH in this system (see Figure 2). One reason might be the effects of the different experimental conditions used for the incubations utilized for BaP metabolite analysis and BaP-DNA adduct formation (i.e., incubation times,

concentrations of BaP, and the presence or absence of DNA; see the Experimental Procedures section).

In summary, this study demonstrates for the first time that NADH, CBR, and cytochrome b_5 can act as sole electron donors for both the first and second reduction of human P450 1A1 during the oxidative metabolism of BaP and formation of BaP-DNA adducts in vitro. These findings confirm our results of a recent study where rat P450 1A1 was utilized to study BaP metabolism.[15] However, although the role of the NADH/cytochrome b_5/CBR system in both reductions of rat[15] and human P450 1A1[present work] is proven in our in vitro studies, further investigations are needed in the future. In this context, the mechanism of both the first and second reduction of P450 1A1 remains to be examined in detail. In addition, as shown by Guengerich and co-workers for the P450 3A4-mediated 6-hydroxylation of testosterone,[57] the question as to whether the NADH/cytochrome b_5/CBR system might also reduce other P450 enzymes needs to be addressed. This might be the case in rat hepatic microsomes where various P450s were induced by their specific inducers; in these microsomes, BaP was oxidized not only in the presence of NADPH but also in the presence of NADH.[15] Another crucial question that remains to be addressed relates to the impact of the NADH/cytochrome b_5/CBR system on BaP metabolism in vivo. Recent in vivo experiments using cytochrome b_5-knockout mouse lines (i.e., HBN and HBRN) provide evidence that in the absence of POR, cytochrome b_5/CBR are capable of supplying electrons for P450 catalytic function (i.e., in the metabolism of the P450 3A substrate midazolam).[27] It is anticipated that these mouse lines will help to elucidate the different mechanisms of P450-catalyzed BaP biotransformation in vivo.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00143.

> Amounts of BaP metabolites generated by human P450 1A1 reconstituted with POR or CBR with or without cytochrome b_5 (cyt b5) (PDF)

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS

BaP, benzo[a]pyrene; BPDE, BaP-7,8-dihydrodiol-9,10-oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-pro-panesulfonate; CBR, NADH:cytochrome b_5 reductase; P450, 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; HEPES, 4-(2-hydroxyethyl)-1-piperazinee-thanesulfonic acid; HBN, hepatic cytochrome b_5 null; HPLC, high performance liquid chromatography; HBRN, hepatic cytochrome b_5/P450 reductase null; HRN, hepatic P450 reductase null; mEH, microsomal epoxide hydrolase; MFO, mixed-function oxidase; POR, NADPH:P450 oxidoreductase; RAL, relative adduct labeling; RCN, reductase conditional null; TLC, thin-layer chromatography

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