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Role of cytochrome P4501B1 in benzo[*a*]pyrene bio-activation to DNA-binding metabolites in mouse vascular smooth muscle cells: Evidence from ^{32}p-postlabeling for formation of 3-hydroxybenzo[*a*]pyrene and benzo[*a*]pyrene-3,6-quinone as major proximate genotoxic intermediates

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Running Title: Benzo[*a*]pyrene metabolic activation in smooth muscle cells

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Abbreviations: SMC, smooth muscle cells; PAH, polycyclic aromatic hydrocarbons; BP, benzo[*a*]pyrene; BPDE, benzo[*a*]pyrene-7,8-dihydrodiol-9,10 epoxide; DMBA, 7,12-dimethylbenz[*a*]anthracene; EpREs, electrophile response elements; ROS, reactive oxygen species; P450, cytochrome P450; AHH, aryl hydrocarbon hydroxylase; 3-OH-BP, 3-hydroxybenzo[*a*]pyrene; BPQ, benzo[*a*]pyrene-3,6-quinone; EP, 1-ethynylpyrene; □-NF, alpha-naphthoflavone; DMSO, dimethylsulfoxide; EH, epoxide hydrolase, ANOVA, analyses of variance, MC, 3-methylcholanthrene;. AHR, Ah receptor; PEI-TLC; polyethyleneimine cellulose thin-layer chromatography

Section Assignment: Cardiovascular

Abstract

Benzo[*a*]pyrene (BP), a polycylic aromatic hydrocarbon (PAH), is a potent atherogen and carcinogen in laboratory animals. Since genotoxic mechanisms may contribute to the development of atherosclerosis by PAHs, here we have tested the hypotheses that: (i) BP induces DNA adducts in mouse aortic smooth muscle cells (SMCs); (ii) 3-hydroxybenzo[*a*]pyrene (3-OH-BP) and benzo[*a*]pyrene-3,6-quinone (BPQ) are proximate genotoxic metabolites; and (iii) cytochrome P4501B1 (CYP1B1) mediates the activation of BP and its metabolites to ultimate genotoxic intermediates. Cultured mouse aortic SMCs were treated with BP, 3-OH-BP, or BPQ for 24 h, and DNA adduct formation was analyzed by ^{32}P-postlabeling. In some experiments, cells were pre-treated with the CYP1B1 inhibitor 1-ethynylpyrene (EP) prior to exposure to BP or its metabolites. BP, 3-OH-BP, and BPQ induced formation of several DNA adducts that were not observed in dimethylsulfoxide (DMSO)-treated cells. Re- and co-chromatography experiments indicated that 3-OH-BP and BPQ were proximate genotoxic metabolites of BP. DNA adduct formation was strongly inhibited by EP, a specific inhibitor of CYP1B1. BP treatment of SMCs resulted in induction of aryl hydrocarbon hydroxylase (AHH) activity and CYP1B1, but not CYP1A1, apoprotein. EP also blocked AHH induction by BP. In conclusion, the results of this study support the hypothesis that in SMCs, which are target sites for the development of atherosclerosis, the major bioactivation pathway of BP entails CYP1B1-mediated formation of the 3-OH-BP and BPQ, which are proximate genotoxic metabolites that may in turn get transformed to ultimate DNA-binding metabolites, which may contribute to atherogenesis by PAHs.

Atherosclerosis is one of the major sequelae of cigarette smoking in humans (Salama et al., 2002; Ross et al., 2001). Atherosclerosis is a complex disease process involving elastic and muscular arteries that, like cancer, involves uncontrolled proliferation and dedifferentiation of vascular smooth muscle cells (SMCs) (Ramos and Parish, 1995). Polycyclic aromatic hydrocarbons (PAHs) are important constituents of cigarette smoke, and animal and human studies have suggested that PAHs may be involved in the etiology of carcinogenesis and atherosclerosis associated with exposure to cigarette smoking (Majesky et al., 1983; Paigen et al., 1986; Izzotti et al., 1995; Ross et al., 2001; Salama et al., 2002). The molecular mechanisms of vascular damage by PAHs are not fully understood. Benzo[*a*]pyrene (BP), a potent PAH carcinogen that induces tumors in a variety of organs in experimental animals, is also a potent atherogen in laboratory animals (Bond et al., 1981). Several studies have suggested that there are similarities between carcinogenesis and atherogenesis in that both processes involve initiation and promotion stages (Ramos and Parish, 1995; Majesky et al., 1983; Ross et al., 2001). The presence of PAH-DNA adducts in atherosclerotic lesions of humans suggests that DNA damage contributes to the development of atherosclerosis (Izzotti et al., 1995; Bond et al., 1981).

Although parent PAHs by themselves are non-toxic, metabolic activation of PAHs by cytochrome P450 (P450) leads to the production of reactive metabolites capable of covalently binding to DNA (Gelboin, 1990). Benzo[*a*]pyrene-7,8-dihydrodiol -9,10 epoxide (BPDE) is the major ultimate carcinogen responsible for the carcinogenicity of BP (Gelboin, 1990). Recent studies have shown that BPDE-induced mutational hot spots are produced in lung tumors of smokers, providing direct evidence for a link between

DNA adduction and cancer (Dennisenko et al., 1996). In addition to BPDE, BP can also undergo one-electron oxidation, and subsequent radical reactions to form BP quinones, which have also been implicated in carcinogenesis (Cavalieri and Rogan, 1995) and atherogenesis (Bond et al., 1981; Kerzee and Ramos, 2000; Miller et al., 2000). Oxidative stress induced by BP has been implicated in atherogenic responses in animal models and cell culture studies (Kerzee and Ramos, 2000; Miller et al., 2000), with electrophile response elements (EpREs) playing an important role in atherogenic mechanisms. Since BP metabolism to quinones and semiquinones can lead to redox cycling and reactive oxygen species (ROS) formation, it is plausible that direct DNA binding of BP semiquinones (Joseph and Jaiswal, 1994) and oxidative DNA damage may contribute to the atherogenicity of BP.

Hepatic and extra-hepatic CYP1 enzymes play major roles in the bioactivation of PAHs to genotoxic metabolites (Guengerich, 1988). In fact, PAH-DNA adduct formation has been demonstrated in several human cancer cell lines and organ explant cultures of extra-hepatic origin (Melendez-Colon et al., 2000). However, BP activation leading to DNA adduct formation in cultured SMCs of aorta, a target site for the development of atherosclerosis, has not been demonstrated. Little progress has been made in the understanding of the relationship between P450 enzymes and DNA adducts in vascular tissues. Evidence to date suggests that BP induces CYP1A1 in rat aortic tissues (Thirman et al., 1994). While CYP1A1 is localized in endothelial cells of porcine aorta (Stegeman et al., 1995), and in vascular SMCs from newborn rats (Giachelli et al., 1991), the enzyme is under negative regulation in adult quail and rodent aortic SMCs (Stegeman et

al., 1995). Thus, other P450 isoforms probably also contribute to PAH metabolism in SMCs.

A number of P450 isoforms are present within the vascular wall, including CYP1B1, which is preferentially expressed in SMCs , as opposed to vascular endothelium, and is co-expressed with CYP1A1 in several extra-hepatic tissues (Kerzee and Ramos, 2001). CYP1A1 and 1B1 activities are frequently determined by measuring the activities of aryl hydrocarbon hydroxylase (AHH), which catalyzes the conversion of BP to 3-OH-BP (Nebert and Gelboin, 1968). PAHs are potent inducers of CYP1B1 (Shimada et al., 1996), and metabolic activation of PAHs by CYP1B1 to oxidative intermediates and carcinogenic precursors (Shimada et al., 1996; Bowes et al., 1996; Moorthy et al., 2002) may have implications for PAH-induced atherogenesis. Taken together, it appears that CYP1B1-catalyzed BP metabolism to oxidative intermediates plays an important role in BP genotoxicity, which may in turn contribute to atherogenesis. To this end, we tested the hypotheses that (i) BP induces DNA adducts in mouse aortic SMCs; (ii) 3-OH-BP and BPQ are proximate genotoxic metabolites; and (iii) CYP1B1 plays an important role in the activation of BP and its metabolites to ultimate genotoxic intermediates.

Materials and Methods

Chemicals

BP (98% purity) was obtained from Aldrich Chemical Co. (Milwaukee, WI), and 3-hydroxybenzo[a]pyrene (3-OH-BP) and benzo[a]pyrene-3,6-quinone (BPQ) (> 99% purity by HPLC) were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories c/o Midwest Research Institute, (Kansas City, MO). 1-Ethinylpyrene (EP) was a kind gift from Dr. William Alworth, Dept. of Chemistry, Tulane University (New Orleans, LA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. BP, 3-OH-BP, BPQ, EP, ellipticine, and alpha-naphthoflavone (α–NF) stock solutions were prepared in dimethylsulfoxide (DMSO). Monoclonal antibody against CYP1A1 was a gift from Dr. Paul E. Thomas, Rutgers University (New Brunswick, NJ). Polyclonal antibodies to rat CYP1B1 that cross-react with mouse CYP1B1 were purchased from Gentest (Woburn, MA). Rat CYP1B1 protein standards were also obtained from Gentest (Woburn, MA).

Cell Culture

Primary cultures of vascular SMCs were isolated from female C57BL/6 mouse aorta and maintained under standard conditions as described by Ramos and Cox (1993). Cells were grown in Medium 199 (Gibco, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (Gibco). Subcultures were prepared by trypsinization (Gibco) of subconfluent primary cultures. Cells were seeded at 100 cells/mm^2 on to 100 mm plates for AHH assays, and at 75 cells/mm^2 on to 150 mm plates

for postlabelling experiments. The cells were allowed to acclimate for 48 hours, and then challenged with BP, 3-OH-BP, BPQ, or EP for the time periods specified in figure legends. Concentrations of DMSO in all experiments were <0.15%.

Aryl Hydrocarbon Hydroxylase Activity

The methodology described by Nebert and Gelboin (1968), with modifications, was used to process samples for measurement of AHH activity. In brief, cells were treated in triplicate with BP, ellipticine, α-NF, or EP, as described in our recent paper (Moorthy et al., 2002). In preliminary concentration-range finding studies, cells were treated in triplicate for 1 or 24 hrs with the CYP inhibitors ellipticine (0.001-0.1 nM), α-NF (1 nM-100 nM) or EP (0.01-1μM). Since no statistically significant differences were observed in the extent of inhibition between the two time points, for metabolism inhibition studies, cells were treated in triplicate first with ellipticine (0.1 nM), α-NF (10 nM), or EP (1 μM) for 1 hr, followed by treatment with BP (3 μM) for 24 hr. Cells were washed with 1ml ice-cold Tris-Sucrose buffer (0.05M Tris-HCl, 0.2M sucrose, pH 8.0), scraped and centrifuged (1100 rpm, 5 min, 4°C). Supernatants were decanted and the pellets resuspended in ice-cold Tris-sucrose buffer. One-third of the sample was combined with 0.1M HEPES (pH 8.0), 0.4 mM NADPH (in 1% sodium bicarbonate) in a borosilicate tube and incubated at 37°C for 2 min, followed by addition of BP (3 μM in methanol), protected from light and further incubated at 37°C for 15 min. The reaction was stopped with ice-cold acetone and hexane, vortexed and the organic (top) layer removed. To the organic layer, 1N NaOH was added, vortexed and the aqueous (bottom) layer removed for analysis. 3-OH BP standards were prepared in 1N NaOH and read using a spectrofluorometer [396 nm excitation, 522 nm emission (slit width, 4 nm)] prior to

analysis of samples. The initial cell sample in Tris-sucrose buffer was analyzed for protein concentration by the method of Bradford (1976). AHH activity was calculated according to the equation:

pmol/min/mg AHH activity = [μM 3-OH-BP formed x 1.702 x 10^6 (dilution factor)]/[mg protein x 15 min]. The factor 1.702 x 10^6 was used to account for dilution of the incubation mixture and conversion of the activity units from μmol/min/mg protein to pmol/min/mg protein. The AHH assay was linear with respect to incubation time and amount of protein (Moorthy et al, 2002).

Western Blotting

CYP1A1 and 1B1 protein expression in the vascular SMCs was determined by Western blotting using CYP1A1 and 1B1-specific antibodies. The cell pellets described above were used as the protein source. Procedures for Western analysis have been described previously (Moorthy et al., 2000). Quantitation of the blots was accomplished by laser densitometric scanning of the photographic negatives of the blots, as described previously (Moorthy et al., 2000). CYP1B1 levels in the cell pellets were estimated from a standard curve that was generated by Western analysis of CYP1B1 standards (0.1-0.8 pmol) that produced band intensities that were in the linear range (r^2 = 0.98).

Epoxide Hydrolase (EH) Activities

EH activities in vascular SMCs were determined spectrophotometrically by a coupled assay (Guengerich and Mason, 1980; Moorthy and Randerath, 1997), which measured the ability of aldehyde dehydrogenase to transfer electrons from styrene-7,8-dihdrodiol to NAD^+, formed as a result of EH-catalyzed hydrolysis of styrene-7,8 oxide.

The EH activities were measured in the same cell pellets that were used for determining CYP1B1 expression.

Cell Isolation for ^{32}P Postlabeling

Vascular SMCs were seeded at 75 cells/mm^2 in 3 separate 150 mm culture plates per treatment to generate ~1x10^7 cells. Cells were treated with DMSO (control), (0.03, 0.3, or 3 μM) BP, 3-OH-BP, or BPQ, alone (24 h) or pre-treated with EP (1 μM, 1 h) prior to chemical treatment. At the end of treatment, cells were harvested by trypsinization, centrifuged (1100 rpm, 5 min), resuspended and counted in a hemocytometer. Cells were then pelleted (1100 rpm, 5 min), resuspended in 1 ml TE buffer (10 mM Tris-Cl, 1mM EDTA, pH 7.65), and stored at -20°C prior to analysis.

DNA Isolation and ^{32}P-Postlabeling

DNA was isolated as reported previously (Reddy and Randerath, 1986; Moorthy et al., 2002). The nuclease-P1-enhanced version of the ^{32}P-postlabeling assay for DNA adducts was also performed as reported previously (Reddy and Randerath, 1986; Moorthy and Randerath, 1997). Briefly, DNA (10 μg) was digested with micrococcal nuclease (0.04 U/ml) and spleen phosphodiesterase (0.4 μg/ml) at pH 6.0 and 37 ^{0}C for 3.5 h. The DNA was then treated with nuclease-P1 (0.6 μg/ml, pH 5.6) at 37^0 C for 40 min, followed by labeling with [γ-^{32}P]ATP (4000 Ci/mmol) and T4 polynucleotide kinase (0.5 U/ml) at pH 9.5 and 37 ^{0}C for 30 min. The labeled products were separated by two-dimensional polyethyleneimine cellulose thin-layer chromatography (PEI-TLC) (Moorthy and Randerath, 1997). The two-dimensional maps were exposed to autoradiography with intensifying screens at a typical exposure of 16 h at -80^0 C, and adduct spots were quantified by scintillation counting. Adduct levels were expressed as relative adduct labeling (RAL)

values, which were calculated by using the formula: RAL = c.p.m. in adduct(s)/specific activity (ATP) x pmol DNA-P labeled (1 μg DNA = 3240 pmol DNA-P) (Reddy and Randerath, 1986). For re-chromatography experiments, spots that were in similar locations were excised from the chromatograms, extracted with isopropanol: ammonia, and chromatographed in different solvents. Co-chromatography experiments were performed similar to re-chromatography, except that mixtures of different spots were chromatographed in different solvents to establish identity or non-identity of the adducts (Moorthy et al., 1996).

Statistical Analyses

Statistical analyses were performed using one-way analyses of variance (ANOVA) or Students' t-test.

Results

Exposure of SMCs to 0.3 μM BP gave rise to eight ^{32}P-postlabeled DNA adduct spots that were not observed in cells treated with the vehicle DMSO (Figure 1A and B). Of these, adducts 2 and 3 were major, while adducts 1, 4, 5, 6, 7, and 8 were minor. Treatment of SMCs with the BP metabolites 3-OH-BP (Figure 1C) and BPQ (Figure 1D) also gave rise to multiple adducts. Adduct 1 of BPQ was in similar chromatographic location to the major 3-OH-BP adduct (spot 1) (compare Figure 1C and D). Our studies showed that total BP-DNA adduct formation was concentration-dependent, with total adduct RAL values increasing from 3.78 x 10^8 for the 0.03 μM concentration to 169.9 x 10^8 for the 3 μM concentration (Table 1). Similarly, when individual BP-DNA adducts were analyzed, a clear concentration-dependent response was observed for adducts 2, 4, 5, 7, and 8 (Table 1). At the 0.03 μM concentration, only adducts 2 and 3 were detectable. On the other hand, adduct 1 was present when SMCs were treated with 0.3 μM BP but undetectable when cells were exposed to 3 μM BP (Table 1). Concentration dependence of adduct formation was also noticed when SMCs were treated with 3-OH-BP (Table 2) or BPQ (Table 3). While adducts induced by 3-OH-BP were detected at 0.03 μM concentration, this was not the case for BPQ, wherein adducts were detected only when 0.3 μM BPQ was used. Taken together, quantitative adduct analyses revealed 3-OH-BP to be more genotoxic than BPQ.

Because CYP1 enzymes play important roles in the metabolic activation of PAHs, we tested the hypothesis that pre-treatment of cells with specific CYP1 inhibitors would prevent DNA adduct formation by BP and its metabolites. We tested three agents for their ability to inhibit aryl hydrocarbon-inducible AHH activity, which is catalyzed by

CYP1A1 as well as CYP1B1 (Kerzee and Ramos, 2001). While ellipticine specifically inhibits CYP1A1 (Annas at al., 2000), α-NF selectively inactivates CYP1B1 (Shimada et al., 1998; Kleiner et al., 2002), and EP is an inhibitor of CYP1B1 in different species, including human (Shimada et al., 1998) and mouse (Alexander et al., 1999).

As shown in our recent study (Moorthy et al., 2002), BP (3 μM) treatment of SMCs caused marked induction of AHH activity. Pre-treatment of cells with ellipticine or α-NF, at the indicated concentrations, prior to BP treatment did not modulate AHH activity. However, pretreatment with EP dramatically inhibited hydrocarbon-inducible AHH activity (Moorthy et al., 2002). In order to confirm that BP induced CYP1B1, but not CYP1A1, vascular SMCs exposed to BP were subjected to Western analyses. As shown in Figure 2A, uninduced cells displayed basal expression of CYP1B1, but not CYP1A1 (Figure 2B). BP at a concentration of 0.03 μM, caused a 1.5-fold induction of CYP1B1 apoprotein, compared to DMSO-treated controls, as determined by densitometric scanning of the blots (Figure 2A). At 0.3 μM BP concentration, the induction was about two-fold higher than control. The extent of CYP1B1 induction in cells treated with 3 μM BP was similar to that observed in cells exposed to 0.3 μM BP (Figure 2A). Pre-treatment of SMCs with EP (1 μM), followed by BP (0.3 μM), resulted in a 20% decrease in the expression of CYP1B1, compared to those that were treated with 0.3 μM BP only (Figure 2A). In contrast, CYP1A1 was not detectable at any BP concentration (Figure 2B).

Since α–NF is known to selectively inhibit CYP1B1 (Shimada et al., 1998; Kleiner et al., 2002), we studied the effect of this compound on CYP1B1 apoprotein expression to determine whether the lack of inhibition of BP-inducible AHH activities in

α-NF + BP-treated cells (Moorthy et al., 2002) was due to induction of CYP1B1 protein by α-NF, which is a partial agonist of the Ah receptor (AHR). As shown in Figure 2C, α–NF elicited significant increase in CYP1B1 apoprotein expression, being 1.4-, 1.7-, and 1.6-fold higher, respectively, in cells that had been treated with 10, 50, and 100 nM α–NF concentrations, compared to DMSO-treated controls. Western analyses of standard rat CYP1B1 proteins yielded concentration-dependent increases in band intensities (Figure 2D), suggesting the validity of the Western blotting approach to quantitatively assess CYP1B1 levels in SMCs.

Due to the strong response observed in the BP-induced AHH activities by EP (Moorthy et al., 2002), we examined the effects of this inhibitor on BP-induced DNA adduct levels. Treatment of SMCs with EP, a specific CYP1B1 inhibitor, followed by BP resulted in strong inhibition (~90%) of total adduct and individual adduct levels (Table 4). There were no qualitative changes in BP-DNA adduct profiles in cells treated by EP, and EP itself did not induce any adducts (not shown). While adduct patterns were quite reproducible in repeat experiments, we did notice some variation in levels of BP-DNA adducts when quantitative data from independent experiments (Tables 1 and 4) were compared. This was probably due to a combination of inter-experimental variability in cell treatments, DNA isolation, and ^{32}P-postlabeling. Pretreatment with EP also almost completely inhibited adducts induced by 3-OH-BP or BPQ (Figure 3).

In order to identify the metabolites that are responsible for DNA adduct formation by BP, re-and co-chromatography experiments were conducted. Re-chromatography experiments (Figure 4) revealed adduct 2 of BP, adduct 1 of 3-OH-BP, and adduct 1 of BPQ adduct to be identical to each other. Similarly, BP adduct 3 and BPQ adduct 2 were

identical (Figure 5). Since the levels of the major adducts 2 and 3 in DNA of SMCs exposed to 3 μM BP, expressed as RAL x 10^8, were 149.6 (4.9 fmol/μg DNA) and 13.2 (0.42 fmol/μg DNA), respectively (Table 1), we did not attempt to perform mass spectral analyses for identification of adduct structures, as at least adduct levels in the pmol range would be required to identify structures of unknown adducts, in the absence of synthetic standards (Tretyakova et al., 2002).

Discussion

The major goal of this investigation was to identify the mechanisms involved in the activation of BP to genotoxic metabolites in SMCs, a response that may contribute to BP-mediated atherogenesis. The observation (Figure 1) showing concentration-dependent formation (Tables 1-3) of multiple DNA adducts in SMCs exposed to BP and its metabolites indicates that BP, 3-OH-BP, and BPQ are potent genotoxic agents in these cells. In fact, these studies indicate the presence in the SMCs of an enzymatic system that activates PAHs (Bowes et al., 1996; Shimada et al., 1996; Moorthy et al., 2002). The rationale for choosing the concentration range (0.03 to 3 μM) of BP was based on previous studies (Lu and Ramos, 1998) showing that treatment of mouse vascular SMCs with 3 μM BP results in activation of L1Md retrotransposon, which when coupled to DNA damage and inhibition of DNA repair, may be linked to the atherogenic response of BP.

The augmentation of AHH activities (Moorthy et al., 2002) in cells treated with BP was most likely due to induction of CYP1B1, since the increases in AHH activities were accompanied by induction of CYP1B1 (Figure 2A), but not CYP1A1 apoprotein (Figure 2B). The observation that the CYP1B1 inhibitor EP, but not ellipticine or α-NF, dramatically inhibited BP-inducible AHH activity (Moorthy et al., 2002), suggested that CYP1B1 was the major enzyme responsible for PAH metabolism in SMCs. Although α-NF also has been shown to markedly inhibit CYP1B1 *in vitro* (Shimada et al., 1998), the fact that this compound did not inactivate BP-inducible AHH activity in intact cells may have been due to differences in the experimental approaches between Shimada et al.'s and our laboratories. While Shimada et al. (1998) performed inhibition experiments *in*

vitro using bacterial membranes containing expressed human CYP1B1, we studied the effect of α-NF pretreatment on BP-inducible AHH activity in intact mouse SMCs. In fact, Alexander et al. (1999) have postulated that a major distinction between inhibition of 7,12-dimethylbenz[*a*]anthracene (DMBA) metabolism in microsomes and intact cells is that in the latter there is a steady state between rate of CYP1B1 inhibition and resynthesis. Since α-NF is a partial agonist of the AHR (Wilhelmsson et al., 1994), it is possible that the inhibitory property of α-NF may have in part been counteracted by the possible inductive effect of α-NF on CYP1B1, resulting in a lack of inhibition of AHH activity in the BP + α-NF-exposed cells.

Although BP-inducible AHH activity was markedly inhibited (~ 80%) in cells pre-treated with EP (Moorthy et al., 2002) the observation that EP+ BP treatment resulted only in a modest attenuation (~20%) of CYP1B1 apoprotein expression, compared to cells treated with BP alone could have been due to the recovery of CYP1B1 resynthesis by EP. This hypothesis is supported by the findings of Alexander et al. (1999) showing marked induction of CYP1B1 by EP in 10T1/2 cells several hours following inhibition of metabolism. The strong inhibition of AHH activity by EP, on the other hand, may have been due to mechanism-based inactivation of CYP1B1 catalytic activity by EP (Alexander et al., 1999). We made similar observations in studies pertaining to the effects in rats of the CYP1A1 inhibitor 1-aminobenzotriazole, which induced CYP1A1 apoprotein on the one hand, and inhibited the catalytic activity on the other (Moorthy et al., 2000).

Our finding that EP markedly suppressed DNA adduct formation by BP (Table 5), 3-OH-BP, and BPQ (Figure 3), demonstrates a pivotal role for CYP1B1 in the metabolic

activation of BP, 3-OH-BP, and BPQ to genotoxic metabolites. These results support earlier findings that CYP1B1 contributes to PAH activation (Bowes et al., 1996; Kerzee and Ramos, 2001; Shimada et al., 1996). The fact that the major BP adduct 2, adduct 1 of 3-OH-BP, and adduct 2 of BPQ were identical (Figure 4) indicates that 3-OH-BP was the proximate metabolite of BP that was further converted to BPQ, which in turn was activated to ultimate DNA-binding intermediate(s). Furthermore, the observation that the major BP adduct 3 was identical to adduct 1 of BPQ (Figure 5) indicates that BP quinones play a major role in the genotoxicity of BP.

BP quinones are formed by one electron oxidation of BP to radical cations, which have been implicated in the carcinogenicity (Cavalieri and Rogan, 1995), mutagenicity (Ramos and Parish, 1995), and atherogenicity (Kerzee and Ramos, 2000; 2001; Miller et al., 2000) of BP. The inhibition of DNA adduct formation of BP and 3-OH-BP by EP suggests that CYP1B1 may have converted BP and 3-OH-BP to DNA-binding intermediates by a series of one electron oxidations. Joseph and Jaiswal (1994) have shown that BPQ, by itself, does not bind to DNA, but has to be reduced by one electron reduction to BP semiquinone, which is the ultimate genotoxic metabolite. The fact that DNA adduct formation by BPQ was also dramatically suppressed suggests that CYP1B1 may have also catalyzed the one electron reduction of BPQ to BP semiquinone (Joseph and Jaiswal, 1994). Alternatively, EP may also have inhibited NADPH P450 reductase, which may catalyze the one electron reduction of BPQ to BP semiquinone, resulting in inhibition of adduct formation.

We previously reported that administration of BP to mice (Moorthy and Randerath, 1997) and rats (Moorthy et al., 1994) leads to production of multiple DNA

adducts in liver and other tissues. In mouse liver, the major BP adducts were derived from BP-7,8-dihydrodiol, suggesting that BPDE was the ultimate carcinogenic metabolite of BP in mice (Moorthy and Randerath, 1997), which was in contrast to the formation of BP quinones as the major genotoxic metabolites of BP in SMCs in the present studies. Since EH also plays an important role in the formation of BPDE, we investigated whether the lack of BPDE adducts in SMCs could have been due to the absence of EH expression in these cells. Interestingly, EH activites were detectable in DMSO-treated SMCs (2.95 ± 0.35 nmol/min/mg protein), and were induced 1.4-, 2.0-, and 2.8-fold by treatment of cells with 0.03, 0.3, and 3 μM BP, respectively, suggesting that BPQ adducts were preferentially formed in the SMCs despite the presence of catalytically active EH. Although 3-OH-BP and BPQ were proximate genotoxic intermediates, the fact that they were less genotoxic than BP in SMCs could have been due to rapid detoxication of these compounds by enzymes other than CYP1B1.

We recently reported that the PAH MC induced DNA adducts in SMCs (Moorthy et al., 2002). Although total DNA adduct levels in SMCs exposed to 3 μM MC (RAL x 10^8) (Moorthy et al., 2002) were comparable to those produced by 3 μM BP [(RAL x 10^8 = 169.9 (Table 1)], distinct differences were observed in adduct patterns and the number of adduct spots induced by these compounds, with MC and BP inducing the formation of 12 and 8 adducts, respectively. While the major BP adducts were slow-moving (non-polar) on TLC plates (Figure 1), MC treatment of SMCs elicited adduct patterns wherein 4 adducts were slow-moving (non-polar) and the remaining 8 were fast-moving (polar), suggesting the existence of at least 2 groups of adducts, presumably being formed from different pathways of metabolic activation of MC (Moorthy et al, 2002). This was in

contrast to BP, which produced adducts that were mainly derived from BP quinones (Figures 1,4,5). Thus, the mechanisms of BP and MC adduct formation appear to be different. In fact, our recent study showing mechanistic differences in the metabolic activation of these compounds to genotoxic metabolites in mouse liver (Kondraganti et al., 2002) lends credence to this hypothesis. The observation that BP (Table 4) as well as MC adducts (Moorthy et al., 2002) were markedly inhibited by EP suggests that CYP1B1 played an important role in the metabolic activation of both the compounds to DNA-binding metabolites in the SMCs.

DNA adduct formation represents a key event in initiation of carcinogenesis (Gelboin, 1990), and, if not repaired, could lead to gene mutations and development of tumors. Because of the close parallelism between carcinogenesis and atherogenesis (Ramos and Parish, 1995; Majesky et al., 1983; Ross et al., 2001), it is likely that DNA adduct formation in SMCs contributes to atherogenesis by PAHs. In fact, increase in unscheduled DNA synthesis and inhibition of DNA repair in SMCs treated with PAHs has recently been reported (Lu et al., 2000), suggesting that DNA damage in SMCs by PAHs might, in fact, contribute to atherogenesis. Although the role of DNA adducts in the development of atherogenesis is not well understood, the mutation theory of atherogenesis (Ramos and Parish, 1995) suggests that DNA adduct formation represents the initiation step of atherogenesis. We recently reported that BP, 3-OH-BP, and BPQ activate L1Md retrotransposon in vascular SMCs, which when coupled to DNA damage and inhibition of DNA repair may be part of the atherogenic response to BP and other PAHs (Lu et al., 2000). Collectively, these findings support the hypothesis that gentoxicity of BP and its metabolites play a causal role in PAH-induced atherogenesis.

Further studies are needed to study the specific role(s) of PAH-DNA adducts in the atherogenic processes.

References

Alexander DL, Zhang L, Foroozesh M, Alworth WL and Jefcoate CR (1999) Metabolism-based polycyclic aromatic acetylene inhibition of CYP1B1 in 10T1/2 cells potentiates aryl hydrocarbon receptor activity. *Toxicol Appl Pharmacol* **161:** 123-139.

Annas A, Granberg AL and Brittebo EB (2000) Differential response of cultured human umbilical vein and artery endothelial cells to Ah receptor agonist treatment: CYP-dependent activation of food and environmental mutagens. *Toxicol Appl Pharmacol* **169**: 94-101.

Bond JA, Gown AM, Yang HL, Benditt RP and Juchau MR (1981) Further investigations on the capacity of polynuclear aromatic hydrocarbons to elicit atherosclerotic lesions. *J Toxicol Environ Health* **7:** 327-335.

Bowes RC III, Parrish AR, Steinberg MA, Willet KL, Zhao W, Savas U, Jefcoate CR, Safe SH and Ramos KS (1996) Atypical cytochrome P450 induction profiles in glomerular mesangial cells at the mRNA and enzyme level. Evidence for CYP1A1 and CYP1B1 expression and their involvement in benzo[*a*]pyrene metabolism. *Biochem Pharmacol* **52**: 587-595.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254.

Cavalieri EL and Rogan EG (1995) Central role of radical cations in metabolic activation of polycylic aromatic hydrocarbons. *Xenobiotica* **25**: 677-688.

Denissenko MF, Pao A, Tang M and Pfeifer GP (1996) Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hot spots in P53. *Science* **274**: 430-432

Gelboin HV (1990) Benzo[alpha]pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes. *Physiol Rev* **60:** 1107-1166.

Giachelli CM, Majesky MW and Schwartz SM (1991) Developmentally regulated cytochrome P4501A1 expression in cultured rat vascular smooth muscle cells. *J Biol Chem* **266**: 3981-3986.

Guengerich FP (1988) Roles of cytochrome P-450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res* **48**: 2946-2954.

Guengerich FP and Mason PS (1980) Alcohol dehydrogenase-coupled spectrophotometric assay of epoxide hydrolase activity. *Anal Biochem* **104:** 445-451.

Izzotti A, De Flora S, Petrilli GL, Gallagher J, Rojas M, Alexandrov K, Bartsch H and Lewtas J (1995) Cancer biomarkers in human atherosclerotic lesions: detection of DNA adducts. *Cancer Epidemiol, Biomarkers Prev* **4**: 105-110.

Joseph P and Jaiswal AK (1994) NAD(P)H:quinone reductase1 (DT diaphorase) specifically prevents the formation of benzo[a]pyrene quinone-DNA adducts generated by cytochrome P4501A1 and P450 reductase. *Proc Natl Acad Sci USA* **91**: 8413-8417.

Kerzee JK and Ramos KS (2000) Activation of c-Ha-ras by benzo(a)pyrene in vascular smooth muscle cells involves redox stress and aryl hydrocarbon receptor. *Mol Pharmacol* **58**: 152-158.

Kerzee JK and Ramos KS (2001) Constitutive and inducible expression of Cyp1a1 and Cyp1b1 in vascular smooth muscle cells: Role of the Ahr bHLH/PAS transcription factor. *Circulation Res* **89:** 573-582.

Kleiner HE, Vulimiri SV, Reed MJ, Uberecken A, and DiGiovanni J (2002) Role of cytochrome P450 1a1 and 1b1 in the metabolic activation of 7,12-

dimethylbenz[a]anthracene and the effects of naturally occurring furanocoumarins on skin tumor initiation. *Chem Res Toxicol* **15**:226-35.

Kondraganti SR, Fernandez-Saluguero, P, Gonzalez FJ, Ramos KS, Jiang W and Moorthy B (2003). Polycyclic aromatic hydrocarbon-inducible DNA adducts: evidence for Ah receptor-independent mechanisms of metabolic activation in mouse liver *in vivo*. *Int J Cancer* **103**: 5-11.

Lu KP, Hallberg LM, Tomlinson J and Ramos KS (2000) Benzo(a)yrene activates L1Md retrotransposon and inhibits DNA repair in vascular smooth muscle cells. *Mutat Res* **454**: 35-44.

Lu KP and Ramos KS (1998) Identification of genes differentially expressed in vascular smooth muscle cells following benzo[a]pyrene challenge: Implications for chemical atherogenesis. *Biochem Biophys Res Commun* **253**: 828-833.

Majesky MW, Yang H-Y, Benditt E.P and Juchau MR (1983) Carcinogenesis and atherogenesis: differences in monooxygenase inducibility and bioactivation of benzo[a]pyrene in aortic and hepatic tissues of atherosclerosis-susceptible versus resistant pigeons. *Carcinogenesis* **4**: 647-652.

Melendez-Colon VJ, Luch A, Seidel A and Baird WM (2000) Formation of stable DNA adducts and apurinic sites upon metabolic activation of bay and fjord region polycyclic aromatic hydrocarbons in human cell cultures. *Chem Res Toxicol* **13**: 10-17.

Miller KP, Chen YH, Hastings VL, Bral CM and Ramos KS (2000). Profiles of antioxidant/electrophile response element (ARE/EpRE) nuclear protein binding and c-

Ha-ras transactivation in vascular smooth muscle cells treated with oxidative metabolites of benzo[*a*]pyrene. *Biochem Pharmacol* **60**: 1285-1296.

Moorthy B, Miller, KP, Jiang W and Ramos KS (2002) The atherogen 3-methylcholanthrene induces multiple DNA adducts in mouse aortic smooth muscle cells: role of cytochrome P4501B1. *Cardiovas Res* **53**:1002-1009.

Moorthy B and Randerath K (1997) Pentachlorophenol enhances 9-hydroxybenzo[a]pyrene-induced hepatic DNA adduct formation in vivo and inhibits microsomal epoxide hydrolase and glutathione S-transferase activities in vitro: likely inhibition of epoxide detoxication by pentachlorophenol. *Arch Toxicol* **70**: 696-703.

Moorthy B, Sriram P, Pathak DN, Bodell WJ and Randerath K (1996) Tamoxifen metabolic activation: Comparison of DNA adducts formed by microsomal and chemical activation of tamoxifen and 4-hydroxytamoxifen with DNA adducts formed in vivo. *Cancer Res* **56**: 53-57.

Moorthy B, Sriram P and Randerath K (1994) Chemical structure- and time-dependent effects of polycyclic aromatic hydrocarbons-type inducers on rat liver cytochrome P450, DNA adducts, and I-compounds. *Fundam Appl Toxicol* **22**: 549-560.

Moorthy B, Parker KM, Smith CV, Bend JR and Welty SE (2000) Potentiation of oxygen-induced lung injury in rats by the mechanism-based cytochrome P-450 inhibitor, 1-aminobenzotriazole. *J Pharmacol Exp Ther* **292**: 553-560.

Nebert DW and Gelboin HV (1968) Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J Biol Chem* **243**: 6242-6249.

Paigen B, Holmes PA, Morrow A and Mitchell D (1986) Effect of 3-methylcholanthrene on atherosclerosis in two congenic strains of mice with different susceptibilities to methylcholanthrene-induced tumors. *Cancer Res* **46**: 3321-3324.

Ramos KS and Parrish AR (1995) Growth-related signaling as a target of toxic insult in vascular smooth muscle cells: implications in atherogenesis. *Life Sci* **57:** 627-635.

Ramos KS and Cox LR (1993) Aortic endothelial and smooth muscle cell cultures (1993) *In Methods in Toxicology*: *In Vitro Biological Systems* (Tyson C and Frazier J, eds) pp 159-168, Academic Press, San Diego.

Reddy MV and Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis* **7**: 1543-1551.

Ross JS, Stagliano NE, Donovan MJ, Breitbart RE and Ginsburg GS (2001) Atherosclerosis and cancer: common molecular pathways of disease development and progression. *Ann NY Acad Sci* **947**: 271-292.

Salama SA, Au WW, Hunter GC, Sheahan RG, Badary OA, Abdel-Naim AB and Hamada FM (2002) Polymorphic metabolizing genes and susceptibility to atherosclerosis among cigarette smokers. *Environ Mol Mutagen* **40**: 153-160.

Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP and Sutter TR (1996) Activation of chemically diverse procarcinogens by human cytochrome P4501B1 *Cancer Res* **56**: 2979-2984.

Shimada T, Yamazaki H, Foroozesh M, Hopkins NE, Alworth WL and Guengerich FP (1998) Selectivity of polycyclic inhibitors for human cytochrome P450s 1A1, 1A2, and 1B1 *Chem Res Toxicol* **11**: 1048-1056.

Stegeman JJ, Hahn ME, Weisbrod R, Woodin BR, Joy JS, Najibi S and Cohen RA (1995) Induction of cytochrome P4501A1 by arylhydrocarbon receptor agonists in porcine aorta endothelial cells in culture and cytochrome P4501A1 activity in intact cells. *Mol Pharmacol* **47**: 296-306.

Thirman MJ, Albrecht JH, Krueger, MA, Erickson RR, Cherwitz DL, Park SS, Gelboin HV and Holtzman JL (1994) Induction of cytochrome CYP1A1 and formation of toxic metabolites of benzo[a]pyrene by rat aorta: A possible role in atherogenesis. *Proc Natl Acad Sci USA* **91**: 5397-5401.

Tretyakova N, Matter B, Jones R and Shallop A (2002) Formation of benzo[a]pyrene diol epoxide-DNA adducts at specific guanines within *K-ras* and *p53* sequences: Stable isotope-labeling mass spectrometry approach. *Biochemistry* **41**: 9535-9544.

Wilhelmsson A, Whitelaw ML, Gustafsson JA, Poellinger L (1994) Agonistic and antagonistic effects of alpha-naphthoflavone on dioxin receptor function. Role of the basic region helix-loop-helix dioxin receptor partner factor Arnt. *J Biol Chem* **269**: 19028-19033.

Foot notes

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TABLE 1

Effect of different concentrations of BP on DNA adduct levels

Adduct #	BP (0.03 μM)	BP (0.3 μM)	BP (3 μM)
1	<0.02.	3.69 ± 0.1	N.D.
2	0.62 ± 0.1	63.70 ± 7.6	149.63 ± 22.8
3	3.16 ± 0.5	28.52 ± 3.4	13.16 ± 2.4
4	<0.02.	0.85 ± 0.1	1.68 ± 0.4
5	<0.02.	1.51 ± 0.3	2.57 ± 0.5
6	<0.02.	2.58 ± 0.3	1.41 ± 0.2
7	<0.02.	0.30 ± 0.04	0.45 ± 0.1
8	<0.02.	0.16 ± 0.02	0.39 ± 0.02
Total	3.78 ± 0.5	101.31 ± 9.4	169.9 ± 28.4

Adduct values represent the means ± S.D. of 3 independent experiments and are expressed as RAL x 10^8. BP treatment of cells, DNA isolation, and ^{32}P-postlabeling were carried out as described in Materials and Methods. The limit of detection, expressed as RAL x 10^8, was 0.02.

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TABLE 2

Effect of different concentrations of 3-OH-BP on DNA adduct levels

Adduct #	3-OH-BP (0.03 μM)	3-OH-BP (0.3 μM)	3-OH-BP (3 μM)
1	2.52 ± 0.3	13.06 ± 1.9	13.90 ± 1.3
2	1.84 ± 0.3	<0.02.	<0.02.
3	<0.02.	0.63 ± 0.1	1.78 ± 0.3
4	<0.02.	0.44 ± 0.04	0.61 ± 0.04
5	<0.02.	<0.02.	1.48 ± 0.2.
Total	4.36 ± 0.3	14.74 ± 1.8	17.18 ± 1.6

Adduct values represent the means ± S.D. of 3 independent experiments and are expressed as RAL x 10^8. 3-OH-BP treatment of cells, DNA isolation, and ^{32}P-postlabeling were carried out as described in Materials and Methods. The limit of detection, expressed as RAL x 10^8, was 0.02.

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TABLE 3.

Effect of different concentrations of BPQ on DNA adduct levels

Adduct #	BPQ (0.03 μM)	BPQ (0.3 μM)	BPQ (3 μM)
1	<0.02	0.14 ± 0.03	2.84 ± 0.1
2	<0.02.	0.11 ± 0.01	2.58 ± 0.1
Total		0.25 ± 0.05	5.42 ± 0.2

Adduct values represent the means ± S.D. of 3 independent experiments and are expressed as RAL x 10^8. BPQ treatment of cells, DNA isolation, and ^{32}P-postlabeling were carried out as described in Materials and Methods. The limit of detection, expressed as RAL x 10^8, was 0.02.

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TABLE 4

Effect of EP on BP-DNA adduct levels

Adduct #	BP (0.3 μM)	EP + BP (0.3 μM)
1	1.70 ± 0.1	0.15[a] ± 0.01
2	38.90 ±2.8	6.43[a] ± 0.9
3	13.80 ± 4. 1	1.20[a] ± 0.3
4	0.59 ± 0.1	0.30[a] ± 0.03
5	1.19 ± 0.1	0.24[a] ± 0.03
6	1.05 ± 0.2	0.30[a] ± 0.04
7	0.83 ± 0.1	0.23[a] ± 0.04
8	0.56 ± 0.1	0.16[a] ± 0.01
Total	58.67 ± 6.4	9.01[a] ± 1.1

Adduct values represent the means ± S.D. of 3 independent experiments and are expressed as RAL x 10^8. BP (0.3 μM), EP (1 μM), and EP (1 μM) + BP (0.3 μM) treatment of cells, DNA isolation, and ^{32}P-postlabeling were carried out as described in Materials and Methods. [a], Statistically significant differences between BP and EP+ BP samples at $P < 0.05$, as determined by Students' t-test.

FIGURE LEGENDS

Figure 1. Effect of BP and its metabolites on DNA adduct formation in SMCs. SMCs were exposed to DMSO (A), BP (0.3 μM) (B), 3-OH-BP (0.3 μM) (C), or BPQ (0.3 μM) (D), as described under Materials and Methods, and DNA adducts analyzed by ^{32}P-postlabeling. TLC plates were autoradiographed for 16 h at room temperature.

Figure 2. Representative Western blot showing effect of BP on CYP1B1 (A) and CYP1A1 (B) protein expression in vascular SMCs. SMCs were treated with DMSO or BP at the indicated concentrations and Western blotting using CYP1A1 or 1B1 antibody was performed using total cellular protein (20 μg), as described in Materials and Methods. In order to the study the effect of EP, cells were treated with 1 μM EP 1 hr prior to treatment with BP (0.3 μM), and Western analyses was performed. As shown in the figure (A), basal and inducible CYP1B1, but not CYP1A1 (B), was detectable. p.c. (positive control), liver microsomes (5 μg protein) from rats treated with MC that show two bands (CYP1A1 and 1A2) in the presence of monoclonal antibodies to CYP1A1. Panel C shows the effect of α-NF at the indicated concentrations on CYP1B1 expression in SMCs. Panel D depicts Western analyses of rat CYP1B1 standards, wherein enhancements in CYP1B1 band intensities were observed when increasing concentrations of rat CYP1B1 standards (0.1 – 0.8 pmols) were loaded on the gel. CYP1B1 levels, estimated from the CYP1B1 standard curve, are expressed as pmol/mg protein.
Data at the bottom of each lane in panels A and C represent mean ± SD (n=5, panel A; n=3, panel C) of CYP1B1 levels (pmol/mg protein). [a, b, c, d,] Statistically significant differences ($P < 0.05$) between DMSO- and BP-treated cells, EP + BP- and BP-treated

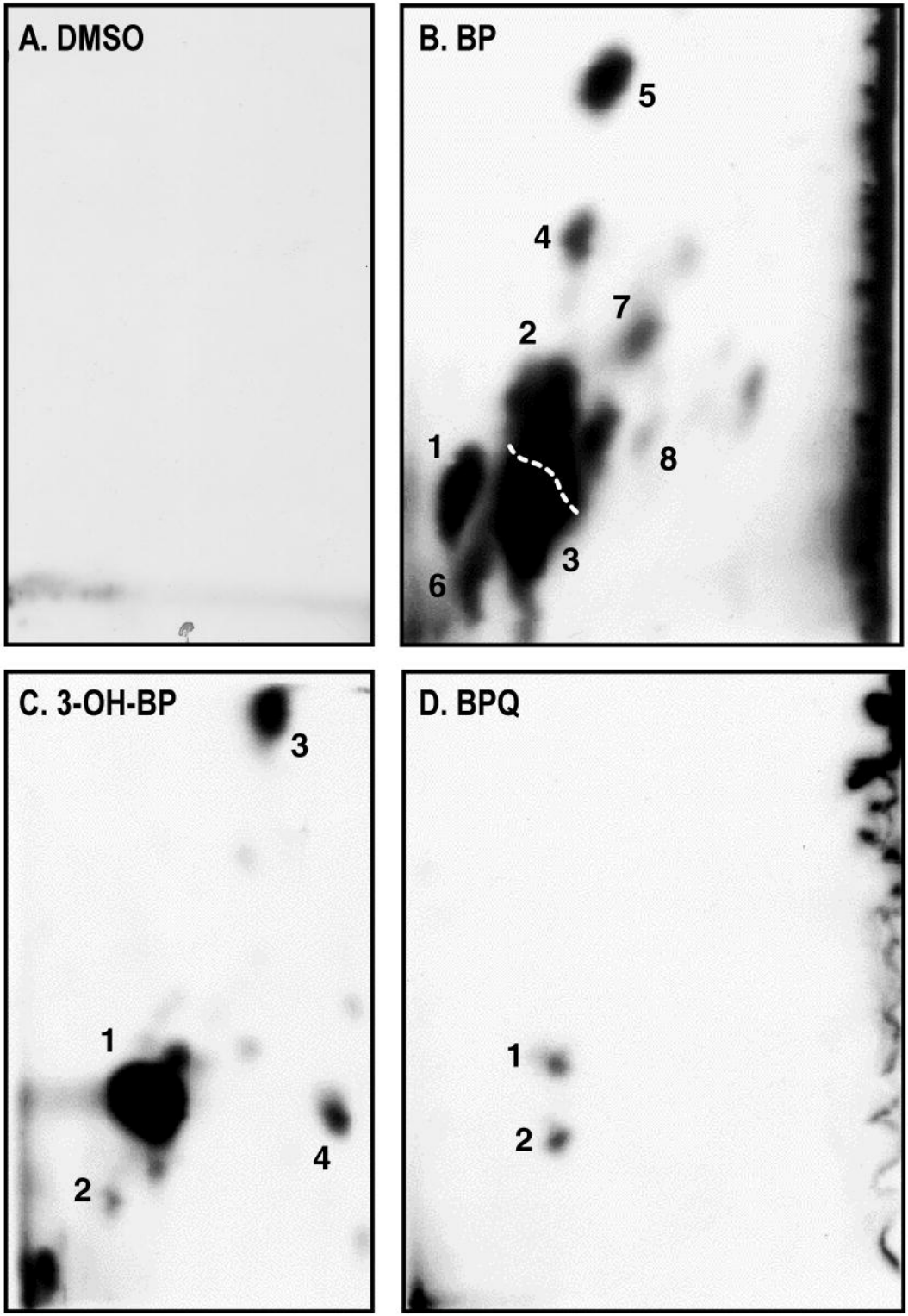
cells, DMSO- and EP+ BP-treated cells, and DMSO- and α-NF-treated cells, respectively, as determined by one-way ANOVA, followed by Tukey's post hoc test.

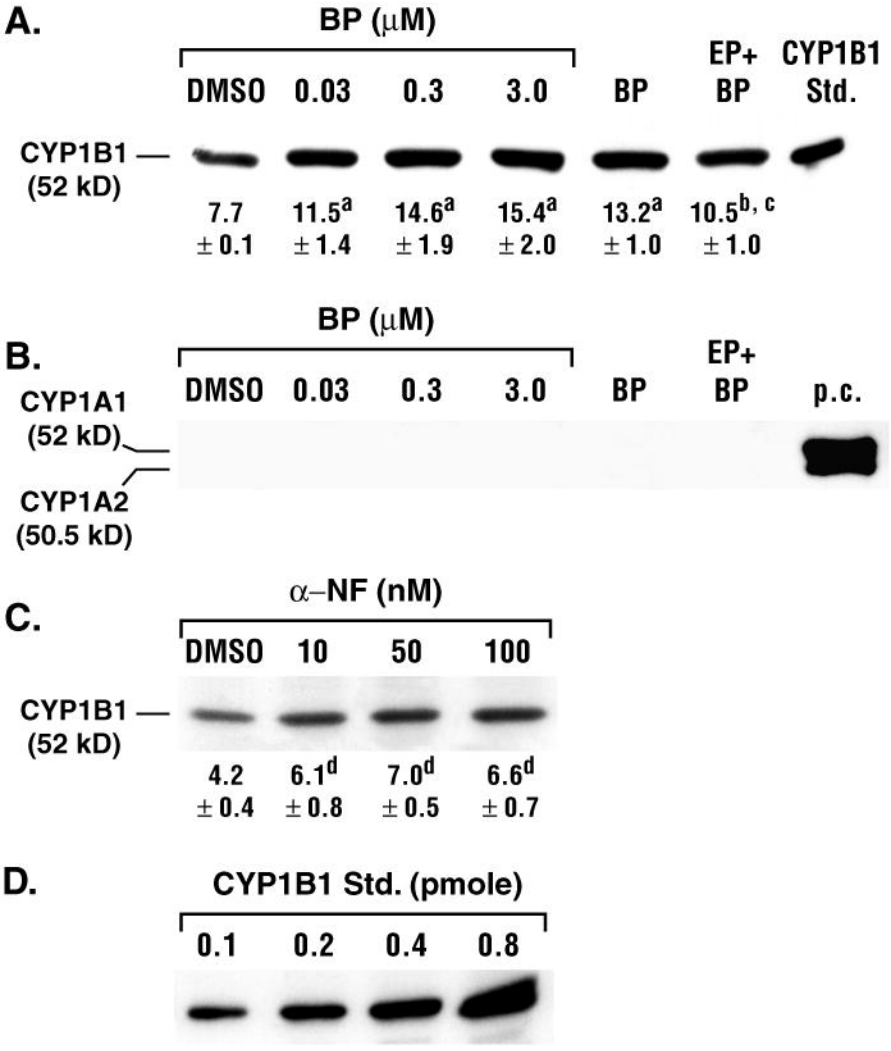
Figure 3. Effect of EP on DNA adduct formation by BP metabolites. SMCs were exposed to DMSO or EP (1 μM) for 1 h, followed by treatment with 3-OH-BP (0.3 μM) or BPQ (0.3 μM) for an additional 24 h. DNA adducts were analyzed by ^{32}P-postlabeling. TLC plates were autoradiographed for 16 h at room temperature.

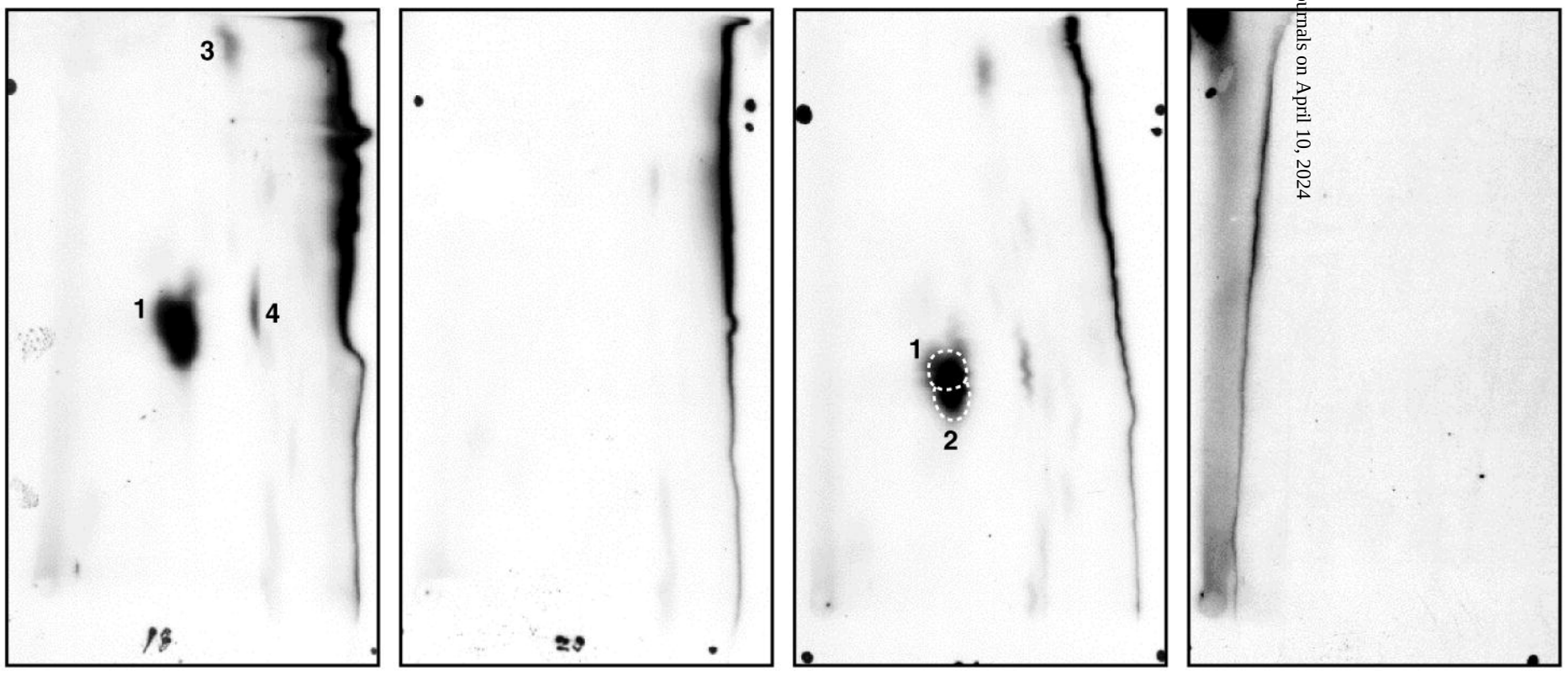
Figure 4. Re-chromatography of major adducts from BP, 3-OH-BP, and BPQ. Major DNA adduct spots from BP, 3-OH-BP, and BPQ were eluted from the TLC plates with 1:1 isopropanol: 4N ammonium hydroxide, and 150 cpm of each spot was applied to PEI-TLC plates, and chromatographed one-dimensionally in different solvents A-E. A, 4.05 M lithium formate, 7.6 M urea, pH 3.3; B, 0.8 M sodium phosphate, 0.45 M Tris HCl, 7.6 M urea, pH 8.2; C, 0.8 M sodium phosphate, 6.3 M urea, pH 6.2; D, 0.8 M lithium chloride, 0.45 M Tris HCl, 7.6 M urea pH 8.2; E, 2-propanol/4 N ammonia (1:2 v/v). Lane a, spot 2 of BP; lane b, spot 3 of BP; lane c, spot 1 of 3-OH-BP; lane d, spot 1 of BPQ; and lane e, spot 2 of BPQ. As can be seen in the figure, spot 2 of BP, spot 1 of 3-OH-BP, and spot 1 of BPQ had identical chromatographic mobilities. Similarly, spot 3 of BP and spot 2 of BPQ appeared to be identical.

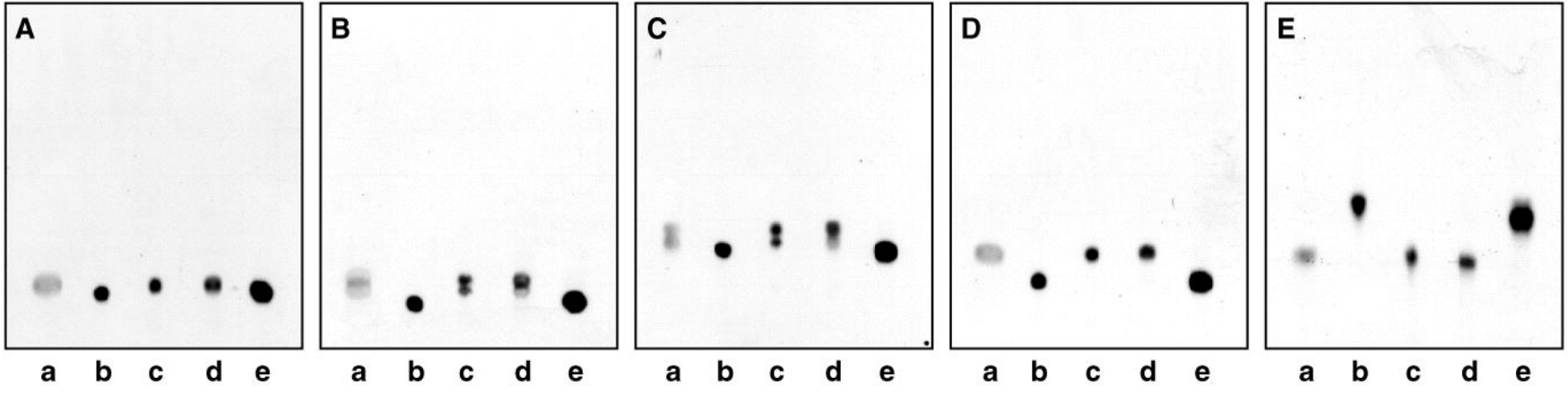
Figure 5. Re- and co-chromatographic comparison of major BP adduct 3 and BPQ adduct 2. Major DNA adduct spot 3 from BP and spot 2 from BPQ were eluted from TLC plates with 1:1 isopropanol: 4N ammonium hydroxide, and 150 cpm of each spot was

applied to PEI-TLC plates, and chromatographed one-dimensionally in different solvents A-D. A, 0.8 M sodium phosphate, 0.45 M Tris HCl, 7.6 M urea, pH 8.2; B, 4.05 M lithium formate, 7.6 M urea, pH 3.3; C, 0.3 M Tris-HCl, 0.3 M boric acid, 0.8 M NaCl, 6 mM EDTA, 4.8 M urea, pH. 8.0; and D, 2-propanol/4 N ammonia (1:2 v/v). Lane a, adduct 3 of BP; lane b, adduct 2 of BPQ; and lane c, mixture of adduct 3 of BP and adduct 2 of BPQ.


A. DMSO
B. BP
5
4
7
2
1
8
3
6
C. 3-OH-BP
3
1
4
2
D. BPQ
1
2


A.
BP (μM)
DMSO
0.03
0.3
3.0
BP
EP+ BP
CYP1B1 Std.
CYP1B1 (52 kD)
7.7 ± 0.1
11.5[a] ± 1.4
14.6[a] ± 1.9
15.4[a] ± 2.0
13.2[a] ± 1.0
10.5[b, c] ± 1.0
B.
BP (μM)
DMSO
0.03
0.3
3.0
BP
EP+ BP
p.c.
CYP1A1 (52 kD)
CYP1A2 (50.5 kD)
C.
α–NF (nM)
DMSO
10
50
100
CYP1B1 (52 kD)
4.2 ± 0.4
6.1[d] ± 0.8
7.0[d] ± 0.5
6.6[d] ± 0.7
D.
CYP1B1 Std. (pmole)
0.1
0.2
0.4
0.8




A
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