Role of Cytochrome P4501B1 in Benzo[a]pyrene Bioactivation to DNA-Binding Metabolites in Mouse Vascular Smooth Muscle Cells: Evidence from 32P-Postlabeling for Formation of 3-Hydroxybenzo[a]pyrene and Benzo[a]pyrene-3,6-quinone as Major Proximate Genotoxic Intermediates

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ABSTRACT

Benzo[a]pyrene (BP), a polycyclic aromatic hydrocarbon (PAH), is a potent atherogen and carcinogen in laboratory animals. Since genotoxic mechanisms may contribute to the development of atherosclerosis by PAHs, we have tested the hypotheses that: 1) BP induces DNA adducts in mouse aortic smooth muscle cells (SMCs); 2) 3-hydroxybenzo[a]pyrene (3-OH-BP) and benzo[a]pyrene-3,6-quinone (BPQ) are proximate genotoxic metabolites; and 3) 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 32P-postlabeling. In some experiments, cells were pretreated 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-treated cells. Reand cochromatography 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 (Ross et al., 2001; Salama et al., 2002). 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 Parrish, 1995). Polycyclic aromatic hydrocarbons (PAHs) are important constituents of cigarette smoke and are major chemotherapeutic agents. 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 (Majesky et al., 1983; Ramos and Parrish, 1995; Ross et al., 2001). The presence of PAH-DNA adducts in atherosclerotic lesions of humans suggests that DNA dam-

ABBREVIATIONS: SMC, smooth muscle cell; PAH, polycyclic aromatic hydrocarbon; BP, benzo[a]pyrene; BPDE, benzo[a]pyrene-7,8-dihydriodiol-9,10 epoxide; P450, cytochrome P450; AHH, aryl hydrocarbon hydroxylase; 3-OH-BP, 3-hydroxybenzo[a]pyrene; BPQ, benzo[a]pyrene-3,6-quinone; EP, 1-ethynylpyrene; α-NF, α-naphthoflavone; DMSO, dimethyl sulfoxide; EH, epoxide hydrolase; MC, 3-methylcholanthrene; PEI-TLC, polyethyleneimine cellulose thin-layer chromatography; RAL, relative adduct labeling.
age contributes to the development of atherosclerosis (Bond et al., 1981; Izzotti et al., 1995).

Although parent PAHs by themselves are nontoxic, 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 (Denissenko 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 (Cavaliere 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 playing an important role in atherogenic mechanisms. Since BP metabolism to quinones and semiquinones can lead to redox cycling and reactive oxygen species 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 extrahepatic 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 extrahepatic 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). Although 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 coexpressed with CYP1A1 in several extrahepatic 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-hydroxybenzo[a]pyrene (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 (Bowes et al., 1996; Shimada 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 1) BP induces DNA adducts in mouse aortic SMCs, 2) 3-OH-BP and benzo-[a]pyrene-3,6-quinone (BPQ) are proximate genotoxic metabolites, and 3) 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 Sigma-Aldrich (St. Louis, MO), and 3-OH-BP and BPQ (>99% purity by high-pressure liquid chromatography) were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories c/o Midwest Research Institute (Kansas City, MO). 1-Ethynylpyrene (EP) was a kind gift from Dr. William Alworth, Department of Chemistry, Tulane University (New Orleans, LA). All other chemicals were purchased from Sigma-Aldrich unless otherwise noted. BP, 3-OH-BP, BPQ, EP, ellipticine, and α-naphthoflavone (α-NF) stock solutions were prepared in dimethyl sulfoxide (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 BD Gentest (Woburn, MA). Rat CYP1B1 protein standards were also obtained from BD Gentest.

**Cell Culture.** Primary cultures of vascular SMCs were isolated from male C57BL/6 mouse aorta and maintained under standard conditions as described by Ramos and Cox (1993). Cells were grown in Medium 199 (In Vitrotext, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B (Invitrogen). Subcultures were prepared by trypsinization (Invitrogen) of subconfluent primary cultures. Cells were seeded at 100 cells/mm² onto 100-mm plates for AH drift assays, and at 75 cells/mm² onto 150-mm plates for postlabeling experiments. The cells were allowed to acclimate for 48 h and then challenged with BP, 3-OH-BP, BPQ, or EP for the time periods specified in the 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 h with the P450 inhibitors ellipticine (0.001–0.1 mM), α-NF (1–100 nM), or EP (0.01–1 µM). 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 h, followed by treatment with BP (3 µM) for 24 h. Cells were washed with 1 ml of ice-cold Tris-sucrose buffer (0.05 M Tris-HCl, 0.2 M 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 0.1 M 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 the 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 was removed. To the organic layer, 1 N NaOH was added, vortexed, and the aqueous (bottom) layer was removed for analysis. 3-OH-BP standards were prepared in 1 N 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 × 1.702 × 10⁶ (dilution factor)]/[mg of protein × 15 min].

The factor 1.702 × 10⁶ was used to account for dilution of the...
incubation mixture and conversion of the activity units from micro- moles per minute per milligrams of protein to picomoles per minute per milligrams of 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 bands was accomplished by laser densitometric scanning of the photog- raphic negatives of the bands, 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), which 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) that measured the ability of aldehyde dehydrogenase to transfer electrons from styrene-7,8-dihydrodiol to NAD$^+$, formed as a result of EH-catalyzed hydroslysis 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 three separate 150-mm culture plates per treatment to generate $\sim 1 \times 10^7$ cells. Cells were treated with DMSO (control), 0.03, 0.3, or 3 $\mu$M BP, 3-OH-BP, or BPQ, alone (24 h) or pretreated with EP (1 $\mu$M, 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 of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.65), and stored at $-20^\circ$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 as- say for DNA adducts was performed as reported previously (Reddy and Randerath, 1986; Moorthy and Randerath, 1997). Briefly, DNA (10 $\mu$g) was digested with micrococcal nuclease (0.04 U/ml) and spleen phosphodiesterase (0.4 $\mu$g/ml) at pH 6.0 and $37^\circ$C for 3.5 h. The DNA was then treated with nuclease-P1 (0.6 $\mu$g/ml, pH 5.6) at $37^\circ$C for 40 min, followed by labeling with [$\gamma$-$^{32}$P]ATP (4000 Ci/ mmol) and T4 polynucleotide kinase (0.5 U/ml) at pH 9.5 and $37^\circ$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 $\sim 80^\circ$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 = cpm in adduct(s)/specific activity (ATP) $\times$ pmol of DNA-P-labeled (1 $\mu$g of DNA = 3240 pmol of DNA-P) (Reddy and Randerath, 1986). For rechromatography experiments, spots that were in similar locations were excised from the chromatograms, extracted with isopropanol-ammonia, and chromatographed in differ- ent solvents. Cochromatography experiments were performed similar to rechromatography, except that mixtures of different spots were chromatographed in different solvents to establish identity or nonidentity of the adducts (Moorthy et al., 1996). Statistical analyses were performed using one-way analysis of variance or Student's $t$-test.

**Results**

Exposure of SMCs to 0.3 $\mu$M BP gave rise to eight $^{32}$P-postlabeled DNA adduct spots that were not observed in cells treated with the vehicle DMSO (Fig. 1, A and B). Of these, adducts 2 and 3 were major, whereas adducts 1, 4, 5, 6, 7, and 8 were minor. Treatment of SMCs with the BP metabolites 3-OH-BP (Fig. 1C) and BPQ (Fig. 1D) also gave rise to multiple adducts. Adduct 1 of BPQ was in a similar chromato- graphic location to the major 3-OH-BP adduct (spot 1) (com- pare Fig. 1, C and D). Our studies showed that total BP-DNA adduct formation was concentration-dependent, with total adduct RAL values increasing from 3.78 $\times$ 10$^8$ for the 0.03 $\mu$M concentration to 169.9 $\times$ 10$^8$ for the 3 $\mu$M 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 $\mu$M concentration, only adducts 2 and 3 were detectable. On the other hand, adduct 1 was present when SMCs were treated with 0.3 $\mu$M BP but undetectable when cells were exposed to 3 $\mu$M 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). Although adducts induced by 3-OH-BP were detected at 0.03 $\mu$M concentration, this was not the case for BPQ, wherein adducts were detected only when 0.3 $\mu$M BPQ was used. Taken together, quantita- tive adduct analyses revealed 3-OH-BP to be more genotoxic than BPQ. 

![Fig. 1. Effect of BP and its metabolites on DNA adduct formation in SMCs. SMCs were exposed to DMSO (A), BP (0.3 $\mu$M) (B), 3-OH-BP (0.3 $\mu$M) (C), or BPQ (0.3 $\mu$M) (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.](image-url)
TABLE 1
Effect of different concentrations of BPQ on DNA adduct levels
Adduct values represent the means ± S.D. of three independent experiments and are expressed as RAL × 10^5. BPQ treatment of cells, DNA isolation, and ^32P-postlabeling were carried out as described under Materials and Methods. The limit of detection, expressed as RAL × 10^5, was 0.02.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>BPQ (0.03 μM)</th>
<th>BPQ (0.3 μM)</th>
<th>BPQ (3 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.69 ± 0.1</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>6.37 ± 0.6</td>
<td>14.69 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>28.52 ± 3.4</td>
<td>13.16 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.05 ± 0.1</td>
<td>1.65 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.51 ± 0.3</td>
<td>2.57 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.58 ± 0.3</td>
<td>1.41 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.30 ± 0.04</td>
<td>0.45 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.16 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>101.31 ± 9.4</td>
<td>169.9 ± 28.4</td>
<td></td>
</tr>
</tbody>
</table>

N.D., not determined.

TABLE 2
Effect of different concentrations of 3-OH-BP on DNA adduct levels
Adduct values represent the means ± S.D. of three independent experiments and are expressed as RAL × 10^5. 3-OH-BP treatment of cells, DNA isolation, and ^32P-postlabeling were carried out as described under Materials and Methods. The limit of detection, expressed as RAL × 10^5, was 0.02.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>3-OH-BP (0.03 μM)</th>
<th>3-OH-BP (0.1 μM)</th>
<th>3-OH-BP (3 μM)</th>
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<tbody>
<tr>
<td>1</td>
<td>13.06 ± 1.9</td>
<td>13.90 ± 1.3</td>
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</tr>
<tr>
<td>2</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.63 ± 0.1</td>
<td>1.78 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.44 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14.74 ± 1.8</td>
<td>17.18 ± 1.6</td>
<td></td>
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</tbody>
</table>

TABLE 3
Effect of different concentrations of BPQ on DNA adduct levels
Adduct values represent the means ± S.D. of three independent experiments and are expressed as RAL × 10^5. BPQ treatment of cells, DNA isolation, and ^32P-postlabeling were carried out as described under Materials and Methods. The limit of detection, expressed as RAL × 10^5, was 0.02.

<table>
<thead>
<tr>
<th>Adduct</th>
<th>BPQ (0.03 μM)</th>
<th>BPQ (0.3 μM)</th>
<th>BPQ (3 μM)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.14 ± 0.03</td>
<td>2.84 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.11 ± 0.01</td>
<td>2.58 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.25 ± 0.05</td>
<td>5.42 ± 0.2</td>
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Because CYP1 enzymes play important roles in the metabolic activation of PAHs, we tested the hypothesis that pretreatment 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 (Kerze and Ramos, 2001). Whereas ellipticine specifically inhibits CYP1A1 (Annas et al., 2000), α-NF selectively inactivates CYP1B1 (Shimada et al., 1999; 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. Pretreatment 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). To confirm that BP induced CYP1B1, but not CYP1A1, vascular SMCs exposed to BP were subjected to Western analyses. As shown in Fig. 2A, uninduced cells displayed basal expression of CYP1B1, but not CYP1A1 (Fig. 2B). BP at a concentration of 0.03 μM caused a 1.5-fold induction of CYP1B1 apoprotein, compared with DMSO-treated controls, as determined by densitometric scanning of the blots (Fig. 2A). At 0.3 μM BP concentration, the induction was about 2-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 (Fig. 2A). Pretreatment of SMCs with EP (1 μM), followed by BP (0.3 μM), resulted in a 20% decrease in the expression of CYP1B1, compared with those that were treated with 0.3 μM BP only (Fig. 2A). In contrast, CYP1A1 was not detectable at any BP concentration (Fig. 2B).

Since α-NF is known to selectively inhibit CYP1B1 (Shimada et al., 1999; 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 aryl hydrocarbon receptor. As shown in Fig. 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 with DMSO-treated controls. Western analyses of standard rat CYP1B1 proteins yielded concentration-dependent increases in band intensities (Fig. 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 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). Although 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, 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 interexperimental variability in cell treatments, DNA isolation, and 32P-postlabeling. Pretreatment with EP also almost completely inhibited adducts induced by 3-OH-BP or BPQ (Fig. 3).

To identify the metabolites that are responsible for DNA adduct formation by BP, re- and cochromatography experiments were conducted. Rechromatography experiments (Fig. 4) revealed adduct 2 of BP, adduct 1 of 3-OH-BP, and adduct 1 of BPQ adduct to be identical to one another. Similarly, BP adduct 3 and BPQ adduct 2 were identical (Fig. 5). Since the levels of the major adducts 2 and 3 in DNA of SMCs exposed to 3 μM BP, expressed as RAL × 10^6, 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 picomole range would be required to identify structures of unknown adducts in the absence of synthetic standards (Tretjakova 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 (Fig. 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 LINMId 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 (Fig. 2A), but not CYP1A1 apoprotein (Fig. 2B). The observation that the CYP1B1 inhibitor EP, but not ellipticine or α-NF, dramatically inhibited 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 mouse SMCs. In fact, Alexander et al. (1999) have postulated that a major distinction between the inhibition of 7,12-dimethylbenz[a]anthracene 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 aryl hydrocarbon receptor (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 pretreated with EP (Moorthy et al., 2002), the observation that EP + BP treatment resulted only in a modest attenuation (−20%) of CYP1B1 apoprotein expression compared with 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 4), 3-OH-BP, and BPQ (Fig. 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; Shimada et al.,

### Table 4

<table>
<thead>
<tr>
<th>Adduct</th>
<th>BP (0.3 μM)</th>
<th>EP + BP (0.3 μM)</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>1.70 ± 0.1</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>38.90 ± 2.8</td>
<td>6.43 ± 0.8</td>
</tr>
<tr>
<td>3</td>
<td>13.80 ± 4.1</td>
<td>1.20 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>0.59 ± 0.1</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>1.19 ± 0.1</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>1.05 ± 0.2</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>0.83 ± 0.1</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>8</td>
<td>0.56 ± 0.1</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Total</td>
<td>58.67 ± 6.4</td>
<td>9.01 ± 1.1</td>
</tr>
</tbody>
</table>

* Statistically significant differences between BP and EP + BP samples at P < 0.05, as determined by Student’s t test.
The fact that the major BP adduct 2, adduct 1 of 3-OH-BP, and adduct 2 of BPQ were identical (Fig. 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 (Fig. 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 Parrish, 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 activities were detectable in DMSO-treated SMCs (2.95 ± 0.35 nmol/min/mg of 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 prefer-
entially 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 × 10⁴) (Moorthy et al., 2002) were comparable to those produced by 3 μM BP (RAL × 10⁸ = 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. Although all the major BP adducts were slow-moving (nonpolar) on TLC plates (Fig. 1), MC treatment of SMCs elicited adduct patterns wherein four adducts were slow-moving (nonpolar) and the remaining eight were fast-moving (polar), suggesting the existence of at least two 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 (Figs. 1, 4, and 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., 2003) lends credence to this hypothesis. The observation that BP (Table 4) as well as MC adducts 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 (Belgin, 1990), and, if not repaired, could lead to gene mutations and development of tumors. Because of the close parallelism between carcinogenesis and atherogenesis (Majesky et al., 1983; Ramos and Parrish, 1995; 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 Parrish, 1995) suggests that DNA adduct formation represents the initiation step of atherogenesis. We recently reported that BP, 3-OH-BP, and BPQ activate LiMd retrotropanoson 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 genotoxicity of BP and its metabolites plays a causal role in PAH-induced atherogenesis. Further studies are needed to identify the specific role(s) of PAH-DNA adducts in the atherogenic processes.

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