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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

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ABSTRACT. Recent studies in this laboratory have shown that benzo[*a*]pyrene (BaP) modulates growth factor-related gene expression and proliferation of renal glomerular mesangial cells (GMCs) *in vitro*. Because many of the toxic and biochemical effects of this polycyclic aromatic hydrocarbon are mediated through oxidative metabolism, the present studies were conducted to examine the patterns of cytochrome P450IA1 (*CYP1A1*) and P450IB1 (*CYP1B1*) inducibility in mesangial cells and the molecular consequences of this response. Exposure of cultured GMCs to BaP (30 μM) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, 10 nM) for 24 hr induced CYP1A1 mRNA levels, a response abolished by cotreatment with 10 μM cycloheximide. The pattern of hydrocarbon inducibility was atypical in that BaP was a more effective inducer of *CYP1A1* gene expression than TCDD, and both hydrocarbons induced aryl hydrocarbon hydroxylase (AHH) activity, but not ethoxyresorufin-O-deethylase activity. Cotreatment with α-naphthoflavone (αNF, 1 μM) or ellipticine (ELLIP, 0.1 nM) only partially inhibited the induction of AHH activity by BaP (30 μM). BaP and TCDD also induced expression of the CYP1B1 protein and the pattern of induction was comparable to that observed for *CYP1A1*. Treatment of GMCs with 30 μM BaP was associated with the formation of eight DNA adducts, and their occurrence could be inhibited by pretreatment with αNF (1 μM), but not ELLIP (0.1 nM). These results demonstrate that CYP1A1 and CYP1B1-related activities are induced in GMCs by BaP and TCDD and this induction is associated with metabolism of BaP to reactive intermediates that bind covalently to DNA. BIOCHEM PHARMACOL 52;4: 587–595, 1996.

KEY WORDS. kidney; glomerular mesangial cells; benzo[*a*]pyrene; TCDD; *CYP1A1*; *CYP1B1*

BaP‖, a PAH formed as a byproduct during the combustion of organic materials, has long been recognized as a mutagen and suspected carcinogen [1]. These effects of BaP have been attributed to bioactivation of the parent compound to BPDE by the sequential actions of CYP1A1 and epoxide hydrolase [2]. This reactive epoxide preferentially binds to guanine nucleotides in DNA to initiate genotoxic damage [2]. CYP1A1 is a member of a large family of proteins that exhibit overlapping substrate specificities and catalyze the oxidation of endogenous and exogenous substrates [3]. The oxidation of PAHs is preferentially catalyzed by CYP1A1, although a role for other CYP isoforms, particularly in extrahepatic metabolism, has long been proposed. Induction of *CYP1A1* is observed in various species and tissues upon exposure to PAHs, as well as polyhalogenated aromatic hydrocarbons such as TCDD [4]. *CYP1B1* is a recently identified PAH-inducible CYP capable of oxidizing PAHs [5]. In the case of PAHs, induction of either CYP1A1 or CYP1B1 may enhance their own metabolism and, as such, represents an important toxification pathway.

The majority of the nephrotoxicity studies to date have focused on the responses of cortico-tubular cells to xenobi-

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‖ *Abbreviations*: AHH, aryl hydrocarbon hydroxylase; BaP, benzo[*a*]pyrene; BPDE, 7,8-diol-9,10-epoxide; CYP1A1, cytochrome P450IA1; CYP1B1, cytochrome P450IB1; ELLIP, ellipticine; EROD, ethoxyresorufin-O-deethylase; GMCs, glomerular mesangial cells; NDGA, nordihydroguararetic acid; PIP, piperonyl butoxide; PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; αNF, α-naphthoflavone; FBS, fetal bovine serum; and TCA, trichloroacetic acid.

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otics. Consequently, the role of other renal cell types in the metabolism and toxicity of exogenous compounds and their participation in nephrotoxic responses have been sparsely investigated. Studies by Goldstein and Linko [6] have demonstrated that treatment of rats with TCDD induces CYP1A1 in the kidney, as reflected by induction of AHH activity, total CYP content, and CYP1A1 protein content. Foster *et al.* [7] have identified CYP1A1 staining in renal tubules using a monoclonal antibody, but not in the glomeruli of Wistar rats treated with β-naphthoflavone. This finding, however, does not preclude the presence of P450 enzymes within the glomerular region of the nephron since antibody-specific recognition of a liver CYP1A1 epitope may be absent and/or modified in other tissues. CYP isozymes from different organs or different cells within the same organ display differences in structure, regulation, and substrate specificity. Studies by Sesardic *et al.* [8] have shown that treatment of Wistar rats with 3-methylcholanthrene (MC) enhances phenacetin-*O*-deethylase activities in both liver and kidney. However, liver phenacetin-*O*-deethylase activities correlated with CYP1A2 but not CYP1A1 expression, while the opposite was true in the kidney. Because CYP1A2 was not detected under basal or induced conditions in rat kidneys, it was concluded that CYP1A1 mediates the renal metabolism of xenobiotics [8].

Recent studies in this laboratory have demonstrated that GMCs are preferential targets of BaP toxicity *in vitro* [9]. Since the ability of BaP to inhibit GMC proliferation and to modulate growth factor-related signal transduction may involve formation of genotoxic intermediates, elucidation of the role of CYP isozymes in this process must be defined. Evidence is presented here demonstrating that cultured GMCs exposed to BaP and, to a lesser extent, TCDD exhibited inducible *CYP1A1* and *CYP1B1* expression and AHH activity without a corresponding increase in EROD activity. GMCs metabolized BaP to reactive intermediates that bound covalently to DNA, a response partially antagonized by αNF. The atypical profiles of mRNA and enzyme induction elicited by BaP and TCDD implicate unique regulation of CYP-related activities in renal GMCs.

MATERIALS AND METHODS

Animals

Female Sprague–Dawley rats between 175 and 200 g (12–21 weeks of age) were purchased from Sasco (Houston, TX). Animals were housed in cages that contained hardwood bedding and received food and water *ad lib.*

Chemicals

BaP (>98% purity) was purchased from the Aldrich Chemical Co. (Milwaukee, WI). FBS was purchased from Intergen (Purchase, NY). TCDD (>99.9% purity) was synthesized by Dr. Stephen H. Safe (Texas A&M University, College Station, TX). BaP stock solution was prepared in DMSO at a concentration of 10 mg/mL; TCDD stock was prepared in DMSO at a concentration of 1 μM. TCA, insulin, antibiotic/antimycotic solution, BSA, DMSO, dicumarol and trypsin/EDTA were obtained from the Sigma Chemical Co. (St. Louis, MO). RPMI 1640 was purchased from Gibco/BRL (Grand Island, NY). Tri-reagent was purchased from Molecular Research Inc. (Cincinnati, OH). Collagenase, holotransferrin, spleen phosphodiesterase, and nuclease P1 were obtained from Boehringer Mannheim (Indianapolis, IN). T4 polynucleotide kinase was purchased from the US Biochemical Corp. (Cleveland, OH). PEI-cellulose thin-layer chromatography sheets were obtained from Scientific Adsorbents Inc. (Atlanta, GA). [^{3}H-*methyl*]Thymidine (56 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA) [γ-^{32}P]ATP (3000–6000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Micrococcal nuclease, potato apyrase, and all other reagents used for the northern analysis, AHH, EROD and ^{32}P post-labeling experiments were purchased from Sigma.

Cell Culture of Glomerular Mesangial Cells

Renal GMCs were isolated and established in culture as described previously [9]. Subcultured GMCs were obtained by trypsinization of confluent cultures. In all experiments, GMCs were used between passages 6 and 14. GMCs were incubated in RPMI 1640 culture medium supplemented with 10% FBS, 1 mg/mL BSA, insulin (0.66 U/mL), transferrin (30 μg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (25 μg/mL), and L-methionine (30 mg/L).

Total RNA Extraction

Total RNA extraction was performed as described previously with modifications [10]. Briefly, RNA was extracted from pre-confluent GMCs, plated in 100 mm tissue culture dishes, exposed to 30 μM BaP or 10 nM TCDD for 24 hr in the presence or absence of 10 μg/mL cycloheximide during the last 4 hr, by the addition of 1 mL Tri-reagent (guanidinium thiocynate/phenol) per dish. After a 5-min digestion, cell lysates were scraped, and the supernatant was collected and placed into microcentrifuge tubes. Following another digestion for 5 min, 200 μL of chloroform was added to the samples; the tubes were vortexed and then centrifuged at 12,000 *g* for 15 min at 4°. The aqueous layer was collected and combined with an equal volume of isopropanol. Following overnight precipitation at −20°, samples were centrifuged at 12,000 *g* for 30 min at 4°, and the RNA pellet was rinsed with cold 70% ethanol and centrifuged at 12,000 *g* for 10 min at 4°. The supernatant was discarded, and the RNA pellet was dried for 15 min using a centrifugal evaporator (Jouan, Houston, TX). The dried RNA pellet was then resuspended in 30 μL of CCS [1 mM sodium citrate, 1 mM CDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid), and 0.1%

SDS] and incubated at 50° for 30 min. Total RNA in each sample was quantified spectrophotometrically at 260 nm.

Separation and Transfer of Total RNA onto a Nylon Membrane

Approximately 15 μg of RNA was resuspended in a total volume of 20 μL of CCS. An equal volume of 2x FPF [63.5% formamide, 20% formaldehyde, 1 M sodium phosphate (pH 6.8), 15% 6x loading buffer] was added to each sample. The samples were vortexed and denatured by heating at 55° for 30 min. RNA was loaded onto a 1.2% agarose/6% formaldehyde/1x SPC (200 mM sodium phosphate and 20 mM CDTA) denaturing gel and electrophoresed in 1x SPC buffer at 50 V for 3–4 hr. The gel was stained with ethidium bromide, destained in 1x SPC, and photographed under UV light for total RNA quantification. After placement on a solid support covered with filter paper (Whatman), the gel was overlaid with a nylon membrane and two sheets of filter paper pre-soaked in 1x SPC. A stack of paper towels was placed on top of the filter paper to facilitate capillary transfer. RNA was allowed to transfer onto the membrane for 48 hr. Following transfer, RNA was cross-linked to the membrane using a UV cross-linker and baked for 1–2 hr at 80°.

Northern Analysis of RNAs

Membranes were incubated in 5 mL of prehybridization buffer [5x SSPE (0.2 M sodium phosphate, 2.98 M sodium chloride, and 0.02 M EDTA), 1% SDS, 10% dextran sulfate, 0.1% polyvinyl pyrolidine, 0.1% Ficoll, and 0.1% BSA] for 18–24 hr at 65° and hybridized for 24 hr at 65° after addition of 5×10^6 cpm of either a CYP1A1 cDNA (1.2 kb) cloned into the *Pst*I site of pU18 or a human β-tubulin cDNA (1.6 kb) cloned into the *Eco*RI site of pBluescript (ATCC) probe. Probes were prepared by mixing 2 μL (25 ng) of denatured cDNA, 3 μL of dATP, dGTP and dTTP (1:1:1), 2 μL of reaction buffer, 5 μL of [α^{32}P]dCTP, 7 μL of sterile distilled water and 1 μL of Klenow fragment for 30 min at 37°. Each probe was separated from free radioactive nucleotides using a Sephadex G-50 Quick spin column (Pharmacia, Piscataway, NJ). After boiling for 10 min, the desired probe was incubated on ice for 5 min and added to the prehybridization buffer as described above. After hybridization, the membrane was washed twice in 1x SSPE for 15 min at room temperature, twice in 0.1x SSPE/2.0% SDS for 45 min at 65°, and once in 0.1x SSPE for 5 min at room temperature.

CYP-Related Enzyme Activities in GMCs Treated with BaP or TCDD

Confluent subcultures of GMCs were grown in 150 mm dishes and, if appropriate, pretreated with 1 μM αNF or 0.1 nM ELLIP for 30 min or 24 hr, respectively. Then GMCs were exposed to 30 μM BaP or 10 nM TCDD for 24 hr in the absence or presence of CYP inhibitors and processed for measurements of AHH or EROD activities. GMCs were scraped and collected after the addition of 5 mL of ice-cold Tris–sucrose buffer (pH 8.0). Then cells were centrifuged for 5 min at 50 *g* (4°), the supernatant was removed, and the pellet was resuspended in 300 μL of Tris–sucrose buffer. Two 100-μL aliquots that contained approximately 100 μg of cellular protein were transferred into new borosilicate tubes for fluorometric enzyme analysis, while 50 μL of sample was used to determine protein concentration by the method of Bradford [11].

AHH ACTIVITY IN GMCs TREATED WITH BaP. GMCs were processed for measurements of AHH activity as described previously [12]. Approximately 100 μg of cellular protein was combined with 1 mL of reaction mixture containing 0.1 M HEPES (pH 8.0) and 0.4 mM NADPH. The samples were preincubated at 37° for 2 min, and the reaction was initiated by the addition of 80 μM BaP dissolved in 40 μL of methanol. Samples were incubated for 10 min and the reaction was terminated by the addition of 1 mL of ice-cold acetone and 3.25 mL of hexane. After vortexing, 2 mL of the organic layer was collected and extracted with 5 mL of 1 N NaOH. Samples were vortexed again, and the NaOH fraction was read on a spectrofluorometer at a wavelength of 396 nm excitation and 522 nm emission. The spectrofluorometer was calibrated using authentic 3-OH BaP standards. AHH activity in cultured GMC preparations was compared with that of BaP-treated H_4IIE rat hepatoma cells to assess relative changes in AHH activity.

EROD ACTIVITY IN GMCs TREATED WITH BaP OR TCDD. GMCs were processed for measurements of EROD activity as previously described [13] with modifications. Briefly, 1.2 mL of 0.1 M HEPES buffer (pH 7.5) containing 0.1 mg of NADH, 0.1 mg of NADPH, 1.5 mg of magnesium sulfate and 1.1 mg of BSA was added to 100 μg of cellular protein. Then the tubes were incubated for 2 min at 37° prior to the addition of 50 μL ethoxyresorufin to give a final concentration of 100 μM. Samples were allowed to incubate at 37° for 15 min, and the reaction was terminated by the addition of 2.5 mL of methanol. Samples were incubated for an additional 2 min to allow for protein flocculation and centrifuged for 10 min at 1500 *g*. EROD activity in the supernatant was measured fluorometrically at a wavelength of 550 nm excitation and 585 nm emission as described by Pohl and Fouts [14]. EROD activity in cultured GMCs or GMC microsomal preparations was compared with samples from BaP-treated cultured hepatocytes, H_4IIE rat hepatoma cells, and rat liver microsomes. For measurements of dicumarol sensitivity, cells were challenged with BaP or TCDD for 24 hr followed by treatment with 5 μM dicumarol for 2 min before processing for enzymatic measurements.

Western Blotting

GMC cultures were plated on 150 mm plastic culture dishes and allowed to attach for 24 hr. Fresh medium containing

30 μM BaP or 10 nM TCDD or vehicle was added for 24 hr before cellular protein was harvested. Cellular protein was precipitated with TCA (15%) and resuspended in buffer to give final protease inhibitor concentrations of 0.9 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, 23 mM EDTA, and 45 μg/mL pepstatin A. Following 24 hr at 4°, proteins were pelleted by centrifugation at 6000 *g*. Pellets were washed two times with ethanol and resuspended in 300 μL of 40% (v/v) glycerol, 25% (w/v) β-mercaptoethanol, 12% (w/v) SDS, 0.31 M Tris–HCl (pH 6.8), 25 mM EDTA, and 0.1% (w/v) pyronin Y. Samples were boiled for 5 min, and 100 μg of protein was electrophoresed on a 12.5% (w/v) polyacrylamide gel. Proteins were transferred to a membrane overnight (15 V) and blocked for 2 hr with 2% Tween/10% nonfat dry milk prior to the addition of a primary antibody (1:5000) to *CYP1B1* for 2 hr. Rabbit anti-IgG horseradish peroxidase conjugate was used as the secondary antibody, and proteins were visualized using the enhanced chemiluminescence method (Amersham).

Isolation of DNA from GMCs Exposed to BaP

GMCs were treated with 30 μM BaP for 24 hr, and DNA was isolated after treatment with RNase A and proteinase K by phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) extraction. The aqueous phase was collected, and DNA was precipitated with cold ethanol and stored at −20° for 24 hr. The supernatant was decanted, and DNA was dissolved in a Tris–EDTA buffer (pH 7.6).

^{32}Post-labeling of Adducted DNA

DNA samples were analyzed using the nuclease P1-mediated enhancement as described by Reddy and Randerath [15]. DNA (12 μg) was digested for 3.5 hr at 37° with 400 mU micrococcal nuclease and 4 μg spleen phosphodiesterase in a total volume of 10 μL containing 10 mM calcium chloride and 30 mM sodium succinate (pH 6.0). A 2-μg aliquot was removed for analysis of nucleotides. Samples were then digested further with 1.4 μg nuclease P1 in a total volume of 15 μL containing 0.1 mM zinc chloride and 60 mM sodium acetate (pH 5.0) for 40 min at 37°. Following the addition of 3 μL of 500 mM CHES (2-[*N*-cyclohexylamino]-ethanesulfonic acid) (pH 9.5), the DNA digest was incubated in the presence of [γ-^{32}P]ATP (160 μCi) and 12 U of T4 polynucleotide kinase for 40 min at 37°. The reaction was terminated over a 3-min period by the addition of 60 mU potato apyrase at 37°. Purification and resolution of ^{32}P-labeled adducts were carried out on PEI-cellulose thin-layer chromatography sheets. Separation was carried out using D1, 2.3 M sodium phosphate (pH 5.7); D3, 4.2 M lithium formate, 8.5 M urea (pH 3.5); D4, 0.8 M sodium phosphate, 0.5 M Tris, 8.5 M urea (pH 8.1); and D5, 1.7 M sodium phosphate (pH 6.0). For further migration of spots from the origin, chromatography paper wicks on D4 and D5 stages were added. Chromatograms were visualized using a Betascope 603 with a 30-min exposure.

Treatment with Cytochrome P450 Inhibitors

Subcultures of randomly cycling GMCs were treated with the CYP inhibitors αNF and ELLIP, or with the general CYP inhibitors PIP and NDGA, or vehicle [16–20]. αNF, ELLIP, and PIP were dissolved in DMSO, while NDGA was dissolved in absolute ethanol at a concentration of 1 mM. GMCs were pretreated with ELLIP (0.01 and 0.1 nM) for 24 hr, while cells exposed to αNF (0.1 and 1 μM), PIP (1 and 10 μM), and NGDA (10 and 100 μM) were incubated for 30 min prior to the addition of 3 μM BaP. The inhibitor concentrations used in these studies have been shown to inhibit CYP in other cell systems ([17, 21]; Zhao W and Ramos KS, unpublished observations). After 24 hr, GMCs were rinsed with PBS, incubated in fresh medium, and grown to confluence. Then GMCs were passed into 8- or 12-well plates at a density of 50 cells/mm^2, growth-arrested in 0.1% FBS for 72 hr, and then processed for DNA synthesis or cell counts following mitogenic stimulation with 10% serum.

[^{3}H-methyl]Thymidine Incorporation into DNA

Cultures of GMCs were evaluated for measurements of [^{3}H]thymidine incorporation as described previously [9]. After 72 hr of growth-arrest, GMCs were stimulated with 10% FBS in the presence of 0.5 μCi/mL [^{3}H]thymidine for 24 hr. At the end of the incubation period, medium was aspirated from culture dishes, and cells were rinsed twice with sterile PBS. A 1-ml aliquot of 5% TCA was placed into each dish, cells were scraped, and cellular components were transferred to 1.5-mL microcentrifuge tubes. After a 5-min centrifugation at 3000 g, the supernatant was removed and the cell pellet rinsed three times with 5% TCA. After another centrifugation (3000 *g*) for 5 min, the supernatant was aspirated and the pellet washed with 750 μL of cold absolute ethanol. Following an overnight precipitation at 4°, samples were centrifuged at 3000 *g* for 5 min, the supernatant was removed, and the pellet was dried in a centrifugal evaporator (Jouan). The cell pellet was dissolved in 500 μL of 1 M sodium hydroxide. Protein concentration was measured according to the method of Bradford [11]. After neutralization of 100 μL of sample with 1 M HCl, [^{3}H]thymidine incorporation into DNA was quantified by liquid scintillation.

Cell Proliferation

Relative cell numbers of GMCs exposed to CYP inhibitors and/or BaP were determined by the Janus green method as described by Rieck *et al.* [22]. Following 72 hr of growth-arrest in medium supplemented with 0.1% FBS, GMCs were stimulated with 10% FBS for 72 hr. Cells were rinsed twice with PBS and fixed by the addition of 1 mL of abso-

lute ethanol for 1.5 min. Ethanol was removed by aspiration, and the cells were allowed to air-dry. Fixed GMCs were then incubated with 1 mL of Janus green dye (1 mg/mL) for 1 min. The dye was removed, and the cells were rinsed twice with 2 mL of PBS. The dye was extracted with 2 mL of absolute ethanol and combined with 2 mL of absolute ethanol in borosilicate glass tubes. Cells were rinsed with an additional 1 mL of ethanol, and the relative amount of Janus green dye was measured spectrophotometrically at a wavelength of 654 nm. Cell numbers were calculated by comparing the relative absorbance in the treated groups to a standard curve.

Data Analysis

ANOVA in conjunction with Fisher's post-hoc test was used to assess the statistical significance of differences between control and treated cultures ($P < 0.05$). Values are the means ± SEM for 3–4 replicates. All experiments were performed in duplicate or triplicate for each of the compounds tested.

RESULTS

The ability of BaP and TCDD to induce CYP1A1 mRNA in subcultured GMCs is shown in Fig. 1. Treatment of GMCs with BaP (30 μM) or TCDD (10 nM) for 24 hr induced *CYP1A1* gene expression relative to DMSO. While cycloheximide (10 μM) alone did not modulate steady-state CYP1A1 mRNA levels, treatment of GMCs with BaP or TCDD in the presence of this protein synthesis inhibitor antagonized CYP1A1 induction. It is interesting to note that, in contrast to cultured rat hepatocytes (Fig. 2), at the concentrations examined BaP was a more effective inducer of the *CYP1A1* gene than TCDD in GMCs. Densitometric analysis of the data revealed that induction of CYP1A1 by BaP was 2-fold greater than by TCDD. Experiments were then conducted to evaluate (i) AHH and EROD activity in GMCs treated with BaP or TCDD, and (ii) the influence of αNF (1 μM) or ELLIP (0.1 nM) in this response. Treatment of GMCs with 30 μM BaP or 10 nM TCDD for 24 hr induced AHH activity (Fig. 3). Consistent with the mRNA profile observed, induction of AHH by BaP was markedly higher than by TCDD. Both αNF and ELLIP partially inhibited the ability of BaP to induce AHH activity. AHH inducibility in GMCs treated with 3 μM BaP was equivalent to that in rat hepatoma-derived H_4IIE cells (data not shown).

Although detectable EROD activity was observed in GMCs, this activity was not BaP- or TCDD-inducible since control cells exhibited 13.61 ± 0.49 pmol/min/mg and BaP- or TCDD-treated cells exhibited 14.49 ± 0.39 or 13.48 ± 0.15 pmol/min/mg protein ($N = 4$), respectively. In addition, microsomal preparations of GMCs exposed to BaP did not exhibit increased EROD activity relative to controls [121.64 ± 6.21 vs 149.88 ± 2.31 pmol/min/mg (N = 4), respectively]. EROD activity, however, was inducible in

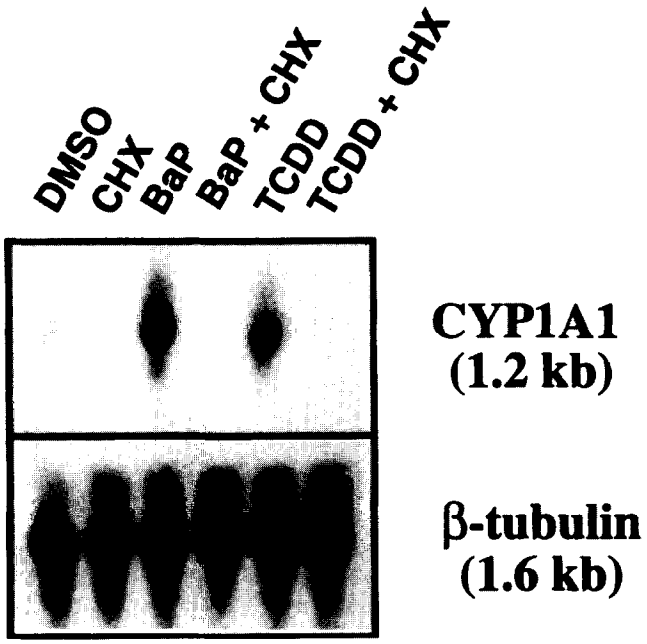

FIG. 1. Steady-state levels of CYP1A1 mRNA in subcultured glomerular mesangial cells treated with BaP or TCDD in the presence or absence of cycloheximide (CHX). Randomly cycling GMCs (P_{10}) were treated with either 30 μM BaP or 10 nM TCDD for 24 hr in the presence or absence of 10 μM CHX during the last 4 hr. Total RNA was extracted and processed as described in Materials and Methods.

BaP-treated rat H_4IIE cells where a 30-fold induction [47.07 ± 3.61 vs 1467 ± 198.53 pmol/min/mg (N = 3)] was observed. To determine if competing enzymatic reduction pathways, such as DT-diaphorase, masked the lack of EROD inducibility by BaP or TCDD, GMCs were incubated in the presence of dicumarol (1 μM), a specific in-

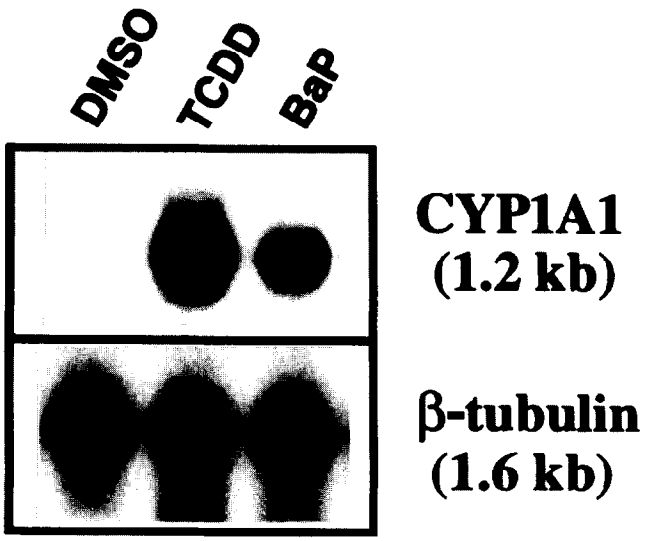

FIG. 2. Steady-state levels of CYP1A1 mRNA in primary cultures of rat hepatocytes treated with BaP or TCDD. Hepatocytes were treated with BaP (3 μM) or TCDD (10 nM) for 24 hr, and total RNA was extracted and processed as described in Materials and Methods.

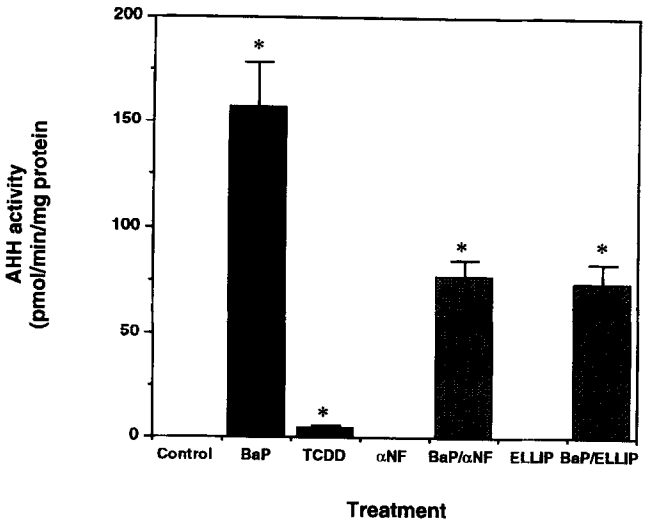

FIG. 3. AHH activity in subcultured glomerular mesangial cells upon exposure to BaP or TCDD. Randomly cycling GMCs (P_{12}) were treated with 30 μM BaP or 10 nM TCDD alone or in the presence of inhibitors for 24 hr and processed for AHH activity as described in Materials and Methods. For cotreatment experiments, cultures were pretreated with 1 μM αNF or 0.1 nM ELLIP for 30 min or 24 hr, respectively. Values are the means ± SEM for four replicate cultures. Similar results were seen in two separate experiments. (Key: (*) significantly different from respective control ($P < 0.05$).

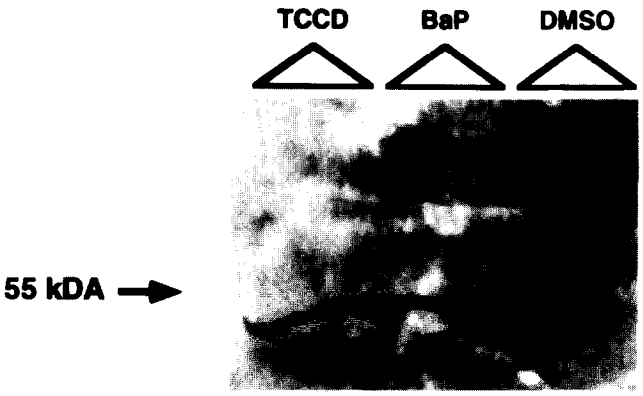

FIG. 4. Induction of CYP1B1 protein in GMCs treated with BaP or TCDD. Randomly cycling GMCs (P_{12}) were exposed to 30 μM BaP or 10 nM TCDD for 24 hr, and cellular proteins were harvested and separated by SDS–PAGE. CYP1B1 protein levels were determined by western blotting as described in Materials and Methods.

hibitor of DT-diaphorase. No increase in EROD activity was observed in BaP-treated GMCs under these conditions (data not shown).

Based on the atypical CYP1A1 mRNA and AHH/EROD induction profiles in GMCs, subsequent studies were conducted to determine if a distinct P450 isozyme capable of metabolizing BaP was expressed by GMCs. Pottenger and Jefcoate [5] have characterized a PAH-inducible CYP capable of catalyzing the oxidation of PAHs in mouse endometrial stromal cells and referred to it as CYP1B1. Treatment of GMCs with BaP (30 μM) or TCDD (10 nM) induced CYP1B1 at the protein level, as demonstrated by western blot analysis using a monoclonal antibody to this protein (Fig. 4). Induction of CYP1B1 protein was 2.5-fold greater in BaP-treated cells relative to TCDD. Then studies were conducted to investigate the metabolism of BaP by GMCs to genotoxic intermediates that covalently bind to DNA. GMCs were incubated with 30 μM BaP for 24 hr, and DNA for ^{32}P post-labeling of DNA adducts was obtained. DNA adducts were not detected in control GMCs (Fig. 5A), whereas four major and four minor DNA adducts were detected in BaP-treated GMCs (Fig. 5B). This same profile was observed in GMCs treated with a lower concentration of (3 μM) BaP (not shown). Pretreatment of GMCs with 1 μM αNF or 0.1 nM ELLIP did not induce adducts, but only αNF inhibited BaP-induced adduct formation (Fig. 5C–F). NDGA (10 μM) also blocked BaP-induced adducts in GMCs; however, this response was likely due to overt cytotoxicity as reflected by extensive cell rounding and detachment (data not shown).

Since the antiproliferative effects of BaP in GMCs may be mediated by oxidative metabolism of the parent compound, the effects of various CYP inhibitors on the antimitogenic effects of BaP were investigated. Consistent with a previous report [9], subcultured GMCs exposed to BaP exhibited decreased [^3H]thymidine incorporation into DNA and lowered cell proliferation rates relative to controls. All P450 inhibitors alone decreased [^3H]thymidine incorporation into GMCs (Table 1). At the two concentrations examined, none of the inhibitors reversed the antimitogenic effects of BaP. Lower concentrations of these inhibitors did not interfere with DNA synthesis in cultured cells, but failed to inhibit CYP1A1-related enzymes (data not shown). Following 72 hr of mitogenic stimulation, GMCs exposed to BaP in the presence of P450 inhibitors exhibited decreased cell numbers relative to controls (Table 1). GMCs treated with BaP and NDGA (10 μM) exhibited the most pronounced decrease in growth rates.

DISCUSSION

The data presented in this study demonstrate that GMCs possess inducible *CYP1A1* and *CYP1B1* expression associated with a corresponding increase in AHH activity and metabolism of BaP to intermediates that bind covalently to DNA. These findings are consistent with previous reports showing that whole kidney microsomes isolated from male or female rats possess CYP1A1-related AHH activity [23], and that TCDD-inducible isoenzymes are expressed in the glomerulus of rabbits [24]. A role for BaP and/or its metabolites in the initiation of renal injury has been suggested by studies showing a comparable profile of DNA adducts in kidney relative to liver in BaP-treated mice [25], and the occurrence of DNA adducts that persist for up to 14–20 days in kidneys of mice treated with BPDE, a key metabolic intermediate in the toxicity and carcinogenicity of BaP

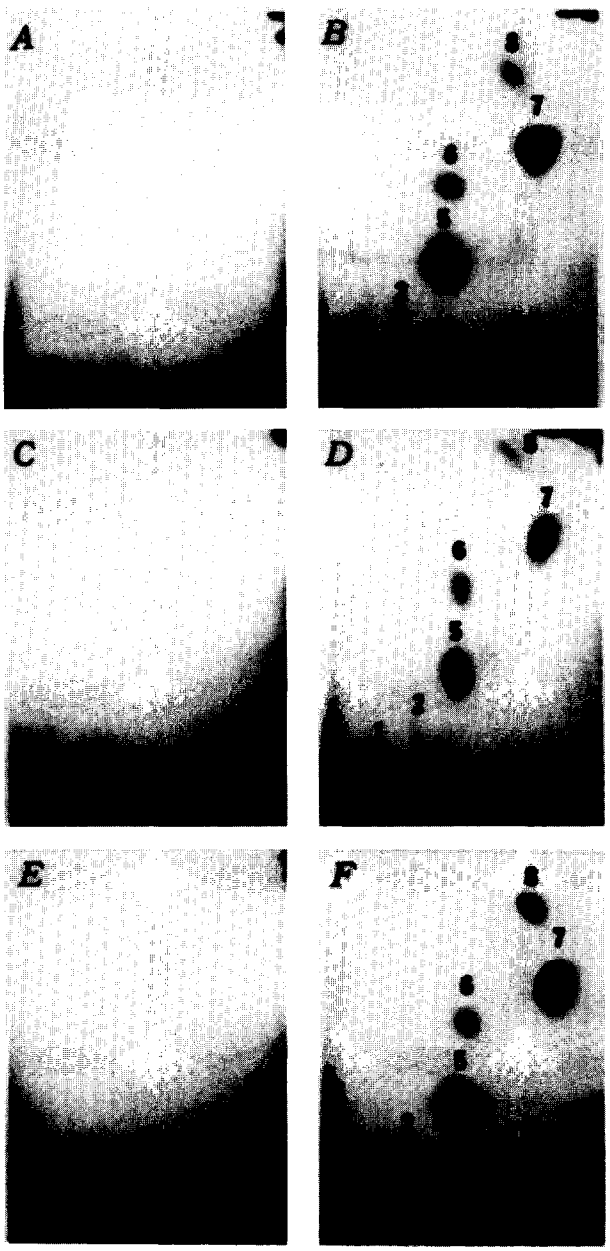

FIG. 5. ^{32}P-Labeled DNA adducts in subcultured glomerular mesangial cells after exposure to BaP in the presence or absence of αNF or ELLIP. Randomly cycling GMCs (P_{10}) were pretreated with either 1 μM αNF or 0.1 nM ELLIP for 30 min or 24 hr, respectively. Mesangial cells were then exposed to 30 μM BaP in the presence or absence of the inhibitors for an additional 24 hr. DNA was extracted for adduct determination as described in Materials and Methods (Key: panel A = control; panel B = BaP; panel C = αNF; panel D = αNF and BaP; panel E = ELLIP; and panel F = ELLIP and BaP. Numbers 1–8 were used to identify individual adducts. Experiments were repeated at least two separate times. A similar profile of adducts was observed in cultures treated with 3 μM BaP.

[26]. Recent *in vitro* studies in this laboratory have identified renal GMCs as preferential targets of BaP [9], observations which, when taken together with previous studies, implicate the kidney as a potential target organ of BaP.

TABLE 1. DNA synthetic rates and cellular growth of glomerular mesangial cells after exposure to BaP in the absence or presence of selected cytochrome P450 inhibitors

Chemical treatment	[^{3}H]Thymidine incorporation (cpm/μg protein)	Cell numbers (× 10^5)
Control	14,140 ± 570	15 ± 0.3
BaP (3 μM)	10,860 ± 440*	12 ± 0.2*
αNF (0.1 μM)	8,700 ± 870*	14 ± 0.2
αNF (1 μM)	7,420 ± 170*	14 ± 0.7*
αNF (0.1 μM) + BaP (3 μM)	7,560 ± 1020*	10 ± 0.3*
αNF (1 μM) + BaP (3 μM)	8,780 ± 800*	11 ± 0.5*
ELLIP (0.01 nM)	9,270 ± 440*	13 ± 0.3*
ELLIP (0.1 nM)	9,720 ± 910*	12 ± 0.5*
ELLIP (0.01 nM) + BaP (3 μM)	7,430 ± 960*	10 ± 0.4*
ELLIP (0.1 nM) + BaP (3 μM)	7,480 ± 200*	12 ± 0.3*
PIP (1 μM)	8,070 ± 250*	15 ± 0.2*
PIP (10 μM)	9,880 ± 420*	12 ± 0.3*
PIP (1 μM) + BaP (3 μM)	8,550 ± 250*	10 ± 0.3*
PIP (10 μM) + BaP (3 μM)	7,400 ± 530*	9 ± 0.1*
NDGA (10 μM)	8,910 ± 630*	15 ± 0.4
NDGA (10 μM) + BaP (3 μM)	3,980 ± 680*	7 ± 0.2*

Results are the means ± the SEM of four replicate samples per group for a representative experiment. Cycling cultured glomerular mesangial cells were exposed to the test compounds as described in Materials and Methods and subsequently processed for measurements of DNA synthesis or cell number. Concentrations of the test compounds utilized are given in parentheses. Experiments were conducted in duplicate.

* Significantly different from control counterparts ($P < 0.05$).

TCDD induces CYP1A1-dependent enzymes more effectively than BaP in most cell types including hepatocytes, and is considered one of the most potent inducers of this family of proteins [4, 27, 28]. Interestingly, the profile of mRNA and protein inducibility by AHs in GMCs was atypical in that BaP induced CYP1A1 mRNA levels and AHH activity more effectively than TCDD, and this induction was not associated with a corresponding increase of EROD activity. This unusual pattern of CYP1A1 inducibility can be accounted for by a CYP isoform that is regulated differentially at the transcriptional level relative to its hepatic counterpart [29]. This possibility is consistent with the ability of a partial CYP1A1 cDNA to recognize inducible mRNAs in GMCs following exposure to AHs and the appearance of ELLIP-sensitive enzymatic activity.

The atypical pattern of induction may also be accounted for by the occurrence of a distinct CYP isoform exhibiting substrate specificity comparable to the enzymatic activity encoded by *CYP1A1*. The identification of *CYP1B1* as a PAH-inducible CYP capable of catalyzing the oxidation of PAHs is consistent with this suggestion [5, 30]. CYP1B1 is highly expressed in human kidney cells [31], and in C3H/10T1/2 cells where benz[*a*]anthracene is a more effective inducer than TCDD [32]. The results of our western blot analysis demonstrate that CYP1B1 is inducible in GMCs by AHs and, as described by others [30], BaP was a more potent inducer than TCDD.

BaP inhibits cell proliferation in Syrian hamster embryo

cells [33], vascular smooth muscle cells [34], CV-1 monkey kidney cells [35], and rat renal GMCs [9]. In GMCs, the inhibitory response involves selective interference with early cell cycle progression [36]. Although the mechanisms that mediate the growth inhibitory effects of BaP remain to be fully defined, it is likely that oxidative metabolites that bind to DNA and other macromolecules contribute to the antimitogenic effect [34, 37]. αNF inhibits the induction of AHH in hamster embryo cells and protects these cells from carcinogen-induced growth suppression [16]. The mechanism by which αNF inhibits AH-inducible CYP1A1 is thought to involve blockade of transcriptionally active nuclear ligand/Ah receptor complexes as well as inhibition of catalytic activity [17]. The ability of αNF to inhibit AHH activity and DNA adduct formation in GMCs may involve either or both of these mechanisms. In contrast, ELLIP is a naturally occurring compound that prevents chemically induced mutagenesis and carcinogenesis, presumably by reversibly binding to the catalytic site of CYP1A1 to inhibit AHH activity [18]. An interesting finding in our studies was that, in contrast to αNF, the ability of ELLIP to inhibit BaP-induced AHH activity did not afford protection from DNA–adduct occurrence in GMCs. These results suggest that these two compounds interfere with CYP-mediated oxidation of BaP via different mechanisms and that the molecular targets of interference for the two compounds are different. The use of PIP and NDGA, two general P450 inhibitors, did not clarify this issue further since both agents failed to modulate BaP-induced DNA-adduct formation.

The ability of BaP to inhibit proliferation likely involves oxidative metabolism by one or more of these CYPs [17, 20]. Thus, experiments were conducted to evaluate the impact of several CYP inhibitors on the acute GMC response to BaP. αNF and ELLIP were used to modulate CYP1A1 and CYP1B1 activities. The insecticide synergist PIP was used to preferentially inhibit CYP1A2 and CYP1B1 following formation of a complex between P450 and a metabolic intermediate, and NDGA to inhibit the CYP monooxygenase and lipooxygenase branches of the arachidonate cascade [20]. Unfortunately, all CYP inhibitors decreased DNA synthesis and enhanced the antiproliferative effects of BaP, thus precluding further elucidation of the atypical response.

Although limited experimental studies have examined the renal effects of BaP, occupational and epidemiologic evidence has emerged suggesting that AHs modulate renal function and contribute to the initiation and/or promotion of renal proliferative and membranous glomerulonephritis (reviewed in Ref. 38). We have shown recently that acute exposure of GMCs to BaP inhibits DNA synthesis [9] and modulates growth factor-related mitogenic signal transduction [36]. The results presented here suggest that these responses are most likely mediated by oxidative metabolism of BaP catalyzed by CYP1A1 and/or CYP1B1.

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