The carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) has been well established, and extensive research over the past 30 years has been directed at elucidating the mechanism of bioactivation of PAHs to yield carcinogenic intermediates (Miller, 1998). These studies have revealed roles for cytochromes P450 (P450), particularly CYP1A and 1B (Guengerich and Shimada, 1998), for microsomal epoxide hydrolase (Guengerich et al., 1996), and for specific structural features of the PAHs in their carcinogenicity (Wood et al., 1981). In the case of benzo[a]pyrene (BAP), a series of reactions catalyzed by P450, epoxide hydrolase, and P450 again, produced the ultimate proximate carcinogenic form of BAP (Shimada et al., 1994). CYP1B1 has also recently been demonstrated to be capable of bioactivating PAH procarcinogens (Shimada et al., 1996; Luch et al., 1999).

The majority of early studies designed to elucidate mechanisms of PAH bioactivation have been performed with rodents. The applicability of the results of such studies to humans has only recently been addressed (Kawajiri and Fujii-Kuriyama, 1991). Thus, human cDNA-expressed CYP1A1 and purified CYP1A1 enhanced the genotoxicity of a proximate carcinogenic form of BAP (Shimada et al., 1994). cDNA-expressed CYP1A1 and CYP1A2 both catalyzed stereoselective epoxidation of a series of PAHs (Shou et al., 1996). Human CYP1B1 has also recently been demonstrated to be capable of bioactivating PAH precarcinogens (Shimada et al., 1996; Luch et al., 1999). The majority of published studies of PAH carcinogenicity have been conducted using individual PAH forms, yet human exposures are usually to mixtures of PAHs and other xenobiotics. PAHs and metals frequently occur as environmental cocontaminants, and several recent reports highlight the ubiquity of PAH/metal mixtures in the environment. Both PAHs and metals are ranked highly on the 1999 list of the most hazardous xenobiotics in the environment, prepared by the Agency for Toxic Substances and Disease Registry and the Environmental Protection Agency (1999). Based on the three criteria of...
frequency of occurrence in the environment, toxicity, and potential exposure to humans, the top listed metals are arsenic, lead, mercury, and cadmium, and the top listed PAHs are BAP, benzo[a]fluoranthene (BFF), dibenzo[a,h]anthracene (DBAHA), benzo[a]anthracene (BAA), and benzo[k]fluoranthene (BKF). These hazard priorities were the basis for the selection of these compounds for this study.

Several reports have implicated metals as modifiers of P450 function and regulation, which implies that such metals could alter P450-mediated PAH mutagenicity and carcinogenicity. The known effects of the listed metals on P450 function and regulation are limited primarily to rodent P450s. Data on metal effects on human P450s are extremely limited; lead body burdens in children decreased 6- to 9-fold after the end of the study, and the top listed PAHs are BAP, benzo[a]anthracene (BAA), dibenzo[a,h]anthracene (DBAHA), benzo[a]anthracene (BAA), and benzo[k]fluoranthene (BKF). These hazard priorities were the basis for the selection of these compounds for this study.

In this article we report our studies to determine the capacity of the readily available human hepatoma cell line HepG2 has proven to be a useful model for investigations of the regulation of human CYP1A1 (Lipp et al., 1992; Krusekopf et al., 1997). Studies of CYP1A2 induction in HepG2 cells have also revealed very low levels of induction of the mRNA by 3-methylcholanthrene (Li et al., 1998), but there are no reports of induction of CYP1A2 protein in HepG2 cells.

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**Experimental Procedures**

**Materials.** BAP, BAA, DBAHA, BKF, and BBF were 99 to 100% pure and were obtained from AccuStandard Inc. (New Haven, CT). A stock solution of each PAH was prepared by dissolving PAHs in dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO), and concentrations were determined in triplicate analyses with a published method (EPA Method 610, 1984). Sodium arsenite was obtained from Sigma, and lead nitrate, cadmium chloride, and mercury chloride (anhydrous beads) were obtained from Aldrich (Milwaukee, WI). All metal salts were 99 to 100% pure. A 10 to 20 mM stock solution of each metal salt was prepared in deionized water, and concentrations of arsenic, cadmium, and lead were determined by inductively coupled argon plasma atomic emission spectroscopy (EPA Method 200.7, 1998), and mercury by cold vapor atomic absorption spectroscopy (EPA Method 245.1 and 245.2, 1998). Stock solutions of metals were stored at room temperature and fresh dilutions were made prior to each experiment.

**HepG2 Cells.** HepG2 cells, obtained from the American Type Culture Collection (Manassas, VA), were grown in medium made up of 1:1 (volume) α-modified Eagle’s medium and Waymouth medium (αEW). The medium was supplemented with 5% fetal bovine serum from HyClone Laboratories (Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM). The HepG2 cell cultures were maintained in 75-cm² surface area, 250-ml tissue culture flasks from Becton Dickinson Labware (Franklin Lakes, NJ), in a 5% CO₂ incubator at 37° C. When needed for confluent cultures of HepG2 cells were removed from the flask following treatment with 0.25% trypsin in phosphate-buffered saline (PBS). A single cell suspension of trypsinized cells in αEW was prepared by repeated passage of cell suspension through an 18-gauge needle. The cell densities were selected based on preliminary studies using a range, which identified the selected densities as providing substantial PAH induction by a 24-h period. Sixteen to 20 h following plating the cultures were 75% confluent and were treated subsequently for various periods with PAHs, metals, or their mixtures. At the end of the treatment, the control or treated cultures exhibiting no toxic effect of treatment were confluent, with occasional pockets of aggregated cells. In studies where PAHs, metals, and their mixtures were added to the medium, serum-free medium was used (see below). The cells plated at cell densities of 5 × 10⁶ cells/well in 96-well plates and 1.5 × 10⁶ cells/well in 6-well plates were found to be optimal for CYP1A induction by PAHs and for metal/PAH cytotoxicity studies, and these cell densities were used in all subsequent studies.

**EROD Assay.** A published EROD activity assay in 96-well plates (Donato et al., 1993) was used with modifications. The culture medium in the wells was replaced with 100 μl/well of culture medium containing 8 μM 7-ethoxresoru- fin from Sigma, and an NADP/PH (quinone acceptor) oxidoreductase inhibitor (Lubet et al., 1985), Dicumarol (10 μM) (Donato et al., 1993) or salicylamide (3 mM) from Sigma. After a 45-min incubation at 37°C in a CO₂ incubator, 75 μl of culture media from each well was transferred to white, opaque 96-well plates supplied by Bioworld Lab Essentials (Dublin, OH). β-Glucuronidase/sulfatase from Roche Molecular Biochemicals (Indianapolis, IN), 15 Fishman units and 120 Roy units, respectively, was dissolved in 15 μl of phosphate-buffered saline, pH 7.2, and added to each well. The plate was covered with SealPlate from Bioworld Lab Essentials to prevent evaporation of media, and incubated for 2 h at 37°C to hydrolyze any hydroxy resorufin conjugates. After 2 h, 100 μl of acetylcholine from J. T. Baker (Phillipsburg, NJ) was added to each well and the plate was centrifuged at 1000g for 5 min. The fluorescence of each well’s content was measured using a luminescence spectrometer LS50B purchased from PerkinElmer (Norwalk, CT), with 530-nm excitation and 590-nm emission filters.

To assess the inhibitory effect of PAHs and metals on EROD activity, assays were carried out using microsomes prepared from baculovirus-infected insect cells expressing human CYP1A1 from GENTEST (Woburn, MA). Reaction mixtures comprised 1 pmol of CYP1A1 in microsomes, 8 μM 7-ethoxresorufin, and 1.3 mM NADPH and were incubated for a range of times at room temperature. The fluorescence in the wells was read as previously described after reactions were terminated with 100 μl of acetonitrile.

**Immunoblot Analysis.** 89 fractions of HepG2 cells in PBS and cDNA-expressed CYP1A1 and 1A2 microsomal standards were separated by the Novex NuPAGE Bis-Tris electrophoresis system using the Bis-Tris-HEC-buffered polyacrylamide gel (with 10% acrylamide) as described by the manufacturer (Novex, San Diego, CA). CYP1A1 and CYP1B2 were loaded at 0.25 and 0.5 ng/well, respectively, and 89 samples were loaded at 25 μg of protein/well. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membrane sheets, which were then treated with a blocking solution (5% Carnation nonfat dry milk in 0.2 M Tris buffer, pH 7.4, containing 0.15 M NaCl and 0.25% Tween 20) for 45 min at room temperature or overnight at 4°C. Membranes were incubated with a polyclonal goat anti-rat CYP1A1/1A2 antibody (catalog no. 242472; GENTEST) in the blocking solution for an additional hour at room temperature or overnight at 4°C, washed with the buffer, and then incubated with a secondary antibody at 1:7500 dilution in the blocking solution. The secondary antibody was peroxi- dase-labeled rabbit anti-goat IgG and was detected with an enhanced chemi- luminescence kit as described by the manufacturer (Pierce, Rockford, IL). Data was quantified with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).
occasional pockets of aggregates of cells. To determine residual PAH, 100 μl of medium was removed from triplicate wells at 0, 1.3, 8, and 24 h, and each sample was solid-phase extracted by direct application to a Sep-Pak C18 cartridge purchased from Waters (Milford, MA). Cartridges were prewashed with methylene chloride (4 × 10 ml), methanol (4 × 10 ml), and water (2 × 10 ml). The PAH-bound cartridge was then dried by drawing air through under vacuum and washed with water (10 ml); then the PAH was eluted with methylene chloride (10 ml). The methylene chloride extract was dried with sodium sulfate and evaporated to 1.0 ml under a stream of dry nitrogen. Acetonitrile (3.0 ml) was added to the methylene chloride extract, which was further concentrated under nitrogen to 0.5 ml. The extract was brought to 1.0 ml with acetonitrile. This solution was analyzed for PAH content by a high-performance liquid chromatography method (EPA Method 610, 1984). Measurement of Cell Viability. HepG2 cell viability after treatment with PAHs or metals was determined by testing the reducing enzymes present in viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals (Liu et al., 1997). In brief, a solution of MTT from Sigma (5 mg/ml) was prepared in PBS, pH 7.2, and 100 μl was added to each well of a 96-well plate containing cells and 100 μl of serum-free medium added directly to the Sep-Pak served as a positive control. To determine the extent of PAHs into HepG2 cells from the medium, the cells at the selected time intervals after removal of medium were washed with fresh PAH-free medium (120 μl). Cells were then incubated at 37°C for 5 min with 0.25% trypsin in PBS. Trypsinized cell suspension was transferred to a microtube with 100 μl of medium, and centrifuged at 13,800 g for 5 min. The cell pellet was suspended in acetonitrile (500 μl) and ultrasonicated for 3 × 5 s. The cell lysate were centrifuged again at 13,800g for 5 min. The supernatant was analyzed for PAH concentration by high-performance liquid chromatography (EPA Method 610, 1984).

Optimization of PAH Induction of CYP1A1 in HepG2 Cells. PAH induction of CYP1A1 was optimized by varying the media, PAH concentrations, and incubation times with HepG2 cells and using EROD activity as a probe. The optimization is functional only, because the probe system, EROD activity, is inhibited by the inducing agent (see below).

Media. One medium containing 5% fetal bovine serum and three without serum were used. dEWS supplemented with 5% fetal bovine serum, penicillin (100U/ml), streptomycin (100 μg/ml), and glutamine (2 mM) prepared by the media department of the Wadsworth Center was used. The other three serum-free media were obtained from In Vitro Technologies (catalog no. Z900009), Clonetics, San Diego, CA (catalog no. CC3198-HCOMBullekit), and the International Institute for the Advancement of Medicine (Scranton, PA) (serum-free hepatocyte culture medium).

Concentrations of PAHs. Each PAH was tested over a range of micromolar concentrations.

Metals. Preliminary experiments were performed to determine the maximum nontoxic concentration of each metal as assessed by the MTT and Calcein methods (described previously). Subsequently, each metal was tested for its effect on PAH-induced EROD activity at nontoxic metal concentrations.

Incubation time. EROD activity of HepG2 cells induced by PAHs was tested at 6, 12, 18, and 24 h after addition of PAH.

Culture conditions. Cultures of HepG2 cells plated for 16 to 18 h were treated with the appropriate culture media containing DMSO (maximum concentration in media was 0.25%), individual PAHs, metals, or their mixtures in 100-μl volume, and were incubated at 37°C in a incubator containing 5% CO₂ for the required duration. After the incubation time, the cells were probed for CYP1A1 induction by the EROD assay as described above.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Conditions used for the isolation of RNA, and reverse transcription (O'Dt priming of 5 μg of total RNA in a 20-μl reaction) with Superscript II were as described previously (Fasco et al., 1995). Preparation of the CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase competitors and description of the nucleotide sequences used as primer sets were also as described (Fasco et al., 1995). Master mixes were used throughout. Amplifications were done in 25-μl aliquots that contained 2.5 μl of a 10× buffer (supplied); 0.1 μl of primer mixture (50 μM each in stock solution); 0.5 μl of dNTP mixture (10 mM each in stock solution); 0.5 μl of Clontech Taq plus antibody; 0.5 μl of cDNA (from 0.125 μg of total RNA); and water to 24 μl. Concentrations of the appropriate competitor were added last in 1 μl of water. Amplification mixtures were heated at 95°C for 1 min followed by 30 cycles of 95°C denaturation for 15 s, annealing at 65°C for 15 s, and extension at 68°C for 30 s. A 5-min incubation at 65°C followed the last cycle. Aliquots (10 μl) were separated in 2% agarose gels. The gel and separation buffer was 0.6% Tris-borate-EDTA containing 0.5 μg of ethidium bromide per milliliter. A Kodak Digital EDAS 120 system was used to digitize and quantitate the bands. A low mass DNA ladder (Life Technologies, Gaithersburg, MD) was used as the external standard.

Additional mRNA Analysis Was Conducted Using Real Time PCR Methods. Real Time RT-PCR reactions were done in a Light Cycler from Roche Molecular Biochemicals using a One-Step RT-PCR kit from Qiagen (Valencia, CA) containing Syber Green from Molecular Probes. Conditions used for the isolation of RNA and the primers for CYP1A1 amplification were as described previously (Fasco et al., 1995). Primers for 36B4, accession number M17885, were 5'-TGC-TCA-ACA-CTC-CCC-CTT-TCT-C (forward primer, starting at 607) and 5'-ACC-AAA-TCC-CAT-ATC-CTC-GTC-C (reverse primer, ending at 1019). Master mixes were used throughout. RT-PCR mixtures contained 4 μl of a 5× buffer, 0.4 μl of primer (25 pmol/μl each in stock solution), 0.8 μl of dNTP mixture, 0.8 μl of reverse transcriptase/DNA polymerase enzyme mix; 1 μl of Syber Green (previously diluted 1:5000 in water), 1 μl of total RNA (1.0–0.001 μg/μl), and water to 20 μl. Mixtures were reverse transcribed by heating at 50°C for 30 min. The DNA polymerase was activated by heating at 95°C for 15 min followed by 45 cycles of 94°C denaturation for 5 s, annealing at 60°C for 5 s, and extension at 72°C for 20 s. Standard curves for 36B4 and CYP1A1 were generated using dilutions of BFK-treated mRNA and software supplied with the Light Cycler. Target gene expression was calculated from the standard curve.

Calculations. The fold induction of CYP1A1 activity following PAH stimulation was expressed as EROD activity (induced/control) and was calculated by determining the mean and standard deviations of the ratios of fluorescence values in wells treated with PAHs to wells treated with DMSO.

Results. Throughout these studies the metal ions investigated were administered as AsO₄²⁻, Cd²⁺, Hg²⁺, and Pb²⁺. For simplicity they are referred to as “metals” in the text. In all cases the salts were readily soluble in aqueous solution at the concentrations used. The data presented are representative examples of at least three experiments.

EROD Assay. To optimize the EROD assay and to determine the potential of the PAHs and the metals to inhibit CYP1A1 activity in the EROD assay in HepG2 cells, CYP1A1 superosomes were used as a model system. EROD rates were saturated at 4 μM substrate concentration and 8 μM ethoxyresorufin was used as substrate in subsequent studies.
The effects of 100 to 165 μM concentrations of the metals individually on the rates of EROD were determined. Arsenic (100 μM) and lead (165 μM) produced no inhibition, while cadmium (100 μM) showed slight inhibition (3%) and mercury (100 μM) completely inhibited the reaction. Additional studies with mercury at lower concentrations showed that at 1 μM mercury the CYP1A1 EROD reaction was inhibited by 28%. The inhibitory effect of the five PAHs individually on CYP1A1 EROD activity varied, with BAA yielding the greatest extent of inhibition and DBAHA the lowest (Table 1).

**Immunoblot Analysis.** Immunoblot analysis (Fig. 1) indicated that under the experimental conditions neither CYP1A1 nor CYP1A2 proteins were detected in DMSO-treated HepG2 cells. Exposure of HepG2 cells to BKF (5 μM) for 24 h at 37°C produced strong induction of CYP1A1 protein, but CYP1A2 was not detected even after extended exposures of the film. The slight shift in migration of CYP1A1 in the S9 fraction from BKF-treated HepG2 cells relative to the CYP1A1 standard was shown to be due to the large difference in total protein content loaded.

**PAH Uptake into HepG2 Cells.** Studies were conducted to determine the time dependence of PAH remaining in media after uptake by HepG2 cells; PAH concentrations within HepG2 cells, and PAH uptake by the walls of the wells on the 96-well plates. The results are presented in Fig. 2 with BAP and BKF as examples of PAHs. At 8 μM BAP there was no significant loss to the well walls from the medium in the absence of HepG2 cells after 24 h of exposure; in contrast, 84% of the BAP in the medium was taken up by the cells by 24 h. In the case of BAP the content in the cells increased over the 24-h period with the maximum level reaching 40% of the available BAP in the medium. The maximum level of BKF in the cells reached 40% of the available BKF and this was achieved at 15 h. At later times the cellular BKF level decreased, probably reflective of its CYP1A1-catalyzed metabolism. All of the cellular levels of BAP or BKF are probably reflective of the levels after depletion by metabolism.

**Effect of Metals and PAHs on HepG2 Viability.** The effects of the metals on HepG2 cell viability were tested at 1, 5, and 25 μM for cadmium, mercury, and arsenic and in addition 50 μM of lead during a 24-h incubation using the MTT assay. The metals differentially affected cell viability. Lead was the least toxic and did not significantly affect viability at 50 μM. In contrast the highest concentrations of cadmium, arsenic, and mercury that did not affect viability (>90% viable) were 5, 25, and 25 μM, respectively. All subsequent studies were conducted at 1 and 5 μM arsenic, cadmium, and mercury, and up to 40 μM lead.

Viability studies with PAHs were limited to PAHs at concentrations of up to 20 μM in excess of the highest level of HepG2 exposure to PAH. HepG2 cells incubated for 24 h with 5 μM concentrations of each of the PAHs exhibited >90% viability except for DBAHA, where viability was 76%. Cell viability studies with calcine AM yielded essentially the same results as with MTT, and the data are thus not shown.

**TABLE 1**

<table>
<thead>
<tr>
<th>PAH</th>
<th>Inhibition of EROD Activity (%)</th>
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<tbody>
<tr>
<td>BAP</td>
<td>27</td>
</tr>
<tr>
<td>BKF</td>
<td>15</td>
</tr>
<tr>
<td>BBF</td>
<td>17</td>
</tr>
<tr>
<td>BAA</td>
<td>39</td>
</tr>
<tr>
<td>DBAHA</td>
<td>5</td>
</tr>
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Data represent the means of triplicate determinations.
Based on the data provided above, PAH induction and all other studies of CYP1A1 in HepG2 cells were conducted for 24 h with serum-free medium from In Vitro Technologies. The extent of CYP1A1 induction was determined using EROD activity. However, to more accurately gauge the relative CYP1A1 induction potencies of the PAHs in HepG2 cells, quantitative immunoblotting was also undertaken. The apparent extent of CYP1A1 induction in HepG2 cells by the five PAHs as a function of PAH concentration, as gauged by EROD activity, is provided in Fig. 4. Induction efficiency (relative to DMSO controls) at 5 μM PAH concentration is in the order BKF (16.1-fold) > DBAHA (13.5-fold) > BAA (3.5-fold) > BAP (2.9-fold) > BBF (1.2-fold). These fold values represent the increase of EROD activity as a consequence of PAH exposure. It is not known whether the basal activity arises from constitutive CYP1A1, which is below detection by immunoblot analysis. The extent of induction by 1, 5, and 10 μM PAH as gauged by quantitation of immunoblots is shown in Fig. 5. Induction efficiency at 5 μM PAH is in the order DBAHA (40-fold) > BKF (24-fold) > BAP (10-fold) > BBF (7-fold) > BAA (1-fold). Since CYP1A1 was not detected by immunoblot analysis in cells that were treated with DMSO only, all relative PAH-mediated induction, using immunoblot analysis, is reported relative to that induced by BAA set at 1.

Effect of Metals on Induction of HepG2 CYP1A1 by PAHs. The effects of the metals (5 μM for arsenic, cadmium, and mercury; 40 μM for lead) on PAH-mediated induction of CYP1A1 in HepG2 cells as determined by EROD activities are shown in Fig. 6. All of the metals (with the exception of mercury in the case of DBAHA) decreased the inductive capacity of the various PAHs in a dose-dependent (data not shown) and metal- and PAH-dependent manner. The relative efficiency of decrease of EROD activity, and thus of CYP1A1 induction by all five PAHs was in the order cadmium > arsenic > lead > mercury. Note that in Fig. 6 lead is at an 8-fold higher concentration than the other metals. BBF induction was least affected by all of the metals, while cadmium most effectively decreased BKF and DBAHA induction, and lead most effectively decreased DBAHA induction. All of these studies were conducted with PAHs and metals coadministered. In studies where metals were administered at the time of addition of the 7-ethoxyresorufin substrate, no inhibitory effects of the metals on EROD activities were observed.

Immunoblot analysis revealed that for BKF (5 μM)-treated HepG2 cells, arsenic (5 μM) and cadmium (5 μM) produced marked decreases in the level of CYP1A1 protein, consistent with the effects on the EROD activities. Addition of mercury (5 μM) or lead (5 μM) to the HepG2 cells together with the BKF, however, increased the induced CYP1A1 protein levels relative to levels obtained with BKF alone (Fig. 7).

RT-PCR Analysis. Triplicate quantitative RT-PCR analysis revealed that BKF (5 μM) for 24 h at 37°C induced CYP1A1 mRNA to a concentration of 8 amol/μg RNA in HepG2 cells. Sodium arsenite (5 μM) when administered concomitantly with BKF in the medium did not decrease CYP1A1 mRNA concentration, which was determined to be 9 amol/μg RNA. The data are presented in Fig. 8. Real time PCR analysis indicated that none of the four metals tested (at 5 μM for arsenic, lead, and mercury and 2.5 μM for cadmium), significantly decreased the levels of CYP1A1 mRNA, which was induced by BKF (5 μM) in the HepG2 cells (Fig. 9).

Discussion

The metals and PAHs selected for investigation represent the most hazardous forms present in the environment (Agency for Toxic Substances and Disease Registry, 1999). They frequently occur as mixtures and assessment of carcinogenic risk from exposure to such mixtures must take into account potential interactions of the components of the mixtures. The generation of PAHs during cigarette smoking, and the presence of high levels of cadmium in tobacco, provide for another potential human exposure to a mixture of PAHs and a heavy metal.

There are many different aspects of the pathways of PAH-mediated to carcinogenesis that could be affected by cocontaminant metals. Metals could influence bioactivation of PAHs by phase I enzymes, inactivation of bioactivated PAHs by phase II enzymes, and induction of the enzymes catalyzing these reactions. Since some of the metals are carcinogenic, they could influence steps subsequent to PAH bioactivation leading to carcinogenicity. In this study we have focused on
only one of the potential interactions, the potential of the metals to affect the induction of human CYP1A1 by PAHs, and thereby influence the extent of bioactivation of the PAHs. EROD activity was selected as a probe for CYP1A1 induction because it provides a functional assessment of induction and because it is readily amenable to rapid throughput analysis (Donato et al., 1993; Kennedy and Jones, 1994). The assessment of CYP1A1 induction by EROD activity can, however, be confounded by the inducing agent or other test compounds inhibiting EROD activity (Willett et al., 1998; Petrulis and Bunce, 1999) and our data support this. Immunoblot analysis was thus also applied to confirm EROD-based observations.

CYP1A1 induction was probed by EROD activity determinations. Values represent mean ± S.D. of triplicate determinations. Experimental methods are presented under Experimental Procedures. The PAH vehicle was DMSO. *, significantly lower than rates in the absence of metals (p < 0.05).

CYP1A1 immunoblot analysis of the HepG2 cells revealed that despite their liver origin, where CYP1A2 expression greatly predominate (Zhang et al., 1995), only CYP1A1 protein was detectable and then only after its induction by a PAH. CYP1A2 protein was not detected even after PAH induction. This is consistent with published observations and occurs despite the detected induction of CYP1A2 mRNA in HepG2 cells after incubation with PAHs (Li et al., 1998). Either CYP1A2 mRNA was not induced to levels that were adequate for translation to immunoblot-detectable CYP1A2 protein, or the relative efficiency of CYP1A2 mRNA translation is low. This implies that HepG2 cells are not a good model for studies of induction of
triplicate experiments.

**Experimental Procedures**

Methods are presented under Experimental Procedures. Data are means ± S.D. of triplicate experiments.

Hepatic CYP1A2. They should rather be considered a model for extrahepatic CYP1A1 induction. Additionally, CYP1B1 protein was not detectable in HepG2 cells, even after the cells were treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (Spink et al., 1994). Thus, in the absence of expressed CYP1A2 and 1B1, the induced EROD activity detected in the HepG2 cells must be catalyzed by human CYP1A1.

Quantitative immunoblot analysis and EROD activity assays revealed that while all five test PAHs induced CYP1A1 in HepG2 cells, DBAHA and BKF were markedly more effective than BAP, BBF, and BAA. The failure to achieve linear increases of CYP1A1 levels with increases in PAH concentrations, as assessed by immunoblot analysis, was not a function of decreased cell viability and is probably a consequence of saturation of binding to the aryl hydrocarbon receptor.

While individual PAH carcinogenicity is determined to a major extent by the molecular structure of the bioactivated PAH (Wood et al., 1981), the extent of the bioactivation is governed by the level of available CYP1A1, which in turn is determined by the extent of its induction (Guengerich, 1988; Guengerich and Shimada, 1998). DBAHA and BKF, because of their superior CYP1A1-inducing efficiency, could thus enhance the carcinogenic potential of a mixture of PAHs.

The induction potency of PAHs for CYP1A1 in rat hepatocyte cultures has been reported to vary in the order BKF > DBAHA > BBF > BAP > BAA with relative potencies of approximately 100: 50:15:10:1 (Till et al., 1999). The rat and human systems thus display similar susceptibilities to CYP1A1 induction by PAHs, although the rat hepatocytes exhibit approximately 1.5- to 2-fold greater extents of CYP1A1 induction than was detected in the HepG2 cells.

Having established the patterns of human CYP1A1 induction by the five PAHs, we investigated the effects of the four metals on this induction. All four of the metals investigated decreased the extent of CYP1A1 induction in HepG2 cells by at least one of the five PAHs, in some cases decreases were marked. The same order of effectiveness of metal-mediated decreases in CYP1A1 were cadmium > arsenic > lead > mercury for all the PAHs. However, CYP1A1 induction in the HepG2 cells was decreased by the metals to varying extents depending on which PAH mediated the induction. Clearly, the observed effects of the metals were on the extents of CYP1A1 protein induction and not on the EROD reactions, since no metal effects were detected when metals were added after the cells were incubated with PAHs for 24 h. This conclusion is strongly supported by the immunoblot data, which indicate that BKF-induced HepG2 CYP1A1 protein levels were markedly decreased by exposure of the cells to arsenic or cadmium concomitant with exposure to the BKF. In the case of lead or mercury exposure, which either did not diminish BKF-induced EROD activity or diminished levels relatively poorly, the BKF-induced CYP1A1 protein levels were in fact increased. This suggests that where mercury and lead decrease PAH-induced EROD activity in HepG2 cells, the mechanisms probably involve loss of heme from the induced CYP1A1, or synthesis of apo enzyme together with holo enzymes.

We plan to conduct extensive studies to determine the mechanisms of metal-mediated decreases in the extent of PAH induction of CYP1A1. To provide a preliminary assessment we determined the effect of the four metals on BKF-induced levels of CYP1A1 mRNA in HepG2 cells using RT-PCR techniques. The study revealed no significant decrease in mRNA concentrations as a consequence of the addition of any of the four test metals to the cells at levels that produced decreased CYP1A1 activities. This is consistent with our studies on the effect of As on CYP1A1 mRNA levels in human hepatocytes induced with PAHs (Vakharia et al., 2000) and on CYP1A1 and 1B1 mRNA levels in a human breast cancer cell line (unpublished data). This suggests that the majority of the effect of the metals on CYP1A1 induction was not at the level of transcription and implies that translational and/or post-translational mechanisms were operative. Published studies on the effects of As on CYP1A1 induction by PAH in chick embryo hepatocytes and rat hepatocytes suggest alternative mechanisms. Sodium arsenite inhibits 3-methylcholanthrene-mediated CYP1A induction in primary cultures of chick embryonic hepatocytes (Jacobs et al., 1998). In this system the arsenite induced heme oxygenase, which could decrease CYP1A levels via degradation of its heme. In a similar study, arsenite inhibited 3-methylcholanthrene-mediated CYP1A induction in primary cultures of rat hepatocytes (Jacobs et al., 1999). Arsenite (5 μM) caused a 55% decrease in CYP1A1 protein and activity and a 25% decrease in CYP1A1 mRNA levels. It was concluded that arsenite-mediated increases in heme oxygenase were not solely responsible for CYP1A1 decreases (Jacobs et al., 1999). The latter study suggests a possible role for arsenic-mediated regulation of CYP1A1 induction at the transcriptional level. We have now demonstrated that arsenic (at the same concentrations used in this study), induces heme oxygenase mRNA in HepG2 cells (unpublished data). The other metals do not induce under the experimental conditions used. While this does not prove that the effects of the metals on HepG2 CYP1A1 induction are due to induced levels of heme oxygenase post-translationally destroying the CYP1A1, they do add some support for such a mechanism.

Based on the marked decreases in PAH-mediated CYP1A1 induction in HepG2 cells brought about by concomitant exposure of the cells to the metals under investigation, it is reasonable to conclude that exposure to mixtures of PAHs and metals would be less effective in inducing CYP1A1 than exposure to PAHs alone. Since increased levels of CYP1A1 will lead to increased extents of bioactivation of PAHs with increased carcinogenic consequences, it is likely that chronic exposure to metal/PAH mixtures could diminish the carcinogenicity of the PAHs in the mixtures. However, many other factors could influence PAH carcinogenicity, and it is not clear whether the effects of metals on PAH induction of CYP1 will be a predominant factor.

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References


