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Regulation of Cytochrome P450 Enzymes by Aryl Hydrocarbon Receptor in Human Cells

CYP1A2 EXPRESSION IN THE LS180 COLON CARCINOMA CELL LINE AFTER TREATMENT WITH 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN OR 3-METHYLCHOLANTHRENE

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ABSTRACT. It has been difficult to study the regulation of cytochrome P4501A2 (CYP1A2) because expression of this enzyme is reported to be limited or absent in cell culture. We found that CYP1A2 can be induced significantly by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene (MC), or benz[*a*]anthracene in the human colon carcinoma cell line LS180. TCDD and MC each caused a dramatic elevation of CYP1A2 mRNA, as assessed by reverse transcription–polymerase chain reaction or by northern blot analysis. TCDD also increased immunoreactive CYP1A2 protein and the activity of phenacetin-*O*-deethylase, a diagnostic catalytic marker for CYP1A2. The induction of CYP1A2 at all levels (mRNA, protein, catalytic activity) was concentration- and time-dependent: the EC_{50} for mRNA induction by TCDD = 0.5 nM, and by MC = 1.4 μM. Inducible CYP1A2 mRNA also was detected at lower levels in two other human cell lines, the hepatoma cell line HepG2 and the breast carcinoma cell line MCF-7. CYP1A1 and CYP1B1, additional CYP1 enzymes regulated by the aryl hydrocarbon receptor (AHR), also were inducible by TCDD and MC in LS180 cells; their concentration-dependent induction was highly correlated with induction of CYP1A2 at mRNA, protein, and catalytic levels. CYP1B1 was constitutively expressed and inducible in the LS180, MCF-7, and HepG2 cell lines as well as in the human choriocarcinoma cell line JEG-3 and the squamous cell carcinoma line A431. CYP1A2 was neither constitutively expressed nor inducible in A431 or JEG-3 cells. The expression of mRNAs encoding the regulators of CYP1 enzymes—the AHR and its heterodimerization partner, the ARNT (AH receptor nuclear translocator) protein—was not altered by treatment with TCDD or MC. However, the cytosolic content of AHR protein and ARNT protein was depleted substantially following treatment with TCDD. The LS180 cell line should constitute a good model for further mechanistic studies on AHR-regulated CYP1A2 expression. BIOCHEM PHARMACOL **56**;5:599–612, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. AH receptor; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3-methylcholanthrene; cytochrome P450; CYP1A2

The large superfamily of cytochrome P450 enzymes metabolizes a vast array of endogenous and exogenous substances. Within the CYP1 family, there are three genes encoding the enzymes CYP1A1, CYP1A2, and CYP1B1 [1]. CYP1A1 metabolizes important polycyclic aromatic hydrocarbon carcinogens such as BP§ and BA. CYP1A2 is a primary pathway for the metabolism of caffeine and also metabolizes carcinogens such as arylamines and aflatoxins (reviewed in Refs. [2–4]). The CYP1B family so far has only one known member, the recently identified enzyme CYP1B1, whose substrates include several PAHs and arylamines, as well as steroid hormones such as estradiol-17β [5–9].

Each member of the CYP1 enzyme family is inducible by TCDD or MC-type compounds via the AHR mechanism. This pathway has been defined most thoroughly for CYP1A1, an enzyme whose expression and induction are maintained in many rodent and human cell lines in culture [10].

Whereas the inducibility of CYP1A1 and CYP1B1 is widely retained in established cell lines and in primary cell cultures [11–15], it has been difficult to study the regulation of CYP1A2 because constitutive or inducible expression of CYP1A2 has been exceptionally low [16–18] or completely absent in human and other mammalian cell lines, even

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§ *Abbreviations:* AHR, aryl hydrocarbon receptor; ARNT, AH receptor nuclear translocator; BA, benz[*a*]anthracene; BP, benzo[*a*]pyrene; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; POD, phenacetin *O*-deethylase; RT–PCR, reverse transcription–polymerase chain reaction; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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though CYP1A2 is one of the most abundant P450s in human liver [19]. In a previous study, we found that there was a high level of functional AHR in the human colon adenocarcinoma cell line LS180, as detected by radioligand binding, photoaffinity labeling, and induction of aryl hydrocarbon hydroxylase activity [20]. In the present study, we further used the LS180 cell line to systematically investigate *in vitro* responses of *CYP1* genes to TCDD and MC. For comparison, these members of the [*Ah*] gene battery also were studied in four other human cell lines: MCF-7, JEG-3, HepG2, and A431 treated with TCDD, MC, or BA. As a secondary goal, we also wished to determine how treatment with TCDD and other AHR agonists might alter expression of the receptor itself or its dimerization partner, ARNT. Our studies demonstrated that CYP1A2 can be induced significantly at the mRNA, protein, and catalytic levels in the human LS180 cell line and that exposure of cells to TCDD and nonhalogenated receptor ligands does not alter the levels of expression of mRNAs encoding the AHR or ARNT protein.

MATERIALS AND METHODS

Chemicals and Reagents

TCDD was purchased from RADIAN (Cambridge Isotope Laboratories, Inc.), and MC, phenacetin, and fluvoxamine were from the Sigma Chemical Co. BA was obtained from the Aldrich Chemical Co., and [α-^{32}P]dCTP (> 3000 Ci/mmol) from Amersham Canada Ltd. α-Minimal essential medium and RPMI 1640 medium were from Media Preparation Services, University of Toronto. Fetal bovine serum was from Life Technologies. The following were gifts: plasmid containing full-length cDNA of CYP1A2 from Drs. M. Bruck and R. Tukey (University of California, San Diego); AHR antibody from Dr. George C. Clark (NIEHS); ARNT antibody from Drs. M. Probst and O. Hankinson (University of California, Los Angeles); rabbit anti-human CYP1A1 and anti-human CYP1A2 antisera from Dr. Robert Edwards (Royal Postgraduate Medical School); yeast microsomes containing human CYP1A1 (14.1 pmol/mg) from Dr. S. W. Ellis (The Royal Halamshire Hospital); and human liver samples (L11, L38, and L75) from Dr. Eve Roberts (The Hospital for Sick Children).

Cell Lines and Cell Culture

The human cell lines LS180 (derived from a colon adenocarcinoma), MCF-7 (from breast adenocarcinoma, pleural effusion), and JEG-3 (from choriocarcinoma) were obtained from the American Type Culture Collection. The HepG2 cell line (derived from hepatocellular carcinoma) was obtained from Dr. D. Grant (The Hospital for Sick Children). The A431 cell line (derived from epidermoid carcinoma) was provided by Dr. D. Sauder (Sunnybrook Health Science Centre). Cells were grown as monolayer cultures in α-minimal essential medium (MCF-7 in RPMI 1640 medium) containing 10% fetal bovine serum (without antibiotics) and were maintained in an atmosphere of 5% CO_2 at 37°.

Treatment of Cells

Cells plated on 100-mm-diameter plates (for RNA preparation) or in 175-cm^2 flasks (for cytosolic and microsomal preparations) were treated with TCDD, MC, or BA at different concentrations and for different time periods as indicated in the figure legends. All inducers were dissolved in DMSO. Control cells received 1% DMSO only.

Isolation of RNA and Reverse Transcription

Total RNA was isolated from the cells using TRIzol Reagent according to the manufacturer (Gibco BRL, Life Technologies). Cells were lysed and homogenized by pipetting with 1.5 mL of reagent per 100-mm plate. One-fifth the volume of chloroform was added, and the cell lysate was centrifuged at 12,000 *g* for 15 min at 4°. The aqueous phase was transferred carefully to a fresh tube, and RNA was precipitated with isopropyl alcohol for 10 min at room temperature. After washing with 75% ethanol, the pellet was dissolved in an adequate amount of diethylpyrocarbonate-treated water and was treated subsequently with RNase-free DNase I (Pharmacia, 15 U/100 μL of RNA) at 37° for 20 min to eliminate potential genomic DNA contamination [21]. The integrity of the isolated RNA was verified visually on 1% agarose gel containing 0.05% ethidium bromide. The intact (non-degraded) RNA clearly showed 28S and 18S ribosomal RNA bands.

The RT technique was performed as described previously [22]. One microgram of total RNA was added to a 40-μL RT reaction containing 8 μM oligo P(dT)$_{15}$ (Boehringer Mannheim), 1 mM dNTP, 60 U RNA guard, 10 mM dithiothreitol, and 400 U of M-MLV reverse transcriptase (Life Technologies). The reaction was incubated at 37° for 1 hr and heated to 70° for 10 min to inactivate the enzyme. The synthesized cDNA was stable at −20° for at least 2 weeks.

Oligonucleotides

Oligonucleotides used for PCR amplification and the putative lengths of the PCR products are shown in Table 1 [23–28]. All primers were commercially synthesized (GSD) and designed to have an intron–exon boundary in order to distinguish the mRNA from contaminating genomic DNA. All primer sets generated PCR products as single bands of the expected size as demonstrated by ethidium bromide staining of products separated in 1% agarose gels (data not shown).

PCR

PCR reactions were carried out as described [22] with slight modifications. A 5-μL sample of the RT mixture was

TABLE 1. Primers used for PCR amplification

Gene	Sequence (5′- to 3′-)	Size (mer)	Source	PCR product size (bp)	Reference
AHR	(S) CATGCTTTGGTCTTTTATGC	20	L19872 /		
	(AS) TTCCCTTTCTTTTTCTGTCC	20	HUMAHREC	368	[23]
ARNT	(S) GGAACAAGATGACAGCCTAC	21	M69238 /		
	(AS) CAGAAAGCCATCTGCTGCC	20	HUMARNTA	226	[24]
CYP1A1	(S) TCTTTCTCTTCCTGGCTATC	20	K03191 /		
	(AS) CTGTCTCTTCCCTTCACTCT	20	HUMCYP145	596	[25]
CYP1A2	(S) CCAACGTCATTGGTGCCATG	21	Z00036 /		
	(AS) GTGATGTCCCGGACACTGTTC	21	HSCYP345	263	[26]
CYP1B1	(S) AACGTCATGAGTGCCGTGTGT	21	U03688 /		
	(AS) GGCCGGTACGTTCTCCAAATC	21	HSU03688	360	[27]
β-Actin	(S) CTACAATGAGCTGCGTGTGG	20	X00351 /		
	(AS) TAGCTCTTCTCCAGGGAGGA	20	HSAC07	450	[28]

subjected to PCR amplification with 0.2 μM specific primers (0.96 μM for CYP1A1) in a 50-μL reaction containing 0.2 mM dNTPs (Pharmacia), 1.25 U *Taq* polymerase (Sangon Ltd.), and 1.5 mM $MgCl_2$ (2.5 mM for CYP1A1). PCR products were labeled by incorporation of [α-^{32}P]dCTP (1 μCi per reaction) in the presence of sufficient unlabeled dCTP. All PCR assays were performed in a GeneAmp PCR System 9600 (Perkin–Elmer). DNA was denatured at 94° for 4 min and cycled immediately 30 times at 94° for 20 sec with specific annealing temperatures chosen by preliminary experiments: 60° (ARNT), 58° (CYP1B1), 52° (AHR, CYP1A2, and β-actin), 49° (CYP1A1) for 20 sec, and extended at 72° for 40 sec. The PCR reaction ended with a 7-min incubation at 72°. PCR amplifications for AHR, ARNT, CYP1A1, CYP1A2, CYP1B1, and β-actin from each ligand treatment were carried out by adding an equal amount of cDNA (5 μL of cDNA product mixture) for each gene amplification generated in one RT tube with 1 μg of RNA in 40 μL of total volume and amplified for the same number of cycles (30 cycles). PCR conditions were optimized by using different amounts of RNA, cDNA, or cycle number to ensure that PCR products for each gene were obtained in the linear range of the reaction (data not shown). β-Actin was used as the internal standard to normalize for RNA loading and PCR variation. The entire 50-μL PCR reaction was electrophoresed on a 10% non-denaturing polyacrylamide gel which was subsequently dried and exposed to a PhosphorImager screen for 2.5 hr, and then visualized using the ImageQuant program in a PhosphorImager SF (Molecular Dynamics). The IPLab Gel program was used to quantitate PCR products by measuring incorporated radioactivity. The dried gel was subsequently autoradiographed (X-Omat AR film, Kodak) at −80° for 30–60 min with two intensifying screens.

Northern Blot Analysis

Total RNA isolated from cells was denatured in formaldehyde/formamide buffer at 55° for 15 min and electrophoresed (30 μg/lane) in a 1% formaldehyde/agarose gel. An RNA ladder (0.24 to 9.5 kb) was used as the size marker. The RNA was blotted overnight onto a nylon membrane (DuPont) in 10× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and immobilized to the membrane by a UV crosslinker (FB-UVXL-1000, Fisher-Biotech). Filter prehybridization was performed for 3 hr at 42° in a buffer containing 5× SSC, 50% (v/v) formamide, 1% (w/v) SDS, 5× Denhardt's solution, and 100 μg/mL salmon sperm DNA. For hybridization, a labeled probe (2.5×10^6 cpm/mL) was added to the fresh prehybridization buffer and hybridized at 42° for 16–24 hr. After twice washing with 2× SSC/0.1% SDS for 5 min at room temperature, twice with 0.2× SSC/0.1% SDS for 5 min at room temperature, and once with 0.2× SSC/0.1% SDS for 20 min at 55°, the blot was exposed to a Kodak XAR film with two intensifying screens at −80° for 1–5 hr. For reprobing, the membrane was washed in boiled 0.5% SDS at 70° for 20 min.

Probes

The full-length cDNA of human CYP1A2 (3.1 kb) was released by *Eco*RI from plasmid DNA and purified using a gel purification method (Qiagen, Canada). The human β-actin (450 bp) probe was the cDNA fragment obtained by RT–PCR and used as an RNA loading control. Probes were radiolabeled with [α-^{32}P]dCTP using Ready-To-Go DNA Labeling Beads (Pharmacia, Canada). The unincorporated ^{32}P-labeled nucleotides were removed by a NICK column (Pharmacia, Canada).

Preparation of Cytosol and Microsomes from Cultured Cells

Cells from three 175-cm^2 flasks were trypsinized and collected by centrifugation at 1000 *g* for 10 min at 4°. After washing twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), the pellet was resuspended in 1.5 mL of HED buffer (25 mM HEPES sodium, 1.5 mM EDTA, and 1 mM dithiothreitol, pH 7.4) and incubated on ice for 15 min. Following a 15-sec homogenization (Polytron, TP 7 tip), an equal volume of HED2×G buffer was added (HED buffer plus 20% glycerol,

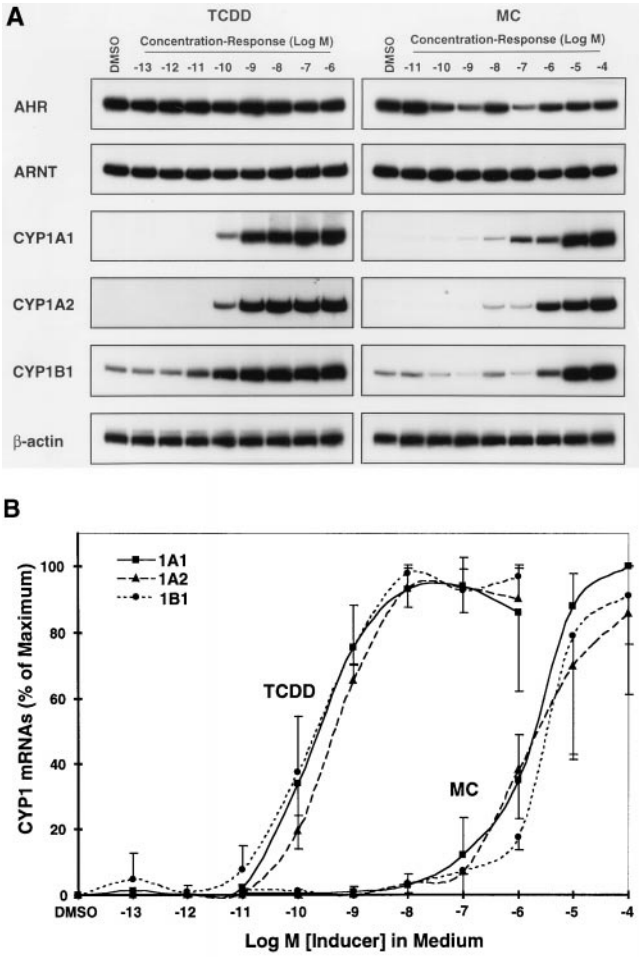

FIG. 1. RT–PCR analysis of mRNAs for AHR, ARNT, and the CYP1 family in LS180 cells treated with TCDD or MC in a concentration–response assay. After exposure for 24 hr (TCDD) or 20 hr (MC), cells were harvested, and total RNA was isolated. One microgram of total RNA from each treatment was used for cDNA synthesis, and one-eighth of cDNA was then subjected to PCR for 30 cycles with different annealing temperatures for different genes as indicated in Materials and Methods. (A) PCR products with [α-^{32}P]dCTP incorporated were separated in 10% acrylamide gel and visualized by exposing to Kodak film for 30–60 min. β-Actin was used as a loading control. Results presented are representative of three separate experiments. (B) mRNA induction patterns of the CYP1 family are plotted as percent of maximal induced response (by subtracting the value of DMSO from each curve) as a function of increased concentrations of inducers (mean ± SD, N = 3). The arbitrary PhosphorImager units for maximal induction by TCDD were: CYP1A1 (3.5), CYP1A2 (3.0), and CYP1B1 (5.0); by MC: CYP1A1 (3.7), CYP1A2 (1.2), and CYP1B1 (2.7).

pH 7.4). The supernatant generated from a 9000 *g*, 20-min centrifugation was spun at 106,000 *g* for 1 hr at 4° (L-80 ultracentrifuge, rotor 70.1 Ti). The final supernatant (cytosol) was divided into aliquots and stored in liquid nitrogen. The pellet (microsomes) was resuspended in storage buffer (10 mM Tris, 20% glycerol, 1 mM EDTA, pH 7.4) using a glass homogenizer on ice, immersed in liquid nitrogen, and then stored at −80° until used. The concentration of cytosolic and microsomal proteins was determined by the method of Bradford [29] using bovine serum albumin as the standard.

Immunoblotting

Immunoblotting assays were optimized for each target protein as described [30, 31] with the following modifications: 50 μg of microsomal protein or 100 μg of cytosolic protein was electrophoresed in 10% SDS polyacrylamide gel (6% for AHR/ARNT) and electrotransferred to nitrocellulose (Schleicher & Schuell, Canada). Twenty micrograms of yeast microsomes expressing human CYP1A1 was loaded onto the CYP1A gels serving as a positive control for CYP1A1 and a negative control for CYP1A2. The blots were blocked with 5% skim milk (3% BSA for AHR/ARNT) in 1× TNT buffer (20 mM Tris-base, 137 mM NaCl, and 0.1% Tween-20, pH 7.6) overnight. Antibodies specifically against human AHR, human ARNT, and human CYP1A1 and CYP1A2 were used to detect their respective proteins. After incubation with either a horseradish-peroxidase-conjugated donkey anti-rabbit IgG or a horseradish-peroxidase-conjugated goat anti-rabbit IgG (as appropriate), the immunopositive bands were visualized by Enhanced Chemiluminescence Reagent (Amersham, Canada). The protein levels were evaluated using a Datacopy Kurzweil 830 scanner (XEROX) and quantitatively analyzed with an IPLab Gel computer program.

POD Assay

POD activity was determined by the method of Gu [32]. Microsomal protein (200 μg) was incubated in a final volume of 500 μL containing 30 mM KCl, 9 mM $MgCl_2$, 60 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 0.2 mM NADPH, 20 μM phenacetin (optimal concentration for CYP1A2 activity), and different concentrations of fluvoxamine as appropriate at 37° for 45 min (30 min for human liver L38) in a shaking water bath. The reaction was terminated by the addition of 100 μL of 0.5 N NaOH. 1,3-Dimethyluric acid (40 ng) was used as an internal standard. After extraction with 3 mL of dichloromethane:isopropanol (95:5, v/v) and centrifugation for 10 min, the aqueous phase was transferred to a fresh tube and pH was brought to about 3 by adding 100 μL of 1 N HCl. After saturation with ammonium sulfate (400 mg per tube), the product of phenacetin *O*-deethylation (paracetamol) was extracted with 4 mL of dichloromethane:isopropanol (95:5, v/v), and the organic phase was evaporated under a stream of N_2 at 38°. The extracts were finally dissolved in 200 μL of HPLC mobile phase solution (0.08% acetic acid, 0.6% acetonitrile, and 1% isopropanol). The product, paracetamol, was eluted from an Ultrasphere ODS column (4.6 mm × 25 cm; Beckman Instruments) at a flow rate of 1.1 mL/min and monitored by UV absorbance at 250 nm. The retention time of paracetamol was 17 min and that of the internal standard 20 min.

TABLE 2. Calculated EC_{50} of TCDD and MC for induction of mRNAs, proteins, and catalytic activity of CYP1 family in LS180 cells

	EC_{50} (nM)					
	mRNA*		Protein†		Catalytic activity†	
	TCDD	MC	TCDD	MC	TCDD	MC
CYP1A1	0.24 ± 0.14‡	1800 ± 800	0.3§	600		
CYP1A2	0.47 ± 0.10	1400 ± 200	3.0	2100	0.338 ± 0.04	300
CYP1B1	0.24 ± 0.15	2350 ± 70				

The EC_{50} values were determined by interpolation from concentration–response plots.
*Cells were exposed to different concentrations of TCDD for 24 hr, or to MC for 20 hr before harvesting.
†Cells were exposed to different concentrations of TCDD for 48 hr, or to MC for 15 hr before harvesting.
‡Mean ± SD, N = 3.
§Result of a single experiment.

RESULTS

Concentration–Response Relationships for mRNA Induction by TCDD or MC in LS180 Cells

Figure 1 (A and B) shows that mRNAs for CYP1A1, CYP1A2, and CYP1B1 were induced in a concentration-dependent fashion by both TCDD and MC. The times chosen for cell incubation with TCDD (24 hr) or MC (20 hr) were based on our previous demonstration that these times produced optimal induction of CYP1A1 [20, 33]. There was no detectable RT–PCR signal for CYP1A1 or CYP1A2 mRNAs in untreated LS180 cells, whereas CYP1B1 was constitutively expressed (Fig. 1A). After subtraction of the uninduced (DMSO-treated control) values, the concentration–response curves for induction of mRNAs encoding CYP1A1, CYP1A2, and CYP1B1 were parallel and essentially superimposable (Fig. 1B). As measured by EC_{50} values (Table 2), the potency of TCDD for CYP1 mRNA induction was more than three orders of magnitude greater than that of MC, as reflected in the shift to the right of the concentration–response curves for MC shown in Fig. 1B. Although treatment with TCDD or MC caused a dramatic elevation in mRNA levels for CYP1A1, CYP1A2, and CYP1B1, these AHR agonists had little effect upon the mRNA levels for the regulatory proteins, AHR and ARNT, over the entire concentration range (Fig. 1A).

Time Course of mRNA Induction in LS180 Cells by TCDD or MC

Elevation of mRNA levels for CYP1A1, CYP1A2, and CYP1B1 was more rapid following exposure to MC than after TCDD. Maximal mRNA induction occurred within 6 hr after MC but the maximal response to TCDD occurred between 6 and 24 hr (Fig. 2). Expression of the mRNAs was sustained for up to 72 hr in cells treated with TCDD; however, in cells treated with MC, the mRNA levels dropped rapidly and returned to near pretreatment levels by 48 hr. As shown in Fig. 2, the mRNAs encoding AHR and ARNT showed no significant changes during exposure to TCDD or MC over the same time period from 0 to 72 hr.

mRNA Levels for CYP1 Family, AHR and ARNT in Four Other Human Cell Lines Treated with TCDD, MC, or BA

Because the LS180 colon carcinoma cell line was highly responsive to induction of CYP1A1, CYP1A2, and CYP1B1 by TCDD and MC, we also studied four other human cell lines to determine if inducibility of each member of the CYP1 family was a general phenomenon in

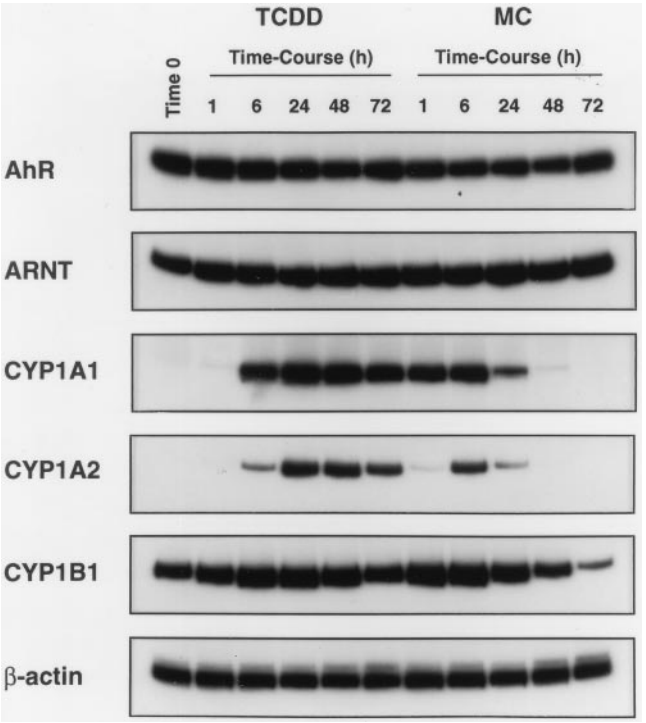

FIG. 2. RT–PCR analysis of mRNAs for AHR, ARNT, and the CYP1 family in LS180 cells treated with TCDD or MC in a time-course assay. After exposure to TCDD (2 nM) or MC (100 nM) for five different time periods, cells were harvested, and total RNA was isolated. RT–PCR was performed as described in Materials and Methods. PCR products with [α-^{32}P]dCTP incorporated were separated in 10% acrylamide gel and visualized by exposing to Kodak film for 30–60 min. β-Actin was used as a loading control. Results presented are representative of three separate experiments.

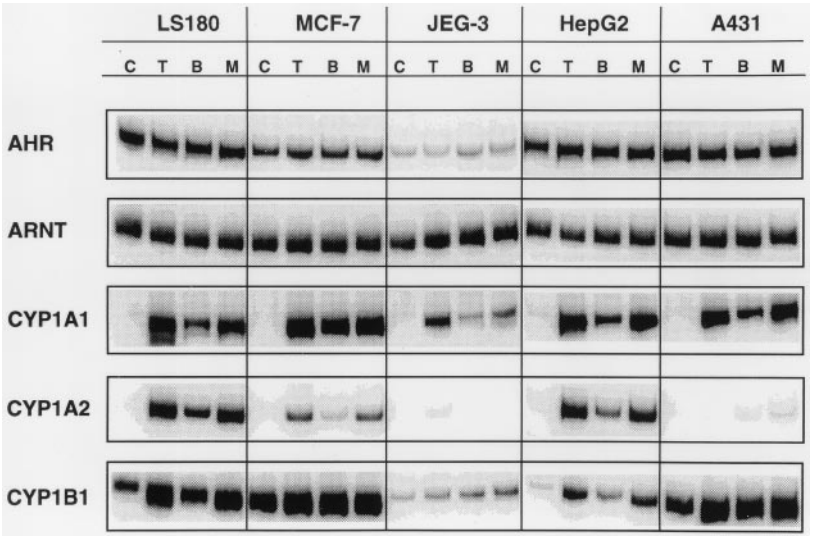

FIG. 3. RT–PCR analysis of mRNAs for AHR, ARNT, CYP1A1, CYP1A2, and CYP1B1 in five different human cell lines treated with TCDD, MC, or BA. One microgram of total RNA isolated from each treatment was used for cDNA synthesis, and one-eighth of cDNA was subjected to PCR as described in Materials and Methods. The result was visualized by exposing to a PhosphorImager screen for 2.5 hr and analyzed using computer program IPLab Gel. C = control; T = TCDD (100 nM, 24 hr); B = BA (10 μM, 24 hr); and M = MC (10 μM, 20 hr).

human cells in culture or if the LS180 cell line was unusual. In addition to TCDD and MC, we also tested the response to another MC-type inducer, BA, in all five cell lines.

Neither CYP1A1 mRNA nor CYP1A2 mRNA was expressed constitutively in any of the five human cell lines tested; however some level of CYP1B1 mRNA was detectable in all untreated cell lines (Fig. 3). CYP1A1 mRNA was induced by treatment with TCDD, MC, or BA in all five cell lines. CYP1A2 mRNA was induced in LS180 cells and to a lesser extent in HepG2 cells and MCF-7 cells but was not detectable after induction in JEG-3 cells or in A431 cells (Fig. 3). CYP1B1 mRNA, constitutively expressed in all five cell lines, was increased further in all the lines by treatment with TCDD, MC, or BA.

ARNT mRNA was uniformly expressed across all the cell lines tested and was not influenced by treatment with TCDD, MC, or BA (Fig. 3). The expression of AHR mRNA was more variable than that of ARNT. AHR mRNA levels were highest in LS180, HepG2, and A431 cells, with lesser levels in MCF-7 cells and only trace amounts in JEG-3 cells.

Evidence of Inducible CYP1A2 Expression by Northern Blotting

RT–PCR can be overly sensitive as a method to detect mRNA. Thus, we verified that CYP1A2 mRNA was expressed at a significant level following induction by also performing Northern blot analyses for CYP1A2 mRNA. As shown in Fig. 4, using a full-length cDNA as the probe, a single band of CYP1A2 mRNA could be detected in cells treated with concentrations of 1×10^{-9} M and above for TCDD and 1×10^{-5} M and above for MC.

The Northern blot (Fig. 4) also indicated that the time course for induction of CYP1A2 mRNA was similar to that observed by RT–PCR (Fig. 2). For comparison, and as a positive control for the CYP1A2 probe, the same amount of total RNA from a healthy human liver (L75) was loaded

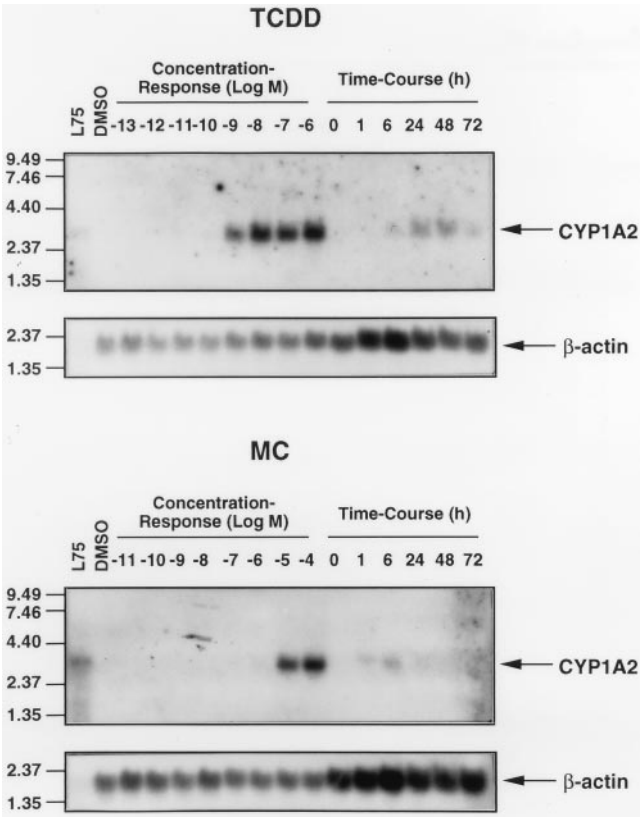

FIG. 4. Northern blot analysis of CYP1A2 mRNA in LS180 cells treated with TCDD or MC in both concentration–response and time-course assays. Thirty micrograms of pooled total RNAs from each treatment of three separate experiments (as shown in Figs. 1 and 2) was loaded in each lane, and the RNA membrane was hybridized with a full-length cDNA probe for CYP1A2. A single band at 3.1 kb was detected after treatment with either inducer in a concentration- and time-dependent manner. The blot was stripped and rehybridized with the β-actin probe, a 450-bp DNA fragment obtained by RT–PCR (see Materials and Methods). The numbers in the left margin indicate size markers (kb) for the mRNA. Human liver (L75) was from a healthy subject and is used for comparison with the signal from LS180 cells.

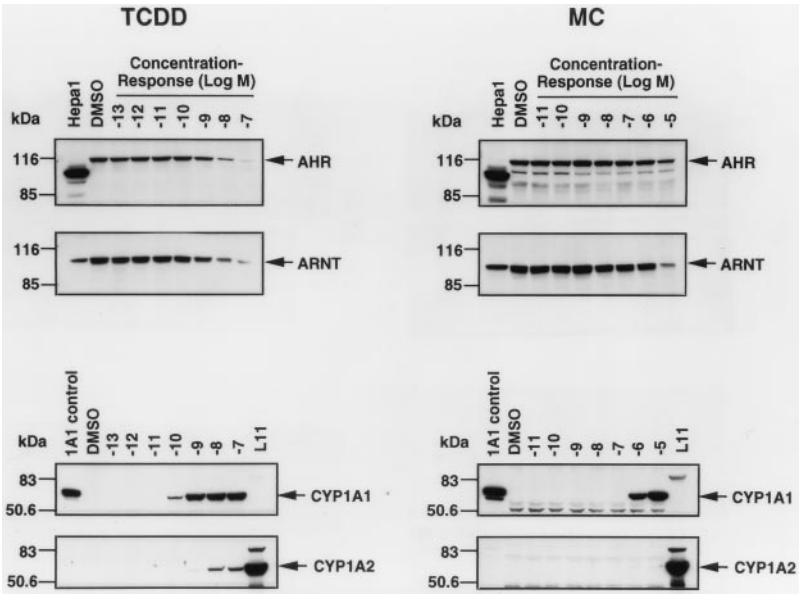

FIG. 5. Immunoblotting analysis of AHR, ARNT, CYP1A1, and CYP1A2 in LS180 cells treated with TCDD or MC in concentration–response assays. After exposure for 48 hr (TCDD) or 15 hr (MC), cells were collected, and cytosol and microsomes were prepared as described in Materials and Methods. The incubation times were chosen based on a report [34] and mRNA expression of CYP1 enzymes in Fig. 2. One hundred micrograms of cytosolic protein (for AHR and ARNT) or 50 μg of microsomal protein (for CYP1A1 or 1A2) was loaded in each lane. Cytosol from mouse Hepa-1 cells (100 μg/lane) was used as a control for AHR (95 kDa) and ARNT (87 kDa). Yeast microsomes containing human CYP1A1 were used (20 μg/lane) as a positive control for human CYP1A1 or as a negative control for human CYP1A2. Human liver (L11) was from a healthy subject. The mouse AHR from Hepa-1 cells has a molecular mass of ≈95 kDa under these electrophoretic conditions, whereas the mass of human AHR is ≈110 kDa.

simultaneously onto the gel. Because this sample was not obtained specifically for molecular experiments, its total RNA was degraded to some extent, leading to an unclear band of β-actin on the Northern blot. However, the CYP1A2 band from liver L75 could still be seen (Fig. 4). Equal total RNA loading and transfer efficiency were verified by post-Northern methylene blue staining (3%, w/v) and blot rehybridization with the β-actin probe (Fig. 4, bottom panel).

Protein Levels for CYP1A1 and CYP1A2: Concentration–Response to TCDD or MC in LS180 Cells

The fact that significant CYP1A2 mRNA was detected after induction with TCDD or MC in LS180 cells prompted us to perform immunoblots to determine if the elevation in mRNA was accompanied by a rise in microsomal CYP1A2 protein. We also performed immunoblotting for CYP1A1 protein in microsomes as well as for AHR and ARNT proteins in cytosols from the same cells after treatment with TCDD or MC in both concentration–response (Fig. 5) and time course studies (Fig. 6).

As positive controls and standards, we used cytosol from mouse Hepa-1 cells as a source for AHR and ARNT proteins; human CYP1A1 protein was obtained from expression in yeast. These were loaded onto SDS–PAGE in protein amounts equal to those of samples from LS180 cells. Protein loading and transfer efficiency were monitored by post-blotting Ponceau S staining (0.2%, v/v; data not shown). Using the specific antibodies, AHR [20], ARNT [35], CYP1A1 [25], and CYP1A2 [36] proteins were able to be detected in LS180 cells at the positions expected from their molecular weights—AHR ≈ 110 kDa; ARNT ≈ 87 kDa; CYP1A1 ≈ 58 kDa; and CYP1A2 ≈ 58 kDa (Fig. 5).

In microsomes from cells treated with TCDD or MC, CYP1A1 protein was detectable at TCDD concentrations above 1×10^{-10} M or at MC concentrations above 1×10^{-6} M. The levels of CYP1A1 immunoreactive protein rose in a concentration-related fashion after treatment with either TCDD or MC as did the levels of immunoreactive CYP1A2 protein after treatment with TCDD (Fig. 5). The absolute amounts of CYP1A1 and CYP1A2 protein are given in Table 3. A sample of human liver from a healthy donor was used for comparison; it showed no detectable CYP1A1 immunoreactive protein but very strong expression of CYP1A2 protein (Fig. 5). The level of CYP1A2 in human liver L11 was 180 (arbitrary densitometer units) compared with 57 units in LS180 cells after maximal induction with TCDD (Fig. 5) or approximately 15% of that observed in microsomes from another human liver specimen (L38; Table 3).

AHR protein and ARNT protein in cytosols from LS180 cells were reduced substantially following treatment with TCDD at concentrations above 1×10^{-9} M (data not shown). Both AHR protein and ARNT protein were reduced to approximately 40% of the control level at the highest concentrations of TCDD. The effect of MC on cytosolic content of AHR and ARNT proteins was more modest and only observable at MC concentrations above 1×10^{-6} M. The concentration–response curve for depletion by TCDD of AHR and ARNT proteins from cytosol was very similar (although in the opposite direction) to the concentration–response curve for induction of CYP1A1 and CYP1A2 proteins (data not shown).

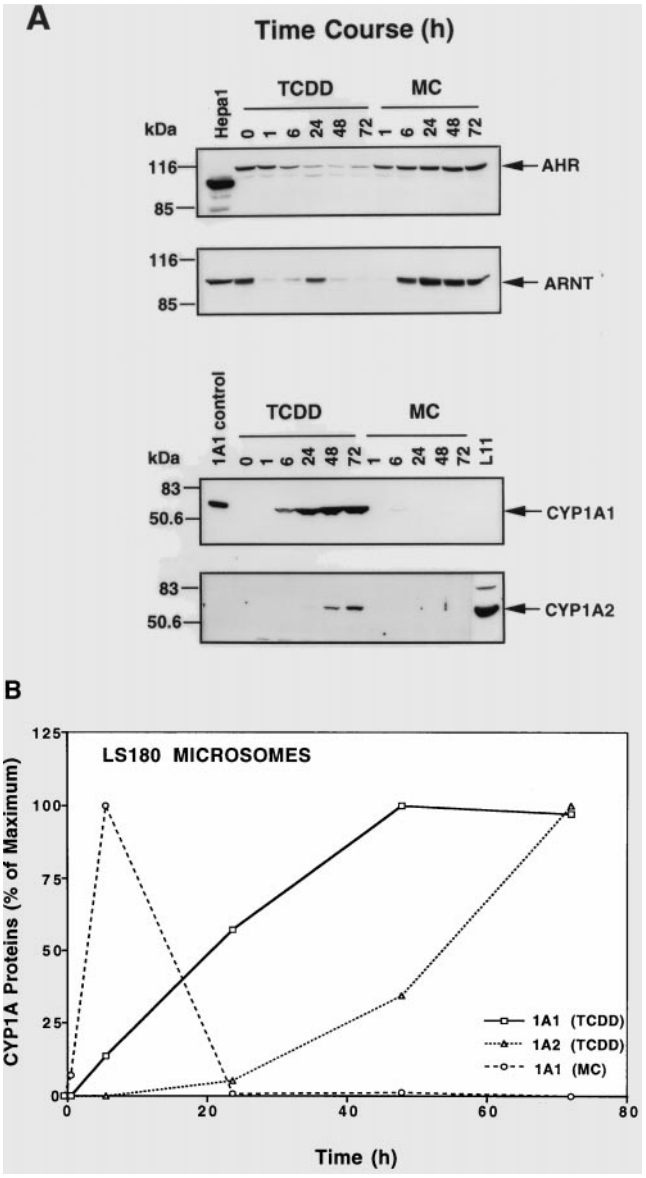

FIG. 6. Immunoblotting analysis of AHR, ARNT, CYP1A1, and CYP1A2 proteins in LS180 cells treated with TCDD or MC in time course assays. Cells were collected at each time point after exposure to TCDD (2 or 10 nM) or MC (100 nM), and cytosol and microsomes were prepared as described in Materials and Methods. (A) One hundred micrograms of cytosolic protein (for AHR and ARNT) or 50 μg of microsomal protein (for CYP1A1 or 1A2) was loaded in each lane. Cytosol from mouse Hepa-1 cells (100 μg/lane) was used as a control for AHR and ARNT. Yeast microsomes containing human CYP1A1 were used (20 μg/lane) as a positive control for human CYP1A1 or as a negative control for human CYP1A2. Human liver (L11) was from a healthy subject. (B) CYP1A microsomal protein levels are plotted as a percent of a maximum induction level as a function of duration of exposure to inducers. The results are representative of at least two separate experiments.

Protein Levels for CYP1A1, CYP1A2, AHR and ARNT: Time Course of Response to TCDD or MC in LS180 Cells

CYP1A1 protein was first detectable on immunoblots at 6 hr after treatment with TCDD and was sustained at its maximum for up to 72 hr (Fig. 6). The CYP1A2 protein was not present at significant levels until 24 hr after treatment with TCDD; however, the CYP1A2 level also was sustained for up to 72 hr.

As shown in Fig. 6, in TCDD-treated cells, cytosolic AHR protein was depleted within 1 hr after the addition of TCDD and remained low—about 50% of control levels—for at least 72 hr. Cytosolic ARNT protein decreased to about 40% of the control level 1 hr after TCDD, but recovered to 80% at 24 hr and then was depleted again thereafter. In MC-treated cells, the cytosolic AHR protein level dropped rapidly within the first hour but then recovered to control levels by 6 hr after MC. The drop in ARNT levels in cytosol likely reflects the association of the ligand · AHR · ARNT complex with the nucleus [37].

TABLE 3. Calculated POD activity and induced CYP1A1 and CYP1A2 contents in LS180 cells treated with TCDD or MC in a concentration- or time-dependent manner

Treatment	CYP1A1*† (pmol/mg)	POD activity (pmol/mg/min)	CYP1A2‡ (pmol/mg)
TCDD (10 nM, 24 hr)	3.2		
TCDD (1 nM, 48 hr)	5.2		
TCDD (2 nM, 48 hr)		4.2	0.8
TCDD (10 nM, 48 hr)	5.7	12.7	2.3
TCDD (100 nM, 48 hr)	5.8	11.8	2.2
TCDD (2 nM, 72 hr)		4.1	0.8
TCDD (10 nM, 72 hr)	5.9	20.6	3.8
MC (0.1 μM, 6 hr)		0.8	0.1
MC (0.1 μM, 24 hr)		0.3	0.1
MC (1 μM, 15 hr)	3.2	2.3	0.4
MC (10 μM, 15 hr)	5.0	0.4	0.1
CYP1A1 (yeast microsomes)	14.1‡		
Human liver (L38)§		145.0	26.5

*CYP1A1 concentrations were calculated based on the known 1A1 amount of yeast microsomes.

†Content of CYP1A1 in human CYP1A1-expressing microsomes was from Dr. Ellis (see Materials and Methods).

‡CYP1A2 content calculation was based on the known 1A2 content of L38 obtained in an EROD assay (Tang B.-K., unpublished data).

§Human liver was from a healthy subject.

Induction of CYP1A2 Catalytic Activity in LS180 Cells by TCDD: Concentration–Response and Time-Course Assays

The *O*-deethylation of phenacetin is a specific index of the catalytic activity of CYP1A2 [38, 39]. To determine if the apparent TCDD-induced CYP1A2 in LS180 cells (observed in RT–PCR, Northern blot, and immunoblot assays) was functional, POD was assessed by HPLC by measuring the conversion of phenacetin to paracetamol (acetaminophen). Because CYP1A1 mRNA and CYP1A1 protein were induced along with CYP1A2 mRNA and protein in TCDD-treated cells, we tested the possibility that POD activity might be contributed by CYP1A1 as well as CYP1A2 [40] by using fluvoxamine, a potent inhibitor of paracetamol production [39, 41]. If CYP1A2 was involved

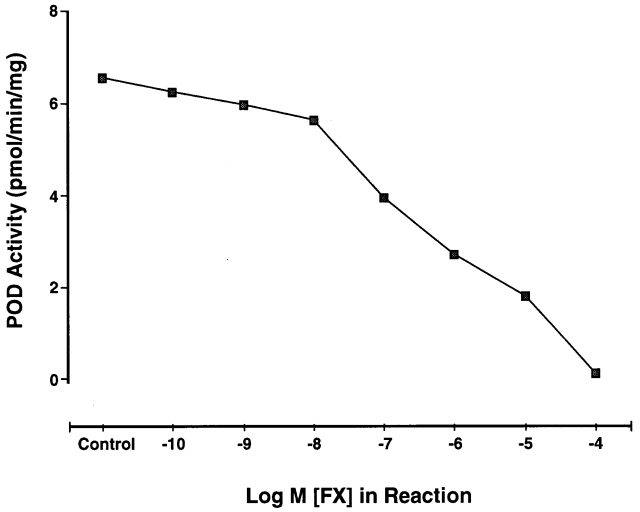

FIG. 7. Inhibitory effect of fluvoxamine on POD activity in microsomal protein from LS180 cells pretreated with TCDD (2 nM) for 72 hr. Microsomes (200 μg) from LS180 cells were incubated with 20 μM phenacetin for 45 min in the presence or absence (control) of different concentrations of fluvoxamine. Results are presented as POD activity as a function of log M fluvoxamine in each reaction. The results are representative of at least two separate experiments.

in POD activity, this activity should be inhibited in the presence of fluvoxamine. Potential inhibition of POD by fluvoxamine was first tested with microsomes from TCDD-treated (2 nM, 72 hr) LS180 cells (200 μg of microsomal protein). The results illustrated in Fig. 7 show that microsomes from TCDD-treated LS180 cells possessed substantial POD activity (6.5 pmol/mg/min), and this activity was inhibited by fluvoxamine in a concentration-dependent manner (Fig. 7). These results, generated from two independent assays, demonstrated that POD activity in microsomes from TCDD-treated LS180 cells was contributed by CYP1A2. Because there was no measurable POD activity in microsomes from untreated LS180 cells even though these cells expressed significant constitutive levels of CYP1B1 mRNA, it is unlikely that CYP1B1 contributes to the POD activity measured in the LS180 cells.

In TCDD-treated cells, POD activity showed a concentration-dependent increase, reaching the maximum level at an inducer concentration of 1×10^{-8} M TCDD (Fig. 8A). In the presence of fluvoxamine (10 μM), POD activity decreased to 30% of its original activity (shown by dashed lines in Fig. 8A), consistent with the result obtained in the inhibition assay (Fig. 7). The calculated EC_{50} for induction of CYP1A2 catalytic activity by TCDD is shown in Table 2.

The time course for induction of POD activity by TCDD in LS180 cells was carried out in two independent assays using both 2 and 10 nM TCDD. As shown in Fig. 8B, the time required for maximal POD activity induction by TCDD was 48–72 hr.

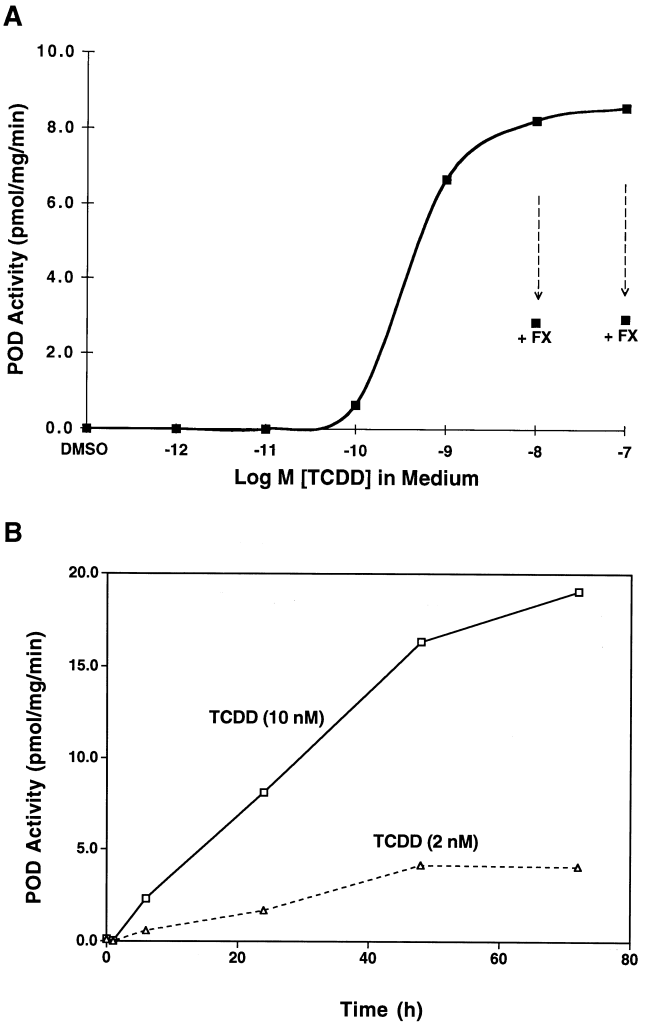

FIG. 8. POD activity of LS180 microsomal protein in TCDD-treated cells. Two hundred micrograms of microsomal protein from each treatment was incubated with 20 μM phenacetin for 45 min as described in Materials and Methods. (A) POD activity in TCDD concentration-dependent assay: cells were incubated with seven different concentrations of TCDD for 48 hr before microsomes were prepared. In the presence of 10 μM fluvoxamine (FX), POD activity in cells treated with higher concentrations of TCDD were inhibited to 30% of original level as indicated by the tip of the arrow for the dashed lines at inducer concentrations of 10^{-8} and 10^{-7} M. The plotted POD values represent the means of duplicate determinations on microsomes prepared from cells exposed to each concentration of TCDD; the values for duplicate determinations were within 20% of each other for each data point. (B) POD activity in TCDD time-dependent assay: cells were incubated with 2 or 10 nM TCDD for five different time periods before microsomes were prepared. The results are representative of at least two separate experiments.

For comparison with the POD activity in LS180 cells, POD activity was measured in microsomes from a human liver from a healthy subject (L38). Maximally induced POD activity after TCDD treatment in LS180 cells was about 15% of that in the human liver microsomes; POD activity in microsomes from MC-treated LS180 cells was only about 1–2% of that in human liver (Table 3).

TABLE 4A. Correlation of induction of CYP1 mRNAs in LS180 by TCDD or MC

	TCDD Concentration–Response				MC Concentration–Response		
	1A1	1A2	1B1		1A1	1A2	1B1
1A1 (9)	1			1A1(9)	1		
1A2 (9)	0.99	1		1A2(9)	0.93	1	
1B1 (9)	0.96	0.97	1	1B1(9)	1.00	0.91	1

Correlation coefficients were computed by the Microsoft Excel program. Numbers in parentheses represent the number of independent measurements for each parameter.

Correspondence of Induction of CYP1 Members at the Levels of mRNA, Protein, and Catalytic Activity

Overall analysis of data from concentration–response assays as well as time-course assays revealed that the responses of mRNAs for CYP1A1, CYP1A2, and CYP1B1 were highly correlated with each other (Table 4A) after treatment with either TCDD or MC. For CYP1A2, where three endpoints could be measured, the extent of mRNA induction was highly correlated with the level of CYP1A2 protein, which, in turn, was highly correlated with the POD catalytic activity (Table 4B). This is further illustrated in Fig. 9 where it can be seen that the concentration–response curves (Fig. 9A) were parallel and nearly overlapping for CYP1A2 mRNA, CYP1A2 protein, and POD activity and in the time course of response (Fig. 9B) where mRNA expression slightly preceded the appearance of CYP1A2 protein and POD activity.

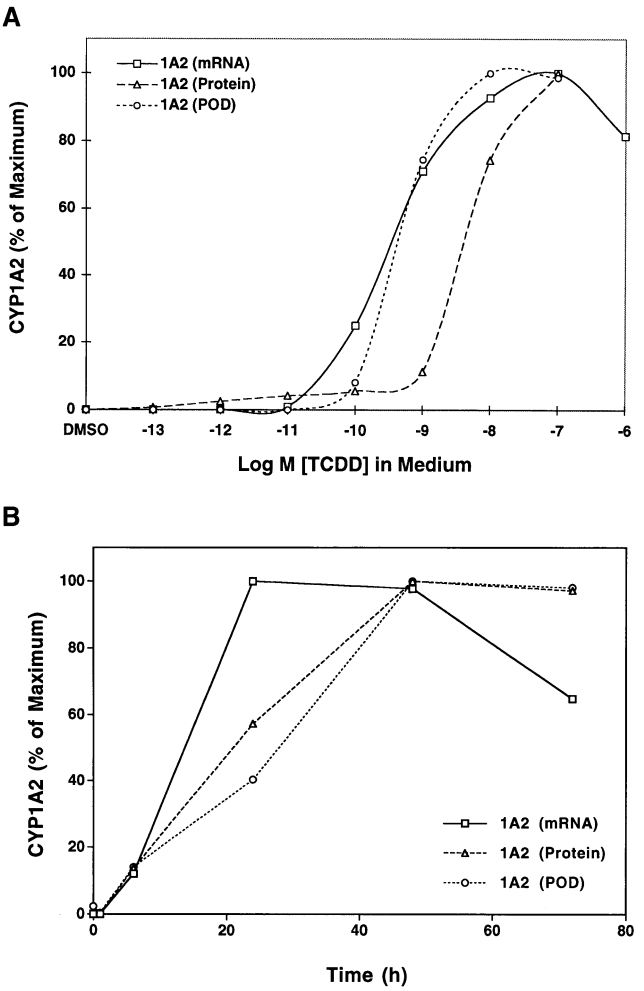

FIG. 9. Expression of CYP1A2 in LS180 cells. Comparison of mRNA, protein, and catalytic activity levels in TCDD concentration–response and time-course assays. (A) CYP1A2 mRNA, protein, and POD activity were detected after treatment of cells with eight (or seven) different concentrations of TCDD; RNA was harvested after 24 hr and microsomes were harvested after 48 hr. Results are presented as percent of maximum induction of expression of CYP1A2 as a function of increased concentrations of TCDD. (B) CYP1A2 mRNA, protein, and POD activity were detected after treatment of cells with 2 nM TCDD for five different time periods. Results are presented as the percent of maximum induction of expression of CYP1A2 as a function of duration of exposure time. The results are representative of at least two separate experiments.

DISCUSSION

Although CYP1A2 is one of the most abundant P450 enzymes in human liver [19, 30, 42], CYP1A2 expression is rarely retained in cells placed in culture (reviewed in Refs. [3] and [43]). This loss of response in cell culture has made studies on the regulation of the *CYP1A2* gene difficult. A few investigators found that some CYP1A2 mRNA can be induced in human hepatoma Hep3B cells [16] and in human hepatoma HepG2 cells [17] using RT–PCR procedures, but expression at protein or catalytic levels was not demonstrated in these previous studies. Quattrochi *et al.* [44] showed, by transient transfection assays of human CYP1A2 constructs into HepG2 cells, that the 5′-flanking region of the human *CYP1A2* gene contains elements that bind the AHR. Both positive and negative regulatory elements have been identified in the human *CYP1A2* gene by transfection of constructs bearing deletions or substitutions in the 5′-flanking region [45]. Our experiments

TABLE 4B. Correlation of induction of CYP1A2 in LS180 at mRNA, protein, and catalytic activity level by TCDD

	TCDD Concentration–Response				TCDD Time Course		
	1A2 (mRNA)	1A2 (Protein)	1A2 (POD)		1A2 (mRNA)	1A2 (Protein)	1A2 (POD)
1A2 (mRNA) (9)	1			1A2(mRNA)(6)	1		
1A2 (Protein) (8)	0.87	1		1A2(Protein)(6)	0.87	1	
1A2 (POD) (8)	0.99	0.87	1	1A2 (POD)(6)	0.79	0.99	1

Correlation coefficients were computed by the Microsoft Excel program. Numbers in parentheses represent the number of independent measurements for each parameter.

demonstrate that native nontransfected LS180 cells contain the complete AHR-mediated mechanism acting on the *CYP1A2* gene and that the CYP1A2 induction mechanism is intact and functional in these cells. Significant levels of CYP1A2 mRNA were induced in the LS180 cell line by TCDD or MC, and this expression at the mRNA level was followed by production of catalytically active CYP1A2 protein.

There is some previous evidence that CYP1A2 expression is not confined to liver in humans *in vivo* but may also occur in the gastrointestinal tract. McDonnell *et al.* [46] reported low levels of CYP1A2 mRNA in human duodenum obtained by biopsy after treatment of the subjects with omeprazole. It seems that, in addition to liver, CYP1A2 is mostly expressed *in vivo* in the lower gastrointestinal tract. Daujat *et al.* [47], working with another human colon adenocarcinoma cells line, Caco-2, found that *CYP1A1* gene transcription was stimulated by omeprazole through the AHR mechanism but did not report any CYP1A2 expression. In our experiments, inducible expression of CYP1A2 was greatest in the LS180 colon carcinoma cells but also occurred to some extent (at the RNA level) in two other human cell lines treated with TCDD or MC—the breast carcinoma cell line MCF-7 and the hepatoma cell line HepG2. Because CYP1A2 plays a central role in the bioactivation of numerous environmental contaminants to potentially mutagenic and carcinogenic forms [48], CYP1A2 expression in the gastrointestinal tract may implicate this enzyme in the formation of gastrointestinal cancers [49].

All three members of the CYP1 family were induced in LS180 cells treated with either TCDD, MC, or BA. Moreover, the concentration–response curves for induction and the time courses of induction were very similar for CYP1A1, CYP1A2, and CYP1B1, implying that regulation of each of these enzymes is via a common pathway in LS180 cells. *In vivo*, CYP1A1 has not been found constitutively in any tissues of humans in the absence of inducers (reviewed in Ref. [50]). CYP1A2 and CYP1B1 are constitutively expressed in human tissues with CYP1A2 mainly in liver [36, 42] and CYP1B1 mainly in kidney and uterus [9] as well as fetal kidney and brain [51]. There are circumstances where CYP1A1, in particular, may be very highly inducible in human cells without a corresponding inducibility of CYP1A2 or CYP1B1, such as in the A431 and JEG-3 cells in our current studies or in JEG-3 cells (our studies; [51]).

CYP1 enzymes all are inducible by aromatic hydrocarbons such as TCDD [13, 52, 53] and MC [54, 55] via the AHR mechanism. Although the AHR mechanism is the primary regulatory template, the underlying bases for tissue-specific differences in constitutive and inducible expression of CYP1 enzymes are not well understood. The AHR and its partner protein ARNT are expressed in a nearly ubiquitous manner in mammalian tissues and cell lines [22, 23, 56]; thus, some factors (other than the AHR or ARNT) that are not ubiquitous must govern tissue-specific expression of CYP1A1, CYP1A2, and CYP1B1. The level of ARNT mRNA was particularly uniform across all the cell lines that we studied; this is consistent with the central role that is increasingly becoming apparent for ARNT as a partner not only of the AHR but also of other proteins in important regulatory pathways [57–61].

In the human cell lines that we studied, the halogenated aromatic hydrocarbon TCDD was far more potent than the non-halogenated hydrocarbons MC or BA at inducing CYP1 enzymes, including CYP1B1. In rodent fibroblasts and mesangial cells, nonhalogenated PAHs such as BA and BP can induce somewhat higher levels of CYP1B1 mRNA or protein than the levels induced by TCDD [8, 62]. However, to achieve maximal CYP1B1 induction with BA or BP requires micromolar concentrations of these nonhalogenated compounds, whereas induction by TCDD is maximal at nanomolar concentrations. In both human cells (our studies) and in rodent cells [8, 62], TCDD is more potent than BA, BP, or MC at inducing CYP1B1. TCDD also was able to sustain the induced levels of mRNA for CYP1A1, CYP1A2, and CYP1B1 over a much longer time-span than was MC, which produced a more transient induction. The greater potency and efficacy of TCDD than MC in the human LS180 cell line were similar to what we observed earlier with the widely studied mouse hepatoma cell line Hepa-1, as is the transient nature of the response to MC [33]; the potency and sustained induction by TCDD appear to be related to its great chemical stability in cell culture, whereas MC is rapidly inactivated by metabolism [33].

As described above, much is known about the regulation of the CYP1 family by the AHR mechanism [63, 64]; however, far less is known about what regulates cellular levels of the AHR protein itself. Our experiments demonstrated that in LS180 cells the cytosolic content of AHR protein (detected by immunoblotting) drops dramatically in the first few hours after treatment with TCDD and that the cytosolic level of AHR remains depressed for up to 72 hr after exposure to TCDD in culture. We and others have observed this same TCDD-induced "down-regulation" of AHR in the mouse Hepa-1 cell line [31, 37, 65, 66]. The initial loss of cytosolic AHR protein is due to translocation of the receptor from cytosol into the nucleus. However, the apparent loss is not due only to translocation; within 6 hr the total cellular content of AHR protein in Hepa-1 cells is reduced and remains low for up to 72 hr [37]. The human LS180 cells exhibit a similar "down-regulation" phenomenon in cytosol; we did not measure AHR protein in nuclei from these cells. The loss of AHR from the cells does not appear to be due to a decrease in the level of mRNA encoding the receptor since, in our experiments, both with LS180 cells (this report) and mouse Hepa-1 cells [37], the AHR mRNA levels were not affected by TCDD treatment nor were the levels of mRNA encoding ARNT affected by TCDD. With either TCDD or MC treatment, CYP1A1 and CYP1A2 proteins reached their maximum level at the concentrations that caused the greatest reduction in AHR and ARNT proteins. This inverse relationship of CYP1A1

levels to AHR levels also has been found by Giannone *et al.* [37] in our laboratory and by Pollenz [66].

The discovery that significant levels of CYP1A2 can be induced in LS180 cells at the mRNA, protein, and catalytic levels indicates that this cell line may be particularly useful for further research into mechanisms regulating human *CYP1A2* gene expression and the functional consequences of this expression in drug metabolism and toxicology.

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