SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 15, No. 4, pp. 582–591, 1996

Printed in the USA
0730-7268/96 $6.00 + .00

RAPID ASSESSMENT OF INDUCED CYTOCHROME P4501A PROTEIN AND CATALYTIC ACTIVITY IN FISH HEPATOMA CELLS GROWN IN MULTIWELL PLATES: RESPONSE TO TCDD, TCDF, AND TWO PLANAR PCBS

MARK E. HAHN,*† BONNIE L. WOODWARD,† JOHN J. STEGEMAN† and SEAN W. KENNEDY‡
†Biology Department, MS-32, Woods Hole Oceanographic Institution, Woods Hole, MA 02543-1049, USA
‡Canadian Wildlife Service, Environment Canada, Hull K1A 0H3, Canada

(*Received* 19 *May* 1995; *Accepted* 15 *September* 1995)

Abstract—Induction of cytochrome P450 1A1 (CYP1A1) in cultured cells can be used to determine taxon-specific relative potencies of Ah receptor agonists. This report describes optimized methods for growth and treatment of PLHC-1 fish hepatoma cells in multiwell plates, in situ analysis of ethoxyresorufin *O*-deethylase (EROD) activity, and measurement of CYP1A protein by immunoblotting of cell lysates. EROD activity was undetectable (<1 pmol min^{-1} mg^{-1}) in untreated or dimethyl sulfoxide-treated cells, but was highly induced (up to 150 pmol min^{-1} mg^{-1}) in cells exposed to Ah receptor agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), or planar chlorobiphenyls (CB). Addition of exogenous NADPH was not required for measurement of EROD activity in PLHC-1 cells. As inducers of EROD activity, TCDD, TCDF, 3,3',4,4',5- pentachlorobiphenyl (CB-126), and 3,3',4,4'-tetrachlorobiphenyl (CB-77) differed both in potency and in apparent efficacy (maximal level of induced activity). In each case, EROD induction was biphasic, with stronger induction at lower concentrations and an attenuated response at higher concentrations. In contrast, the content of immunodetectable CYP1A protein increased monotonically with dose of CB, and the maximum level achieved was similar for all inducers. The discrepancy in results obtained for EROD activity versus CYP1A protein may result from inhibition or inactivation of catalytic function at high concentrations of inducer. By reducing peak EROD values, this inhibition leads to lower apparent EC50 values and thus the overestimation of relative potencies or toxic equivalency factors (TEFs) for many inducers. These studies demonstrate the necessity of measuring both EROD activity and immunodetectable CYP1A protein for the accurate assessment of CYP1A induction and relative potencies in cultured cells.

Keywords—Dioxin PCB TEF Cytochrome P450 Ah receptor

INTRODUCTION

Induction of cytochrome P450 1A1 (CYP1A1) is a well-known response of vertebrate animals exposed to planar halogenated aromatic hydrocarbons (PHAH) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [1]. This response, which is mediated by the Ah receptor (AhR), has been used extensively to study the mechanism of PHAH action [2], to establish inducer structure– activity relationships (I-SAR) [3,4], and as a marker of exposure to PHAH [5–7]. Hepatoma cell lines (e.g., Hepa-1, H4IIE) derived from mice and rats have been particularly useful in investigating mechanisms of cytochrome P450 induction [2]. Studies of the I-SAR for induction of CYP1A-dependent catalytic activities such as aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin *O*-deethylase (EROD) in rat hepatoma cells have contributed to an understanding of the varied biological potencies of individual PHAH congeners in mammals [8,9]. Such information has supported the use of cell culture bioassays for detecting PHAH in complex mixtures of chemicals extracted from tissues or the environment [10,11].

Evidence from several studies shows that I-SAR may differ between classes of vertebrate animals [12–15]. Thus, as noted previously [16–19], the relative potencies established in mammalian cells may not apply to other animal groups, indicating the need for taxon-specific model systems that can be used to define such differences. One such system is the PLHC-1 fish hepatoma cell line, which expresses an AhR [19] and an inducible CYP1A [19,20]. These cells, and cultured cells derived from other nonmammalian species, are currently being used by several laboratories to determine taxon-specific I-SAR [21–26].

The substantial effort required to conduct experiments on cells grown in flasks or dishes has stimulated the development of methods for growth, treatment, and assay of cultured cells in multiwell plates. Kennedy et al. [27,28] described multiwell-based assays for EROD activity and porphyrin content in primary cultures of chick embryo hepatocytes. Donato et al. [29] and Lubinski et al. [30] presented methods for assay of alkoxyresorufin *O*-dealkylase activities in multiwell plates of rat or human hepatocytes.

Most studies of CYP1A induction in cell culture have measured CYP1A catalytic rates, primarily EROD and AHH. However, it has become evident that CYP1A catalytic rates do not always reflect the amount of catalyst (CYP1A protein). For example, studies of CYP1A induction in vivo or in cultured cells often report dose–response relationships characterized by an increase in catalytic activity at low doses followed by inhibition or inactivation at higher doses of inducer (reviewed in Hahn et al. [19]). This finding has indicated the need for routine measurement of CYP1A enzyme protein in addition to its activity, if relative potencies of PHAH are to be determined accurately. In this report, we establish optimal conditions and methods for measuring both CYP1A catalytic activity (EROD) and immunodetectable CYP1A protein in PLHC-1 fish hepatoma cells grown in multiwell plates. This system was used to analyze CYP1A induction by a set of PHAH, illustrating the utility of such in vitro techniques for determining taxon- or cell-

* To whom correspondence may be addressed.
Presented in part at the 45th Annual Meeting of the Tissue Culture Association, Research Triangle Park, NC, June 4–7, 1994 and at the 15th Annual Meeting, Society of Environmental Toxicology and Chemistry, Denver, CO, October 30– November 3, 1994.

specific I-SAR. The results further substantiate the importance of using complementary measures of CYP1A induction in cell culture systems.

METHODS

Chemicals and solutions

The 2,3,7,8-TCDD (10 μg/ml in toluene, >98% purity) was obtained from Ultra Scientific (Hope, RI, USA). 3,3',4,4'-Tetrachlorobiphenyl (CB-77) was obtained from Pathfinder Labs (St. Louis, MO, USA). Its purity was >99% as determined by gas chromatography–electron capture detection (GC-ECD) and GC-mass spectrometry (MS). Further analysis by high-resolution MS showed that 3,3',4,4',5-pentachlorobiphenyl (CB-126) was not present (<0.001%) [31]. 3,3',4,4',5-Pentachlorobiphenyl (>99% pure) and 2,3,7,8-tetrachlorodibenzofuran (TCDF; >98% pure) were purchased from Ultra Scientific. Cell culture medium, calf serum, trypsin, dimethylsulfoxide (DMSO), and bovine serum albumin (BSA) were from Sigma. Other reagents were obtained as described previously [19].

Solutions of TCDD were prepared by drying an aliquot of TCDD in toluene under N_2 and dissolving the residue in DMSO. Stock solutions of CB-77 (10 mM), CB-126 (1 mM), and TCDF (1 mM) were prepared by dissolving the crystalline compound in DMSO. Aliquots of these stock solutions, or dilutions made from them, were used to treat the cells.

Growth, plating, and treatment of cells

The PLHC-1 fish hepatoma cells [32,33] were strain HC-16-1_{20}, obtained from L.E. Hightower in March of 1993. Cells were grown in tightly capped 75-cm² flasks at 30°C in minimal essential medium containing Earle's salts, nonessential amino acids, L-glutamine, and 10% calf serum, as described previously [19]. Hepes buffer (25 mM) was present to maintain pH at ~7.0. The cells were from various passages (10 to 75, beginning with receipt in our laboratory), as described in the Results; most of the experiments were performed using cells from passages 20 to 35. One day prior to dosing, cells were subcultured into 48-well plates (Costar). Cells from one or two confluent 75-cm² flasks were detached by trypsin treatment as described previously [19]. The detached cells were diluted in a volume of cold medium, typically 50 ml, and counted with a hemacytometer. The cells were then further diluted to a cell density (cells/ml) that would result in the desired plating density (cells/well) at a volume of 0.5 ml per well. While plating, cells were maintained in a sterile beaker with constant stirring at moderate speed. Cells were plated using an Eppendorf repeating pipettor with a sterile 12.5-ml tip. The tip was refilled after each set of eight wells to prevent settling of cells in the tip. After plating, the plates were wrapped in plastic wrap to minimize evaporation.

Twenty-four hours after plating, the medium was removed and the cells received fresh medium (0.5 ml) warmed to 20–30°C. The cells were then dosed with solutions of the inducing compounds dissolved in DMSO (2.5 μl/well; four wells per dose). Control wells received DMSO only. Plates were returned to the 30°C incubator for 24–72 h, after which the medium was aspirated from the wells and each well was washed with 0.5 ml phosphate-buffered saline (PBS) prior to analysis of EROD activity as described below. Plates used for analysis of CYP1A protein by immunoblots or for total protein measurement were frozen at −80°C.

Assays

EROD activity was measured using a multiwell plate reader by a modification of the method described by Kennedy et al. [27]. The EROD reaction was initiated by the addition (100 μl/well) of a solution of 7-ethoxyresorufin (2 μM final concentration) in TN buffer (50 mM Tris, 0.1 M NaCl, pH 7.8 at room temperature). In some experiments, NADPH (1.67 mM final concentration) was also included in the reaction mixture. Reactions were carried out at room temperature for 5 min or other times as noted in figure legends. Reactions were stopped by the addition of 150 μl cold methanol, and the plates were allowed to stand for 5 min. Blank wells contained DMSO-treated cells and complete reaction mixtures but were stopped immediately with methanol. Resorufin fluorescence was measured using the Cytofluor 2300 fluorescent plate reader (Millipore) with 530 nm and 590 nm excitation and emission filters, respectively, at sensitivity settings 2, 3, and 4. Resorufin standard curves were prepared by the addition of various concentrations of resorufin in methanol to complete reaction mixtures, including untreated cells. The extinction coefficient for resorufin in methanol was determined to be 49 mM^{-1} cm^{-1} at 573 nm.

In some experiments, the EROD reaction was followed kinetically. The reactions were initiated as above and the plate was placed in the Cytofluor and scanned at 1- or 2-min intervals. EROD reaction rates (pmol resorufin formed per minute) were determined by dividing the rate of resorufin production (change in fluorescence per minute) by the slope of the resorufin standard curve (change in fluorescence per pmol resorufin).

Cell protein was measured on separate plates containing cells treated with DMSO only, using the fluorescent protein assay described by Lorenzen and Kennedy [34], with BSA as standard.

Measurement of CYP1A protein

CYP1A protein was measured by immunoblotting of whole cell lysates. To prepare lysates from cells grown in 48-well plates, the cells were solubilized on ice in 100 μl of either sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample treatment buffer (0.25 M Tris-HCl, pH 6.8, 40% [v/v] glycerol, 4% [w/v] sodium lauryl sulfate, 0.008% bromphenol blue, and 5% [v/v] 2-mercaptoethanol) or PBS containing 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.5% Nonidet P-40 (NP-40). After 5 min on ice, the plates were shaken briefly on a vortex mixer with plate attachment, and the lysates were pipetted into 0.5-ml Eppendorf tubes (one well per tube). Lysates in sample treatment buffer were boiled for 5 min or placed in a thermocycler (Perkin Elmer) and heated to 95°C for 5 min to solubilize the cells completely and inactivate proteases. To lysates in the PBS solution, ⅓ volume (25% final concentration) of the sample treatment buffer was added before thermal treatment.

Samples and CYP1A standards (purified CYP1A1 from scup, *Stenotomus chrysops*) were analyzed by denaturing gel electrophoresis on 6–15% acrylamide gradient gels by methods described earlier [19,35], with slight modifications. Proteins were electrophoretically transferred onto 0.2-μm nitrocellulose and incubated with monoclonal antibody 1-12-3 (anti-scup CYP1A1) [36] at 10 μg/ml, then with goat anti-mouse IgG linked to alkaline phosphatase (Schleicher and Schuell; 1/5,000 dilution). Color was developed by enhanced chemiluminescence as directed for the Schleicher and Schuell Rad- Free Chemiluminescence Detection Kit, using Kodak X-AR film. Fluorographs were digitized with a Kodak DCS200 digital camera and Adobe Photoshop, and band intensities were quantified by videoimaging densitometry

using NIH Image software. Values for CYP1A equivalents were determined from the integrated optical density of the MAb 1-12-3 cross-reactive proteins relative to that of scup CYP1A1 standards.

Data analysis

Fluorescence data obtained from the Cytofluor plate reader were imported into SigmaPlot (Jandel Scientific) for analysis and curve fitting. Data obtained from EROD assays and densitometry of immunoblots were normalized to total cellular protein and analyzed by nonlinear regression using the curve-fitting subroutine of SigmaPlot. Data were fitted to a modified Gaussian function (Eqn. 1, for biphasic relationships) or to a logistic function (Eqn. 2, for sigmoid relationships) as described by Kennedy et al. [27].

The Gaussian function was

$$y(d) = Y_b + (Y_m - Y_b)\exp\{-C[\ln(d) - \ln(d_m)]^2\} \quad (1)$$

where $C = \ln(2)/[\ln(EC50) - \ln(d_m)]^2$, $y(d)$ is EROD activity or CYP1A1 content at inducer concentration d, Y_b is basal EROD activity or CYP1A1 content, Y_m is maximal EROD activity or CYP1A1 content, d_m is the inducer concentration producing maximal EROD activity or CYP1A, and EC50 is the inducer concentration producing 50% of maximal EROD activity or CYP1A1 content.

The logistic function was

$$y(d) = Y_b + (Y_m - Y_b)\{1 + \exp[-g(\ln(d) - \ln(EC_{50}))]\}^{-1} \quad (2)$$

where $y(d)$, Y_b, Y_m, and EC50 are as for Equation 1 and g is a slope parameter [27].

RESULTS

To develop the PLHC-1 cell system for measuring CYP1A induction, it was important to establish optimal conditions and methods for growth, treatment, and assay of cells grown in multiwell plates. The 48-well plate format (1 cm^2 well surface area) was selected over 24- or 96-well formats based on results of previous studies in chick embryo hepatocytes [27] and on the yield of cellular protein needed for immunoblotting (see below).

Conditions for growth and treatment of PLHC-1 cells in 48-well plates

Cell density. In initial experiments, cells were grown at densities ranging from 2.6×10^5 to 2.1×10^6 cells per well. The amount of cell protein per well after 1 d in culture was linearly related to initial plating density, indicating that attachment efficiencies were independent of density over this range (data not shown). In subsequent experiments, the effect of plating density on protein yield and induced CYP1A activity was determined in cells plated at initial cell densities between 1×10^5 and 5×10^5 cells/well. Protein assays were conducted at 1 d or 3 d after plating, corresponding to the typical times for dosing and EROD measurement, respectively. The amount of cell protein in the wells was directly proportional to the plating density at each time (Fig. 1A). Between 1 and 3 d, cell protein content approximately doubled. The plating density chosen for subsequent experiments, 4×10^5, was one that typically produced approximately 80 μg protein per well.

EROD activity was measured in cells plated at 1×10^5 to 5×10^5 cells/well and treated with DMSO or with TCDF, a potent inducer of CYP1A in fish [37,38]. For these studies, a kinetic (continuous) assay was used. EROD activity was not detected in DMSO-treated cells plated at 5×10^5 cells/well (Fig. 1B, lower curve). In contrast, a time-dependent increase in resorufin formation occurred in cells treated with 1 nM TCDF. The rate of resorufin production was proportional to the initial cell density (Fig. 1B, upper curves), as well as to the amount of cell protein.

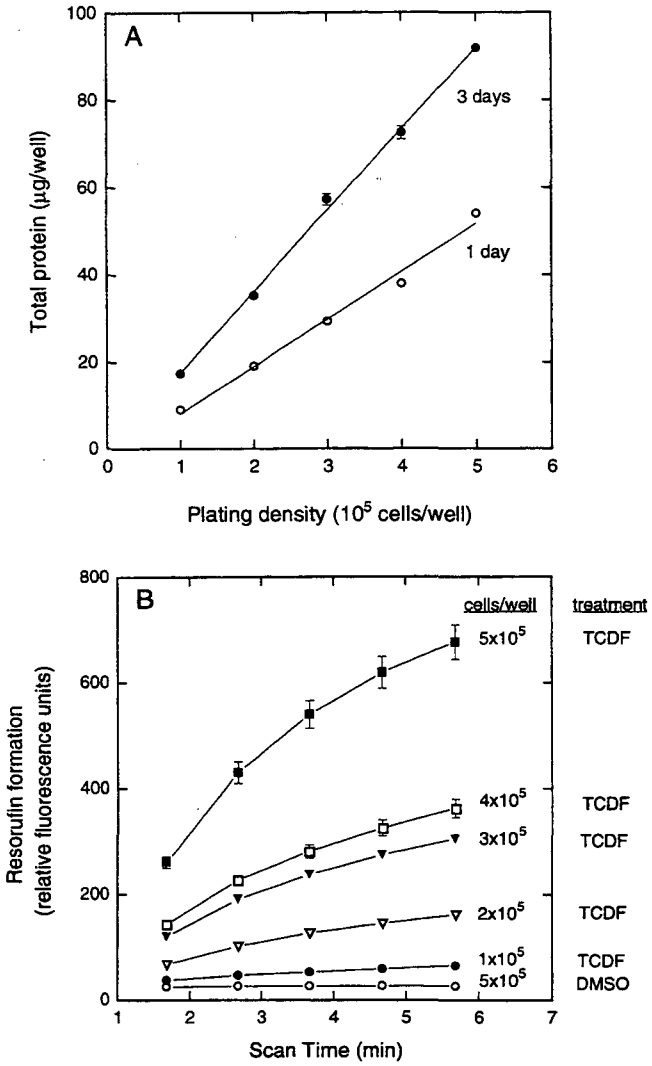

Fig. 1. Effect of plating density on cell protein content and induced EROD activity. The PLHC-1 cells were plated at 1–5 × 10^5 cells/well and used for measurement of total protein (A) or resorufin formation (EROD activity) induced by TCDF (1 nM) (B). (**A**) Assays for total cell protein were conducted at 1 d or 3 d after plating. (**B**) Cells were treated with DMSO (5×10^5 cells/well only) or 1 nM TCDF 1 d after plating, and EROD activity was measured 2 d later using the kinetic assay described in the Methods; NADPH was not present in the reaction mixture. Each point represents the mean ± SE of four wells. The *y*-axis shows resorufin formation, in units of relative fluorescence, as measured on the cytofluor at sensitivity 2. The *x*-axis indicates time after addition of substrate (7-ethoxyresorufin).

Volume of culture medium. An unexpected finding was that the volume of medium in contact with PLHC-1 cells during induction strongly influenced the degree of induction observed. When cells were treated with CB-77 in the presence of various volumes of culture medium (0.25, 0.5, or 1.0 ml, with the concentration of CB-77 held constant), the magnitude of induction was inversely proportional to volume. These results will be described in detail elsewhere (M.E. Hahn and A.B.

Table 1. Effect of exogenous NADPH on rates of resorufin production in PLHC-1 cells treated with TCDF

TCDF concentration (nM)	Resorufin production (Δ fluorescence min^{-1})	
	(−) NADPH	(+) NADPH
0	0.55 ± 0.06	0.29 ± 0.05[a]
0.1	30.6 ± 3.4	24.3 ± 4.5
1	73.9 ± 5.8	63.2 ± 2.0[a]
10	61.8 ± 4.4	58.5 ± 6.7
100	33.4 ± 2.4	38.8 ± 5.0

The PLHC-1 cells were plated at 4 × 10^5 cells/well, treated with the indicated concentrations of TCDF, and assayed 2 d after treatment as described in the Methods. Kinetic EROD assays were performed in the absence or presence of exogenous NADPH (1.67 mM). Results shown are the means ± SD of four wells per group. Statistical analysis was performed using an unpaired *t*-test in StatView (Abacus Concepts).

[a] $p < 0.05$ when compared to the −NADPH group.

Patel, manuscript in preparation; [39]). For the present studies, the 0.5-ml volume was used because induction was maintained for at least 2 d at this volume, whereas induction was more transient at the lower volume [39].

EROD assay conditions and induction by PHAH

Requirement for exogenous NADPH. In the experiment described above (Fig. 1B), formation of resorufin occurred in the absence of added NADPH, suggesting that PLHC-1 cells have sufficient endogenous NADPH-generating capacity to support CYP1A catalytic function. To determine directly the necessity of including added cofactor in our system, the EROD activity of PLHC- 1 cells treated with various concentrations of TCDF was measured in the presence and absence of added NADPH. EROD activity was not increased by addition of exogenous NADPH, at any TCDF concentration; in some cases there was a slight decrease (Table 1). Although statistically significant, the decreases were not large and were not consistently observed at all TCDF concentrations; the biological significance of these decreases is thus uncertain.

Other components of EROD reaction. The optimal concentration of substrate for use in the EROD assay was determined by measuring activity at various concentrations of 7-ethoxyresorufin up to 4 μM. The K_M for 7-ethoxyresorufin in cells treated with 0.3 nM TCDF was 0.33 ± 0.07 μM. Subsequent assays were performed at a 7-ethoxyresorufin concentration of 2 μM.

We also evaluated the effect of sonication to lyse the cells prior to assay, and the effect of adding BSA, which may help solubilize some monooxygenase substrates [40] or inhibit conjugation of resorufin [41]. EROD rates were similar in the presence and absence of BSA (5.32 mg/ml). Similarly, sonication of cells did not increase EROD activity, even in the presence of added NADPH (data not shown).

Salicylamide, an inhibitor of sulfate and glucuronide conjugation, has been shown to be necessary for accurate detection of EROD rates in primary cultures of rat hepatocytes [30]. The EROD activities of PLHC-1 cells treated with various concentrations of TCDF were not increased by inclusion of salicylamide at 1.5 mM in the EROD reaction mixture (not shown). Previously, we showed that the DT-diaphorase inhibitor dicumarol did not affect EROD activity in these cells [19].

Time course of assay. EROD activity can be measured either by kinetic (continuous) or stopped (single time) assays. Stopped assays provide significant efficiencies in data handling and calculations, but it must be established that reaction rates are linear with time under the conditions used. The time course of the EROD reaction measured with a stopped assay was determined using cells treated for 48 h with DMSO or with maximal EROD-inducing concentrations of CB-77 or TCDF. Assay times between 30 s and 15 min were evaluated. Resorufin production was not detected in cells treated with DMSO alone, even after 30 min of incubation with substrate (Fig. 2; data for longer times not shown). Resorufin production in TCB- and TCDF-treated cells was linear through at least 10 min (Fig. 2). EROD activities in cells treated with TCDF were eightfold greater than in cells treated with TCB (see *Potency and efficacy of AhR agonists as inducers of CYP1A activity,* next page).

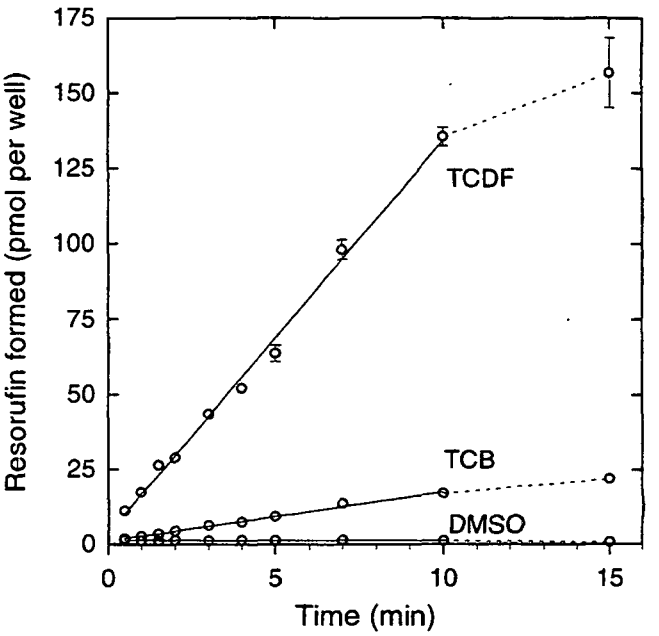

Fig. 2. Time course of EROD activity using a stopped assay. The PLHC-1 cells were plated at 4 × 10^5 cells/well and treated with DMSO, 3,3',4,4'-TCB (CB-77; 10 μM), or TCDF (1 nM) 1 d after plating, and EROD activity was measured 2 d later. Each point represents the mean ± SE of four wells.

Time course of induction. The time course of EROD induction was evaluated using PLHC-1 cells continuously exposed to TCDF for 24, 48, or 72 h. Dose–response curves for induction of EROD activity in cells treated with TCDF for 24 or 48 h were similar, reaching identical maxima (~120 pmol/min mg) (Fig. 3). In contrast, after 3 d of exposure the maximal activity was about half of that at the earlier time points. The EC50 values increased slightly with time of treatment (1 d: 0.069 ± 0.026 nM; 2 d: 0.103 ± 0.020 nM; 3 d: 0.131 ± 0.019 nM; mean ± SE based on four replicates per dose, one plate per treatment time); additional experiments would be required to determine the statistical significance of this apparent trend.

Effect of passage number on EROD responsiveness. The characteristics of cell lines may change with repeated subculturing. To determine if the responsiveness of PLHC-1 cells is different for early passage versus late passage cells, induction of EROD by TCDD was compared in a single experiment using cells from passages 10 and 75. For each group of cells, duplicate plates were prepared, treated with a range of TCDD concentrations, and EROD activity was measured after 2 d. Both groups

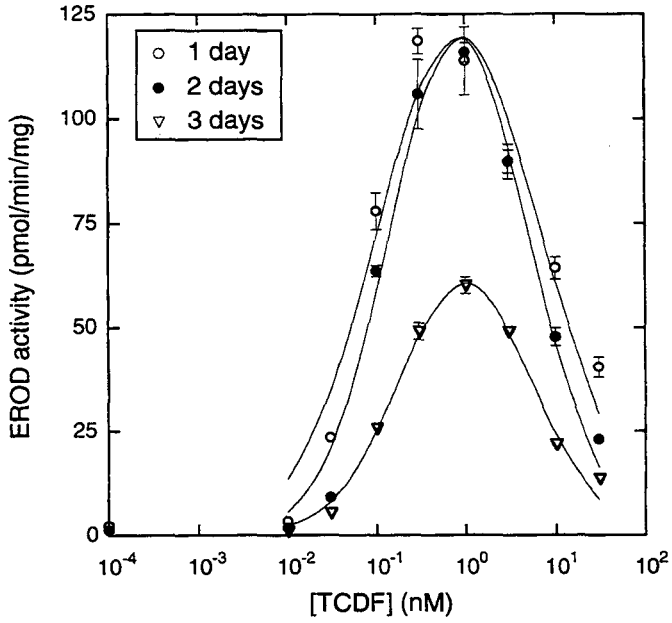

Fig. 3. Time course of EROD induction by TCDF. Cells were plated at 4 × 10^5 cells/well. Twenty-four hours later the cells were exposed to various concentrations of TCDF, and induced EROD activity was measured at 1, 2, or 3 d after dosing. Points represent the mean ± SE of four wells per dose. Points at 10^{-4} nM TCDF represent DMSO-treated cells.

of cells were highly responsive to EROD induction by TCDD. The maximal induced EROD activity in passage 10 cells was slightly greater than that in passage 75 cells (65 pmol min^{-1} mg^{-1} vs. 57 pmol min^{-1} mg^{-1}, respectively [mean of two plates]) and the passage 10 cells were slightly more sensitive (EC50 values: 0.33 nM [p10] and 0.58 [p75]). These results indicate that "old" cultures retain responsiveness to CYP1A inducers, but that there may be some change in sensitivity as cultures age.

Potency and efficacy of AhR agonists as inducers of CYP1A activity

To evaluate the potential of PLHC-1 cells grown in 48-well plates for determining inducer structure–activity relationships, the dose-dependent induction of CYP1A catalytic activity (EROD) was determined for three commonly used AhR agonists: TCDD and two planar chlorobiphenyls (CB-77, CB-126). As shown in Figure 4A, the compounds differed both in potency (concentration needed to produce a defined effect [42,43]) and apparent efficacy (maximal level of induction). In all cases, EROD induction was biphasic, with stronger induction at lower concentrations and an attenuated response at higher concentrations. Similar biphasic patterns of EROD induction were seen following exposure to several other chlorobiphenyl congeners (manuscript in preparation).

Measurement of immunodetectable CYP1A protein in cells grown in 48-well plates

The presence of biphasic EROD dose–response curves (e.g., Fig. 4A; see also [27,28]) has been shown to occur when CYP1A is inhibited or inactivated by residual inducer present in the cells [19]. To evaluate both CYP1A activity and CYP1A protein in cells treated with inducers, duplicate plates were treated with each inducer. One plate was used for determination of EROD activity as described above, while the other was used for immunochemical detection of CYP1A protein. For the latter, total cell protein was extracted from the wells using SDS-PAGE sample treatment buffer (containing SDS) or a PBS solution containing the nonionic detergent NP-40. Visual inspection after

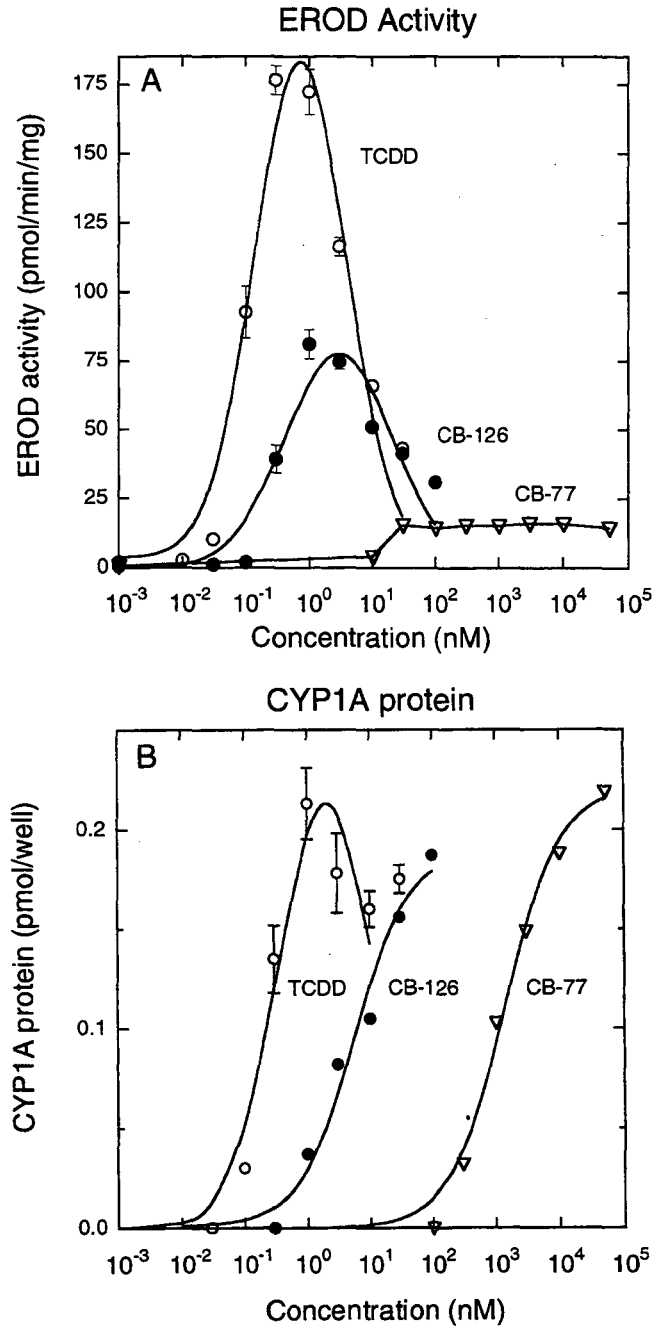

Fig. 4. Induction of EROD activity and CYP1A protein by TCDD and two planar PCB congeners. Cells were plated at 4 × 10^5 cells/well. Twenty-four hours later duplicate plates were exposed to TCDD, 3,3',4,4',5- pentachlorobiphenyl (CB-126) or 3,3',4,4'-TCB (CB-77). Induced EROD activity and CYP1A protein were measured 2 d after dosing. (**A**) EROD activity measured using the stopped assay. Points represent the mean ± SE of four wells per dose. Points at 10^{-3} nM inducer represent DMSO- treated cells. (**B**) Immunodetectable CYP1A protein. Data shown here were obtained by densitometric analysis of fluorograms like those shown in Figure 5 and are presented in units of "scup CYP1A-equivalents." Points represent a single immunoblot (CB-126) or the means of two (CB-77) or three (TCDD) immunoblots on lysates from a single plate. Bars on the TCDD data indicate SE.

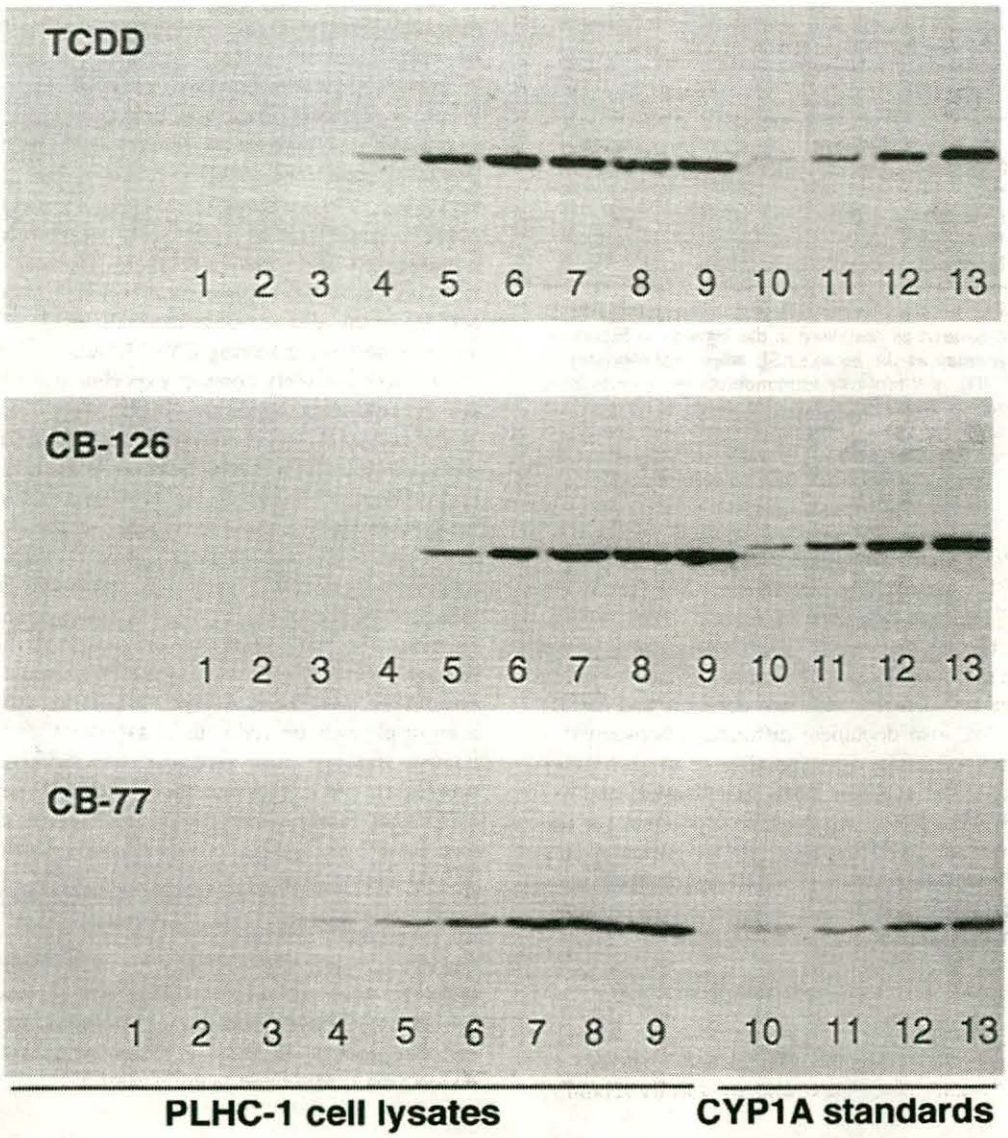

Fig. 5. Immunochemical detection of CYP1A protein induced by TCDD or planar PCB congeners. Cells were treated as described in the legend to Figure 4, and cell lysates were analyzed by immunoblotting using monoclonal antibody 1-12-3 and chemiluminescent detection as described in the Methods. Lanes 1–9 of each blot represent lysates of cells treated with the following concentrations of inducer: TCDD: 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30 nM; CB-126: 0, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30, 100 nM; CB-77: 0, 10, 30, 100, 300, 1000, 3000, 10,000, 50,000 nM. In each blot, lanes 10–13 contained purified scup CYP1A1 at 0.025, 0.050, 0.1, and 0.2 pmol/lane, respectively.

extraction confirmed that both solutions completely removed the cells from the wells of the plate.

CYP1A protein was determined by applying the entire cell lysate from one well to one lane of a denaturing polyacrylamide gel. Staining of the nylon membrane with Ponceau *S* after electrophoretic transfer from the gel indicated that the protein loading of lanes was approximately equal, consistent with results of total protein analyses performed on duplicate plates. Fluorograms produced by enhanced chemiluminescent detection of immunoreactive CYP1A protein from cells treated with TCDD, CB-126, or CB-77 are shown in Figure 5, and the data obtained by densitometric analysis of the film are plotted in Figure 4B. TCDD produced a dose-dependent increase in immunodetectable protein, with a slight decrease in CYP1A content at the three highest concentrations. For the CB congeners, CYP1A protein increased monotonically with dose, in contrast to the biphasic EROD dose–response curves. Importantly, and also in contrast to the EROD results, the maximally induced level of CYP1A protein was similar for the three compounds (Fig. 4).

The EC50 values for induction of CYP1A protein and catalytic activity were calculated using Equation 1 for biphasic curves and Equation 2 for sigmoid curves as described in the Methods and in Kennedy et al. [27]. For both endpoints, TCDD was the most potent inducer, followed by CB-126 and then CB-77 (Table 2). For each compound, the EC50 for EROD induction was lower than that for induction of CYP1A protein. Using the mean EC50 values, the relative potencies of the CB congeners as compared to TCDD could be estimated. Relative potencies calculated from EROD data were an order of magnitude greater than those calculated from CYP1A data (Table 2).

DISCUSSION

This report describes methods for growth and treatment of PLHC-1 fish hepatoma cells in 48-well plates, in situ analysis

Table 2. Potencies of TCDD, CB-126, and CB-77 as inducers of EROD activity and CYP1A protein in PLHC-1 cells

	EROD			CYP1A		
	N	EC50 (nM)	Relative potency	*R*	EC50 (nM)	Relative potency
TCDD	12	0.13 ± 0.04	1.0	5	0.21	1.0
CB-126	3	0.37 ± 0.07	0.35	1	7.9	0.027
CB-77	5	78 ± 112	0.0017	2	1300	0.00016

The PLHC-1 cells were treated with the indicated inducers, and EROD and CYP1A were measured as described in the legends to Figures 4 and 5. Data are presented as the mean (±SE where appropriate) of *N* experiments (EROD) or *R* replicate immunoblots using cells from one or more experiments. In the latter case, the means of all replicate CYP1A values at each inducer concentration were used for EC50 calculations. Relative potencies were estimated by dividing the mean EC50 for TCDD by the mean EC50 for each chlorobiphenyl.

of EROD activity of intact cells, and measurement of immunodetectable CYP1A protein. The plating and EROD protocols are adaptations of methods described by Kennedy and coworkers for chick embryo hepatocytes [27]. Optimal conditions are established for performing these procedures in PLHC-1 cells, including effective cell density, treatment duration, and EROD assay conditions. We also document differences between this fish line and primary cultures of mammalian or avian hepatocytes with respect to the need for BSA, salicylamide, and exogenous NADPH. In addition, we describe a protocol for determining the content of CYP1A protein, and we present results that demonstrate how measurement of CYP1A protein can provide complementary information that is important for accurate assessment of CYP1A induction in cultured cells.

Conditions for growth, treatment, and EROD assay in PLHC-1 cells

Our earlier paper described the growth and treatment of PLHC-1 cells in 75- cm^2 flasks, harvesting of cells by scraping, preparation of subcellular fractions, and analysis of induced EROD activity and CYP1A protein in cell lysates or microsomes [19]. In the present study it was found that PLHC-1 cells are easily adaptable to experiments performed entirely within the wells of 48-well plates, resulting in significant reduction in effort needed to assess CYP1A induction. Cells plated at various densities continued to grow over 3 d in culture, doubling in protein content between 1 and 3 d after plating. It should be noted that the cell densities used in these studies are relatively high, resulting in confluency being reached within 1 d of plating; PLHC-1 cells grow best at such high densities.

In the original method for assessing CYP1A induction in chick embryo hepatocytes grown in 48-well plates, exogenous NADPH was included in the EROD reaction mixture [27]. In that chick hepatocyte system, NADPH is essential for detecting EROD activity in cells frozen before assay; in cells analyzed without freezing, this cofactor is necessary for full activity (S.W. Kennedy and S.P. Jones, unpublished data). In contrast to those results, we found that PLHC-1 cells have sufficient endogenous NADPH-generating capacity to support full EROD activity in the absence of added cofactor. We have obtained identical results using mammalian endothelial cells in culture [31]. Similarly, Lubinski et al. found that addition of NADPH was not neccesary for measuring EROD activity in rat hepatocytes [30]. The requirement for exogenous NADPH will need to be determined for other cell types used in CYP1A induction experiments. Significant reductions in the cost of performing these assays may be realized for cell systems in which NADPH can be omitted.

EROD activity in untreated or DMSO-treated PLHC-1 cells grown in 48-well plates was undetectable in the experiments described here (<1 pmol min^{-1} mg^{-1}). EROD activities of DMSO-treated cells reported in our earlier paper (~10 pmol min^{-1} mg^{-1} [19]) were artifacts of the spectrophotometric assay used, as confirmed by performing a cytofluor-based assay on homogenates from that experiment. Because of the extremely low (undetectable) expression of CYP1A protein and catalytic activity in the absence of inducer, PLHC-1 cells provide a sensitive system for detecting CYP1A induction by AhR agonists.

We now routinely conduct experiments in PLHC-1 cells under the following conditions (Appendix): Cells are plated at 4 × 10^5 cells/well in 0.5 ml medium. After 24 h, the medium is replaced with fresh medium and cells are treated with inducer (2.5 μl/well). EROD activity is determined after 24 or 48 h, using a stopped assay (5–10 min incubation) and 2 μM 7-ethoxyresorufin, without the addition of BSA, dicumarol, salicylamide, or NADPH. If desired, porphyrin accumulation (an indicator of alterations in the heme biosynthetic pathway) can be measured immediately following EROD [44]. For each inducing compound, plates for EROD/porphyrins and CYP1A protein are treated and assayed in parallel. In each experiment, a set of plates is treated with TCDD (0.01–30 nM) as a positive control and one plate is devoted to measuring total cellular protein. (In our experience, the peak EROD activity induced by TCDD has varied somewhat among experiments [53–176 pmol min^{-1} mg^{-1} in eight experiments], underscoring the need for a TCDD-treated plate as a positive control in each set of plates.) Recent improvements that allow measurement of EROD activity, porphyrins, and total cell protein in the same wells of chick embryo hepatocytes [28] should also be applicable to PLHC-1 cells. We have not determined the optimal passage number for conducting CYP1A induction experiments. Although both early and late passage PLHC-1 cells are responsive to CYP1A inducers, we recommend using earlier passage cells to minimize any changes that might occur as the cultures age.

Measurement of CYP1A protein

EROD activity is the most commonly used indicator of CYP1A induction. However, there are circumstances under which EROD activity may be misleading as a measure of CYP1A content. For example, certain CYP1A inducers are able to inhibit or inactivate the induced CYP1A enzyme, leading to biphasic dose– response curves that underestimate CYP1A content at high doses (reviewed in Hahn et al. [19]). By reducing peak EROD values, inhibition of EROD activity at high doses leads to lower apparent EC50 values, affecting determination of relative induction potencies such as those used in the toxic equivalency factor (TEF) approach [23]. Consistent with this, EC50s for EROD induction in PLHC-1 cells were lower than those for induction of CYP1A protein, and relative potencies (TEFs) calculated using EROD data were higher than those calculated from CYP1A (Table 2 and M.E. Hahn, unpublished data). In such cases, EC50s for induction of CYP1A protein may provide a more accurate measure of biological potency.

In the present study, and in a study of additional CB congeners to be described elsewhere, disparate maximal EROD responses following treatment with certain congeners suggested that some of those congeners may act as partial AhR agonists, inducing CYP1A with an apparent efficacy less than that of TCDD. (The concept of partial agonism is defined in Jenkinson

et al. [43].) Congener- specific differences in apparent efficacies for CB induction of the CYP1A activities EROD or AHH have also been observed in other cell systems, including rat hepatoma cells [8] and chick embryo hepatocytes [25–28] (however, see also Lambrecht et al. [45]). In PLHC-1 cells, we were able to test the hypothesis of partial agonism by measuring CYP1A protein. The results indicate that TCDD, TCDF, CB-77, and CB-126 are equally efficacious as inducers of CYP1A in these cells, contrary to the results based on EROD alone. The CYP1A protein results therefore suggest that these CB congeners are full, not partial, agonists in this system. We have obtained similar results for two other non- *ortho*-substituted CB congeners, CB-169 (3,3′,4,4′,5,5′- hexachlorobiphenyl) and CB-81 (3,4,4′,5-tetrachlorobiphenyl) (M.E. Hahn, manuscript in preparation). Whether measurement of CYP1A protein content in rat or chick hepatocytes would reveal CB congeners as full agonists in these other systems as well is not known but should be tested. Given our present results, we speculate that, at least for some compounds, EROD or AHH data reported previously may underestimate the true efficacies and overestimate the true potencies of certain CB congeners.

For examining CYP1A induction, measurement of both EROD activity and CYP1A protein is preferable to determination of either alone. In this study (Fig. 4, Table 2) as in several others [15,46–48], EROD was found to be more sensitive than CYP1A protein as an index of induction. Moreover, EROD activity can be measured easily and rapidly. Measurement of CYP1A protein is more involved but provides important information, as noted above. The method described here (performing immunoblots on cell lysates extracted from single wells) is an improvement over earlier methods that required scraping of cells and preparation of homogenates or microsomes before immunoblotting. In addition, the chemiluminescent detection procedure is approximately 10-fold more sensitive than the colorimetric method used previously [19]. This method for determining CYP1A protein in cells grown in 48-well plates has also been applied successfully to mammalian endothelial cells [31] and chick embryo hepatocytes (S.W. Kennedy, A. Lorenzen, and M. Hahn, unpublished results). Further improvements in analyzing CYP1A protein are possible. Enzyme-linked immunosorbent assay (ELISA) methods, such as the one described in the accompanying paper [48], will permit even more efficient determination of CYP1A protein in cells grown in multiwell plates [48–50]. Although the immunoblotting procedure described here may be less sensitive than a CYP1A ELISA, immunoblotting may be advantageous when information is required on antibody specificity, CYP1A size, or the presence of proteolytic degradation products.

Alternative bioassay systems using recombinant cell lines that respond to AhR agonists are also being developed [51,52]. These systems utilize reporter genes under control of mammalian CYP1A promoter elements and, in so doing, avoid the complications of CYP1A inhibition and inactivation. The reporter constructs respond through binding of inducer to the endogenous AhR, supporting their use in species-specific bioassays. However, the mammalian dioxin response elements that drive expression in these systems may differ in function (e.g., number or context) from those of the species in question, arguing that results obtained in recombinant cells should be confirmed using an endogenous response such as CYP1A.

Cell culture systems utilizing the CYP1A induction response or other AhR-dependent responses show great potential for determining taxon-specific or cell-specific I-SAR. The methods described here for measuring CYP1A protein and catalytic activity in PLHC-1 fish hepatoma cells may be useful for studying PHAH action in these or other fish cells. Using these procedures, we have determined the relative potencies of several chlorobiphenyls for induction of EROD activity and CYP1A protein in PLHC-1 cells (manuscript in preparation, see also Hahn [23]). The PHAH-induced accumulation of porphyrins also occurs in these cells and is being used as an additional endpoint for studying the effects of AhR agonists [44]. Determining the relative potencies of PHAH in PLHC-1 cells will establish the feasibility of using these cells in a bioassay to measure the amount of dioxin equivalents in environmental samples, such as extracts of sediment or aquatic biota. Results obtained in such a piscine system would have greater relevance to fish than estimates of dioxin equivalents measured in rat hepatoma cells or dioxin equivalents calulated using mammalian TEFs. In addition to its use in establishing I-SAR and screening extracts, the system described here may be useful for studying the interactive effects (e.g., synergism, antagonism) of PHAH in fish.

Acknowledgement—This research was supported in part by grants from the U.S. Environmental Protection Agency (Grants R817988 and R823889), the National Oceanic and Atmospheric Administration (NOAA) National Sea Grant College Program Office (Grant NA90-AA-D-SG480, Woods Hole Oceanographic Institution [WHOI] Sea Grant project R/P-49), and the the Penzance Endowed Fund in Support of WHOI Scientific Staff. We thank Larry Hightower for providing the PLHC-1 cells and Bruce Woodin for advice on the chemiluminescent western blot procedure. We also thank Alka Patel and Kartik Chandran for technical assistance with portions of the study and Malin Celander for comments on the manuscript. Contribution 8981 from the Woods Hole Oceanographic Institution.

REFERENCES

1. **Okey, A.B.** 1990. Enzyme induction in the cytochrome P-450 system. *Pharmacol. & Ther.* **45**:241–298.
2. **Whitlock, J.P.** 1990. Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin action. *Annu. Rev. Pharmacol. Toxicol.* **30**:251–277.
3. **Poland, A.** and **E. Glover.** 1977. Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: A study of the structure–activity relationship. *Mol. Pharmacol.* **13**:924–938.
4. **Goldstein, J.A., P. Hickman, H. Bergman, J.D. McKinney** and **M.P. Walker.** 1977. Separation of pure polychlorinated biphenyl isomers into two types of inducers on the basis of induction of cytochrome P-450 or P-448. *Chem.-Biol. Interact.* **17**:69–87.
5. **Payne, J., L. Fancey, A. Rahimtula** and **E. Porter.** 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp. Biochem. Physiol.* **C86**:233–245.
6. **Goksøyr, A.** and **L. Forlin.** 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquat. Toxicol.* **22**:287–312.
7. **Stegeman, J.J.** and **M.E. Hahn.** 1994. Biochemistry and molecular biology of monooxygenases: Current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. In D. C. Malins and G. K. Ostrander, eds., *Aquatic Toxicology: Molecular, Biochemical and Cellular Perspectives.* CRC, Boca Raton, FL, USA, pp. 87–206.
8. **Sawyer, T.** and **S. Safe.** 1982. PCB isomers and congeners: Induction of aryl hydrocarbon hydroxylase and ethoxyresorufin *O*-deethylase enzyme activities in rat hepatoma cells. *Toxicol. Lett.* **13**:87–94.
9. **Safe, S.** 1990. Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *CRC Crit. Rev. Toxicol.* **21**:51–88.
10. **Bradlaw, J.A.** and **J.L. Casterline.** 1979. Induction of enzyme activity in cell culture: A rapid screen for detection of planar polychlorinated organic compounds. *J. Assoc. Off. Anal. Chem.* **62**: 904–916.
11. **Tillitt, D.E., J.P. Giesy** and **G.T. Ankley.** 1991. Characterization

of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. *Environ. Sci. Technol.* **25**:87–92.

12. **Gooch, J.W., A.A. Elskus, P.J. Kloepper-Sams, M.E. Hahn** and **J.J. Stegeman.** 1989. Effects of *ortho* and non-*ortho* substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicol. Appl. Pharmacol.* **98**:422–433.
13. **Walker, M.K.** and **R.E. Peterson.** 1991. Potencies of polychlorinated dibenzo-*p*-dioxin, dibenzofuran, and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **21**:219–238.
14. **Zabel, E.W., P.M. Cook** and **R.E. Peterson.** 1995. Toxic equivalency factors of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyl congeners based on early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* **31**:315–328.
15. **Newsted, J.L., J.P. Giesy, G.T. Ankley, D.E. Tillitt, R.A. Crawford, J.W. Gooch, P.D. Jones** and **M.S. Denison.** 1995. Development of toxic equivalency factors for PCB congeners and the assessment of TCDD and PCB mixtures in rainbow trout. *Environ. Toxicol. Chem.* **14**:861–871.
16. **Niimi, A.J.** and **B.G. Oliver.** 1989. Assessment of relative toxicity of chlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls in Lake Ontario salmonids to mammalian systems using toxic equivalent factors (TEF). *Chemosphere* **18**:1413–1423.
17. **Hahn, M.E., T.M. Lamb, M.E. Schultz, R.M. Smolowitz, A. Poland** and **J.J. Stegeman.** 1990. Induction of cytochrome P450IA1 in a teleost hepatoma cell line. *Abstracts,* 11th Annual Meeting, Society of Environmantal Toxicology and Chemistry, Arlington, VA, USA, November 11–15, p. 87.
18. **Smith, L.M., T.R. Schwartz, K. Feltz** and **T.J. Kubiak.** 1990. Determination and occurrence of AHH-active polychlorinated biphenyls, 2,3,7,8- tetrachlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzofuran in Lake Michigan sediment and biota. The question of their relative toxicological significance. *Chemosphere* **21**:1063–1085.
19. **Hahn, M.E., T.M. Lamb, M.E. Schultz, R.M. Smolowitz** and **J.J. Stegeman.** 1993. Cytochrome P4501A induction and inhibition by 3,3′,4,4′-tetrachlorobiphenyl in an Ah receptor- containing fish hepatoma cell line (PLHC-1). *Aquat. Toxicol.* **26**:185–208.
20. **Babich, H., D.W. Rosenberg** and **E. Borenfreund.** 1991. In vitro cytotoxicity studies with fish hepatoma cell line, PLHC-1 (*Poeciliopsis lucida*). *Ecotoxicol. Environ. Saf.* **21**:327–336.
21. **Tillitt, D.E.** and **S.M. Cantrell.** 1992. Planar halogenated hydrocarbon (PHH) structure activity relationship in a teleost (PLHC) cell line. *Abstracts,* 13th Annual Meeting, Society of Environmental Toxicology and Chemistry, Cincinnati, OH, USA, November 8–12, p. 45.
22. **Lee, L.E.J., J.H. Clemons, D.G. Bechtel, S.J. Caldwell, K.-B. Han, M. Pasitschniak-Arts, D.D. Mosser** and **N.C. Bols.** 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450- dependent monooxygenase activity. *Cell Biol. Toxicol.* **9**:279– 294.
23. **Hahn, M.E.** 1994. Cytochrome P450 induction and inhibition by planar halogenated aromatic hydrocarbons in a fish cell line: Promise and pitfalls for environmental testing. *In Vitro Cell & Dev. Biol.* **A30**:39–40.
24. **Clemons, J.H., M.R. van den Heuvel, J.J. Stegeman, D.G. Dixon** and **N.C. Bols.** 1994. Comparison of toxic equivalent factors for selected dioxin and furan congeners derived using fish and mammalian liver cell lines. *Can. J. Fish. Aquat. Sci.* **51**:1577–1584.
25. **Kennedy, S.W., A. Lorenzen** and **S.P. Jones.** 1994. Sensitivity of various primary avian embryo hepatocyte cell cultures to cytochrome P4501A induction by TCDD, TCDF, and PCBs. *Organohalogen Compounds* **21**:475–480.
26. **Kennedy, S.W., A. Lorenzen** and **R.J. Norstrom.** 1996. Chicken embryo hepatocyte bioassay for measuring cytochrome P4501A-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent concentrations in environmental samples. *Environ. Sci. Technol.* (in press).
27. **Kennedy, S.W., A. Lorenzen, C.A. James** and **B.T. Collins.** 1993. Ethoxyresorufin-*O*-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multi-well plate reader. *Anal. Biochem.* **211**:102–112.
28. **Kennedy, S.W., S.P. Jones** and **L.J. Bastien.** 1995. Efficient analysis of cytochrome P4501A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Anal. Biochem.* **226**:362–370.
29. **Donato, M.T., M.J. Gomezlechon** and **J.V. Castell.** 1993. A microassay for measuring cytochrome-P450IA1 and cytochrome-P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Anal. Biochem.* **213**:29–33.
30. **Lubinski, J., O.P. Flint** and **S.K. Durham.** 1994. In vivo and in vitro studies of rat liver cytochrome P450 induction II. In vitro induction by phenobarbital and 3-methycholanthrene measured in an automated 24-well plate assay for cytochrome P450-dependent activity (pentoxyresorufin *O*- depentylase and ethoxyresorufin *O*-deethylase). *In Vitro Toxicol.* **7**:13–23.
31. **Stegeman, J.J., M.E. Hahn, R. Weisbrod, B.R. Woodin, J.S. Joy, S. Najibi** and **R.A. Cohen.** 1995. Induction of cytochrome P4501A1 by aryl hydrocarbon receptor agonists in porcine aorta endothelial cells in culture, and CYP1A1 activity in intact cells. *Mol. Pharmacol.* **47**:296– 306.
32. **Hightower, L.E.** and **J.L. Renfro.** 1988. Recent applications of fish cell culture to biomedical research. *J. Exp. Zool.* **248**:290–302.
33. **Ryan, J.A.** and **L.E. Hightower.** 1994. Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. *Environ. Toxicol. Chem.* **13**:1231–1240.
34. **Lorenzen, A.** and **S.W. Kennedy.** 1993. A fluorescence-based protein assay for use with a microplate reader. *Anal. Biochem.* **214**: 346–348.
35. **Kloepper-Sams, P.J., S.S. Park, H.V. Gelboin** and **J.J. Stegeman.** 1987. Specificity and cross-reactivity of monoclonal and polyclonal antibodies against cytochrome P450E of the marine fish scup. *Arch. Biochem. Biophys.* **253**:268–278.
36. **Park, S.S., H. Miller, A.V. Klotz, P.J. Kloepper-Sams, J.J. Stegeman** and **H.V. Gelboin.** 1986. Monoclonal antibodies to liver microsomal cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): Cross-reactivity with 3-methylcholanthrene induced rat cytochrome P-450. *Arch. Biochem. Biophys.* **249**:339–350.
37. **Muir, D.C.G., A.L. Yarechewski, D.A. Metner** and **W.L. Lockhart.** 1992. Dietary 2,3,7,8-tetrachlorodibenzofuran in rainbow trout: Accumulation, disposition, and hepatic mixed-function oxidase enzyme induction. *Toxicol. Appl. Pharmacol.* **117**:65–74.
38. **Hahn, M.E.** and **J.J. Stegeman.** 1994. Regulation of cytochrome P4501A1 in teleosts: Sustained induction of CYP1A1 mRNA, protein, and catalytic activity by 2,3,7,8-tetrachlorodibenzofuran in the marine fish *Stenotomus chrysops*. *Toxicol. Appl. Pharmacol.* **127**: 187–198.
39. **Hahn, M.E.** and **A.B. Patel.** 1995. Rapid assessment of cytochrome P4501A induction in fish hepatoma cells grown in multi-well plates. *Mar. Environ. Res.* **39**:354.
40. **Alvares, A.P., G. Schilling, A. Garbut** and **R. Kuntzman.** 1970. Studies on the hydroxylation of 3,4-benzpyrene by hepatic microsomes. Effect of albumin on the rate of hydroxylation of 3,4-benzpyrene. *Biochem. Pharmacol.* **19**:1449–1455.
41. **Burke, M.D.** and **S. Orrenius.** 1978. The effect of albumin on the metabolism of ethoxyresorufin through *O*-deethylation and sulphate-conjugation using isolated rat hepatocytes. *Biochem. Pharmacol.* **27**:1533–1538.
42. **Kenakin, T.** 1993. *Pharmacologic Analysis of Drug–Receptor Interaction.* Raven Press, New York, NY, USA.
43. **Jenkinson, D.H., E.A. Barnard, D. Hoyer, P.P.A. Humphrey, P. Leff** and **N.P. Shankley.** 1995. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. 9. Recommendations on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **47**:255–266.
44. **Hahn, M.E.** and **K. Chandran.** 1994. Increased porphyrin accumulation in PLHC-1 fish hepatoma cells treated with TCDD or 3,3′,4,4′-TCB. *Abstracts,* 15th Annual Meeting, Society of Environmental Toxicology and Chemistry, Denver, CO, USA, October 30–November 3, p. 135–136.
45. **Lambrecht, R.W., P.R. Sinclair, W.J. Bement** and **J.F. Sinclair.** 1988. Uroporphyrin accumulation in cultured chick embryo hepatocytes: Comparison of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3,4,3′,4′-tetrachlorobiphenyl. *Toxicol. Appl. Pharmacol.* **96**:507–516.
46. **Forlin, L.** and **M. Celander.** 1993. Induction of cytochrome P450 1A in teleosts: Environmental monitoring in Swedish fresh, brackish and marine waters. *Aquat. Toxicol.* **26**:41–56.
47. **van der Weiden, M.E.J., M. Celander, W. Seinen, M. van den Berg, A. Goksoyr** and **L. Forlin.** 1993. Induction of cytochrome

P450 1A in fish treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or chemically contaminated sediment. *Environ. Toxicol. Chem.* **12**: 989–999.

48. **Bruschweiler, B.J., F.E. Wurgler** and **K. Fent.** 1996. An ELISA assay for cytochrome P4501A in fish liver cells. *Environ. Toxicol. Chem.* **15**:592–596.
49. **Herrero, M.E.** and **J.V. Castell.** 1994. Quantification of CYP1A1 and 2B1/2 in rat hepatocytes cultured in microwells by immunological methods. *Toxicol. In Vitro* **8**:1167–1175.
50. **Saad, B., F.A. Scholl, H. Thomas, H. Schawalder, V. Streit, F. Waechter** and **P. Maier.** 1993. Crude liver membrane fractions and extracellular matrix components as substrata regulate differentially the preservation and inducibility of cytochrome P450 isoenzymes in cultured rat hepatocytes. *Eur. J. Biochem.* **213**:805–814.
51. **Postlind, H., T.P. Vu, R.H. Tukey** and **L.C. Quattrochi.** 1993. Response of human CYP1-luciferase plasmids to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **118**:255–262.
52. **El-Fouly, M.H., C. Richter, J.P. Giesy** and **M.S. Denison.** 1994. Production of a novel recombinant cell line for use as a bioassay system for detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-like chemicals. *Environ. Toxicol. Chem.* **13**:1581–1588.

APPENDIX

Conditions for growth, treatment, and assay of PLHC-1 cells in 48-well plates

Initial plating density	4×10^5 cells/well
Volume of medium	500 μl
Volume of inducer	2.5 μl
Treatment duration	1–2 days
Substrate concentration (7 ethoxyresorufin)	2 μM
Reaction time	≤10 min
NADPH	Not needed
BSA	Not needed
Dicumarol	Not needed
Salicylamide	Not needed