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Uroporphyrin Accumulation Associated with Cytochrome P4501A Induction in Fish Hepatoma Cells Exposed to Aryl Hydrocarbon Receptor Agonists, Including 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Planar Chlorobiphenyls[1]

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Hepatic uroporphyria is a well-known effect of halogenated aromatic hydrocarbons in mammalian and avian systems, including primary cell cultures, but attempts to produce uroporphyria in vertebrate (mammalian) hepatoma lines have been unsuccessful. In this study, the ability of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzofuran (TCDF), and selected chlorobiphenyl congeners to cause uroporphyria was examined in a fish hepatoma cell line (PLHC-1) that expresses aryl hydrocarbon (Ah) receptors and an inducible cytochrome P4501A (CYP1A). Dose-dependent accumulation of porphyrins was observed in cells treated for 48 h with TCDD or 3,3′,4,4′-tetrachlorobiphenyl (3,3′,4,4′-TCB; IUPAC 77) when the heme precursor δ-aminolevulinic acid (ALA) was present during the last 5 h of treatment. HPLC analysis identified the porphyrins as uroporphyrin (~80%) and heptacarboxylporphyrin (~20%). Uroporphyria did not occur in cells treated with TCDD or 3,3′,4,4′-TCB in the absence of added ALA. ALA-dependent porphyrin accumulation was also seen following treatment of PLHC-1 cells with TCDF or with the non-*ortho*-substituted chlorobiphenyls 3,4,4′,5-tetrachlorobiphenyl (IUPAC 81) and 3,3′,4,4′,5-pentachlorobiphenyl (IUPAC 126). Neither of the mono-*ortho*-substituted chlorobiphenyls 2,3,3′,4,4′-pentachlorobiphenyl (IUPAC 105) or 2,3′,4,4′,5-pentachlorobiphenyl (IUPAC 118) increased the porphyrin content of PLHC-1 cells. The ability of the PCB congeners to cause porphyria correlated with their ability to induce the CYP1A catalytic activity ethoxyresorufin *O*-deethylase (EROD) and immunodetectable CYP1A protein in these cells, suggesting direct or indirect regulation of porphyrin accumulation via the Ah receptor and/or the induced CYP1A. Induction of EROD activity by TCDD, TCDF, and the planar polychlorinated biphenyls was biphasic, with increases at lower concentrations of inducer followed by decreased induction at higher concentrations, as seen previously. EC_{50} values for porphyrin accumulation were similar to, or slightly higher than, the concentrations at which peak EROD activities were obtained, suggesting a relationship between the decline in EROD activity and enhanced porphyrin accumulation. α-Naphthoflavone inhibited TCDD-induced EROD activity and porphyrin accumulation, providing further evidence for the involvement of a fish CYP1A in the mechanism of this porphyria. Addition of 3,3′,4,4′-TCB to TCDD-treated cells also inhibited EROD activity, but enhanced porphyrin accumulation, suggesting that an interaction between the halogenated inducer and the induced CYP1A is necessary for the porphyrogenic response. PLHC-1 cells grown in medium supplemented with ALA may be a useful model system for studying mechanisms of chemical uroporphyria induced by Ah receptor agonists.

Key Words: **heme; porphyrin; porphyria; Ah receptor; dioxin; polychlorinated biphenyl; cytochrome P450; CYP1A; fish hepatoma cells.**

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Exposure of mammals and birds to planar halogenated aromatic hydrocarbons (PHAH)[4] such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), certain polychlorinated biphenyls (PCBs), or hexachlorobenzene causes a disruption in heme biosynthesis leading to the development of a hepatic porphyria that mimics the human disease *porphyria cutanea tarda* (1, 2). This porphyria is characterized by the hepatic accumulation and urinary excretion of uroporphyrin (URO) and heptacarboxylporphyrin, oxidation products of the heme precursors uroporphyrinogen (URO'gen) and heptacarboxylporphyrinogen.

The mechanism of PHAH-induced porphyria is not completely understood. In inbred mice, susceptibility to uroporphyria caused by TCDD and hexachlorobenzene is controlled in part through the *AHR* locus, which encodes the Ah receptor (AhR) (3–5). The role of the AhR in PHAH-induced porphyria may be exerted through the induction of cytochrome P450 1A1 (CYP1A1) and/or 1A2, both of which are regulated by the AhR. Studies in avian systems have shown an essential role of CYP1A forms in PHAH-induced porphyrin accumulation (6, 7). One possible mechanism involves the CYP1A-dependent oxidation of URO'gen to URO (8). Hepatic microsomes from mice, rats, and chickens treated with CYP1A inducers catalyze URO'gen oxidation *in vitro* (9–12), and immunoinhibition and reconstitution studies have demonstrated that in mammals this activity is catalyzed primarily by CYP1A2 (12, 13).

Primary cultures of avian or mammalian hepatocytes have been instrumental in the study of PHAH-induced porphyria. Much of our understanding of this porphyria and its relationship to CYP1A has come from studies in primary cultures of avian (chicken embryo) hepatocytes, which are highly responsive to this effect (6, 10, 14–17). PHAH-induced porphyria also occurs in primary cultures of mouse hepatocytes (18, 19). In contrast, rat and human hepatocytes and established mammalian liver cell lines appear to be refractory to PHAH-induced porphyria (10, 18, 20), despite the *in vivo* sensitivity of these mammals to this response. It has been suggested (18) that this lack of responsiveness is linked to the poor inducibility of CYP1A2 in these *in vitro* systems (21, 22).

In contrast to the abundant information concerning PHAH-induced porphyria in mammals and birds, almost nothing is known about the susceptibility of other vertebrate groups to uroporphyria. There is a single report of hepatic porphyria in wild fish exposed to environmental PHAH (23), but experimental treatment of fish with PHAH has not resulted in altered porphyrin levels. For example, porphyria was not observed in carp (24) or rainbow trout (J. P. Giesy and S. W. Kennedy, personal communication) treated with TCDD, in carp injected with a PCB mixture (J. Krijt, personal communication), or in channel catfish exposed to iron plus hexachlorobenzene (R. T. DiGiulio, personal communication). Fish might be expected to be sensitive to PHAH-induced porphyria, since they express Ah receptors (25–27) and exhibit other PHAH effects, including CYP1A induction (28). Unlike mammals and birds, however, most fish possess only a single known form of CYP1A, which is more closely related in structure, function, and regulation to mammalian CYP1A1 than to CYP1A2 (29, 30).[5] Thus, the susceptibility of fish to PHAH-induced porphyria is uncertain.

The objective of the present study was to determine if Ah receptor agonists could cause porphyrin accumulation in a fish hepatoma cell line. We chose the PLHC-1 line (34) because these cells express Ah receptors (35) and respond to PHAH exposure with induction of cytochrome P4501A catalytic activity (35, 36) and immunodetectable CYP1A protein (35, 37, 38). Here we show that these cells are also sensitive to PHAH-induced accumulation of uroporphyrin and heptacarboxylporphyrin, and provide evidence consistent with the involvement of the Ah receptor and/or the induced CYP1A in the mechanism of this response.

METHODS

Chemicals and solutions. TCDD (10 μg/ml in toluene, >98% purity) was obtained from Ultra Scientific. 3,3',4,4'-Tetrachlorobiphenyl (3,3',4,4'-TCB; IUPAC 77) was obtained from Pathfinder Laboratories (St. Louis, MO). Its purity was >99% as determined by GC-ECD and GC-MS. Further analysis by high resolution mass spectrometry showed that 3,3',4,4',5-pentachlorobiphenyl (3,3',4,4',5-PCB; IUPAC 126) was not present (<0.001%) (39). 3,3',4,4',5-PCB (>99% pure), 3,4,4',5-TCB (IUPAC 81; >99%), 2,3,3',4,4'-PCB (IUPAC 105; >99%), 2,3',4,4',5-PCB (IUPAC 118; >99%), and 2,3,7,8-tetrachlorodibenzofuran (TCDF; >98%) were purchased from Ultra Scientific. Porphyrin standards (type I series free acids) and uroporphyrin I standards were purchased from Porphyrin Products (Logan, Utah). Cell culture medium, calf serum, trypsin, dimethylsulfoxide (DMSO), and bovine serum albumin were from Sigma. 5-Amino-

[4] Abbreviations used: AhR, Ah (aryl hydrocarbon) receptor; ALA, δ-aminolevulinic acid; ALAS, ALA synthase; ANF, α-naphthoflavone; CYP1A, cytochrome P450 1A; EC_{50}, estimated concentration needed to produce 50% of the maximal response; EROD, ethoxyresorufin *O*-deethylase; P450, cytochrome P450; PCB, polychlorinated biphenyl; PHAH, planar halogenated aromatic hydrocarbon; TCB, tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; URO, uroporphyrin; URO'gen, uroporphyrinogen; DMSO, dimethyl sulfoxide; MEM, minimal essential medium.

[5] The gene duplication that led to the divergence of CYP1A1 and CYP1A2 is thought to have occurred after the divergence of lineages leading to present day fish and tetrapods (29–31). All of the CYP1A forms that have been cloned so far from fish, including the two trout forms designated CYP1A1 and CYP1A2 (32), are more closely related to mammalian CYP1A1 than to mammalian CYP1A2 (30). Fish P450 forms that have not been sequenced but are recognized by anti-CYP1A1 monoclonal antibody 1-12-3 (33) are presumed to be in the CYP1A subfamily and are designated CYP1A (rather than CYP1A1 or CYP1A2) to indicate that their exact identity has not yet been established.

levulinic acid hydrochloride (ALA) and α-naphthoflavone (ANF) were obtained from Aldrich Chemical Co. Methanol and acetonitrile (both HPLC grade) were from J. T. Baker. Other reagents were obtained as described previously (35).

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 3,3',4,4'-TCB (10 mM), 3,3',4,4',5-PCB (1 mM), 3,4,4',5-TCB (2 mM), 2,3,3',4,4'-PCB (10 mM), 2,3',4,4',5-PCB (10 mM), ANF (10 mM), and TCDF (1 mM) were prepared by dissolving crystalline compound in DMSO. Aliquots of these stock solutions, or dilutions made from them, were used to treat the cells. Sterile solutions of ALA were prepared by dissolving ALA in 0.9% NaCl to 3 mM ALA and filtering though a 22-μm filter.

Growth and treatment of cells. PLHC-1 fish hepatoma cells (34) were grown in 75-cm^2 flasks at 30°C in MEM containing Earle's salts, nonessential amino acids, L-glutamine, and 10% calf serum, as described previously (35, 37). One day prior to dosing, cells were subcultured into 48-well plates (Costar) at a density of 4×10^5 cells/well, in a volume of 0.5 ml per well. Plating cells at this density resulted in total protein concentrations of 80–115 μg/well after 72 h of growth. Twenty-four hours after plating, the cells received fresh medium (0.5 ml) and were then dosed with solutions of 2,3,7,8-TCDD (0.01–30 nM final concentrations), 3,3',4,4'-TCB (1–50,000 nM), or other PHAH dissolved in DMSO (2.5 μl/well; 4 wells per dose). Control plates (and wells) received DMSO only. Standard plates received no treatment. Plates were placed in the 30°C incubator for another 43 h, at which time they received 100 μl of 3 mM ALA in 0.9% NaCl (final concentration of ALA = 0.5 mM) or 0.9% NaCl alone. Plates were then incubated for another 5–6 h before analysis. At that time, medium was aspirated from the wells and each well was washed with 0.5 ml phosphate-buffered saline prior to analysis of ethoxyresorufin *O*-deethylase (EROD) activity and total intracellular porphyrin content as described below. Plates used for HPLC analysis of porphyrin patterns, immunoblotting, and total protein measurement were frozen and stored at −80°C.

Inhibition experiments. Two sets of experiments were conducted to determine the effect of CYP1A inhibition on the porphyrogenicity of TCDD. These experiments were similar to those described above (48 h of 10 nM TCDD, with ALA present during the last 6 h) except that ANF (final concentration 50 μM), 3,3',4,4'-TCB (10 μM), or an equivalent amount of DMSO was added at the same time as ALA; EROD activity and total cellular porphyrin content were determined as described below.

Assays. EROD activity and total porphyrin concentrations were measured using a multiwell plate reader with modifications (37) of the methods described by Kennedy *et al.* (15, 40). Briefly, the EROD reaction was initiated by the addition (100 μl/well) of a solution of 7-ethoxyresorufin (2 μM final concentration) and NADPH (1.67 mM final concentration) in TN buffer (50 mM Tris, 0.1 M NaCl, pH 7.8 at room temperature). Plates were then incubated for 5 min at room temperature. Reactions were stopped by the timed 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 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.

Immediately following the measurement of EROD activity, a 0.5-ml aliquot of 3 M HCl was added to each well without removing the EROD reaction mixture. After a brief (1 s/well) treatment with a probe sonicator (Virsonic 475), porphyrin fluorescence was measured using the Cytofluor with 409 and 590 nm excitation and emission filters at sensitivity settings 4 and 5. Porphyrin standard curves were prepared using a uroporphyrin I fluorescence standard (Porphyrin Products) added to wells containing cells and complete EROD reaction mixtures.

Total cellular protein was measured on separate plates containing cells treated with DMSO only, using the fluorescent protein assay described by Lorenzen and Kennedy (41), with bovine serum albumin as standard.

Extraction and HPLC analysis of porphyrins. Plates for porphyrin extraction were treated as above with either DMSO, TCDD (10 nM), or TCB (10 μM) in the presence or absence of added ALA (12 wells per group). Porphyrins were extracted from the cells as described by Kennedy *et al.* (15, 42). Briefly, trypsin/EDTA (0.05% (w/v) trypsin, 0.5 mM EDTA in phosphate-buffered saline) was added to the wells (50 μl/well), and the plates were incubated at room temperature for 4–5 min to detach the cells. A solution of HCl (1 M)/acetonitrile (1/1, 150 μl) was added to each well and the wells were briefly sonicated. Extracts from each group of 12 wells were pooled and diluted with deionized water to 20 ml. After pretreatment of Sep-Pak tC18 cartridges (Waters) with 10 ml acetonitrile followed by 15 ml water, extracts were slowly applied to the cartridge (<1 drop/s). The flow-through fraction was discarded, and porphyrins were eluted with 2.5 ml acetonitrile (<1 drop/s), dried under nitrogen, and frozen at −20°C. The entire extraction procedure was carried out under red light to minimize light-induced degradation of porphyrins.

Dried samples were dissolved in 50 or 100 μl concentrated HCl, sonicated in a sonicator water bath, diluted to 500 μl or 1 ml, respectively, and filtered through 0.45-μm filters. Porphyrins were analyzed by reverse-phase HPLC using an Isco Model 2350/2360 chromatograph equipped with a 50-μl injection loop, a C_{18} column (Rainin Instrument Co.), a Varian 2070 spectrofluorometer, and a Shimadzu Chromatopac C-R3A integrator. Excitation and emission filters in the spectrofluorometer were set at 418 and 620 nm, respectively. Solvents and gradients were as described earlier (43). A 25- or 50-μl aliquot of each porphyrin–HCl solution was injected onto the column. Porphyrins were quantified from a linear standard curve generated with porphyrin I series chromatographic markers over a 5- to 40-pmol range for each porphyrin. Separate standard curves were prepared for uroporphyrin I, hepta-, hexa-, and pentacarboxylporphyrin, coproporphyrin, and mesoporphyrin IX.

Recovery experiments. Recovery experiments were performed to test the efficiency of porphyrin extraction from cells in 48-well plates. A plate of cells was spiked with 120 pmol HPLC standard markers/well (20 pmol of each standard) in a volume of 4 μl. Trypsin/EDTA was then added to the wells (50 μl/well), and the porphyrins were extracted as before, in groups of 12 wells. Extracted porphyrins were resolved and quantified by HPLC. Recoveries of URO through coproporphyrins from cells ranged from 75 to 85%.

Data analysis. Fluorescence data obtained from the Cytofluor plate reader were imported into SigmaPlot (Jandel Scientific) for analysis and curve fitting. Data obtained from porphyrin and EROD assays were normalized to total cellular protein and analyzed by nonlinear regression using the curve-fitting subroutine of SigmaPlot. Data were fitted to the modified Gaussian function (Eq. [1], for biphasic relationships) or to a logistic function (Eq. [2], for sigmoid relationships) as described earlier (15).

The Gaussian function was

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

where $C = \ln(2)/[\ln(EC_{50}) - \ln(d_m)]^2$, $y(d)$ 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, and EC_{50} is the inducer concentration producing 50% of maximal EROD activity or CYP1A1 content.

The logistic function was

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

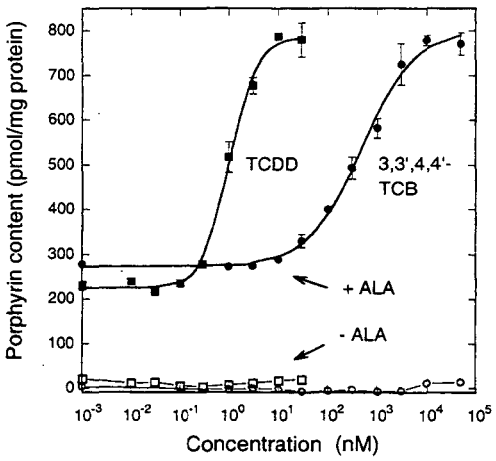

FIG. 1. Porphyrin accumulation in PLHC-1 cells treated with TCDD or 3,3',4,4'-TCB ± ALA. PLHC-1 cells were exposed to 3,3',4,4'-TCB or TCDD for 48 h. ALA (0.5 mM) or 0.9% NaCl was present during the last 5–6 h of treatment. Total intracellular porphyrins were measured using the Cytofluor as described under Methods. Data are presented as means ± SE of values from four wells per dose. Points at 10^{-3} nM concentration indicate the response of cells in the absence of PHAH, i.e., treated with DMSO ± ALA.

where $y(d)$, Y_b, Y_m, and EC_{50} are as for Eq. [1] and g is a slope parameter (15).

RESULTS

Porphyrin Accumulation in PLHC-1 Cells Exposed to TCDD or 3,3',4,4'-TCB

The ability of TCDD and 3,3',4,4'-TCB to cause porphyrin accumulation in PLHC-1 cells was examined using the method recently developed by Kennedy and coworkers for measuring porphyrin content of cells grown in multiwell plates (15). Since ALA, the initial intermediate in the heme biosynthetic pathway, has been shown to enhance the PHAH-dependent porphyrin accumulation in avian and mammalian hepatocytes (10, 18, 44, 45), we added ALA (0.5 mM) to some cultures during the last 5–6 h of PHAH treatment. No changes in intracellular porphyrin content were observed in cells exposed to TCDD (0.01 to 30 nM) or 3,3',4,4'-TCB (1 to 50 μM) in the absence of added ALA (Fig. 1). When ALA was present in the medium, however, treatment with TCDD or 3,3',4,4'-TCB increased the cellular porphyrin content in a dose-dependent manner (Fig. 1). Porphyrin accumulation was also seen, though to a lesser extent, in cells exposed to ALA alone. The maximum level of porphyrin accumulation was similar for TCDD and 3,3',4,4'-TCB (~800 pmol/mg protein), but the potency of TCDD was approximately 500-fold greater than that of 3,3',4,4'-TCB (EC_{50} values 1.0 and 477 nM, respectively).

There was variation between experiments in the porphyrin content of cultures treated with ALA alone (range of porphyrin accumulation 100–300 pmol/mg) or PHAH+ALA (300–800 pmol/mg) but the PHAH-dependent increase and its requirement for ALA was consistently observed. If the duration of exposure to ALA was increased, greater amounts of porphyrin accumulation were observed. Figure 2 shows a comparison of cellular porphyrin content following treatment with TCDD (48 h) with ALA present for the final 11.5 or 6.0 h of exposure. Maximal porphyrin content after 6 h of ALA (48 h TCDD) was approximately 600 pmol/mg, whereas after 11.5 h of ALA (48 h TCDD), the content of total porphyrins was greater than 1200 pmol/mg. Despite this difference in maximal porphyrin level, the EC_{50} values for TCDD-induced porphyrin accumulation were similar for the two plates (6-h ALA exposure, 0.84 nM; 11.5-h ALA exposure, 0.91 nM).

Porphyrin Profiles in PLHC-1 Cells Treated with TCDD or 3,3',4,4'-TCB ±ALA

The identity of porphyrins accumulated in PLHC-1 cells treated with TCDD (0 and 10 nM, ±ALA) or 3,3',4,4'-TCB (0 and 10 μM, ±ALA) was determined by extraction of the cells and analysis of porphyrin patterns by HPLC with fluorescence detection. The lack of accumulation of total porphyrins in cells treated with DMSO, TCDD, or 3,3',4,4'-TCB in the absence of ALA was confirmed by HPLC analysis (Fig. 3). Treatment of cells with 3,3',4,4'-TCB or TCDD in the presence of ALA produced large increases in URO (~80% of total) and, to a lesser extent, heptacarboxylporphyrin (~20%) (Fig. 3), similar to patterns of PHAH-dependent porphyrin accumulation reported for avian and mammalian systems (15, 18, 44, 46).

In cells treated with DMSO and ALA without PHAH,

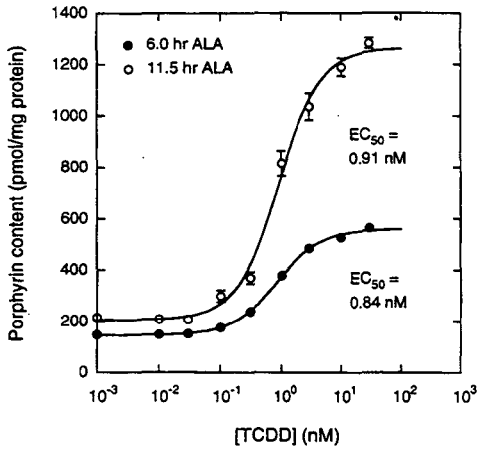

FIG. 2. Time dependence of ALA effect on porphyrin accumulation after TCDD treatment. PLHC-1 cells were exposed to TCDD for 48 h. ALA (0.5 mM) was present during the last 6 or 11.5 h of treatment. Total intracellular porphyrins were measured using the Cytofluor as described under Methods. Data are presented as means ± SE of values from four wells per dose. Points at 10^{-3} nM concentration indicate the response of cells treated with DMSO + ALA.

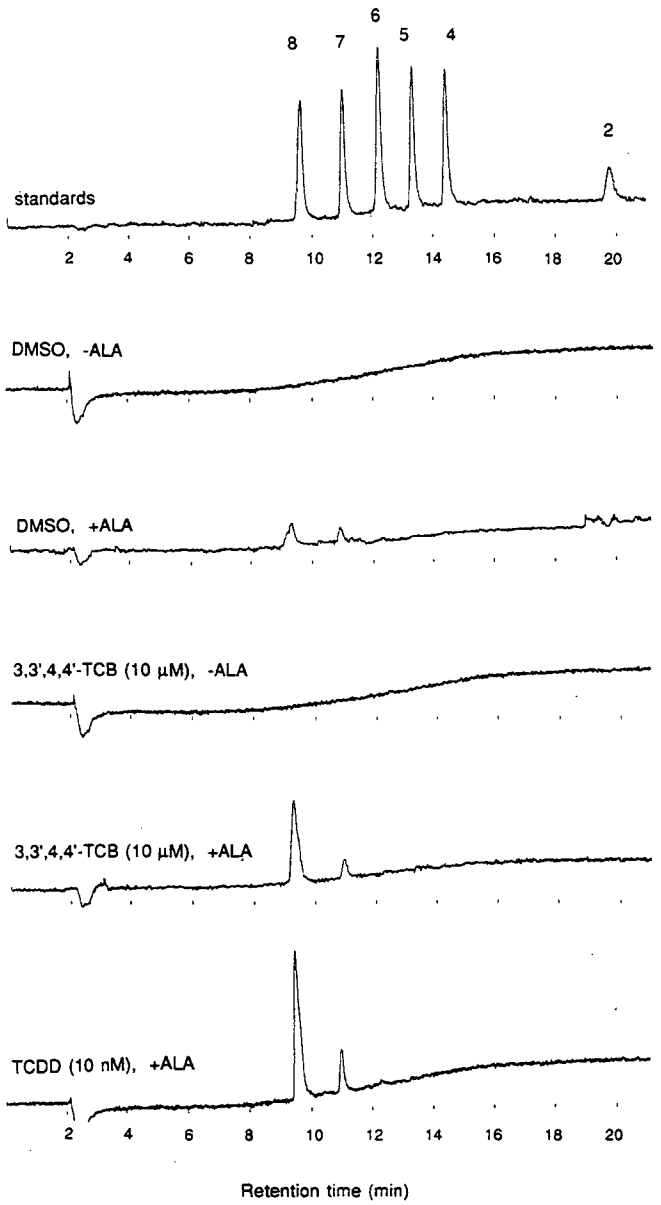

FIG. 3. HPLC profiles of porphyrins in cells treated with 3,3',4,4'-TCB or TCDD ± ALA. PLHC-1 cells were exposed to 3,3',4,4'-TCB or TCDD for 48 h as described under Methods. ALA (0.5 mM) or 0.9% NaCl was present during the last 5–6 h of treatment. Porphyrins were extracted from the cells and individual porphyrins determined by HPLC with fluorescence detection. Identity of porphyrins is indicated by the number of carboxyl groups: 8, URO; 7, heptacarboxylporphyrin; 6, hexacarboxylporphyrin; 5, pentacarboxylporphyrin; 4, coproporphyrin; 2, mesoporphyrin.

small amounts of uroporphyrin and heptacarboxylporphyrin were present, but other porphyrins were not evident (Fig. 3). Studies performed in mouse and chick embryo hepatocytes exposed to ALA have shown that protoporphyrin predominates when cells and medium are analyzed together (18, 46, 47), but protoporphyrin was not detected in PLHC-1 cells incubated for 6 h with ALA. The extraction method that we used (42) provides quantitative recovery of this porphyrin (S. W. Kennedy, personal communication) and, although we cannot exclude the possibility that small amounts of protoporphyrin were present in the cells, the amounts of URO and heptacarboxylporphyrin detected by HPLC account for most if not all of the total porphyrin content measured with the Cytofluor. Kawanishi *et al.* (47) reported that the protoporphyrin formed in ALA-treated chick embryo hepatocytes accumulated primarily in the culture medium, which was not analyzed in our experiments.

Structure–Activity Relationships for PHAH-Induced Porphyrin Accumulation

In light of the proposed involvement of the AhR and CYP1A forms in PHAH porphyria in avian and mammalian systems, we examined the structure–activity relationship for porphyrin accumulation in PLHC-1 cells. Cells were treated with a range of concentrations of TCDD, TCDF, or each of three non-*ortho*-substituted ("planar") chlorobiphenyls, all of which induce CYP1A activity (EROD) and CYP1A protein in these cells (35, 37, 48). Two mono-*ortho*-substituted chlorobiphenyls that do not induce CYP1A in PLHC-1 cells (37, 38, 48) were also evaluated. Figure 4 shows the dose–response relationships for EROD induction and porphyrin accumulation following PHAH treatment of PLHC-1 cells. For these figures, the porphyrin content of cells exposed to ALA alone (114–277 pmol/mg) has been subtracted from each value to give the PHAH-dependent component of porphyrin accumulation. The amount of immunodetectable CYP1A protein determined in separate experiments (M. E. Hahn, unpublished studies) is also shown for comparison.

Induction of EROD activity by TCDD, TCDF, and the planar PCBs was biphasic, with increases at lower concentrations of inducer followed by decreased induction at higher concentrations. There were large differences among these compounds in the apparent efficacies with which they induced EROD activity, with the chlorobiphenyls producing between 10 and 50% of the maximal induction produced by TCDD or TCDF. The causes and significance of the biphasic curves and differing efficacies have been discussed previously (35, 37, 48).

All of the PHAH that induced CYP1A activity and protein were also porphyrogenic. Thus, TCDD, TCDF, 3,3',4,4',5-PCB, 3,4,4',5-TCB, and 3,3',4,4'-TCB all caused accumulation of porphyrins to a similar degree (maximum of 500–600 pmol/mg over that produced by ALA alone). In all cases, the increase in porphyrin accumulation with dose was monotonic (Fig. 4) and dependent on the presence of ALA (not shown).

Unlike the marked porphyrogenicity of the non-*ortho*-substituted PCBs, the two mono-*ortho*-substituted chlorobiphenyls were inactive or nearly so. A very slight increase in EROD activity and porphyrin accu-

mulation was seen at the highest concentration (50 μM) of 2,3′,4,4′,5-PCB, whereas 2,3,3′,4,4′-PCB did not induce EROD or increase porphyrin accumulation at any concentration.

The relative potencies of TCDD, TCDF, and the PCBs for increasing EROD activity and porphyrin accumulation are compared in Table I. EC_{50} values for porphyrin accumulation were determined using a logistic equation; EC_{50} values for EROD induction were determined using the same logistic equation (3,3′,4,4′-TCB) or the modified Gaussian equation described by Kennedy *et al.* (15). It should be noted that for some of the compounds the EROD EC_{50} values are likely to be underestimates of the true EC_{50} values for CYP1A induction, due to inhibition of EROD activity by residual inducer present in the cells (37). For each of the porphyrogenic PHAH, the EC_{50} for porphyrin accumulation was greater than the EC_{50} for EROD induction (Table I). In most cases, porphyrin EC_{50} values were similar to, or slightly higher than, the concentrations at which peak EROD activities were obtained. Thus, porphyrin accumulation did not occur until substantial EROD induction was evident. The one apparent exception, 3,3′,4,4′-TCB, is known to be a potent inhibitor of EROD activity (49, 50). In contrast to the low EROD activity induced by this compound, the increase in CYP1A protein was similar to that caused by other inducers, and induction of CYP1A protein and porphyrin accumulation occurred over the same range of 3,3′,4,4′-TCB concentrations (Fig. 4).

Effect of EROD Inhibition on Accumulation of Porphyrins

The CYP1A-dependent oxidation of URO'gen to URO reported by other investigators suggests that a functional CYP1A enzyme is required for porphyrin accumulation in response to PHAH (9, 10, 12, 13, 51). In order to determine if the porphyrogenicity of PHAH in PLHC-1 cells has a similar requirement for CYP1A, we examined the effect of α-naphthoflavone on PHAH-dependent porphyrin accumulation. ANF is a potent inhibitor of CYP1A forms, including those in fish (28). This experiment was similar to those previously described (48 h of 10 nM TCDD, with ALA present during the last 6 h) except that ANF (final concentration 50 μM) or an equivalent amount of DMSO was added at the same time as ALA; EROD activity and total cellular porphyrin content were determined as before.

The results of one set of experiments are shown in Fig. 5. As expected, ANF added 6 h prior to assay inhibited EROD activity by greater than 98%. In addition, ANF inhibited the (TCDD+ALA)-dependent accumulation of porphyrins to levels near those of cells exposed to ALA alone.

In a second set of experiments, the effects of EROD inhibition by ANF and by 3,3′,4,4′-TCB were compared (Fig. 6). ANF inhibited EROD activity (99%) and TCDD-induced porphyrin accumulation (72% of total, 90% of the TCDD-inducible component) as before. Interestingly, ANF had only a slight effect on the accumulation of porphyrins caused by ALA alone. 3,3′,4,4′-TCB was also a good inhibitor of TCDD-induced EROD activity (80–81% reduction), but in contrast to ANF, addition of the chlorobiphenyl enhanced porphyrin accumulation in TCDD-treated cells in the presence of ALA (Fig. 6).

DISCUSSION

The PHAH-induced accumulation of highly carboxylated porphyrins such as URO and heptacarboxylporphyrin has been observed in mammals and birds *in vivo* and in primary hepatocyte cultures isolated from mice and chickens. Hepatic porphyria associated with PHAH exposure has been observed in wild fish (23) but this finding has not been confirmed experimentally (24), and the sensitivity of fish to PHAH-induced porphyria remains uncertain. In this paper, we present the first experimental evidence that PHAH are able to interfere with the heme biosynthetic pathway in a piscine system, causing an intracellular accumulation of highly carboxylated porphyrins. These findings establish uroporphyria as a shared vertebrate response to PHAH exposure and suggest that PLHC-1 cells might be a useful model for examining its mechanism, complementing the avian and mammalian models that currently exist. Some aspects of the porphyrogenic response in these fish hepatoma cells are discussed below in relation to porphyria in other vertebrate systems.

Requirement for Exogenous ALA

In PLHC-1 cells, PHAH-induced porphyrin accumulation was only seen in the presence of added ALA (Figs. 1 and 3). The dependence of porphyrin accumulation on an exogenous source of ALA varies among the different types of cultured cells in which this effect has been examined. For example, ALA loading enhances PHAH-induced uroporphyrin accumulation in chick embryo hepatocytes, but is not absolutely required (e.g., 6, 10, 44, 45). In primary cultures of mouse hepatocytes treated with 3,3′,4,4′,5,5′-hexachlorobiphenyl, uroporphyrin accumulation did not occur in the absence of ALA, but was substantial after 9 days of exposure to HCB+ALA (18). In contrast to the results in mouse hepatocytes, porphyrin accumulation did not occur or was minimal in rat hepatocytes exposed to PCBs, even in the presence of added ALA (10, 18).

Differences in the effect of ALA on PHAH-induced porphyrin accumulation most likely reflect variation among vertebrate species in the relative activities and regulation of enzymes in the heme biosynthetic pathway. ALA synthase (ALAS) is thought to be the rate-limiting enzyme for hepatic heme synthesis in mammals, birds, and possibly fish (52, 53). The regulation

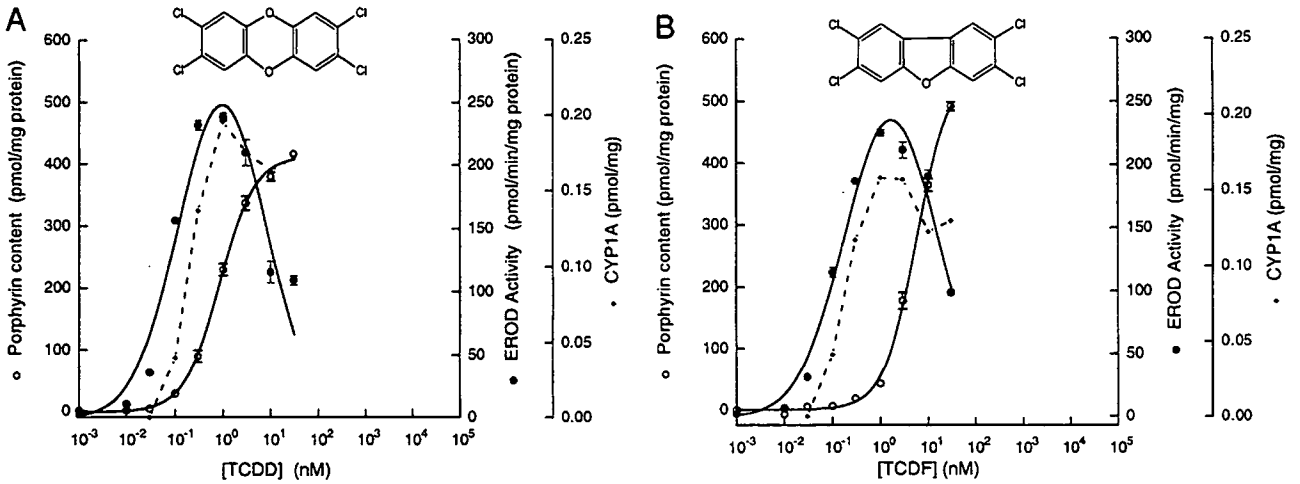

FIG. 4. Dose–response relationships for EROD induction and PHAH-dependent porphyrin accumulation in PLHC-1 cells treated with TCDD, TCDF, or selected chlorobiphenyls. PLHC-1 cells were exposed to PHAH for 48 h as described under Methods. ALA (0.5 mM) was present during the last 5–6 h of treatment. EROD activity (filled circles) and total intracellular porphyrins (open circles) were measured on the same plate using the Cytofluor. Data are presented as means ± SE of values from four wells per dose. For porphyrin content, the porphyrin accumulation due to ALA alone (114–277 pmol/mg) has been subtracted from all values for each experiment to reveal the PHAH-dependent component of porphyrin accumulation. Note the different ranges for EROD activity in each graph. The amount of immunodetectable CYP1A protein determined in separate experiments (M. E. Hahn, unpublished results) is shown for comparison (broken line). Neither 2,3,3′,4,4′-pentachlorobiphenyl (IUPAC 105) nor 2,3′,4,4′,5-pentachlorobiphenyl (IUPAC 118) induced detectable amounts of CYP1A protein in PLHC-1 cells.

of ALAS activity is complex, involving feedback control by heme at several levels, as well as direct control by specific inducing chemicals. In chick embryo liver or cultured avian hepatocytes, this enzyme is highly inducible by a variety of compounds, including PHAH (45, 54–56), through a mechanism that is not fully understood but may (in the case of PHAH) involve the AhR, either directly or indirectly (54). ALAS activity may be less inducible in mammals than it is in avian systems (4, 57, 58). The inducibility of this enzyme in fish is not well understood, but in one study using primary cultures of toadfish hepatocytes, a concentration of 3,3′,4,4′,5-PCB (100 nM) that strongly induced CYP1A activity caused a slight but consistent 50% increase in ALAS activity (59). In preliminary experiments, the ALAS activity of PLHC-1 cells was increased approximately two- to threefold following 24-h exposure to 3,3′,4,4′,5-PCB at 1, 10, or 100 nM (N. W. Cornell and M. E. Hahn, unpublished observations). We suggest that differences in sensitivity of avian, mammalian, and piscine ALAS to induction by PHAH may contribute to the observed differences in the requirement for added ALA in the various cell culture systems. A more rigorous evaluation of ALAS inducibility and its relationship to porphyria in these systems is warranted.

Relationship between Porphyrin Accumulation and CYP1A

Several lines of evidence support the hypothesis that one or more P450 forms in the CYP1A subfamily is involved in the mechanism of PHAH-induced porphyrin accumulation in mammalian and avian systems. (i) Studies in inbred mice that differ at the *AHR* locus have shown a relationship between "Ah responsiveness" (sensitivity to induction of CYP1A forms due to the presence of a high-affinity AhR allele) and susceptibility to PHAH porphyria. Although in several different mouse strains the correlation appears to be incomplete (3, 60), experiments conducted using congenic mice that differ *only* at the *AHR* locus have conclusively established the importance of the AhR (5). (ii) In chick embryo hepatocytes, the PCB structure–activity relationship for porphyrin accumulation closely parallels the structure–activity relationship for induction of CYP1A activities, non-*ortho*-substituted congeners being the most active (14, 16, 61). (iii) Inhibitors of CYP1A catalytic activity block the accumulation of URO (6, 7, 45). (iv) Hepatic microsomes (9, 10) or purified, reconstituted CYP1A forms (13) catalyze the *in vitro* oxidation of URO'gen to URO, and this oxidation is inhibited by antibodies to CYP1A2 (12).

The data presented here demonstrate that, as in the other vertebrate systems, there is a strong relationship between porphyrin accumulation and CYP1A induction in PLHC-1 fish hepatoma cells exposed to PHAH plus ALA. Chlorobiphenyl congeners that induced the CYP1A-dependent catalytic activity of EROD (Fig. 4) and CYP1A protein [(37) and M. E. Hahn, unpublished results] also caused porphyrin accumulation, whereas those that did not induce CYP1A (or did so only weakly) did not cause substantial increases in porphyrins in these cells. In addi-

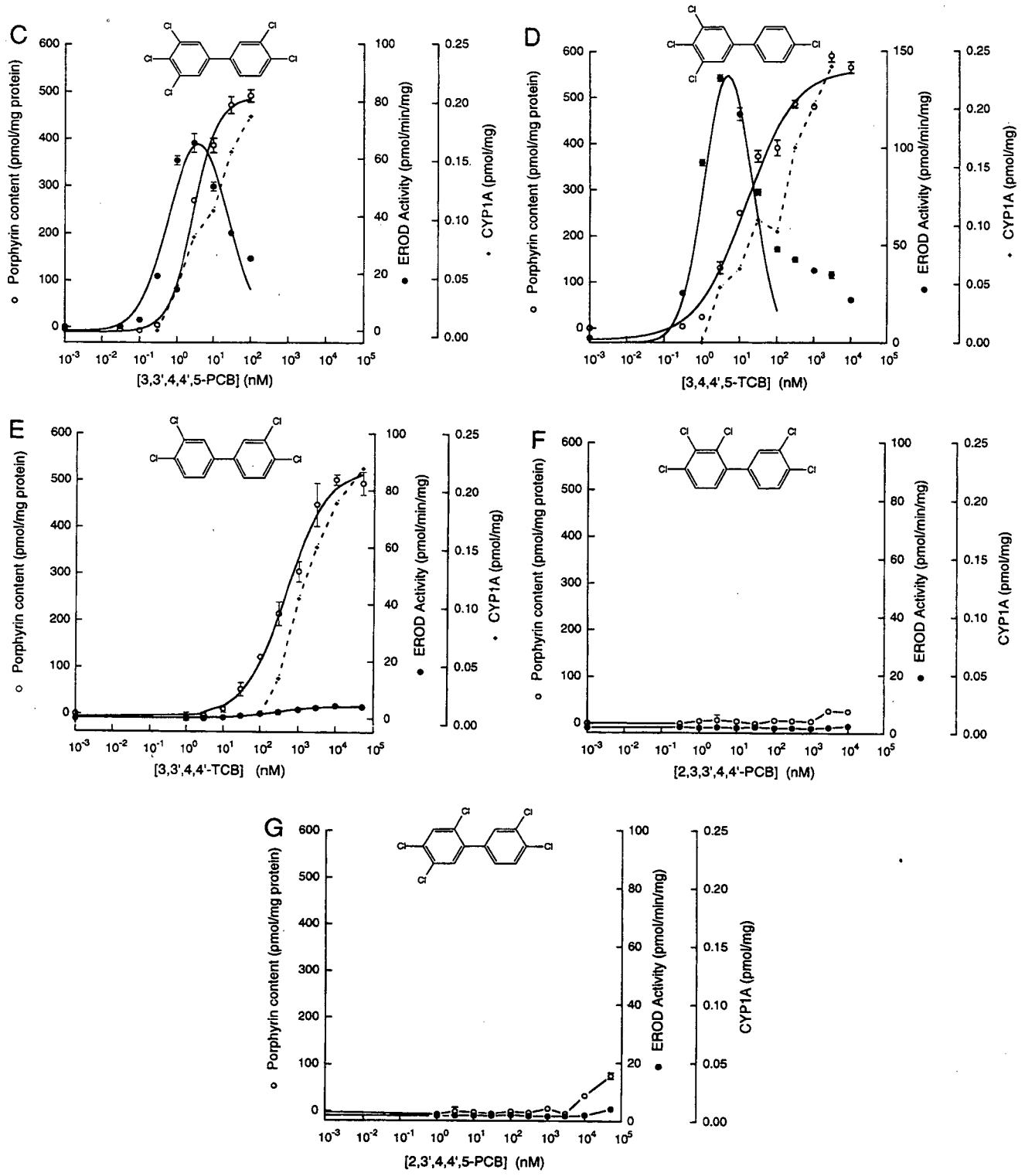

FIG. 4—*Continued*

tion, the rank order potencies for CYP1A induction and porphyrin accumulation were similar: CYP1A induction, TCDD ≅ TCDF > 3,3′,4,4′,5-PCB > 3,4,4′,5-TCB > 3,3′,4,4′-TCB ⩾ 2,3′,4,4′,5-PCB ≅ 2,3,3′,4,4′-PCB; porphyrin accumulation, TCDD > 3,3′,4,4′,5-PCB ≅ TCDF > 3,4,4′,5-TCB > 3,3′,4,4′-TCB ⩾ 2,3′,4,4′,5-PCB ≅ 2,3,3′,4,4′-PCB. The inability of the latter two congeners to cause porphyrin accumulation is especially interesting. Although these two mono-*ortho*-substituted congeners induce CYP1A in mammals and some birds (40, 62, 63)

TABLE I

Relative Potencies for EROD Induction and Porphyrin Accumulation in PHAH-Treated PLHC-1 Cells

	EC_{50} for EROD induction (nM)	[PHAH] at maximum EROD activity (nM)	EC_{50} for porphyrin accumulation (nM)
TCDD	0.091	1	0.85
TCDF	0.12	1	5.7
3,3′,4,4′,5-pentachlorobiphenyl (CB-126)	0.38	3	2.8
3,4,4′,5-tetrachlorobiphenyl (CB-81)	0.68	3	14.7
3,3′,4,4′-TCB (CB-77)	184	10,000	477
2,3,3′,4,4′-pentachlorobiphenyl (CB-105)	>50,000	NA	>50,000
2,3′,4,4′,5-pentachlorobiphenyl (CB-118)	>50,000	NA	>50,000

Note. PLHC-1 cells were cultured in 48-well plates and treated with PHAH+ALA as described under Methods and in the legend to Fig. 4. Dose–response relationships for EROD induction and porphyrin accumulation were determined by sequential analysis of the same plate. EC_{50} values were determined by fitting the data to a modified Gaussian function or to a logistic function as described under Methods. IUPAC numbers are given in parentheses for each chlorobiphenyl congener. NA, not applicable.

and cause porphyrin accumulation in chick embryo hepatocytes (40), they are inactive or only weakly active at inducing CYP1A in several fish systems (48, 49, 64, 65).

Additional evidence for the involvement of induced CYP1A in PHAH-dependent porphyria in PLHC-1 cells was obtained by using ANF, a specific inhibitor of CYP1A forms. Both EROD activity and PHAH-dependent porphyrin accumulation were blocked by ANF (Figs. 5, 6). The ANF was added at a time (43 h) when maximal CYP1A induction had already been achieved, so it was acting to inhibit catalytic activity of the induced CYP1A rather than the AhR-mediated induction itself or other AhR-mediated processes. These results are consistent with the ability of other CYP1A inhibitors to block URO accumulation in chick embryo hepatocytes (6, 7, 45) and suggest that a functional CYP1A is necessary for porphyria in these cells.

Unlike ANF, 3,3′,4,4′-TCB did not inhibit PHAH-dependent porphyrin accumulation and may have enhanced it, despite strong inhibition of EROD activity (Fig. 6). The contrast between results obtained with these two CYP1A inhibitors may be related to the proposed mechanism of PHAH-induced porphyria. DeMatteis, Sinclair, and others (7, 8) have hypothesized that the role of CYP1A in uroporphyria is to catalyze the oxidation of URO'gen to URO; such a reaction has been demonstrated *in vitro* using hepatic microsomes from chicks or rodents (9, 10, 13). In the avian system, CYP1A-dependent URO'gen oxidation is stimulated by 3,3′,4,4′-TCB, supporting the proposal that an interaction of PHAH with the CYP1A is necessary for the oxidation, and therefore the uroporphyria, to occur (9, 10, 12). Consistent with this, PHAH are highly porphyrogenic in cultured chick embryo hepatocytes and stimulate URO accumulation when added to 3-methylcholanthrene-treated cells, while nonhalogenated CYP1A inducers are only slightly porphyrogenic and block accumulation of URO in PHAH-treated chick cells (6, 7, 16). The discrepancy between the effects of halogenated versus nonhalogenated compounds in this system may reflect differences in the nature of their interaction with CYP1A and thus in their ability to participate in the URO'gen oxidation reaction. However, it should be noted that there are important species differences in the PHAH-dependence of this reaction; unlike results obtained in the chick system, the *in vitro* URO'gen oxidation catalyzed by microsomes from rats and mice is not stimulated by 3,3′,4,4′-TCB (10, 12). Whether the induced CYP1A in PLHC-1 cells is capable of URO'gen oxidation and whether such a process is stimulated by 3,3′,4,4′-TCB or other compounds is not yet known, but the slight increase in porphyrin accumulation that occurred when 3,3′,4,4′-TCB was added to cultures of TCDD-treated PLHC-1 cells (Fig. 6) suggests that this may be so.

The idea that an interaction of PHAH with the induced CYP1A is involved in the mechanism of uroporphyria in PLHC-1 cells is also supported by the relationship between porphyrin and CYP1A dose–response curves shown in Fig. 4. The PHAH concentrations at which cellular porphyrin content began to increase were at or near the concentrations resulting in maximal EROD activity (Table I). At higher concentrations, EROD activities declined while porphyrins continued to accumulate. Similar findings have been reported for chick embryo hepatocytes (6, 16, 40, 45). In contrast to the declining EROD activities, levels of immunodetectable CYP1A protein continue to increase with dose in PLHC-1 cells [Fig. 4; see also (35, 37)]. Previous studies have shown that the biphasic EROD curves are due in part to inhibition or inactivation of CYP1A activity by residual PHAH (35, 49, 50, 66), evidence for the PHAH-CYP1A interaction suggested earlier. However, establishing the mechanistic relationship between the EROD inhibition, the URO'gen oxidation, and the URO accumulation in PLHC-1 cells will require further study.

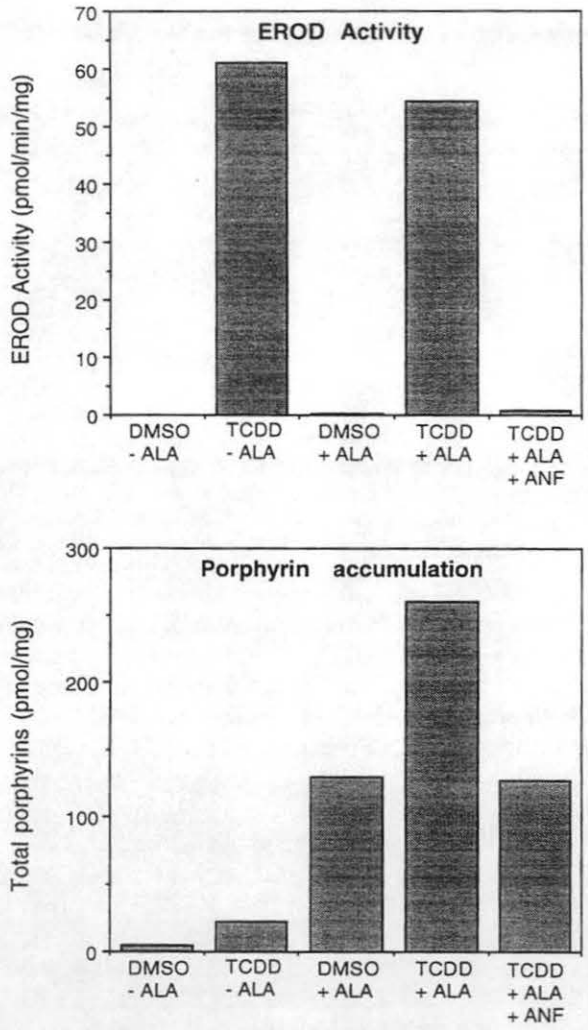

FIG. 5. Effect of ANF on EROD activity and porphyrin accumulation in PLHC-1 cells treated with TCDD±ALA. PLHC-1 cells were exposed to 10 nM TCDD for 48 h. ALA (0.5 mM) or 0.9% NaCl was present during the last 6 h of treatment. ANF (50 μM final concentration) or an equivalent amount of DMSO was added at the same time as ALA. EROD activity and total intracellular porphyrins were measured using the Cytofluor. Data are presented as means of two experiments, each of which was performed using four wells per dose. In each experiment, the SE for each set of four wells was ≤7% of the mean, except for values near the limits of detection.

PLHC-1 Cells as a Model System

The results of our studies suggest that PLHC-1 fish hepatoma cells might be a useful model system for investigating mechanisms of PHAH-induced porphyria, including the role of cytochrome P450 forms. To our knowledge, the PLHC-1 line is the only vertebrate hepatoma line in which PHAH-induced porphyrin accumulation has been demonstrated. The lack of porphyria in mammalian hepatoma lines is hypothesized to be related to their lack of CYP1A2 expression (18). In contrast to mammals and birds, most fish express only a single CYP1A form (28). The CYP1A form in fish has been designated a CYP1A1 based on its greater structural, functional, and regulatory similarities to mammalian CYP1A1 compared to CYP1A2 (29, 30). We suggest that the CYP1A induced by PHAH in PLHC-1 cells is also a CYP1A1, based on its recognition by MAb 1-12-3 (35, 37), which recognizes all vertebrate CYP1A1 but not CYP1A2 (39, 67). However, we cannot exclude the possibility that these cells also express a second CYP1A with properties similar to those of mammalian CYP1A2. If there is a single CYP1A in PLHC-1 cells and it is a CYP1A1, the sensitivity of these cells to PHAH-induced porphyria would stand in contrast to (1) the proposed predominant role of CYP1A2 versus CYP1A1 in porphyria in other vertebrate systems

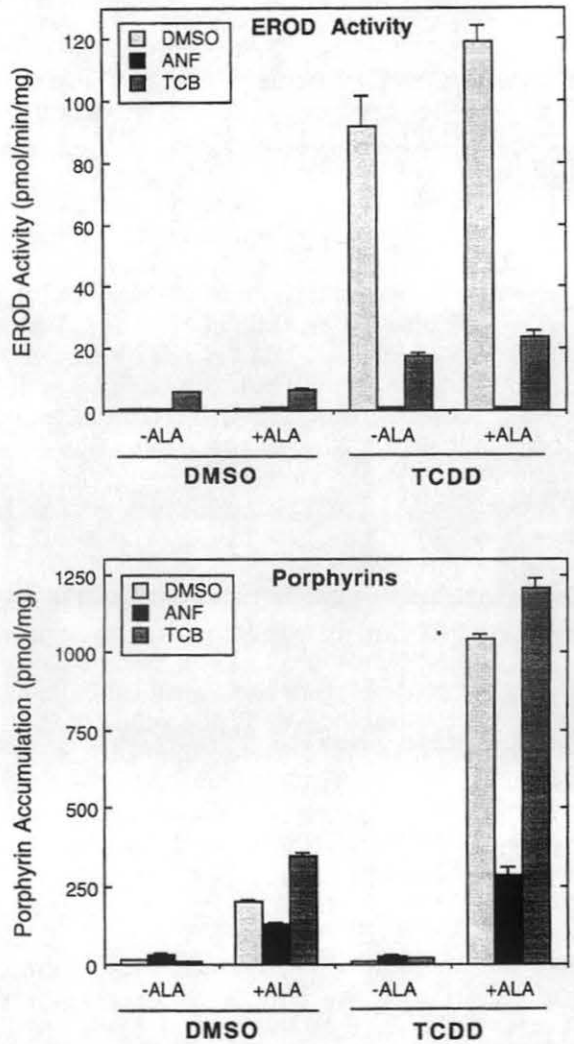

FIG. 6. Effect of ANF or 3,3′,4,4′-TCB on EROD activity and porphyrin accumulation in PLHC-1 cells treated with TCDD±ALA. PLHC-1 cells were exposed to 10 nM TCDD for 48 h. ALA (0.5 mM) or 0.9% NaCl was present during the last 6 h of treatment. ANF (50 μM), 3,3′,4,4′-TCB (10 μM), or an equivalent amount of DMSO was added at the same time as ALA. EROD activity and total intracellular porphyrins were measured using the Cytofluor. Data are presented as means ± SE of values from four wells per dose.

(12, 13) and (2) the apparent lack of PHAH-induced porphyria in other cells expressing only CYP1A1 (10, 18, 20).

Additional work will be needed to establish the features of the heme biosynthetic pathway and P450 expression in PLHC-1 cells. In particular, it will be important to determine whether more than one CYP1A form is expressed in PLHC-1 cells and to determine the relationship of *Poeciliopsis* CYP1A form(s) to CYP1A and CYP1B forms that have been identified in mammals and birds. The inducibility of ALAS, likely a key factor in sensitivity to chemical porphyria, should also be examined further in these cells. These questions notwithstanding, our initial studies of PHAH-induced porphyrin accumulation in PLHC-1 fish hepatoma cells establish this cell line as a useful model for studying PHAH porphyrogenicity in fish. The correspondence between CYP1A induction and porphyrin accumulation provides evidence for similar mechanisms of porphyrin accumulation in diverse vertebrate species, and suggests a more general utility of these cells as a vertebrate model for examining the relationship between these two parameters. Differences between PLHC-1 cells and avian cell cultures in structure–activity relationships for CYP1A induction and porphyria, particularly for the mono-*ortho*-substituted PCBs, may prove especially interesting in this regard.

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