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Cytochromes P450 (CYP) in the *Poeciliopsis lucida* Hepatocellular Carcinoma Cell Line (PLHC-1): Dose- and Time-Dependent Glucocorticoid Potentiation of CYP1A Induction without Induction of CYP3A

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Glucocorticoids are being found to influence expression of cytochrome P450 (CYP) genes in multiple subfamilies in mammals (J. S. Sidhu, and C. J. Omiecinski (1995) *Pharmacogenetics* 5, 24–36). In the present study we investigated CYP1A and CYP3A expression in the fish *Poeciliopsis lucida* hepatocellular carcinoma cell line (PLHC-1) after coadministration of CYP1A and CYP3A inducers, including glucocorticoids. A putative CYP3A protein is expressed in PLHC-1 cells but its content was not altered by exposure of cultures to the prototypical mammalian CYP3A inducers dexamethasone (DEX), pregnenolone-16α-carbonitrile (PCN), or rifampicin (RIF). However, when coadministered with 3,3′,4,4′-tetrachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), DEX but not PCN or RIF caused increases in the degree of CYP1A induction by these aryl hydrocarbon receptor (AHR) agonists. This increase was seen both in CYP1A protein content and rates of ethoxyresorufin-*O*-deethylase (EROD) activity. DEX alone caused no induction of CYP1A, indicating that the enhancement of CYP1A induction caused by DEX + AHR agonists was not an additive effect but rather a potentiation. The dose of DEX required for maximal potentiation was three orders of magnitude greater at 48 h than the dose required at 24 h. Moreover, the degree of potentiation of CYP1A induction was much greater at the lower doses than at the highest doses of TCDD. There was up to 20-fold potentiation of EROD induction in cultures exposed to 0.1 nM TCDD. Two other glucocorticoid receptor (GR) agonists, cortisol and prednisone, also produced a strong potentiation of CYP1A induction, but other mammalian CYP3A inducers that are not GR agonists, such as the anti-glucocorticoid PCN, the anti-mineralocorticoid spironolactone, or the macrolide antibiotics RIF and troleandomycin, did not potentiate the CYP1A induction in PLHC-1 cells. Addition of the mammalian GR antagonists PCN or RU 38486 reduced the DEX-mediated potentiation of CYP1A induction, whereas spironolactone had no effect on the potentiation. RU 38486 also potentiated the induction of EROD activity by TCDD, which suggests that RU 38486 acts as a partial GR agonist in PLHC-1 cells. These results suggest that potentiation of CYP1A induction in this nonmammalian cell line proceeds by a classical GR-mediated pathway, independently of the expression of CYP3A. However, the complex interaction between doses of both GR and AHR agonists and duration of exposure, suggests that additional processes influence this potentiation. The unusually strong potentiation at lower doses of TCDD may make PLHC-1 cells particularly suitable in exploring further the consequences of this potentiation.

Key Words: **gene regulation; potentiation; P450; CYP1A; CYP3A; AHR agonists; GR agonists; RU 38486; TCB; TCDD; teleost fish; hepatoma cell line.**

Members of several cytochrome P450 (CYP)[3] gene families (1) code for the major enzymes responsible for

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[3] Abbreviations used: AHR, aryl hydrocarbon receptor; BNF, β-naphthoflavone; CYP, cytochrome P450; DEX, dexamethasone; DMSO, dimethylsulphoxide; EROD, ethoxyresorufin-*O*-deethylase; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; NADPH, reduced nicotine amide dinucleotide phosphate; PAb, polyclonal antibodies; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate buffer saline; PCN, pregnenolone-16α-carbonitrile; PHAH, planar halogenated aromatic hydrocarbon; PLHC-1, *Poeciliopsis lucida* hepatocellular carcinoma cell line; RIF, rifampicin; TAO, troleandomycin; TAT, tyrosine aminotransferase; TCB, 3,3′,4,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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metabolism of lipophilic xenobiotics, including various drugs and pollutants. The regulation of these *CYP* genes is an important feature of their role in xenobiotic metabolism.

Proteins in the CYP1A subfamily metabolize and activate procarcinogenic polycyclic aromatic hydrocarbons (PAHs) and planar-halogenated aromatic hydrocarbons (PHAHs) (2, 3). CYP1As also are induced by these compounds, mediated via the aryl hydrocarbon receptor (AHR), which binds to specific consensus sequences in *CYP1A* gene promotor regions and thereby activates transcription (4). CYP3A enzymes are the major catalysts of steroid 6β-hydroxylation (5), they metabolize a variety of therapeutic agents (6, 7), and they activate several procarcinogens including aflatoxin B_1 and 7,8-dihydroxy-7,9-dihydrobenzo[*a*]pyrene (3). CYP3As are induced predominantly by glucocorticoids and synthetic analogues, through a nonclassical glucocorticoid receptor (GR) pathway, which differs from the classical GR-mediated pathway in terms of specificity, dose of glucocorticoid required, and lack of inhibition by antiglucocorticoids (8). In a recent study, the same *cis*-acting element was shown to be involved in induction of the rat *CYP3A1* gene by both a glucocorticoid and an antiglucocorticoid (9). CYP3As in mammals are inducible also by nonglucocorticoids, including macrolide antibiotics such as rifampicin (RIF) and troleandomycin (TAO), the antiglucocorticoid pregnenolone-16α-carbonitrile (PCN), the antimineralocorticoid spironolactone, and other therapeutic drugs such as phenobarbital and cyclosporin A (8–12). Thus, the role of the GR in CYP3A induction remains to be established.

Several lines of evidence indicate that CYP1A and CYP3A subfamily members may share regulatory features involving glucocorticoids. Glucocorticoids, and synthetic analogues such as dexamethasone (DEX), can potentiate induction of CYP1A1 by PAH in cultures of hepatocytes from fetal human and rat, by a classical GR-mediated mechanism (13, 14). Glucocorticoid responsive element (GRE) consensus sequences have been identified in the first intron of the human and rat *CYP1A1* genes, and this region was found to be involved in the stimulation of *CYP1A1* transcription by GR agonists (15, 16). In the rat H4IIEC3/T hepatoma cell line, DEX treatment increased the AHR content in the cytosol, suggesting another avenue for interaction between GR agonists and CYP1A1 expression (17). Thus, despite distinct pathways for induction of CYP1A and CYP3A, there seems to exist an intimate relationship between GR agonists and induction of certain genes in both subfamilies. Determining the degree of similarity in GR agonist regulation of homologous CYP in different taxonomic groups may help to achieve a general understanding of the nature and significance of that relationship.

Teleost fish possess well-known CYP1A proteins (18). As in mammals, the CYP1As in fish are inducible by PAH/PHAH, apparently via AHR-mediated mechanisms (19). Recently, prominent CYP forms in several teleost species have been identified as probable members of the CYP3A subfamily (20). In this paper we investigate CYP1A and CYP3A expression in a teleost hepatoma cell line, the *Poeciliopsis lucida* hepatocellular carcinoma cell line, exposed to prototypical CYP1A and CYP3A inducers, including GR agonists. Defining the general features of how these CYP3A inducers affect expression of CYP1A will be important for the interpretation of PAH/PHAH effects in laboratory and clinical studies as well as in natural populations of fish and other vertebrates, in which induction of CYP1A increasingly is found to occur at high levels, reflecting exposure to AHR agonists in the environment.

MATERIALS AND METHODS

Chemicals. 3,3′,4,4′-Tetrachlorobiphenyl (TCB), PCB congener 77, was obtained from Pathfinder Laboratories (St. Louis, MO) and its purity was >99% as determined by GC-ECD and GC-MS. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (10 μg/ml in toluene, >98% purity) was obtained from Ultra Scientific (Hope, RI). 7-Ethoxyresorufin was obtained from Pierce. The progesterone receptor and glucocorticoid type II receptor antagonist, RU 38486 (mifepristone or RU 486), was a generous gift from Dr. Russell Prough, University of Louisville (Louisville KY), as a part of studies to evaluate glucocorticoid receptor-dependent regulation of drug metabolizing enzymes. Sterile 75-cm^2 flasks and 48-well polystyrene tissue culture clusters were obtained from Costar. Bovine serum albumin, calf serum, cortisol (hydrocortisone (98%)), dexamethasone (DEX) (98.2% as determined by HPLC), dimethylsulphoxide (DMSO) (99.9%), Eagle's Minimal Essential Medium, fluorescamine, α-ketoglutarate, prednisone, pregnenolone-16α-carbonitrile (PCN), pyridoxal-5′-phosphate, rifampicin (RIF), spironolactone, troleandomycin (TAO), trypsin, and L-tyrosine were from Sigma Chemicals. Polyclonal rabbit anti-human CYP3A4 antibody was purchased from Oxygene Dallas (Cat. No. 003A0-P-HU). Nylon sheets, rad-free lumi-phos 530 chemiluminescent substrate sheets, blocking powder, and alkaline phosphatase-conjugated secondary antibodies were obtained from Schleicher & Schuell (NH). All other chemicals were of the highest purity available from Bio-Rad, Curtin Matheson Inc., EM Science, ICN Biochemicals, and Sigma Chemicals.

Treatment of cells. The *Poeciliopsis lucida* hepatocellular carcinoma cell line (PLHC-1) strain HC-16-1_{20} (21) was obtained from Dr. Larry E. Hightower (University of Connecticut), in March of 1993. PLHC-1 cells (passages 12 to 16 and 51 to 72) were grown in tightly capped 75-cm^2 polystyrene flasks at 30°C in Eagle's Minimal Essential Medium in 25 mM Hepes buffer pH 7.1, containing 10% calf serum (v/v) as previously described (22). Prior to each experiment, PLHC-1 cells were subcultured into 48-well polystyrene plates as recently described (23), at density of 13×10^5 cells/ml. After 24 h incubation at 30°C, the media was changed and various doses (ranging between 10^{-12} to 10^{-4} M) of either TCB, TCDD, DEX, cortisol, prednisone, PCN, RU 38486, spironolactone, RIF, or TAO were dissolved in DMSO and added to the media. The concentration of DMSO in the media never exceeded 0.5% (v/v).

Assays. The cells were exposed for 24 or 48 h at 30°C, whereupon the media was aspirated and the cells were rinsed with 10 mM Na-phosphate buffer, pH 7.4, including 0.9% (w/v) NaCl (PBS). The cells were immediately assayed for ethoxyresorufin-*O*-deethylase (EROD)

activity *in situ* using a kinetic assay (23, 24). The assay solution consisted of 2.6 μM 7-ethoxyresorufin in 50 mM Na-phosphate buffer, pH 8.0. No exogenous NADPH or other cofactors were added to the assay solution. In agreement with previous studies (23, 24), addition of NADPH had no effect on EROD activities in intact cells, implying that PLHC-1 cells possess an endogenous electron-generating system sufficient for deethylation of exogenous 7-ethoxyresorufin. EROD assays were followed by measurements of total protein content in the same wells, basically according to the procedure described before (25) using a CytoFluor 2300 Fluorescence System. EROD activities are presented as absolute values (pmol/min/mg protein). Means ± SE; $n = 4$ or $n = 8$; statistical analysis was performed using a two-tailed Mann–Whitney U test, $P < 0.05$, when compared to control (DMSO) or TCB/TCDD-treated cells.

Tyrosine aminotransferase (TAT) activities in sonicated PLHC-1 cells were measured at 30°C according to the method earlier described (26).

Immunochemical analyses. After removal of media and a single wash with 500 μl PBS, 80 μl of sample treatment buffer (0.25 M Tris:HCl, pH 6.8, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 4% (w/v) sodium dodecyl sulfate, 0.008% (w/v) bromphenol blue) was added to each well. The plate was placed on a shaking platform for 10 min at room-temperature. The solubilized cell lysates were next transferred to 0.25 ml polypropylene tubes, boiled for 5 min, and immediately placed on ice. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 12% continuous acrylamide gels using a "Mighty Small" gel electrophoresis unit from Hoefer Scientific Instruments. Aliquots of cell lysate (20 μl) were added to each well, giving approximately 25–30 μg cellular protein per lane. The proteins were electrotransferred to nylon membranes using a "Trans Blot Cell" from Bio-Rad. Equal loading of cellular protein on each gel was confirmed by staining the nylon membranes with 0.2% (w/v) Ponceau *S* in 3% (w/v) trichloracetic acid, 3% (w/v) sulfosalicylic acid. Remaining protein binding sites on the nylon membrane were blocked with 1% Schleicher & Schuell blocking powder in 10 mM Tris:HCl, pH 7.4, including 0.9% (w/v) NaCl (blocking solution). The monoclonal antibody 1-12-3 (27) (10 μg/ml in blocking solution) was used for CYP1A protein analyses. CYP3A-like proteins were detected in PLHC-1 cells, using polyclonal antibodies (PAb) to either P450con from rainbow trout (*Oncorhynchus mykiss*) (28) (rabbit serum diluted 1:5000 in blocking solution), to P450A from scup (*Stenotomus chrysops*) (29) (10 μg IgG/ml in blocking solution) or to human CYP3A4 (67.2 mg/ml diluted 1:50 in blocking solution). Rainbow trout P450con and scup P450A are suggested members of the CYP3A subfamily, based on properties including immunochemical relationships established, using PAb to these fish proteins and to mammalian CYP3A proteins (20). Alkaline phosphatase-conjugated (AP) anti-mouse IgG or AP-conjugated anti-rabbit IgG were used as secondary antibodies diluted in blocking solution. The protein bands were detected according to the "Schleicher & Schuell Rad-Free Kit for Chemiluminescent Detection of Western Blots" and the bands were visualized by fluorography on Kodak X-OMAT AR films. Fluorographs were digitized with a Kodak DCS200 digital camera and Adope Photoshop, and protein band intensities were measured by video imaging densitometry using NIH Image software.

RESULTS

Initial studies were carried out to establish the responses of PLHC-1 cells to prototypical CYP1A and CYP3A inducers, to define the optimal conditions for examining the GR-dependent effects on CYP1A induction, and to investigate expression and responsiveness of CYP3A-like proteins in this fish hepatoma cell line.

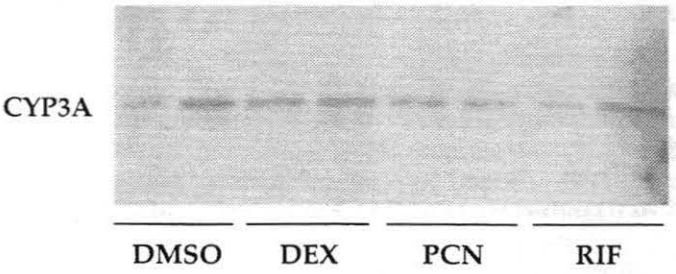

FIG. 1. Expression of CYP3A-like proteins in PLHC-1 cells. Cultures were treated with DMSO (control, vehicle), 10 μM DEX, 10 μM PCN, or 10 μM RIF for 48 h. Western blot analysis was performed on cell lysate from individual wells ($N = 2$ for each treatment). CYP3A-like proteins were detected using PAb against P450con in rainbow trout. This protein band was also recognized by PAb to a CYP3A-like protein in scup (P450A) and by PAb to human CYP3A4 (not shown).

CYP3A Expression

Polyclonal antibodies to the apparent CYP3A-like protein from rainbow trout (P450con) recognized one major protein band in Western blots of lysates from DMSO (vehicle)-treated PLHC-1 cells (Fig. 1). This band also was recognized by PAb to a scup CYP3A-like protein (P450A) and by PAb to human CYP3A4 (not shown). Compounds that induce CYP3A in mammals and mammalian cells in culture, DEX, PCN, or RIF, at 10 μM did not alter the levels of CYP3A-like proteins in the PLHC-1 cells after 48 h exposure (Fig. 1). There also was a lack of induction of CYP3A-like proteins in cells treated with doses of PCN ranging from 5 pM to 50 μM, for 48 h (not shown). Thus, expression of CYP3A-like proteins in PLHC-1 cells appears to be unaffected by exposure to prototypical mammalian CYP3A-inducers under the conditions used here.

CYP1A Induction

AHR agonists. Treatment of PLHC-1 cells with TCB at 0.005 to 50 μM or TCDD at 0.01 to 100 nM resulted in EROD induction that reached a plateau at 1 μM TCB and peaked at 1 nM TCDD, similar to our prior results (23). EROD activity was not detected (<0.5 pmol/min/mg protein) in PLHC-1 cells treated with 0.5% (v/v) DMSO (Fig. 2A), nor was CYP1A-immunoreactive protein detected in DMSO-treated cells (Fig. 2B).

In 5 of 10 experiments a slight increase of EROD activity (about 0.8 pmol/min/mg protein) was observed after 48 h exposure of PLHC-1 cells to 10 μM DEX alone. These rates are near the limit of detection and negligible in comparison with rates achieved after treatment with TCDD. Treatment of PLHC-1 cells with 10 μM of either cortisol, PCN, prednisone, RIF, RU 38486, or TAO for 48 h did not cause any detectable increase in EROD activity.

Potentiation of CYP1A Induction

When 10 μM DEX was coadministered with a maximally inducing dose of TCB, there was approximately a 2-fold enhancement of EROD induction (Fig. 2A). Similarly, at a maximally inducing dose of TCDD (1 nM), DEX elicited approximately a 2-fold increase in EROD induction (Fig. 2A). For induction of CYP1A protein levels, these doses of TCB and TCDD still were submaximal and in the presence of DEX there was about a 9-fold potentiation of induction of CYP1A protein (Fig. 2B). As in cultures treated with DEX alone, levels of expression of CYP3A-like protein remained unchanged in these cells (Fig. 2C).

TCDD dose-response. Addition of 10 μM DEX resulted in elevation of TCDD-induced CYP1A expression, measured as EROD activity, at doses of TCDD ranging from 0.01 to 100 nM (Fig. 3A). The degree of potentiation of EROD induction differed substantially between different doses of TCDD, being strongest at submaximal doses, with 7- to 20-fold potentiation at 0.1 nM (Fig. 3A). As above, at the maximal dose of TCDD for EROD induction (1 nM) there was a 2-fold potentiation of EROD activity, and at a supra-maximal dose (100 nM), the potentiation was only 1.2- to 1.4-fold (Fig. 3A).

When induction was measured at the level of CYP1A protein, potentiation by DEX was observed at all tested doses of TCDD above 0.01 nM (Fig. 3B). As seen with EROD activity, a greater degree of potentiation at doses of TCDD less than 100 nM was evident also at the level of CYP1A protein, with approximately 9-fold potentiation at 1 and 10 nM, compared to 4-fold at 100 nM (Fig. 3B).

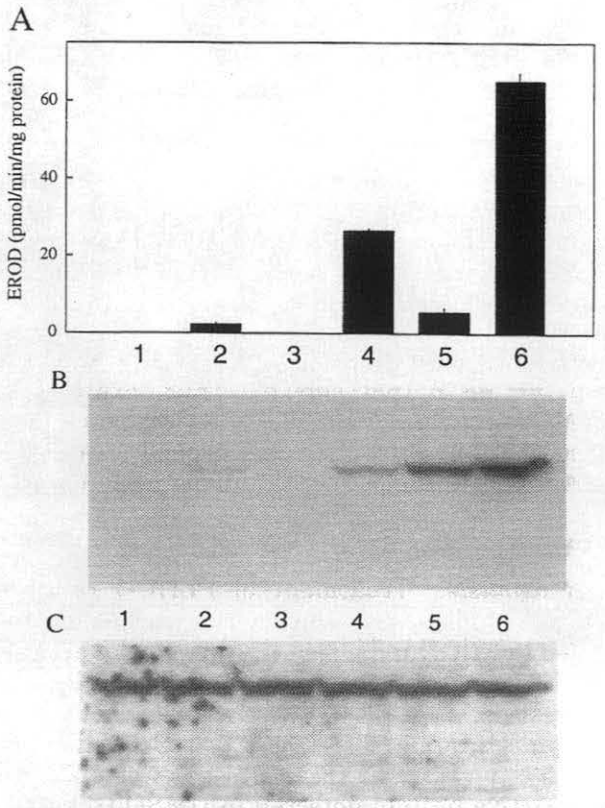

FIG. 2. Potentiation of CYP1A induction in PLHC-1 cells. (A) EROD activities, (B) Immunoblot of CYP1A protein, and (C) Immunoblot of CYP3A-like protein. EROD activities are expressed as pmol/min/mg protein and each value represents the mean of activities measured in eight wells ± SE. Western blot analysis was performed on cell lysate from individual wells (N = 1). CYP1A proteins were detected using the monoclonal antibody 1-12-3 and CYP3A-like proteins were detected using PAb to P450con in rainbow trout. The three analyses were performed on the same samples. Sample treatments were: (1) DMSO (control, vehicle); (2) 1 μM TCB; (3) 10 μM DEX; (4) 1 nM TCDD; (5) 1 μM TCB/10 μM DEX; (6) 1 nM TCDD/10 μM DEX.

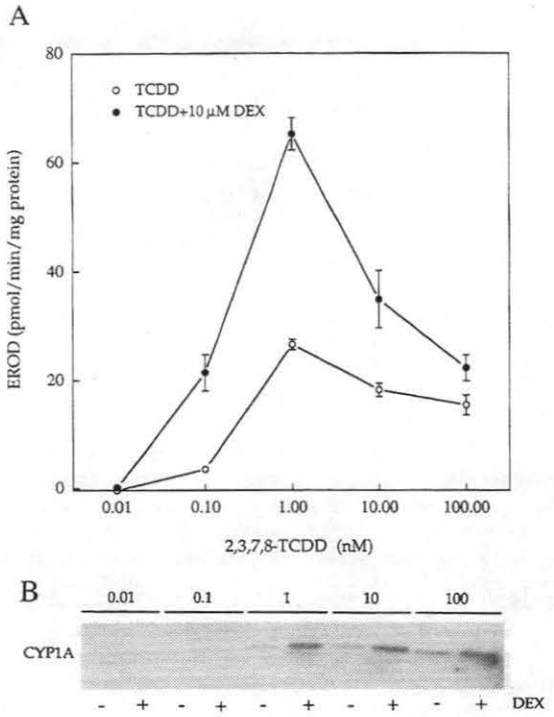

FIG. 3. Dose-response for induction of CYP1A by TCDD in the absence or in the presence of DEX. PLHC-1 cells were exposed for 48 h to 0.01 to 100 nM TCDD without (○) or with (●) 10 μM DEX. (A) EROD activities are expressed as pmol/min/mg protein and each value represents the mean of activities measured in four wells ± SE. In this experiment the degree of potentiation of EROD induction with DEX was 7-fold in cells treated with 0.1 nM TCDD, 2-fold in cells treated with 1 or 10 nM TCDD, and 1.4-fold in cells treated with 100 nM TCDD. However, we have repeatedly observed up to 20-fold degree of potentiation in other experiments of PLHC-1 cells treated with 0.1 nM TCDD and 10 μM DEX (not shown); (B) Western blot analysis of CYP1A protein levels in cell lysate from individual wells (N = 1) and detection was performed using the monoclonal antibody 1-12-3. The degree of DEX-mediated potentiation of CYP1A induction was 9-fold in cells treated with 1 or 10 nM TCDD and 4-fold in cells treated with 100 nM TCDD.

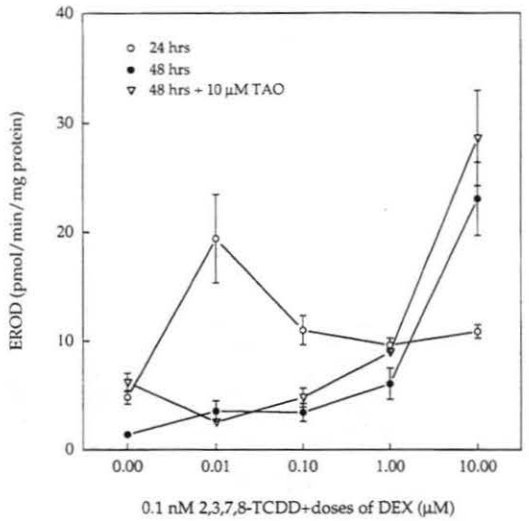

FIG. 4. Time-course and DEX dose-response for the potentiation of CYP1A induction. EROD induction was measured in PLHC-1 cells treated with TCDD and DEX. Cultures were treated with 0.1 nM TCDD and doses of DEX ranging from 0.01 to 10 μM for 24 h (○), for 48 h (●), and for 48 h in the presence of 10 μM TAO (▽). EROD activities are expressed as pmol/min/mg protein. Each value represents the mean of activities measured in four wells ± SE.

There was approximately a 50% decrease observed in responsiveness of EROD activity to 0.1 nM TCDD between early and late passages of PLHC-1 cells. However, the strong DEX-mediated potentiation was evident and was similar in magnitude in PLHC-1 from earlier passages (passage 12 to 16) and in cells from later passages (passage 51 to 72).

Time-course and DEX dose-response. The GR agonist concentration-dependence for potentiation of EROD induction was investigated at 24 and 48 h after 0.1 nM TCDD was coadministered with doses of DEX ranging from 0.01 to 10 μM. At 24 h exposure, potentiation was obtained with 0.01 μM DEX, whereas at 48 h exposure a dose of DEX that was three orders of magnitude greater (10 μM) was required to obtain a degree of potentiation comparable to that seen at 24 h (Fig. 4). Similar results were obtained when cortisol replaced DEX in the media, i.e., potentiation of EROD induction by 0.1 nM TCDD was observed at a lower dose of cortisol at 24 than at 48 h. These results could be due to metabolism of GR agonists over time in PLHC-1 cells. In a subsequent experiment, the CYP3A enzyme inhibitor TAO was coadministered with 0.1 nM TCDD and various doses of DEX. However, the presence of 10 μM TAO did not alter the dose of DEX required to elicit potentiation of EROD induction at 48 h (Fig. 4).

GR agonists and antagonists. Two other GR agonists, cortisol and prednisone, also potentiated induction of EROD activities with TCDD (Figs. 5A and 5B). As seen with DEX, a greater degree of potentiation of EROD activity with these GR agonists was observed at lower doses of TCDD (Figs. 5A and 5B). To further explore the possibility that the mechanism of potentiation involved a GR, two mammalian type II GR (glucocorticoid receptor) antagonists, PCN and RU 38486, and one mammalian type I GR (mineralocorticoid receptor) antagonist, spironolactone, each were coadministered with 10 μM DEX and 0.1 nM TCDD. Addition of 10 μM PCN or 10 μM RU 38486 reduced the DEX-mediated potentiation of EROD induction by approximately 50% at 48 h (Fig. 6), whereas addition of 10 μM spironolactone had no significant effect on the potentiation. At 24 h, addition of 10 μM RU 38486 abolished the potentiation (not shown).

Coadministration of 10 μM RU 38486 with 0.1 nM TCDD caused potentiation of induction of EROD activity (Fig. 7), although this response was slight in comparison with that obtained with DEX. No detectable EROD activities were observed in cultures exposed to 0.1, 1, or 10 μM RU 38486 alone. The rank order in

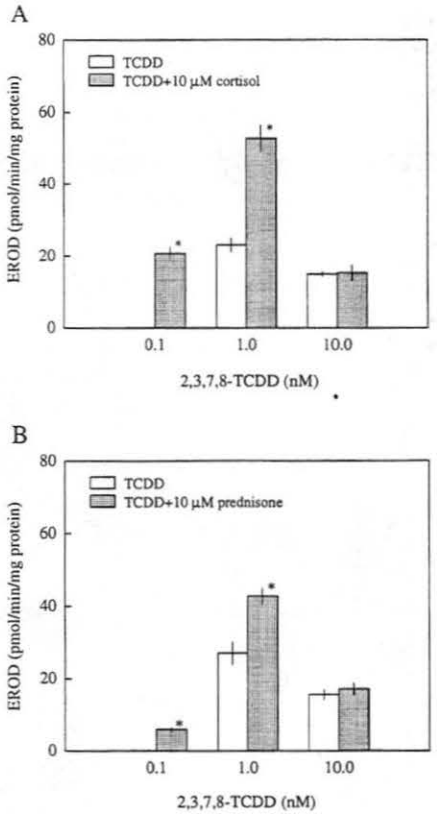

FIG. 5. Potentiation of CYP1A induction with GR agonists. EROD activities were measured in PLHC-1 cells treated with 0.1, 1.0, or 10 nM TCDD in the absence or in the presence of GR agonists for 48 h. (A) 10 μM Cortisol; (B) 10 μM Prednisone. EROD activities are expressed as pmol/min/mg protein. Each value represents the mean of activities measured in eight wells ± SE; $^*P < 0.05$ when compared to cells that were exposed to TCDD alone.

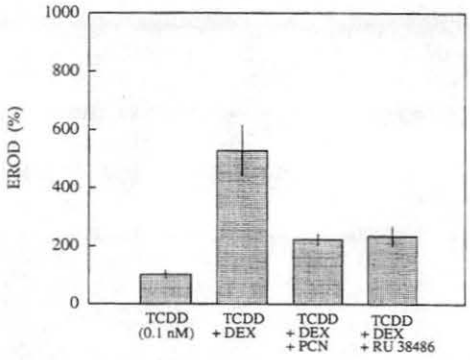

FIG. 6. Effect of GR antagonists on potentiation of CYP1A induction. EROD activities were determined in PLHC-1 cells treated with 0.1 nM TCDD and 10 μM DEX in the absence or the presence of 10 μM PCN or 10 μM RU 38486 for 48 h. EROD activities are expressed as percentage of that with 0.1 nM TCDD (100%). Each value represents the mean of activities measured in eight wells ± SE.

which the various compounds tested, all at 10 μM, potentiated EROD induction in 0.1 nM TCDD-exposed PLHC-1 cells is DEX > cortisol > prednisone ≥ RU 38486 (Table I). Other prototypical mammalian CYP3A-inducers that are not GR agonists, such as PCN, RIF, spironolactone, or TAO, all at 10 μM, did not potentiate CYP1A induction (Table I). Analogous to cells treated with TCDD, EROD induction caused by 1 μM TCB was not enhanced in the presence of either 10 μM PCN or RIF (Table I).

TABLE I

Ethoxyresorufin-*O*-deethylase Activities[a] in PLHC-1 Cells Treated with 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) or 3,3′,4,4′-Tetrachlorobiphenyl (TCB), for 48 h in the Absence or in the Presence of various Types of Steroids and Macrolide Antibiotics

	CYP1A inducers		
Additives[b]	0.1 nM TCDD	1 nM TCDD	1 μM TCB
0.5% (v/v) DMSO	2.8 ± 0.9	31.8 ± 7.0	0.9 ± 0.1
10 μM DEX	30.8 ± 0.4*	67.2 ± 4.7*	2.0 ± 0.1*
10 μM Cortisol	20.6 ± 1.8*	—	—
10 μM Prednisone	6.0 ± 0.5*	—	—
10 μM RU 38486	4.9 ± 1.0*	—	—
10 μM PCN	3.2 ± 0.2	33.9 ± 6.4	0.6 ± 0.1
10 μM Spironolactone	2.6 ± 0.3	—	—
10 μM RIF	2.7 ± 0.4	34.0 ± 3.6	0.8 ± 0.1
10 μM TAO	1.9 ± 0.2	—	—

Note. —, not analyzed.

[a] Ethoxyresorufin-*O*-deethylase activities are expressed as pmol/min/mg protein. Each value represents means of eight wells ± SE; *$P < 0.05$ when compared to cells that were exposed to TCDD or TCB alone.

[b] Abbreviations used: DEX, dexamethasone; DMSO, dimethylsulphoxide; PCN, pregnenolone-16α-carbonitrile; RIF, rifampicin; TAO, troleandomycin.

DISCUSSION

By using protocols for treating cells and measuring EROD activities *in situ* in multiwell plates (23–25), we performed extensive dose-response and time-course studies on the synergistic action of glucocorticoids or synthetic analogues and PHAH on expression of CYP1A in PLHC-1 cells, a teleost hepatoma cell line. Potentiation of CYP1A induction by GR agonists proceeded without changes in the expression of CYP3A-like proteins. The degree of potentiation of CYP1A induction was greater than previously reported in any system, up to 20-fold, and depended strongly on the dose of AHR agonist. Moreover, the doses of GR agonists required for maximal potentiation of CYP1A induction varied substantially with duration of exposure.

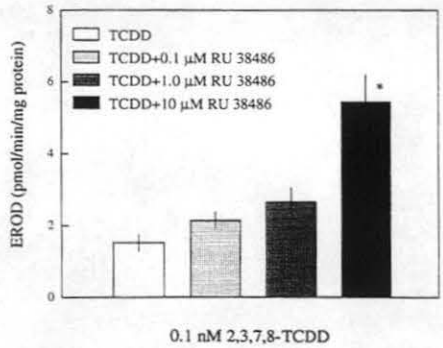

FIG. 7. Potentiation of CYP1A induction with RU 38486. EROD activities were measured in PLHC-1 cells treated with 0.1 nM TCDD and doses of RU 38486 ranging from 0.1 to 10 μM for 48 h. EROD activities are expressed as pmol/min/mg protein. Each value represents the mean of activities measured in eight wells ± SE; *$P < 0.05$ when compared to cells that were exposed to TCDD alone.

CYP3A Expression

Immunoblot analyses showed that CYP3A-like proteins are expressed in vehicle-treated PLHC-1 cells. However, the amounts of these piscine CYP3A-like proteins remained unchanged upon treatment with either a GR agonist (DEX), an antiglucocorticoid (PCN), or a macrolide antibiotic (RIF), all of which induce CYP3As in mammalian systems. Our data suggest that a CYP3A-like protein is constitutively expressed in PLHC-1 cells. The cells used in the present study were hepatocellular carcinoma cells and CYP3A might be inducible in fish liver. Earlier *in vivo* studies showed a slight increase in content of an hepatic microsomal CYP3A-like protein in rainbow trout treated with cortisol or PCN (28) and in tilapia (*Oreochromis niloticus*) treated with PCN (30). However, levels of this protein seem not to be affected by DEX in rainbow trout *in vivo*

TABLE II

Potentiation of CYP1A Induction and Expression and Inducibility of CYP3A in the *Poeciliopsis lucida* Hepatocellular Carcinoma Cell Line (PLHC-1) and in Mammalian Hepatic Cell Lines

		CYP3A expression		Potentiation of CYP1A induction		
Cell line	Species	Forms expressed	Inducibility	CYP1A inducers	Additives	Reference
PLHC-1	*Poeciliopsis lucida*	CYP3A-like protein	No[a]	TCB TCDD β-Naphthoflavone[b]	Cortisol, DEX, Prednisone, RU 38486[c]	Present study
Hepa-1	Mouse	Cyp3a proteins(s)[d]	No[e]	—	—	33
H4IIEC3	Rat	CYP3A protein(s)[d]	No[e]	Benz[a]anthracene TCDD	DEX	17 and 33
HTC	Rat	CYP3A protein(s)[d]	No[e]	—	—	33
FAZA	Rat	CYP3A protein(s)[d]	No[e]	—	—	33
HepG2	Human	CYP3A7 protein	Yes[f] (CYP3A7)	3-Methylcholanthrene	DEX	15 and 34
TONG/HCC	Human	CYP3A3-, 3A4- and 3A5 mRNA CYP3A5 protein	Yes[g] (CYP3A3- and 3A4 mRNA)	—	—	34

Note. —, not studied. Abbreviations used: DEX, dexamethasone; TCB, 3,3′4,4′-tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCN, pregnenolone-16α-carbonitrile; RIF, rifampicin; TAO, troleandomycin.

[a] Inducers tested were DEX, PCN, and RIF (Fig. 1).

[b] Results from studies in PLHC-1 cells using β-naphthoflavone are presented elsewhere (M. Celander, J. Bremer, M. E. Hahn, and J. J. Stegeman, manuscript).

[c] Addition of DEX, cortisol, prednisone, or RU 38486 caused potentiation of CYP1A induction, whereas neither PCN, spironolactone, RIF, nor TAO had any effect on the induction (Table I).

[d] Antibodies used were anti-rat $P450_{PCN}$, which may recognize more than one CYP3A form in rat and mouse liver (L. C. Quattrochi, personal communication).

[e] Inducers tested were DEX and PCN (33).

[f] CYP3A7 was induced by DEX, erythromycin, phenobarbital, RIF, SKF-525A, and TAO, whereas PCN had no effect on the expression of this protein (34).

[g] CYP3A mRNAs were induced by DEX, phenobarbital, and RIF, whereas PCN and TAO had no effect on the expression of these genes (34).

(28, 31, 32). It is possible that the PLHC-1 cell line has lost the capacity to respond to CYP3A inducers. Lack of CYP3A inducibility has been described in several mammalian hepatic cell lines (33, 34) (See Table II). Studies in serum-free culture media may be required to conclusively establish the influence of GR agonists on the expression of CYP3A-like proteins in PLHC-1 cells. Despite the lack of CYP3A induction, our data indicate that PLHC-1 cells have retained responses to GR agonists, particularly the potentiation of CYP1A induction (see below).

CYP1A Induction

As seen earlier (22, 23), no CYP1A expression was detected in DMSO-treated PLHC-1 cells, whereas treatment with TCB or TCDD resulted in a strong induction of CYP1A. With TCDD-treatment, EROD induction was attenuated at the highest doses, although CYP1A protein levels still were elevated with increasing TCDD doses, implying that inhibition and/or inactivation of the CYP1A protein occurs at higher concentrations of TCDD. Furthermore, EROD rates normalized to the amount of CYP1A protein detected were higher in TCDD-treated cells than in TCB-treated cells, consistent with a strong inhibition of CYP1A by TCB even at the lower doses of TCB tested (23). Inhibition and/or inactivation of EROD activities by TCB with a K_i of 0.02 to 0.3 μM, has been reported in scup hepatic microsomes *in vitro* (35, 36).

Potentiation of CYP1A Induction

TCDD dose-response. When PLHC-1 cells were exposed to 10 μM DEX there was occasionally a slight elevation of EROD rates, but CYP1A protein content remained below the limit of detection. When the same dose of DEX was coadministered with either TCB or TCDD, EROD rates and CYP1A protein content were much greater than levels obtained with the CYP1A inducer alone. This indicates that the increase of CYP1A induction caused by DEX + AHR agonists was not an additive effect but rather a potentiation mediated by DEX.

The observed degree of potentiation in PLHC-1 cells was dependent not only the concentration of DEX but also on the dose of TCDD and the endpoint measured. At maximal doses of TCB or TCDD, the degree of potentiation for EROD induction was much less than that for CYP1A protein. This discrepancy is probably a result of inhibition by TCB or TCDD present in the cells. The highest degree of potentiation of EROD induction in PLHC-1 cells was seen at submaximal doses of TCDD. We repeatedly observed as much as a 20-fold potentiation of EROD induction when DEX was added with 0.1 nM TCDD. Potentiation of CYP1A induction by DEX has been demonstrated in mammalian hepatocytes and hepatoma cell lines *in vitro* (13–17, 37), in rat liver *in vivo* (38), and in rainbow trout hepatocytes *in vitro* (39). However, in contrast to PLHC-1 cells, no more than 2- to 5-fold potentiation of CYP1A activities by DEX or by other GR agonists have been reported in these systems (13–17, 38–40).

The apparent efficacy for CYP1A induction was increased in the presence of DEX, and the threshold dose for detectable CYP1A induction by TCDD was approximately 10 times lower when DEX was present. In rat hepatocytes, the presence of DEX lowered the dose of β-naphthoflavone (BNF) required to achieve detectable induction of CYP1A1 mRNA. In addition, the degree of potentiation of CYP1A1 mRNA induction with DEX was greater at submaximal doses of BNF in these cells (37). If the greater potentiation seen at lower doses of AHR agonists *in vitro* also occurs *in vivo*, there could be biologically relevant impacts of elevated glucocorticoid levels on CYP1A expression and function in animals in the environment, where exposure to CYP1A inducers is common and generally at levels that result in submaximal induction.

Time-course and DEX dose-response. The potentiation of CYP1A induction by DEX was dependent not only on the doses of DEX and TCDD, but also on the duration of exposure. Thus, a 1000-fold higher dose of DEX was required to obtain maximal potentiation at 48 h than at 24 h. The possibility of a CYP3A-dependent metabolism of DEX was considered. Addition of a mammalian CYP3A inhibitor, TAO, which is also an inhibitor of CYP3A-like activity (steroid 6β-hydroxylase) in fish liver microsomes *in vitro* (32), had no effect on the observed temporal pattern of potentiation. A lack of effect of addition of TAO could occur if TAO does not inhibit DEX metabolism in PLHC-1 cells, or if the time-dependent effect does not involve DEX metabolism. In preliminary experiments, rates of tyrosine aminotransferase (TAT) activity, which is inducible by a classical GR-mediated mechanism, increased from 15 to 30 nmol/min/mg protein following 48 h exposure of PLHC-1 cells to 1 μM DEX. However, no significant increase of TAT activity was observed at 24 h in cells treated with 0.01 to 10 μM DEX. Thus, the distinction between 24 and 48 h in the amount of DEX required for potentiation of EROD induction was not evident for TAT induction. The basis for the distinction in doses of DEX required for potentiation between 24 and 48 h remains unknown, but we cannot yet exclude metabolism of the GR agonist as a reason.

GR agonist specificity. The potentiation of CYP1A induction in this teleost system was specific to GR agonists, whereas other mammalian CYP3A inducers that are not GR agonists, such as PCN, RIF, spironolactone, and TAO, had no effect on the induction of CYP1A. Addition of a mammalian GR antagonist, RU 38486, which also exhibits GR antagonist properties in rainbow trout fibroblasts and hepatocytes (41, 42), markedly reduced the DEX-mediated potentiation in PLHC-1 cells, consistent with a GR-dependent mechanism. The suggestion of a GR-mediated potentiation was supported by the observation that another antiglucocorticoid, PCN, also reduced this potentiation. Interestingly, RU 38486 itself was shown to potentiate EROD induction, although the response was considerably weaker than that seen with DEX or cortisol. These data suggest that RU 38486 acts as a partial GR agonist in PLHC-1 cells.

The dose of DEX sufficient for potentiation at 24 h exposure, 10 nM, is generally associated with classical GR-mediated responses. The specificity for GR agonists for potentiation, established at 48 h, also is characteristic of classical GR-mediated responses. Moreover, at both 24 and 48 h GR antagonists strongly reduced the potentiation by DEX, and TAT activity also was induced at 48 h, consistent with a GR-mediated response. Taken together these results imply that potentiation of CYP1A induction at 24 and 48 h in PLHC-1 cells is mediated by a classical GR-dependent pathway, similar to that in mammals (13). The potentiation of CYP1A induction by DEX in primary cultures of rat hepatocytes involves increased transcription of the *CYP1A1* gene, presumably via the GR and the GRE consensus sequences identified in mammalian *CYP1A1* genes (15, 16). Putative GREs have been identified in teleost *CYP1A1* genes (43, 44), and thus similar mechanisms may be involved in potentiation of CYP1A in diverse vertebrate taxa. Despite the evidence for a classical GR response, the concentration of DEX required for potentiation of CYP1A induction in PLHC-1 cells at 48 h, 10 μM, is a dose generally associated with nonclassical GR mechanisms. Some time-dependent process appears to be influencing the potentiation.

The possible relationship between mechanisms of induction of CYP3A and of potentiation of CYP1A induction is not clear in any vertebrate system. As cell lines increasingly are used in studies of CYP regulation we found it useful to summarize our results regarding ex-

pression and inducibility of CYP3A and potentiation of induction of CYP1A1 in PLHC-1 cells, as compared to responses in immortalized mammalian cell lines of hepatic origin (Table II). This comparison indicates that lack of inducibility of CYP3As occurs also in several mammalian cell lines, although potentiation of CYP1A induction by GR agonists is prominent. By measuring CYP1A and CYP3A simultaneously, we have further shown that CYP1A induction with GR agonists can proceed independently of changes in the expression of putative CYP3A in PLHC-1 cells and that the GR agonists seem to be the only mammalian CYP3A inducers able to potentiate CYP1A induction.

Our results provide strong evidence that interactions between GR- and AHR-mediated pathways occur generally in vertebrate systems. Given the role of CYP1A in metabolism and activation of toxic chemicals, a 20-fold enhancement of CYP1A-dependent activities like that seen here could have important consequences for toxicity of CYP1A substrates. GR agonists also might affect other AHR-dependent processes, a possibility supported by the observed synergistic induction of cleft palate in mice by cortisol and TCDD (45). Further investigations, including studies *in vivo,* should address questions regarding the significance of GR-mediated potentiation of CYP1A induction. This could open new areas for investigation in our attempts to understand the mechanism(s) by which AHR agonists or CYP1A substrates exert their toxicity.

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