Phenobarbital Induction of CYP1A1 Gene Expression in a Primary Culture of Rainbow Trout Hepatocytes*

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In mammals, phenobarbital (PB) is an in vivo inducer of the cytochrome P4502B (CYP2B) family, whereas in teleosts PB induction of cytochrome P450 is unclear. We show that teleost cytochrome P4502K1 (CYP2K1) protein levels and 7-pentoxyresorufin-O-deethylase activity were not induced by exposure of primary cultures of rainbow trout hepatocytes to PB. Instead, cytochrome P4501A1 (CYP1A1) gene expression was strongly induced by PB, based upon observations of marked increases in CYP1A1 mRNA, CYP1A1 protein, and 7-ethoxyresorufin-O-deethylase activity. In accordance with these data we provide a temporal study employing antibodies for the aromatic hydrocarbon (Ah) receptor that showed an increase in Ah receptor in nuclear extracts prepared from cells exposed to PB. Employment of the electrophoretic mobility shift assay (EMSA) showed PB to cause activation or "transformation" of the Ah receptor in nuclear extracts. Studies employing actinomycin D and cycloheximide indicated that PB induction of CYP1A1 was regulated at both the transcriptional and post-transcriptional levels. Nuclear run-off experiments confirm that PB causes an increase in CYP1A1 transcription. Inhibition of protein synthesis with puromycin in rainbow trout hepatocytes and examined various parameters of gene expression. While fish hepatic cytochrome P4501A1 is similar to its mammalian counterpart in its ability to be induced by numerous PAHs (for reviews see Refs. 17, 18), little is understood about its molecular mechanism. To date, an Ah receptor has been identified in the hepatic cytosol of rainbow trout (19), and TCCD has been shown to induce its translocation to the nucleus (20). DNA sequence analysis has resulted in the detection of two XREs in the 5'-flanking region of the rainbow trout CYP1A1 gene (21). In vivo studies of induction by PAH-like compounds with various fish species have resulted in conflicting results. This may in part be due to the high mortalities seen in fish exposed to PB (22). However, some investigations have shown induction of various cytochrome P450 monooxygenases by PAH-like compounds (23–28). Others suggest that fish cytochrome P450 may be refractory to induction by PAH-like compounds (29–32) despite the presence of CYP2B-related genes that have been detected in rainbow trout (31, 33) and scup liver (34).

To define phenobarbital inducibility of cytochrome P450 in fish hepatocytes and clarify previous inconclusive in vivo studies, we have employed primary cultures of rainbow trout hepatocytes and examined various parameters of CYP1A1 and CYP2 gene expression. Once we clearly observed CYP1A1 induction by PB, we conducted further experiments in order to elucidate a possible mechanism for PB induction of CYP1A1. To our knowledge, this is the first study in either mammalian or teleost research that has examined possible mechanisms of PB induction of CYP1A1 gene expression.

Cytochrome P450 (CYP)3 comprises a superfamily of hemo-

proteins found in large abundance in the liver that catalyze the monooxygenation of both lipophilic exogenous (xenobiotics) and endogenous compounds. The genes encoding for the various CYP isozymes may be expressed constitutively or may be induced by various chemicals (1). In the past, exogenous inducers were broadly classified as either "polyaromatic hydrocarbon (PAH)-like" or "phenobarbital (PB)-like." In mammals, PAH-like inducers can be characterized by an induction of CYP1A1 that is mediated through the aromatic hydrocarbon (Ah) receptor. PB-like inducers result in the induction of CYP2B. To date, a PB receptor has not been identified.

The molecular mechanisms involved in CYP1A1 induction have been extensively studied in mammals. Planar, lipophilic PAHs, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), have been shown to be ligands for the cytosolic Ah receptor (2). Induction of CYP1A1 is initiated with the binding of the ligand to the Ah receptor. The ligand-bound complex translocates to the nucleus (3) and, subsequent to dimerization with the Ah receptor nuclear translocator protein, binds to specific DNA sequences termed xenobiotic response elements (XREs) in the regulatory region of the gene (4). PB appears not to compete with TCDD for the Ah receptor (5). However, PB has been shown to induce CYP1A1 or cytochrome P4501A1-dependent enzymes in various mammalian hepatocyte cell cultures (6–12). In addition, other compounds that fail to show good affinity for the Ah receptor have also induced CYP1A1 (13–16).

While fish hepatic cytochrome P4501A1 is similar to its mammalian counterpart in its ability to be induced by numerous PAHs (for reviews see Refs. 17, 18), little is understood about its molecular mechanism. To date, an Ah receptor has been detected in the hepatic cytosol of rainbow trout (19), and TCDD has been shown to induce its translocation to the nucleus (20). DNA sequence analysis has resulted in the detection of two XREs in the 5'-flanking region of the rainbow trout CYP1A1 gene (21). In vivo studies of induction by PAH-like compounds with various fish species have resulted in conflicting results. This may in part be due to the high mortalities seen in fish exposed to PB (22). However, some investigations have shown induction of various cytochrome P450 monooxygenases by PAH-like compounds (23–28). Others suggest that fish cytochrome P450 may be refractory to induction by PAH-like compounds (29–32) despite the presence of CYP2B-related genes that have been detected in rainbow trout (31, 33) and scup liver (34).

To define phenobarbital inducibility of cytochrome P450 in fish hepatocytes and clarify previous inconclusive in vivo studies, we have employed primary cultures of rainbow trout hepatocytes and examined various parameters of CYP1A1 and CYP2 gene expression. Once we clearly observed CYP1A1 induction by PB, we conducted further experiments in order to elucidate a possible mechanism for PB induction of CYP1A1. To our knowledge, this is the first study in either mammalian or teleost research that has examined possible mechanisms of PB induction of CYP1A1 gene expression.

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The abbreviations used are: CYP, cytochrome P450; PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MeSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin-O-deethylase; EMSA, electrophoretic mobility shift assay; XRE, xenobiotic response elements; PAH, polyaromatic hydrocarbon; Ah, aromatic hydrocarbon; PROD, 7-pentoxyresorufin-O-dealkylase; ECOD, 7-ethoxycoumarin-O-deethylase; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
Materials and Methods

Cell Culture and Treatments—All chemicals were purchased from Sigma, unless stated otherwise. Hepatocytes were isolated and cultivated from juvenile, cultured rainbow trout (Oncorhynchus mykiss) as described previously (35), except for an additional supplement of 1% newborn calf serum (Life Technologies, Inc.) in the culture medium, and the cultures were incubated at 12 °C. Cells were counted in a Bürker chamber, assayed for cell viability by the trypan blue exclusion test, and seeded at 30° cells per plate (10 cm) 24 h before inducers were added. PB was added to cultures in Medium 199 (Life Technologies, Inc.), cycloheximide in water, while TCDD (Cambridge Isotope Laboratories) and actinomycin D were dissolved in dimethyl sulfoxide (Me2SO; final concentration of 0.1%). Control cultures for actinomycin D and TCDD received Me2SO only (0.1%). All experiments were performed at least three times using different cell culture preparations, and the data presented for monoxygenase activities represent a single experiment using duplicates, triplicates, etc. of culture plates. Hepatocytes were harvested in salmon buffer (36), centrifuged (5 min at 60,000 × g) and were resuspended in 0.1 M phosphate buffer, pH 7.4, containing 20% glycerol and harvested in saline buffer (36), centrifuged (5 min at 60,000 × g) and were resuspended in 0.1 M phosphate buffer, pH 7.4, containing 20% glycerol and 0.15 M KCl. Cell suspensions were immediately frozen and stored in liquid nitrogen. Monoxygenase assays were conducted within 1 week of harvesting.

Assays of Monoxygenase Activities—7-Ethoxyresorufin-O-deethylase (EROD), 7-pentoxyresorufin-O-dealkylase (PROD), and 7-ethoxycoumarin-O-deethylase (ECOD) activities were measured in cell homogenates as described earlier (37–38).

RNA Isolation and Analysis—Total RNA was extracted from cultured hepatocytes using the RNAzol (Cinna/Biotecx Lab. Inc.) solution method. DNAse —acid guanidinium thiocyanate/phenol/chloroform, as described (39–40). Total RNA (10 μg) was fractionated by agarose gel electrophoresis with formaldehyde as a denaturing agent (41). Equal loading and the integrity of each sample was assessed by staining the gel with ethidium bromide and quantification of 18S rRNA. Fractionated RNA was transferred to nylon membranes by vacuum transfer. Membranes were hybridized with a cDNA probe that was specific for rainbow trout CYP1A1 mRNA (pSG-15 plasmid with 2553 base pairs of the cDNA for rainbow trout CYP1A1). This probe amplified by polymerase chain reaction with the upper primer recognizing position 661 base pairs of the gene (5′-GGC TTG GTG AAC ATG AGT-3′) and the lower primer recognizing the sequence at 1433 base pairs of the gene (5′-GCC AAG AAG ACC ACC-3′) and was labeled with digoxigenin for detection of CYP1A1 mRNA with the DIG Luminescent Detection Kit (Boehringer Mannheim). After high stringency washes (2 × SSC, 0.1% SDS, and 0.5 × SSC, 0.1% SDS at room temperature and 68 °C, respectively), the chemiluminescent signal was recorded on x-ray film, and the CYP1A1 mRNA bands were quantitated by scanning with a densitometer (LKB 2222-020 Ultra scan XL Laser Densitometer).

Immunoblots—Isolation of cytosol and nuclear was performed as described earlier (42). Briefly, four culture plates of hepatocytes were pooled for each sample. Cell plasma and nuclei were lysed by the addition of 1 ml of Nonident P-40 lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40) and incubated for 5 min (on ice) before centrifuging, removing the supernatant (cytosol). Nuclei were washed twice and nuclear proteins in HEDG buffer (25 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM dithiothreitol, 10% glycerol) by shaking nuclei on ice for 30 min before centrifugation for 1 h (105,000 × g at 4 °C). Western blots were performed using 10–20 μg of protein from cell homogenates, or nuclear extracts, as described previously (43). Nitrocellular ose blots were blocked for 30 min with 5% dry milk (w/v) in 20 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, TBS. The membranes were incubated with 10% dry milk in TBS (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml of GTP, CTP, and ATP (11 μM) for 1 hour at 37°C, and the nitrocellulose membranes were washed with 6 × SSC. The nitrocellulose membranes with CYP1A1 and GAPDH cDNAs were hybridized inside of 5-ml scintillation vials for 72 h at 65 °C in TES/NaCl (final NaCl concentration of 300 mM). Following hybridization the membrane was washed twice in 2 × SSC for 1 h at 65 °C. Background was corrected by measuring the counts on a nitrocellulose strip without application of either CYP1A1 or GAPDH cDNAs. All membranes were transferred to a new scintillation vial and counted in a scintillation counter. The transcriptional activity of GAPDH was used as a standard to correct for differences in labeling of each RNA sample. The CYP1A1 transcriptional activity of the control value was arbitrarily set to 1.0.

Results—The Student’s t-test was used for statistical analysis. The significance levels were set at p < 0.05, p < 0.01.

Results

Lack of 7-Pentoxysresorufin-O-dealkylase Induction by Phenobarbital—7-Pentoxysresorufin-O-dealkylase (PROD) activity is considered to be a specific indicator of CYP2B activity in PB-induced mammalian cells (46–48). 7-Ethoxyresorufin-O-deethylase (EROD) activity has been generally accepted to specifically reflect CYP1A activity in vertebrates (49). Therefore, to determine if PB can induce a CYP2 family isozyme, we exposed primary cultures of rainbow trout hepatocytes to 2 μM PB for 24 h prior to measuring PROD activities in cell homogenates. To ensure our cell cultures were responsive to cytochrome P450 induction, we included 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a positive control since it is known to potently induce EROD activity and CYP1A1 in fish (35). PROD activity was not detectable in untreated (control) cells (detection limit 0.5 pmol/min/mg cell protein). PB increased PROD activity to barely detectable levels, and TCDD caused only a slight increase in PROD activity (Table I). EROD activity to barely detectable levels, and TCDD caused only a slight increase in PROD activity (Table I). EROD activity was increased 9-fold by TCDD and 3-fold by PB, as compared with control levels. Low PROD activities in TCDD- and PB-treated cells may be an indication of 7-pentoxysresorufin being a weak substrate of CYP1A1 rather than indicating metabolism by a CYP2 family isozyme (48, 50).

Cells Treated with Varying Concentrations of Phenobarbital Increased EROD Activity in a Dose-dependent Manner—EROD activity was examined in cells exposed for 24 h to varying concentrations of PB. An increase in EROD activity was shown...
to be dose-dependent over the range of 0.5 to 4 mM PB, with an EC50 estimated to be approximately 1 mM (Fig. 1). Treatment of cells with 0.5 mM PB showed a 3.6-fold increase in EROD activity, as compared with control values. The dose-response curve for EROD activity was linear in the concentration range of 0.5 to 2.0 mM PB and plateaued between 2 mM and 4 mM PB (17.5-fold higher than the control levels). Further increases in the concentration of PB (>4 mM) was shown to decrease the EROD activity when compared with maximum induced levels. The decline in EROD activity at high concentrations of PB may indicate cytotoxic effects. Optimal induction of EROD activity occurred at a concentration of 2 mM PB; therefore, this concentration was employed in all the following experiments.

Temporal Patterns of Phenobarbital Induction of CYP1A1 Gene Expression—We performed a time-course analysis during which PB was added to cultures 24 h after hepatocytes were seeded. PB induction proceeded for a period of 76 h during which, at various time points, samples were harvested and analyzed for EROD activity and levels of CYP1A1 mRNA. Hepatocytes exposed to PB showed an increase in EROD activity 6 h after the addition of the inducer, when compared with control values (Fig. 2). Maximal PB induction of EROD activity (19-fold induction compared with control levels) was attained 34 h after PB addition to the cells. Thereafter, PB-induced EROD activity slowly decreased but still remained elevated (9-fold) over control levels upon the duration of the experiment. Controls showed a slight increase in EROD activity that peaked at approximately 25 h after medium change.

At various sampling times after the addition of PB to the hepatocytes, the levels of accumulated CYP1A1 mRNA were determined by Northern blot analysis using the trout pSg-15 probe. Northern blot bands of CYP1A1 mRNA (2.8 kilobase pairs) were quantitated by scanning with laser densitometry. Control levels of CYP1A1 mRNA slightly increased 6 h after medium change and remained elevated for the duration of the experiment (Fig. 2). Treatment of hepatocytes with PB caused a 10-fold increase in CYP1A1 mRNA levels at 25.5 h, as compared with control levels at the same time point. PB-induced accumulation of CYP1A1 mRNA decreased after peaking, and by 48 h the CYP1A1 mRNA levels remained static until the duration of the experiment (76 h). PB induction of EROD activity lagged behind the elevated CYP1A1 mRNA levels by approximately 9 h. The kinetics of PB induction of EROD activity and CYP1A1 mRNA levels were similar to that of β-naphthoflavone (βNF) in primary cultures of rainbow trout hepatocytes (51). Conversely, the kinetics of TCDD induction of CYP1A1 mRNA in primary cultures of rainbow trout hepatocytes has been shown to increase linearly up to 12 h after TCDD addition and then remain at a plateau for up to 96 h (51).

Nuclear Translocation of Cytosolic Ah Receptor in Cells Exposed to Phenobarbital—To determine if the mechanism of PB induction of CYP1A1 involves the nuclear translocation of the Ah receptor, or alters the levels of nuclear Ah receptor, we performed experiments using specific antibodies for the Ah receptor. Cells were exposed to TCDD (a positive control) or PB, and at various time points the cells were harvested, cytosol and nuclear components isolated, and immunoblots performed. The protein band detected for the Ah receptor was calculated to be at approximately 145 kDa which approximates the reported value for rainbow trout (52–53). Specificity of the 145-kDa band was confirmed by competition experiments with antigen (1 mg/ml) for the antiserum which resulted in blocking this band. Immunoblots as in Fig. 3 clearly show the accumulation of the Ah receptor in nuclear extracts from both PB- and TCDD-treated hepatocytes after exposure of cells to these compounds for 6 h. However in TCDD-treated cells, there was an increase in nuclear Ah receptor levels (as compared to time 0) after only 3 h, while this was not the case in PB-treated cells. These transient increases in nuclear Ah receptor with prolonged CYP1A1 gene expression are identical to that reported for TCDD exposure in mammals (54).

Activation of DNA Binding Activity of the Ah Receptor in Nuclear Extracts of Cells Exposed to PB—To assess if PB caused the transformation of the Ah receptor, we analyzed the DNA binding activity of the nuclear Ah receptor following treatment of hepatocytes with PB, as well as TCDD. Cells were exposed to TCDD and PB for 6 h before harvesting, and nuclear extracts were prepared. DNA binding activity of the Ah receptor was monitored by EMSA with a synthetic XRE sequence as a specific probe. The in vivo-activated receptor generated a complex with the XRE probe in both PB- and TCDD-treated cells that was not detected in the nuclear extracts from control cells (0.1% Me2SO) (Fig. 4, compare lanes 1 and 2 to lane 4). The specificity of the TCDD- and PB-induced XRE complex was characterized by DNA competition experiments where 100-fold
and TCDD varied strongly depending on the time period. After the determined parameters, ECOD activity, and CYP2K1 protein levels were measured by Northern blot analysis using a cDNA, pSG-15 probe from rainbow trout, and pooling two culture plates. Control cells; PB-treated cells. EROD values represent the means ± S.D. of three or four plates.

Fig. 2. Temporal patterns of changes in EROD activity (A) and CYP1A1 mRNA levels (2.8 kilobase pairs) (B) in a primary culture of rainbow trout hepatocytes. Cells were seeded for 24 h before changing the medium and adding PB (2 mM). CYP1A1 mRNA levels were measured by Northern blot analysis using a cDNA, pSG-15 probe from rainbow trout, and pooling two culture plates. Control cells; PB-treated cells. EROD values represent the means ± S.D. of three or four plates.

Excess unlabeled XRE abolished the formation of the Ah receptor-dependent XRE complex (Fig. 4, compare lanes 2 and 3). Additive induction of EROD activity when primary cultures of rainbow trout were exposed to mixtures of PB and 1,2-benzanthracene (56). These results are similar to an earlier mammalian study noting additive induction of EOD activity when primary cultures of rat hepatocytes were exposed to mixtures of PB and 1,2-benzanthracene (8).

ECOD activity is considered to represent the activities of numerous CYPs both in mammals (55) and in fish (17). We found additive increases in ECOD activity in cells exposed for 48 h to mixtures of PB and TCDD (Fig. 3C). At this time point, ECOD activity was substantially induced by PB (8-fold over control values) and TCDD (15-fold over control levels). When cells were incubated in a medium containing a mixture of PB and TCDD, the induced ECOD activity approximated the sum of the two individual responses (24-fold over control levels). These results are similar to an earlier mammalian study noting additive induction of ECOD activity when primary cultures of rat hepatocytes were exposed to mixtures of PB and 1,2-benzanthracene (56).

Semi-quantitative Western blots of CYP1A1 protein generally reflected EROD activities. Control levels of CYP1A1 protein were not detectable after 24 or 48 h (Fig. 6A). After 24 h the mixture of TCDD and PB induced CYP1A1 protein levels 2.5-fold the PB individual response. TCDD CYP1A1 protein levels were 6.2-fold the PB response. After 48 h of incubation, PB induction of CYP1A1 protein was 6-fold the Me$_2$SO response, while the TCDD-induced level of CYP1A1 protein was 52-fold the Me$_2$SO response. The mixture of TCDD and PB caused a marginal increase of CYP1A1 protein, over the individual response for TCDD. CYP2K1 protein levels, a constitutively expressed P450 isozyme in fish (57), were not affected by PB, TCDD, or the mixture of the two compounds (Fig. 6B).

Analyses of Northern blots of CYP1A1 mRNA (Fig. 7A) showed PB to strongly induce CYP1A1 mRNA levels at 24 h (7.3-fold over control levels) while after 48 h PB-induced levels of CYP1A1 mRNA were decreased to 1.5-fold over control levels. TCDD induction of CYP1A1 mRNA accumulation was 21-fold over control levels at both 24 and 48 h. The level of accumulated CYP1A1 mRNA was equivalent in cells treated with a mixture of TCDD and PB, as compared with the TCDD-
only treated cellular levels. Cells exposed to mixtures of TCDD and PB for 24 h (A) or 48 h (B) with inducers before they were harvested and EROD activity determined. Values represent the means ± S.D. of three plates. Cells were treated for 48 h with inducers before harvesting and EROD activity determined (C). Values represent the averages ± range of two plates. Cells were treated for 48 h with inducers before harvesting and EROD activity determined (D). Values represent the means ± S.D. of three plates. *, significantly increased over TCDD-treated values, p < 0.05. Both TCDD and βNF were added to the culture medium in Me2SO (final concentration of 0.1%). Me2SO (DMSO), 0.1%; phenobarbital (PB), 2 mM; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 100 pM; β-naphthoflavone (βNF), 360 nM.

**Fig. 6.** Immunoblots of CYP1A1 (A) and CYP2K1 (B) protein levels in hepatocytes treated with TCDD and PB or a mixture of TCDD and PB. Primary cultures of rainbow trout hepatocytes were treated for 48 h (lanes 1–6) or 24 h (lanes 7–12) before the cells were harvested and immunoblots prepared using antibodies for teleost CYP1A1 and CYP2K1 proteins. Me2SO (DMSO, 0.1%); phenobarbital (PB), 2 mM; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 100 pM.

**Fig. 7.** Northern blots of CYP1A1 mRNA levels in hepatocytes. Cells were treated with TCDD, PB, or a mixture of TCDD and PB (A) or with βNF, TCDD, or a mixture of βNF and TCDD (B) for 24 or 48 h before the cells were harvested and Northern blots prepared using a cDNA, pSG-15 probe from rainbow trout. Me2SO (DMSO, 0.1%); phenobarbital (PB), 2 mM; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 100 pM; β-naphthoflavone (βNF), 360 nM.

Transcriptional and Post-transcriptional Regulation of Induction of EROD and ECOD Activities—To determine the contribution of transcriptional and post-transcriptional regulation of PB induction of CYP1A1, we undertook studies employing cycloheximide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of transcription, and followed two different protocols. The first protocol involved the simultaneous addition of these inhibitors with inducers for an incubation period of 12 h, whereas the second protocol investigated the effects of the inhibitors on preinduced cells.

Following the first protocol, after 12 h of exposure of hepatoctyes to either PB or TCDD, EROD and ECOD activities were strongly induced over control values. The simultaneous addition of cycloheximide with either PB or TCDD for 12 h completely prevented EROD and ECOD induction (Fig. 8). EROD activity was not detectable (detection limit 0.5 pmol/min/mg cell protein) in cells treated only with cycloheximide. Cells exposed simultaneously to PB plus cycloheximide had EROD activities higher than that of TCDD plus cycloheximide (17 and 3% of control values, respectively). The effects of cycloheximide on ECOD activity was not as potent as seen with EROD activity. Cells treated only with cycloheximide had ECOD activities that were 38% of the control levels, and mixtures of cycloheximide and PB, or cycloheximide and TCDD, expressed 47 and 40%, respectively, of the ECOD levels in control cells. These results suggest that the induction of EROD and ECOD activity...
by both PB and TCDD requires de novo protein synthesis. PB may stabilize EROD activity as indicated by residual EROD activities still detectable after treatment of cells with a mixture of PB and cycloheximide.

The effects of inhibition of transcription on PB and TCDD induction of EROD and ECOD activities were investigated. Rainbow trout hepatocytes exposed to actinomycin D for 12 h maintained 55% of control levels of EROD activity and 62% of control levels of ECOD activity (Fig. 8). The simultaneous addition of actinomycin D with PB blocked PB induction of EROD and ECOD activities. PB levels of EROD activity were 117% of control levels when actinomycin D was present, whereas ECOD activity was 67% of control levels. Actinomycin D blocked the TCDD induction of EROD and ECOD activities. EROD activity was 87%, whereas ECOD activity was 76%, of the control levels when TCDD was co-incubated with actinomycin D. These results imply the transcriptional activation of EROD and ECOD activities by PB and TCDD.

The effects of cycloheximide and actinomycin D on hepatocytes preinduced for 24 h with either TCDD or PB showed dramatic differences in the regulation of CYP1A1. Hepatocytes were induced for 24 h with PB or TCDD before actinomycin D, or cycloheximide, was added directly to the cells without removing the inducer, and incubation continued for an additional 54 h (Fig. 9). Both actinomycin D and cycloheximide had little effect upon the PB induction of EROD activity in PB-preinduced hepatocytes. Although not statistically significant, EROD activity in PB-preinduced cells did appear to be reduced by cycloheximide, as compared with PB-induced levels. In TCDD-preinduced cells the addition of actinomycin D caused a 44% decrease in EROD induction, as compared with the TCDD response. Cycloheximide was even more potent in TCDD-preinduced cells and caused a 75% decrease of EROD activity, as compared with the TCDD response. These data suggest that the regulation of PB induction of CYP1A1 involves post-transcriptional contributions as indicated by the lack of significant effect of cycloheximide upon PB-preinduced EROD activities. TCDD induction of EROD activity does not appear to involve stabilization of EROD activity, as compared with the PB results.

Cycloheximide Elevates the Levels of CYP1A1 mRNA, While Actinomycin D Blocks Accumulation of CYP1A1 mRNA—The kinetics of PB induction of CYP1A1 mRNA involves the decrease in levels of CYP1A1 mRNA after 25.5 h of exposure to PB (Fig. 2). This decrease did not occur when cycloheximide was added to PB-preinduced cells (Fig. 10). Cycloheximide enhanced induction of CYP1A1 mRNA accumulation within 3 h after its addition to both PB- and TCDD-preinduced cells. The cycloheximide-induced elevated levels of accumulated CYP1A1 mRNA was maintained for the duration of the time course examined (36 h). Three hours after cycloheximide was added to PB-preinduced cells, the levels of accumulated CYP1A1 mRNA were 1.5-fold higher than the amount in PB-induced cells not exposed to cycloheximide at the same time point. In TCDD-preinduced cells, TCDD plus cycloheximide showed a 2.3-fold increase in CYP1A1 mRNA over TCDD-treated cell mRNA levels after 3 h of cycloheximide treatment. These levels of enhanced accumulation of CYP1A1 mRNA (superinduc-
PB Increases the Transcription of CYP1A1 mRNA—To ensure transcriptional regulation of CYP1A1 by PB, we performed nuclear run-off experiments with isolated nuclei from cells exposed for 6 h to either control (vehicle), PB, or TCDD (positive control). Results presented in Table II clearly show a significant increase in CYP1A1 transcription in cells exposed to PB (1.43 ± 0.22) over control levels (1.00 ± 0.08) when normalized against GAPDH transcription. TCDD exposure increased CYP1A1 transcription to a value of 2.40 ± 0.74.

**DISCUSSION**

We have shown the lack of PB induction of the CYP2 family in a primary culture of rainbow trout hepatocytes by the measurement of CYP2K1 protein levels and PROD activity. These results support the contention that rainbow trout hepatic cytochrome P450 may be refractory to PB induction. However, it is unclear whether our results are a reflection of employing primary cultures of hepatocytes, since CYP2B activity is often lost in mammalian cell cultures (for reviews see Refs. 60–61), or due to physiological differences between fish and mammals.

Conversely, CYP1A1 gene expression was strongly induced by PB in primary cultures of hepatocytes. The present studies investigated the regulation of CYP1A1 induction by PB and revealed the following. (i) PB induction of CYP1A1 gene expression is regulated at both the transcriptional and post-transcriptional levels. (ii) PB exhibited superinduction of CYP1A1 mRNA when used in association with cycloheximide. (iii) PB exposure resulted in the nuclear accumulation of Ah receptor. (iv) PB caused activation of the Ah receptor to a DNA-binding form in nuclear extracts. (v) PB induction of CYP1A1 was in a dose-dependent manner with PB being effective at the same concentration range as in mammalian studies of CYP2B. In mammals, the induction of CYP1A1 is considered to be principally regulated at the level of transcription (62). However, there are reports of post-transcriptional as well as transcriptional contributions in the regulation of induction of CYP1A1, depending upon the biological model studied (63–66). Our studies showed PB induction of CYP1A1 gene expression to involve contributions at both the transcriptional and the post-transcriptional levels. Post-transcriptional contributions were observed as a stabilization of CYP1A1 catalytic activity (EROD) in hepatocytes preinduced by PB and then exposed to cycloheximide. Additional support for PB-stabilizing CYP1A1 gene expression at the protein level can be drawn from the data involving co-incubation of PB with a saturating concentration of TCDD. This data showed a mixture of PB and TCDD to induce an additive increase in EROD activity, and synergistic elevation of CYP1A1 protein levels, without any increase in accumulation of CYP1A1 mRNA over the TCDD individual response. Therefore, our observations point to PB induction of CYP1A1 involving post-transcriptional contributions in its regulation.

Transcriptional activation of CYP1A1 by PB was shown by nuclear run-off experiments. In addition, there was a rapid increase in CYP1A1 mRNA in response to PB addition. This
increase in CYP1A1 mRNA was followed by pronounced enhancement of EROD activity. Employment of actinomycin D completely inhibited PB-induced accumulation of CYP1A1 mRNA and CYP1A1 protein and EROD levels. Thus, PB does not appear to stabilize CYP1A1 mRNA and PB induction of CYP1A1 protein, and EROD activity requires transcription. Parallel studies employing actinomycin D with TCDD showed similar results in blocking CYP1A1 parameters, although actinomycin D was not as efficient in blocking TCDD-induced accumulation of CYP1A1 mRNA as it was for blocking PB-induced accumulation. This suggests that TCDD may slightly stabilize CYP1A1 mRNA. In addition, actinomycin D was shown to stabilize TCDD-preinduced CYP1A1 mRNA while having no effect on PB-preinduced CYP1A1 mRNA levels. These TCDD results are similar to an earlier study in mammals noting an actinomycin D-insensitive phase of induction of CYP1A1 (67). Our data suggest that PB induction of CYP1A1 gene expression is regulated at both the transcriptional and post-transcriptional levels.

In HeLa cells, superinduction of CYP1A1 mRNA is a transcriptional event involving a liganded Ah receptor (68) and is considered to reflect the existence of a labile repressor protein that inhibits the response of the receptor-enhancer system through protein-protein interactions (69). Our studies showed superinduction of CYP1A1 mRNA in primary cultures of rainbow trout hepatocytes when protein synthesis was inhibited in PB- and TCDD-treated cells. This suggests that teleost CYP1A1 gene expression may involve a labile negative regulatory protein. In addition, contrary to what is seen in HeLa cells, we show superinduction of CYP1A1 mRNA to occur in the absence of an additional exogenous inducing agent when protein synthesis is inhibited. This finding of cycloheximide alone, in the absence of an inducer, elevating CYP1A1 mRNA has been noted in various mammalian cells (13, 59, 70) suggesting species-specific and cell-specific differences in the regulation of CYP1A1.

The finding that CYP1A1 gene expression in primary cultures of rainbow trout hepatocytes may be regulated by a labile repressor protein suggests an alternative mechanism for induction of this system through derepression of the gene, as found to be the case in mammalian cells for mevinolin (14). PB does not induce CYP1A1 via this mechanism as revealed through the lack of enhanced levels of CYP1A1 mRNA in cells treated to mixtures of PB and TCDD. If PB derepressed the CYP1A1 gene, we should have observed an increase in CYP1A1 mRNA levels in cells treated with mixtures of TCDD and PB, as compared with the individual response for TCDD, which we did not observe. Therefore, the mechanism of PB induction of CYP1A1 does not occur through derepression of the gene.

The mechanism for CYP1A1 induction in mammals requires the translocation ligand-receptor complex from the cytosol to the nucleus where it dimerizes with the Ah receptor nuclear translocator protein, and the ligand-receptor complex then binds to xenobiotic responsive elements (XREs) on the nucleus and transcription is initiated (for review see Ref. 71). Similar to the mammalian mechanism of CYP1A1 induction, we observed accumulation of Ah receptor in nuclear extracts from rainbow trout hepatocytes exposed to PB. The kinetic pattern of accumulation of the Ah receptor was slightly delayed in PB-treated cells, as compared with that shown for TCDD. In addition, the nuclear accumulation of the Ah receptor in TCDD-exposed rainbow trout hepatocytes appears to be much slower than that reported for the TCDD-induced nuclear translocation of the Ah receptor in mammals (54, 72). In support of the nuclear translocation of the Ah receptor in PB- and TCDD-treated cells, we show activation of the Ah receptor to a DNA binding form in nuclear extracts obtained from both PB- and TCDD-treated cells. These data strongly suggest that PB induction of CYP1A1 involves the nuclear translocation of the Ah receptor and does not support a theory that PB enhances the levels of cytosolic Ah receptor protein (73).

The potency of a compound to induce CYP1A1 gene expression has been correlated to the binding affinity of the compound to the Ah receptor (74–75). However, resistance of the compound to metabolism also plays a key role in its potency (76). TCDD is known to be a poorly metabolized compound with strong affinity for the Ah receptor. PB has been shown to be a poor ligand of the Ah receptor and is relatively quickly metabolized. In primary cultures of rainbow trout hepatocytes, the optimal concentration at which TCDD induces CYP1A1 is in the pM range (35). The present data shows PB to induce CYP1A1 in the nM range. This concentration is identical to PB concentrations used to induce CYP2B (77–78) and CYP1A1 (6–12) in mammalian cells. Mevalonate, a poor ligand for the Ah receptor, has also been shown to induce CYP1A1 in the nM range (14).

Signal transduction and CYP1A1 induction has just begun to be investigated in mammals. In liver cells, TCDD alters intracellular calcium concentrations (79–80), an important second messenger, as well as increases protein kinase A levels (81), protein kinase C levels (80–82), and various protein tyrosine kinases (82). PB concentrations in the mM range (35). The present data shows PB to induce CYP1A1 in the nM range. This concentration is identical to PB concentrations used to induce CYP2B (77–78) and CYP1A1 (6–12) in mammalian cells. Mevalonate, a poor ligand for the Ah receptor, has also been shown to induce CYP1A1 in the nM range (14).

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