Biochemical Pharmacology, Vol. 53, pp. 1029–1040, 1997.
Copyright © 1997 Elsevier Science Inc.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00902-1

Induction and Post-Transcriptional Suppression of Hepatic Cytochrome P450 1A1 by 3,3′,4,4′-Tetrachlorobiphenyl

Renee D. White, Damian Shea,† Andrew R. Solow‡ and John J. Stegeman*§*

*Biology Department and ‡Marine Policy Center, Woods Hole Oceanographic Institution, Woods Hole, MA 02543; and †Department of Toxicology, North Carolina State University, Raleigh, NC 27607, U.S.A.

ABSTRACT. 3,3′,4,4′-Tetrachlorobiphenyl (TCB) can induce and inhibit cytochrome P450 1A1 (CYP1A1) in vertebrates. TCB may also suppress CYP1A1 protein levels, but the mechanism is unknown. This study examined transcriptional and translational aspects of hepatic CYP1A1 regulation in the fish scup (*Stenotomus chrysops*) given single intraperitoneal injections of low (0.1 mg/kg) or high (5 mg/kg) doses of TCB, and sampled over 16 days. The low dose strongly induced hepatic CYP1A1 mRNA (25-fold), protein (12-fold), and activity [ethoxyresorufin O-deethylase (EROD)] (15-fold). The high dose also strongly induced CYP1A1 mRNA (29-fold), in a pattern like that at the low dose, but microsomal CYP1A1 protein content was induced only 4-fold and EROD rates were near control levels. Both TCB doses caused similar increases in microsomal cytochrome b_5 content, and in rates of NADPH–cytochrome *c* (P450) reductase and UDP-glucuronosyltransferase (with *p*-nitrophenol). The contents of CYP forms other than CYP1A1 (putative CYP2B or CYP3A) were only weakly affected by TCB at either dose. The strong and largely specific post-transcriptional suppression of CYP1A1 content was associated with high concentrations of TCB measured in the liver. Incubation of scup hepatic microsomes with TCB plus NADPH led to a time-dependent inactivation of CYP1A1 that was distinct from catalytic inhibition, and appeared not to involve reactive metabolites of TCB. This *in vitro* result suggests that TCB may inactivate CYP1A1 *in vivo*, which could account for the apparent antagonistic effect of TCB on CYP1A1 induction. BIOCHEM PHARMACOL 53;7:1029–1040, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cytochrome P450; CYP1A1; tetrachlorobiphenyl; induction; inhibition; fish

Regulation of the cellular content and function of cytochrome P450 1A (CYP1A) [1] enzymes can affect the capacity of animals to metabolize and activate numerous toxic and carcinogenic compounds, thereby influencing the susceptibility to the effects of these compounds that often occur as environmental pollutants. In general, substrates can induce CYP enzymes by transcriptional activation of *CYP* genes [2] or by protein stabilization [3, 4]. Transcriptional activation of the *CYP1A* genes by polynuclear aromatic hydrocarbons and planar halogenated aromatic hydrocarbons is well established in most vertebrate taxa, and appears to occur via the AhR‖ that has been identified in vertebrates from elasmobranch fish to mammals [5].

The coplanar PCB congener TCB is an AhR agonist that appears to regulate CYP1A1 at several levels simultaneously. TCB is a potent CYP1A1 inducer in both mammals [6, 7] and fish [8–12]. TCB is also a CYP1A1 substrate in mammals [13–15] and a probable CYP1A1 substrate in fish.¶ In addition to being an inducer and a substrate of CYP1A1, TCB inhibits CYP1A1 catalytic activity. Thus, relatively low doses of TCB strongly induce hepatic CYP1A1 activities, but increasing doses are associated with a decline in those rates. This was observed initially in several fish species [8–12]. Similarly, in cultured hepatocytes or hepatoma cells from rat [16], fish (*Poeciliopsis*) [17], and chick [18, 19], and *in vivo* in rat and trout [20], TCB or other coplanar PCB congeners (PeCB and HCB) have maximally inducing doses, above which induction of CYP1A enzyme activity declines.

Decreased CYP1A activities at high doses of TCB may be explained, in part, by catalytic inhibition of CYP1A1.

§ Corresponding author: Dr. John J. Stegeman, Biology Department, Redfield 342, Woods Hole Oceanographic Institution, Woods Hole, MA 02543. Tel. (508) 289-2320; FAX (508) 457-2134; E-mail: jstegeman@whoi.edu

‖ *Abbreviations:* AhR, aryl hydrocarbon receptor; PCB, polychlorinated biphenyl; TCB, 3,3′,4,4′-tetrachlorobiphenyl; PeCB, 3,3′,4,4′,5-pentachlorobiphenyl; HCB, 3,3′,4,4′,5,5′-hexachlorobiphenyl; EROD, ethoxyresorufin O-deethylase; PROD, pentoxyresorufin O-dependentylase; UGT, UDP-glucuronosyltransferase; TCDF, 2,3,7,8-tetrachlorodibenzofuran; 3-MC, 3-methylcholanthrene; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; and SSPE, saline–sodium phosphate–EDTA buffer.

Received 25 June 1996; accepted 27 September 1996.

¶ White *et al.*, manuscript submitted for publication.

TCB is a competitive inhibitor of hepatic microsomal CYP1A1 activity in scup, rat, and chick embryo [8, 21]. Additional studies have suggested that TCB may suppress CYP1A1 at levels other than catalytic inhibition. In the teleost fish scup, administration of high doses of TCB is associated not only with a lesser induction of CYP1A1 catalytic activity, but also with a lesser induction of CYP1A1 protein, than is seen after treatment with a lower TCB dose [8]. This has been observed also in chick embryo hepatocytes [22–24], and in porcine aorta endothelial cells [25]. Other studies have suggested that the content of CYP1A1 mRNA is less in fish exposed to repeated doses of mixed PCBs than in fish exposed to lower PCB concentrations [26, 27]. Whether the effects of TCB on CYP1A1 protein content are due more to effects at transcriptional or post-transcriptional levels, and the mechanism(s) involved, are not known. In this study, transcriptional and translational aspects of hepatic CYP1A1 regulation by TCB were examined in the teleost fish scup (*Stenotomus chrysops*). Scup were treated with low or high doses of TCB, and samples were examined for the specificity of enzyme suppression by TCB, the dose- and time-dependence of such suppression, and the possible mechanisms by which TCB suppresses CYP1A1.

MATERIALS AND METHODS

Chemicals

3,3′,4,4′-Tetrachlorobiphenyl (IUPAC No. 77) and [^{14}C]TCB (52.1 mCi/mmol) were from Pathfinder (St. Louis, MO). Purity of the TCBs was greater than 99% by gas chromatography with electron capture detection (GC/ECD) and gas chromatography with mass spectrometry (GC/MS) analysis. High resolution (5000 resolution) mass spectrometry (VG Autospec) and selective ion monitoring showed that the AhR agonist 3,3′,4,4′,5-PeCB was not a contaminant of the TCB, at a detection limit of 0.001%. Ethoxyresorufin and pentoxyresorufin were from Molecular Probes (Eugene, OR). Other chemicals were the highest grades commercially available.

Animals and Dissection

Scup (*Stenotomus chrysops*) were caught by angling in Vineyard Sound, MA. Fish were held in flowing seawater at 20° for 14 months prior to experimental use, and were maintained on a diet of Purina trout chow and squid. Prior studies in our laboratory have shown that these fish have little CYP1A1 expression, implying that environmental inducers are depurated from the fish held under these conditions for a year or more. At the time of injection, experimental animals (mixed sex) were gonadally immature and weighed 76–276 g.

Fish were injected intraperitoneally either with corn oil (vehicle control) or with 3,3′,4,4′-TCB dissolved in corn oil, at doses of 0.1 or 5 mg TCB/kg body weight. A group of un-injected fish was used as a "zero" point in the time-course. Individuals were held as above until sampling at 1–16 days after treatment or until death from toxicity. Fish were fed Purina trout chow once weekly, and were not fed within the 3 days prior to killing. At each sampling time, a group of four or five animals from each treatment group were killed by cervical transection and dissected immediately. Portions of liver for GC analysis were rinsed in methanol and stored in acid-washed vials at −20° until analysis. Liver sections for RNA preparation and microsome preparation were removed and frozen in liquid nitrogen within 1–2 min of death. Tissues remained in liquid nitrogen until used; enzyme degradation does not occur to any measurable degree in scup tissues stored at liquid nitrogen temperatures (unpublished observations). Microsomal fractions were prepared as before [28], and protein content was measured with the bicinchoninic acid method [29], using bovine serum albumin as a standard.

Cytochromes P450 and b_5

The content of hepatic microsomal P450 was determined from dithionite difference spectra of CO-treated samples, and cytochrome b_5 content was measured from NADH difference spectra, as previously described [30, 31].

Immunoblotting

Western blot analysis of microsomal samples was performed as described previously [32, 33]. The three primary antibodies used were raised against the three principal P450 forms isolated from scup liver: monoclonal antibody 1-12-3 [34] to scup P450E, a CYP1A1 homologue [35]; polyclonal antibody 7-94 to scup P450B, a putative CYP2B homologue [36, 37]; and polyclonal antibody 7-93 to scup P450A, a putative CYP3A homologue [38]. Each antibody reacts strongly with its own immunogen in western blots, and does not cross-react with the other two scup P450 forms ([32]; and unpublished observations). Secondary antibodies were alkaline phosphatase linked goat-anti-mouse IgG and goat-anti-rabbit IgG. The relative content of proteins that were immunoreactive was measured by scanning with a video imaging system (Masterscan, Scanalytics/CSPI, Billerica, MA). CYP1A1 protein was quantified by comparison of optical density of the sample bands to optical density of standards with known P450E (scup CYP1A1) content.

Enzyme Assays

EROD activity was measured in hepatic microsomes at 32° with a fluorometric kinetic assay, using a Cytofluor 2300 fluorescence plate reader (Millipore), as described previously [39]. PROD activity was measured similarly, with 5 μM nominal substrate concentration. NADPH–cytochrome *c* (P450) reductase activity was measured at 30° by monitoring absorbance at 550 nm, as described previously [40]. UGT (EC 2.4.1.17) was measured at room temperature, according to published procedures [41]. The assay con-

tained 0.3 M KH_2PO_4 (pH 7.0), 1.3 mg/mL digitonin to solubilize microsomes, 0.2 mM *p*-nitrophenol (4-nitrophenol) as the aglycone substrate, 2.7 mg/mL UDPGA (omitted from blanks), and 1.1 to 3.7 mg microsomal protein/mL. Phenol UGT activity is induced in fish by Ah-receptor agonists [42].

RNA Preparation

Liver pieces weighing 100–150 mg were collected from each fish for RNA isolation. Two pooled RNA samples were prepared from each treatment group, each pool consisting of equally sized pieces of liver from two or three fish. Frozen liver was powdered in liquid nitrogen, and total RNA was isolated using the procedures of Chirgwin [43] and Clemens [44]. Modifications were as follows: instead of two high-speed centrifugation steps to clarify the RNA solution, RNA pellets were washed once in 4 M LiC to remove glycogen, followed by two washes in 95% ethanol. Total RNA was quantified on a Shimadzu UV-260 spectrophotometer.

Northern Blotting

Total RNA samples (10 μg each, as determined spectrally) were denatured in formamide/formaldehyde and separated on a 1% agarose/2.2 M formaldehyde gel, in 20 mM MOPS running buffer, according to standard protocols [45]. RNA was transferred to a positively charged nylon membrane (Amersham Hybond) in 20× SSC and cross-linked to the membrane with a Fotodyne DNA transfer lamp. The dried membrane was pre-hybridized for 1 hr at 60°, in a buffer composed of 6× SSPE, 0.5% SDS, 10× Denhardt's solution, and 100 mg/mL calf thymus DNA. The membrane was then hybridized at 42° for approximately 20 hr, with a ^{32}P-labeled probe (see below) in a buffer composed of 5× SSPE, 0.1% SDS, 2× Denhardt's solution, and 50% formamide. The membrane was washed for 30 min at 42° with 2× SSC, 0.1% SDS, and exposed to Kodak X-OMAT AR film. The relative amount of CYP1A1 mRNA in each probe-reactive band was determined by measuring the pixel density with software from NIH, Image 1.54.

A cDNA probe containing 75% of the scup CYP1A1 coding region [35] was used for quantification of CYP1A1 mRNA. The probe was radiolabeled with the Boehringer-Mannheim random-primed DNA labeling kit, using [α-^{32}P]dCTP (Amersham, Arlington Heights, IL), and was cleaned with an NACS Prepac column (Gibco/BRL, Inc. (Gaithersburg, MD) according to manufacturers' instructions.

Poly(T) Dot Blotting

To verify that northern blots were loaded with equal amounts of poly(A) mRNA in each lane, duplicate aliquots of each total RNA sample were subjected to dot blotting with a poly(T) probe. Samples consisted of 10 μg of total RNA (determined spectrally) brought to a volume of 50 μL in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA), 30 μL of 20× SSC, and 20 μL of 37% formaldehyde. Samples were heated to 60° for 15 min and then chilled on ice. Wells of the dot blot apparatus (Millipore Milliblot) were pre-washed with 20× SSC, samples were applied (each at 4, 2, 1, 0.5, 0.25, and 0.125 μg total RNA), and wells were washed with 20× SSC. RNA was cross-linked to the membrane as above. ^{35}S-Labeled poly(T) probes were prepared and blots were hybridized according to published procedures [46]. Total poly(A) mRNA in each dot was determined by measuring the ^{35}S-labeled poly(T) on a Beckman LS5000TD scintillation counter using a wide window (0–670). The content of poly(A) mRNA varied by 10–30% among samples.

TCB Residues

The content of 3,3′,4,4′-TCB was quantified in pooled samples, consisting of equally sized liver sections from all fish within a treatment group. Tissue extraction followed AOAC Method 983.21 [47]. Samples were mixed with sodium sulfate and petroleum ether, homogenized, and serially extracted with petroleum ether (boiling range 30–60°). Extracts were cleaned up on a Florisil column and concentrated under nitrogen. Extracts were analyzed for TCB by GC/ECD using an HP 5890 Series II GC/ECD. Confirmation of the TCB was performed by GC/MS using an HP 5890/5970 GC/MS by monitoring molecular ions 110, 150, 184, 220, 222, 290, 292, and 294. The fragmentation pattern of each sample was compared to the TCB standard and TCB was quantified with m/z 292. Measured concentrations of TCB were corrected for extraction efficiency using the recovery of 4,4′-dibromooctafluorobiphenyl (DBOFB), which was spiked into each sample prior to extraction, and were quantified relative to the internal standard 2,4,5,6-tetrachloro-*m*-xylene (TCMX). Recoveries of DBOFB ranged from 60 to 96%, averaging 76%. The recovery of TCB matrix spikes was 100%. The precision of duplicate analyses was within 3%.

Statistics

The statistical significance of differences between treatments was assessed using a nonparametric randomization procedure [48]. The basic model considered was:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \epsilon_{ijk}$$

where X_{ijk} is the measurement for fish k at time j under treatment, i, μ is the overall mean, α_i is the effect due to treatment i, β_j is the time effect, and ϵ_{ijk} is the error. The statistic used to test the null hypothesis H_o that the distribution of X_{ijk} is independent of treatment (but not of time) against the alternative hypothesis that the distribution of X_{ijk} depends on treatment (and time) was:

$$T = \Sigma\Sigma\Sigma\ (X_{ijk} - X_{.j.})^2 - \Sigma\Sigma\Sigma\ (X_{ijk} - X_{ij.})^2$$

where the summations are taken over all fish and where the dot notation indicates averaging over the corresponding subscript. This statistic is the reduction in residual sum of squares achieved by using treatment means, instead of overall means, at each time. This is equivalent to the usual F statistic for testing the null hypothesis of no treatment effect.

The distribution of T under H_o was estimated by randomly permuting the treatment labels on the fish at each time point and recalculating T. The significance level (or *P* value) was estimated by the proportion of permutations for which the recalculated value of T exceeded the observed value. The same approach was used to test for overall differences in treatment effects and differences between pairs of treatments. Two treatment effects were considered to be statistically significantly different if the corresponding *P* value, reported in the figure legends, was less than 0.05.

In Vitro *Incubations*

Hepatic microsomes from freshly caught scup were incubated at 1 mg/mL in 50 mM Tris, pH 7.6, with 0.1 mM EDTA. To the incubations was added either 3,3′,4,4′-TCB (1.5 μM), 2,2′,5,5′-TCB (1.5 μM), benzo[*a*]pyrene (1.5 μM), or acetone (vehicle control). Half of the incubations contained NADPH (1.5 mg/mL), and half lacked NADPH. Incubations were at 30° [40], under air in a shaking incubator. At 15, 30, and 60 min, aliquots of each incubation were removed for measurement of microsomal EROD activity. To the aliquot was added EROD buffer (100 mM Tris, 100 mM NaCl, pH 8) and 7-ethoxyresorufin substrate to a final concentration of 2 μM. Incubations that had been conducted with NADPH contained sufficient cofactor for the EROD reaction; NADPH was added to the microsomal incubations that had been carried out without NADPH. The rate of EROD activity was monitored spectrally for 3 min, as previously described [49]. Experiments were conducted twice, each time in triplicate.

Covalent Binding

The method of Shimada and Sato [50] was used to assess covalent binding of TCB to microsomal protein. Reaction mixtures (1 mL) contained 1 mg of microsomal protein from scup treated with TCDF or rat treated with 3-MC, 2 μM [^{14}C]TCB cleaned by extraction in hexane-NaOH and dissolved in acetone, 50 mM Tris, pH 7.6, and 0.1 mM EDTA. Reactions were initiated by adding 1 mM NADPH and run for 60 min at 30° or 37°. Reactions were stopped with 10% ice-cold trichloroacetic acid (TCA), protein was pelleted at 5000 *g* for 30 min, and pellets were washed with 1.5 mL of 10% TCA, 80% MeOH (2×), MeOH:diethyl ether (1:1, 2×), *n*-hexane (2×), and 80% MeOH (2×). The final wash had ^{14}C levels near background. Pellets were dissolved in 1 N NaOH and [^{14}C] was counted in a Beckman LS5000TD counter. Blank reactions had no NADPH added.

RESULTS

Gross Effects

Neither the low dose nor the high dose of TCB produced a change in hepatosomatic index, gonadosomatic index, or weight gain, as measured with a morphometric index of body weight to surface area (data not shown). Average microsomal protein yield was 14 ± 5 mg/g liver, and yield did not differ between treatment groups. Some fish treated with 5 mg TCB/kg died over the course of the experiment, possibly from TCB toxicity, as discussed in detail elsewhere;* none of the fish treated with corn oil or with 0.1 mg TCB/kg died prior to sampling.

Hepatic Monooxygenase Components

The content of total P450 in hepatic microsomes was strongly induced by 0.1 mg TCB/kg on days 2–16 after treatment (Fig. 1A). Maximal induction (3-fold increase over vehicle controls) was attained by day 5, and was sustained until day 16. In fish treated with 5 mg TCB/kg, total P450 content of hepatic microsomes was not induced significantly on days 1 through 12 after treatment. On day 16, two of the four fish treated with 5 mg TCB/kg had total P450 content similar to that in control fish, and two had strongly induced P450 content, similar to the levels in fish treated with 0.1 mg/kg TCB. The specific content of cytochrome b_5 was increased approximately 2-fold at both doses of TCB (Fig. 1B).

CYP Proteins

Hepatic microsomes were immunoblotted with antibodies to three major P450 forms expressed in scup liver: P450E, a CYP1A1 homologue [34, 35]; P450B, a probable CYP2B homologue [36, 37]; and P450A, a possible CYP3A homologue [38]. Each antibody recognized a single protein band in scup liver microsomes. CYP1A1 content was strongly induced by 0.1 mg TCB/kg, reaching a peak on day 5 and then declining slowly (Fig. 2A). CYP1A1 was induced in fish treated with 5 mg TCB/kg, also reaching a peak on day 5, but there was only a 4-fold induction at this dose, in contrast to the 12-fold induction at the low dose. Among the animals treated with 5 mg TCB/kg and sampled on day 16, the two with induced total P450 content expressed two times more CYP1A1 than did the other two fish in this group.

In each TCB treatment group the molar increases in CYP1A1 accounted for approximately 85% of the molar increases in total P450 (Table 1). Thus, both the strong induction of total P450 at 0.1 mg/kg, and the lesser induc-

* White *et al.*, manuscript in preparation.

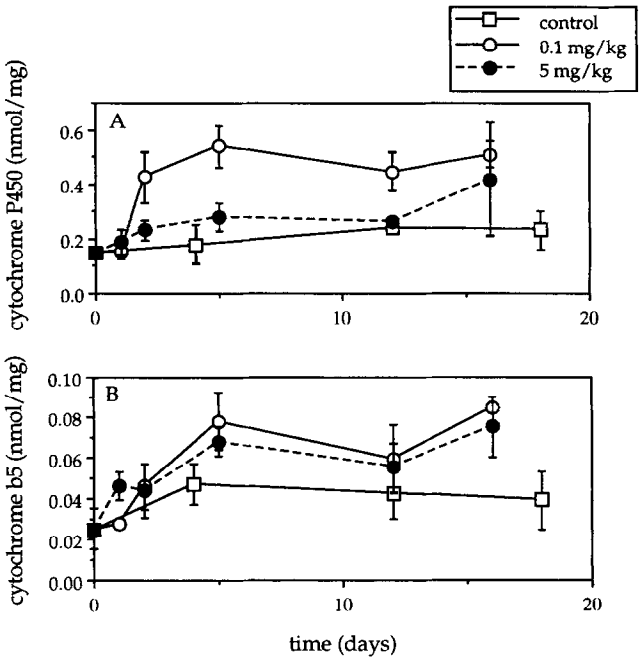

FIG. 1. Effect of TCB on hepatic microsomal cytochrome P450 (A) and cytochrome b_5 (B) content. Enzymes were quantified spectrally as described in Materials and Methods. X-axis is the number of days subsequent to injection; y-axis is the enzyme content in nmol/mg microsomal protein. Each point is the mean ± SD (N - 1) of measurements on four or five fish per treatment group. Key: (□) control, (○) 0.1 mg TCB/kg, and (●) 5 mg TCB/kg. Cytochrome P450 was significantly greater in the 0.1 mg/kg treatment than in the other two treatments ($P \leq 0.0006$). The b_5 content was significantly greater in TCB-treated than in control fish ($P \leq 0.0002$).

tion of total P450 at 5 mg/kg, appeared to be due primarily to differences in CYP1A1 content.

Like the content of CYP1A1, the contents of hepatic microsomal P450B (Fig. 2B) and P450A (Fig. 2C) both showed an initial increase (days 2, 5) followed by a decline in the fish treated with 0.1 mg/kg, while the content of these P450s was closer to control levels in fish given 5 mg/kg (day 5). However, the relative differences between treated and control groups were far less for P450B and P450A than for CYP1A1. Furthermore, content of both P450A and P450B varied widely within each treatment group (large standard deviations about the means), suggesting that the content of these enzymes was determined largely by factors other than TCB treatment.

Microsomal Enzyme Activities

Several enzyme activities were measured in hepatic microsomes (Figs. 3 and 4), to evaluate the specificity of enzyme inhibition and/or suppression by the high dose of TCB. EROD activity, catalyzed principally by CYP1A1 in scup [51], was strongly induced by 0.1 mg TCB/kg, reaching maximal levels by day 5 (15-fold induction) that were sustained until the end of the experiment (Fig. 3A). EROD activity was not induced in the fish treated with 5 mg TCB/kg (days 1–12), even though there was a significant induction of CYP1A1 protein content in these samples. As with total P450 content, an increase in mean EROD activity was seen on day 16 in the fish given 5 mg/kg. In this group, the two fish that had elevated P450 and CYP1A1 levels (see above) also had strongly induced EROD activity, whereas the other two fish had EROD activities near control levels.

PROD activity was strongly induced by the low dose of TCB, with a pattern of response almost identical to that of EROD activity (Fig. 3B). Also like EROD, PROD activity was not elevated in the fish receiving the high dose of TCB (days 1–12).

NADPH–cytochrome *c* (P450) reductase activity was increased by approximately 50% on days 5–16 in scup treated with either dose of TCB (Fig. 4A). Hepatic microsomal UGT activity with *p*-nitrophenol was increased approximately 2-fold in fish treated with TCB and sampled on day 5 (Fig. 4B). UGT rates were statistically equivalent in fish treated with either dose of TCB.

Hepatic CYP1A1 mRNA Expression

A cDNA probe containing 75% of the scup CYP1A1 coding region hybridized to a single band in each RNA sample on northern blots (Fig. 5). Equivalent loading of samples was verified by measuring total poly(A) mRNA content in each sample with a poly(T) probe (see Materials and Methods; data not shown). CYP1A1 mRNA content peaked on days 1–5 and declined by day 12 at both doses (Fig. 6). In fish treated with 0.1 mg TCB/kg, mRNA content continued to decline through day 16, whereas some fish receiving the higher dose of TCB showed a second rise in CYP1A1 mRNA content on day 16. Of the four high-dose fish sampled on day 16, CYP1A1 mRNA content was elevated in the same two fish that had induced CYP1A and EROD levels (described above).

TCB Residues

TCB content was measured in pooled liver samples from each treatment group (Fig. 7). The average dry weight of liver samples was determined to be 25% of the wet weight; TCB content is reported on a dry weight basis. In fish treated with 0.1 mg TCB/kg, the TCB content in liver reached a maximum concentration of 0.015 μg/g liver on day 5. In fish treated with 5 mg TCB/kg, the TCB content in liver reached a maximum concentration of 2.2 μg/g liver on day 12. Hepatic TCB content had declined substantially by day 12 at the low dose, and by day 16 at the high dose. Analysis of TCB residues (unpublished) indicated that this apparent depuration was due to metabolism and transport of metabolites and parent compound into bile.

In Vitro *Incubations*

To further analyze TCB/CYP1A1 interactions, scup hepatic microsomes were preincubated with TCB and/or

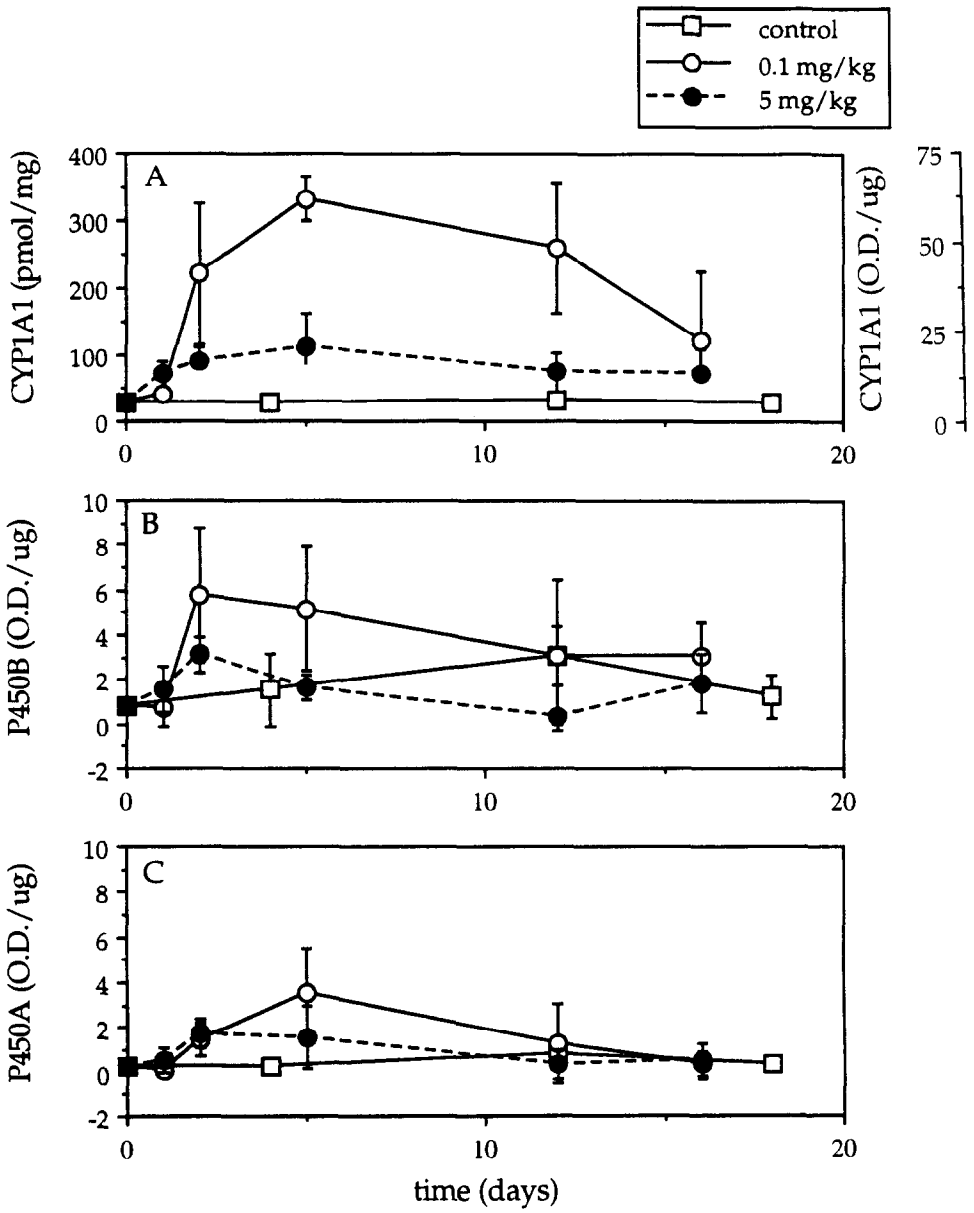

FIG. 2. Effect of TCB on hepatic microsomal CYP1A1 (A), P450B (B), and P450A (C) content. Proteins were quantified by immunoblotting as described in Materials and Methods. X-axis is the number of days subsequent to injection; y-axis is the CYP content in pmol/mg microsomal protein (A) or relative optical density/μg microsomal protein (A, B, and C). Each point is the mean ± SD (N - 1) of measurements on four or five fish per treatment group. Key: (□) corn oil control, (○) 0.1 mg TCB/kg, and (●) 5 mg TCB/kg. CYP1A1 was significantly different between each of the three treatments ($P \leq 0.0003$). P450B was significantly different between each of the three treatments ($P \leq 0.02$). P450A was significantly different between control and TCB-treated ($P \leq 0.03$).

NADPH, and aliquots of those reaction mixtures were withdrawn at various times and analyzed for CYP1A1 catalytic activity (EROD activity). Microsomes preincubated for 60 min with NADPH alone showed no change in the capacity to catalyze EROD activity (Fig. 8). Addition of TCB alone to the preincubation caused an immediate 50% decrease in the capacity of the samples to catalyze EROD activity. This loss was neither time- nor cofactor-dependent, and presumably was due to competitive inhibition of EROD activity by TCB [8]. Preincubation of microsomes with both TCB and NADPH, a condition that supports TCB metabolism *in vitro*, caused a time-dependent decrease in the capacity to catalyze EROD activity. This is indicative of a loss of CYP1A1 catalytic capacity, or CYP1A1 inactivation. To verify the specificity of the inactivation, the same series of incubations was conducted with benzo[*a*]pyrene (a rapidly metabolized CYP1A1 substrate) or with 2,2',5,5'-TCB (not a CYP1A1 substrate) substituted for the 3,3',4,4'-TCB. Neither benzo[*a*]pyrene nor 2,2',5,5'-TCB caused a time-dependent loss of CYP1A1 catalytic capacity (data not shown).

Analysis of microsomes from these incubations revealed a loss of spectral P450 and the appearance of spectral P420, only in the incubations containing both TCB and NADPH. Immunoblot analysis of microsomes from those incubations did not show any loss of immunodetectable CYP1A1, nor were there any lower molecular weight bands that might be attributed to breakdown products generated in the *in vitro* incubation.

Incubation of TCDF-induced scup liver microsomes for 1 hr with [^{14}C]TCB plus NADPH followed by exhaustive extraction with multiple solvents was able to remove all but a trace of radioactivity. The amount remaining, the fraction possibly covalently bound to CYP, was 1.5 to 1.8 pmol

TABLE 1. Contribution of CYP1A1 to the induction of total hepatic cytochrome P450 by TCB

	Total P450* (pmol/mg)	Increase in P450⁺ (pmol/mg)	CYP1A1* (pmol/mg)	Increase in CYP1A1⁺ (pmol/mg)	% P450 induction due to CYP1A1
Control	178 ± 71		27 ± 0.1		
TCB (0.1 mg/kg)	538 ± 75	360	332 ± 33	305	85
TCB (5 mg/kg)	278 ± 53	100	111 ± 51	84	84

* Values repesent means ± SD (N−1) for five fish (TCB-treated) or four fish (control). Animals were sampled on day 5 (TCB) or day 4 (control) after treatment.

⁺ Increase in total P450 or CYP1A1 content over control level. Determined by subtracting enzyme content in control fish from enzyme content in TCB-treated fish.

bound/hr/mg microsomal protein. Similar incubations with 3-MC-induced rat liver microsomes resulted in about 99.3 pmol bound/hr/mg. Reactions minus NADPH showed no [^{14}C] bound to microsomal protein. Thus, the formation of reactive metabolites that might participate in the inactivation of scup CYP1A1 was very limited, and could not account for the loss of EROD activity *in vitro*, or of CYP1A1 *in vivo*.

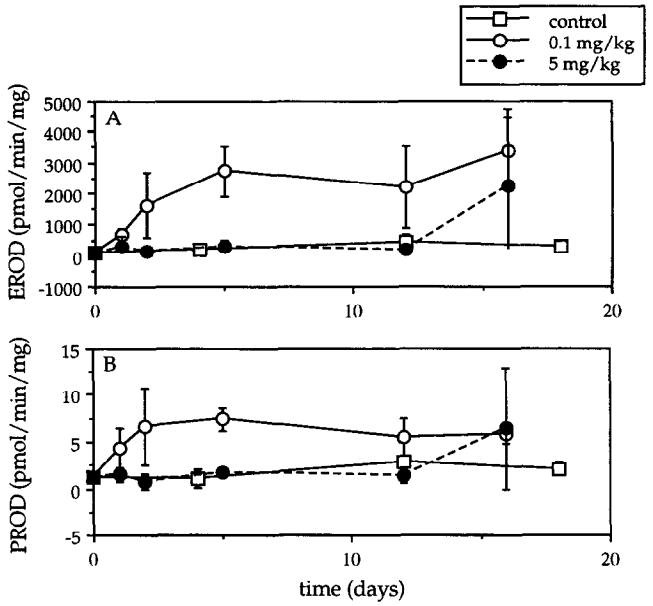

FIG. 3. Effect of TCB on hepatic microsomal EROD (A) and PROD (B) activities. Enzyme activities were quantified fluorometrically as described in Materials and Methods. EROD rates are expressed as pmol/min/mg to highlight the comparison with the PROD rates, which are much lower. X-axis is the number of days subsequent to injection; y-axis is the rate of enzyme activity in pmol/min/mg microsomal protein. Each point is the mean ± SD (N − 1) of measurements on four or five fish per treatment group. Key: (□) corn oil control, (○) 0.1 mg TCB/kg, and (●) 5 mg TCB/kg. EROD and PROD rates in the 0.1 mg/kg treatment were significantly greater than controls ($P \leq 0.0001$) and were significantly greater than the 5 mg/kg treatment ($P \leq 0.005$).

DISCUSSION

The inhibition of hepatic CYP1A1 catalytic activity by 3,3',4,4'-TCB was first suggested in studies of rainbow trout [9], and then in scup [8]. The present results show that the strong negative effect of high levels of 3,3',4,4'-TCB on

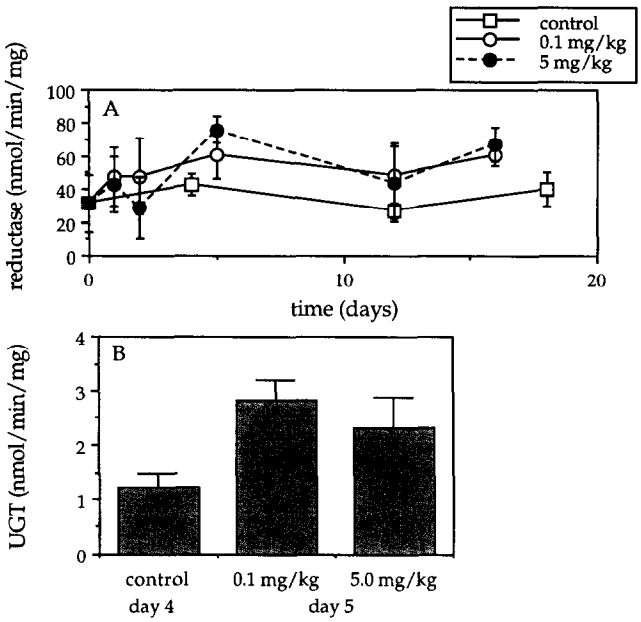

FIG. 4. Effect of TCB on hepatic microsomal NADPH–cytochrome c (P450) reductase activity (A) and UDP-glucuronsyltransferase activity (B). Enzyme activities were quantified spectrophotometrically as described in Materials and Methods. The substrate for UGT activity was *p*-nitrophenol. X-axis is the number of days subsequent to injection (A), or is treatment on day 4 (control) or 5 (TCB) (B). Y-axis is the rate of enzyme activity in nmol/min/mg microsomal protein. Each point or bar is the mean ± SD (N − 1) of measurements on four or five fish per group. Key: (□) corn oil control, (○) 0.1 mg TCB/kg, and (●) 5 mg TCB/kg. Reductase rates were significantly greater in both TCB treatments than in controls ($P \leq 0.004$). UGT rates in the two TCB treatments were statistically equivalent and were elevated over controls ($P \leq 0.01$) based on a one-way ANOVA.

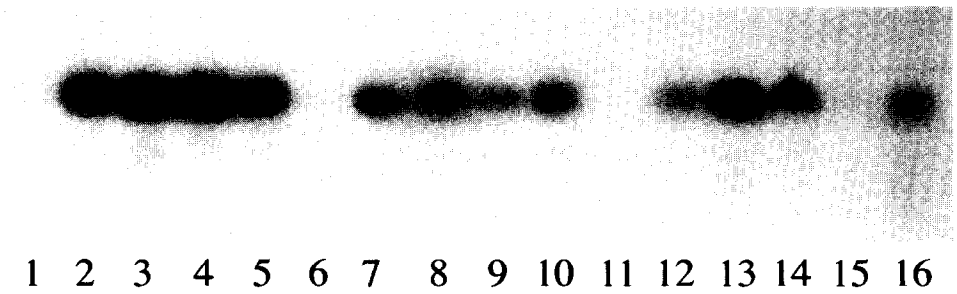

FIG. 5. Northern blot of CYP1A1 mRNA in scup liver. Liver samples were pooled samples (three fish each). Total RNA (10 μg/lane) was separated on an agarose gel, transferred to nylon, and probed with a ^{32}P-labeled cDNA containing 75% of the scup CYP1A1 coding region, as described in Materials and Methods. Exposure time and TCB doses of samples were as follows: lane 1-time zero (uninjected); lane 2-day 1, 0.1 mg/kg; lane 3-day 1, 5 mg/kg; lane 4-day 2, 0.1 mg/kg; lane 5-day 2, 5 mg/kg; lane 6-day 4, corn oil; lane 7-day 5, 0.1 mg/kg; lane 8-day 5, 5 mg/kg; lane 9-day 12, 0.1 mg/kg; lane 10-day 12, 5 mg/kg; lane 11-day 12, corn oil; lane 12-day 16, 0.1 mg/kg; lanes 13 and 14-day 16, two different samples at 5 mg/kg; lane 15-day 18, corn oil; lane 16-day 34, 0.1 mg/kg. Equivalent loading was established on the basis of poly(A) RNA, determined by scintillation counting of dot blots of poly(A) RNA in these samples.

hepatic CYP1A1 *in vivo* involves not only inhibition of CYP1A1 catalytic activity, but also a reduction in CYP1A1 protein content that persists over time while high concentrations of TCB remain in the liver. A relatively low dose of TCB (0.1 mg/kg) strongly induced the levels of CYP1A1 mRNA, protein, and catalytic activity, consistent with previous observations [52]. A 50-fold higher dose of TCB (5 mg/kg) also strongly induced CYP1A1 mRNA by 1–2 days after treatment, but levels of CYP1A1 protein and catalytic activity were only weakly elevated. This weak increase in CYP1A1 protein *in vivo* at the high dose of TCB is referred to here as an apparent, post-transcriptional "suppression" of enzyme induction.

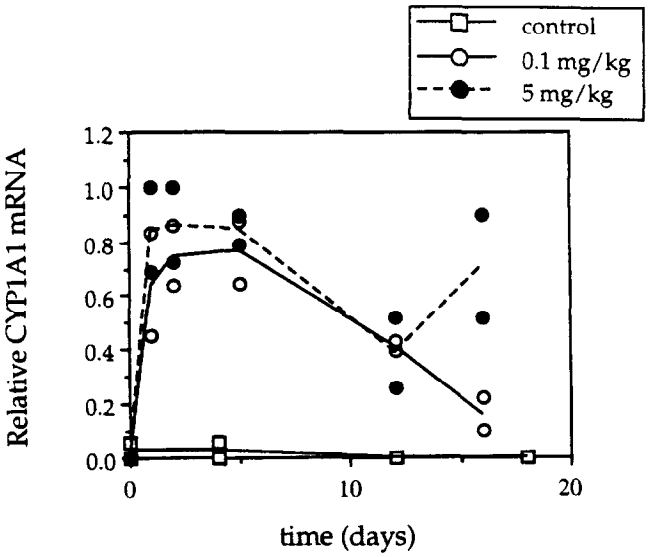

FIG. 6. Effect of TCB on hepatic CYP1A1 mRNA content. X-axis is the number of days subsequent to injection; y-axis is the relative content of CYP1A1 mRNA, expressed as pixel density of cDNA-reactive bands. RNA was isolated and analyzed as described in Materials and Methods and in the legend of Fig. 5. Each point is a measurement on one pooled sample, and lines indicate the mean values for each treatment. Key: (□,—) corn oil control, (○,—) 0.1 mg TCB/kg, and (●,---) 5 mg TCB/kg.

Specificity of Enzyme Suppression by TCB

Analysis of other microsomal enzymes suggests that the effect of high dose TCB is largely specific to CYP1A1. Thus, the content or activity of cytochrome b_5, P450 reductase, and UGT each were elevated to the same extent by both low and high doses of TCB. The content of total P450 was less in fish given the high dose of TCB than in those given the low dose. However, the negative effect on CYP1A1 can account for the majority of that difference (Table 1). Consistent with that, TCB had only a slight effect on the expression of two other scup hepatic CYP forms. Histological examination of liver from fish treated with either dose of TCB showed no degenerative or necrotic damage (unpublished), indicating that the suppression of CYP1A1 by the high dose did not result from gen-

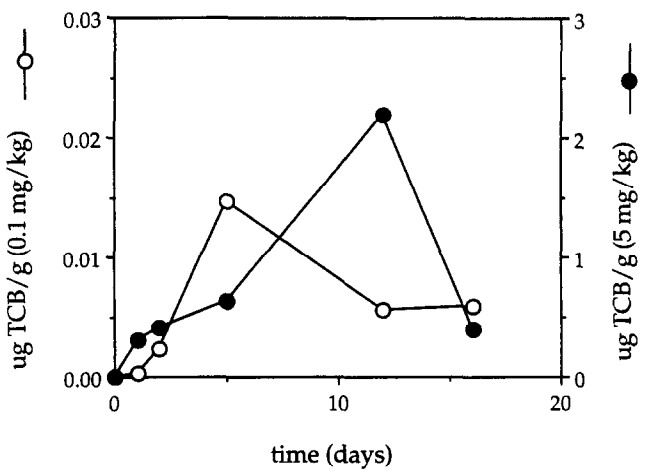

FIG. 7. GC/MS analysis of 3,3',4,4'-TCB content in pooled liver samples from TCB-treated fish. X-axis is the number of days subsequent to injection; y-axis is the TCB concentration in μg/g liver dry weight. Left y-axis is the concentration in fish treated with 0.1 mg/kg; right y-axis is the concentration in fish treated with 5 mg TCB/kg: note the difference in scale. Each point is a measurement on a single sample, consisting of tissue sections from all four or five fish in each treatment group. Key: (○) 0.1 mg TCB/kg, and (●) 5 mg TCB/kg.

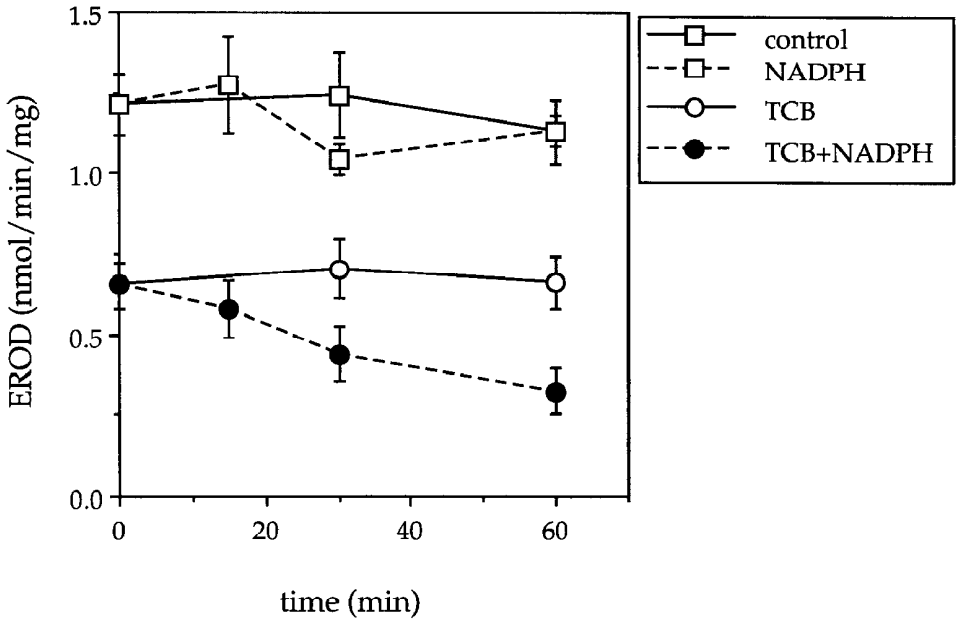

Fig. 8. *In vitro* inhibition and inactivation of scup hepatic microsomal CYP1A1 by 3,3',4,4'-TCB. Scup hepatic microsomes were from untreated, freshly caught fish that had induced levels of CYP1A1 (compare EROD rates with those in induced fish in Fig. 3). Microsomes were preincubated with or without TCB (1.5 μM) and with or without NADPH. At the indicated times, an aliquot of the incubation was removed for measurement of EROD activity. X-axis is the length of time of the microsomal incubation prior to removal of an aliquot for EROD analysis; y-axis is the rate of EROD activity in the aliquot. Each point represents the mean ± SD (N - 1) of six separate incubations.

eral toxicity, which would be expected to lead to a loss of other proteins as well. Earlier, Gooch *et al.* [8] reported that scup treated with 5 mg TCB/kg had lower rates of hepatic microsomal EROD activity than did scup treated with 1 mg TCB/kg, but that rates of aminopyrine *N*-demethylase and estradiol 2-hydroxylase, non-CYP1A activities in scup, were similar in fish treated with these doses of TCB. The present results and those of Gooch *et al.* [8] suggest that the suppressive effect of TCB is largely specific to CYP1A1.

Given the evidence for specificity of the effect, results obtained here for PROD activity bear on the identity of the catalyst for that activity. Although PROD is largely catalyzed by CYP2B enzymes in mammals [53], the catalyst(s) for this activity in teleosts has not been identified. The CYP2B-like scup P450B was induced slightly by low dose TCB, and it is possible that TCB could have exerted an effect on some unidentified, minor P450 that we did not examine, that might include a different PROD catalyst. Yet, the patterns of induction and decline for EROD and PROD seen here were parallel, suggesting that CYP1A1 contributes substantially to PROD activity in scup.

Dose-Dependence of CYP1A1 Suppression by TCB

In fish treated with the low dose of TCB, the hepatic TCB content and immunodetectable CYP1A1 content both peaked on day 5 and declined by day 12, and TCB content did not exceed 0.015 μg/g. In fish given 5 mg TCB/kg, the hepatic TCB concentrations on days 5 and 12 (0.6 and 2.2 μg/g) were associated with suppressed CYP1A1 content, while the lesser TCB concentration on day 16 (0.4 μg/g in the pooled sample) coincided with an elevation of CYP1A1 content and activity in two of the four animals. Thus, suppressed CYP1A1 protein content was associated with TCB concentrations in excess of 0.4 μg/g liver dry weight, or approximately 0.1 μg/g liver wet weight. These data suggest that the suppression of CYP1A1 content may be more sensitive to TCB than was inferred by Gooch *et al.* [8], who reported inhibition of microsomal EROD activity in scup livers containing ≥2 μg TCB/g liver wet weight. Determining the intrahepatic distribution of TCB and CYP1A1 could establish the relationship between CYP1A1 suppression and TCB doses *in vivo*.

The decline in TCB concentrations in liver of high dose fish on day 16 was associated with a release of EROD inhibition, and an increase in CYP1A1 protein and mRNA contents in some fish in that group. The second rise in CYP1A1 mRNA content on day 16 suggests that the highest concentrations of TCB in liver may suppress the induction of CYP1A1 at a transcriptional level. This has been suggested in other studies of fish exposed to mixed PCBs [26, 27]. However, transcriptional regulation alone cannot account for the extent to which CYP1A1 protein content was suppressed by high concentrations of TCB on days 2 through 12 in the present study.

Mechanism of Post-transcriptional Suppression

The corresponding decline of both immunodetectable CYP1A1 protein and spectrally detectable P450 holoenzyme in TCB-treated fish suggests the possible involvement of several mechanisms, including: (a) reduced translation of CYP1A1 mRNA, (b) reduced availability of heme, necessary for the synthesis of P450 holoenzyme, or (c) enhanced CYP1A1 protein degradation. In northern blot analysis (Fig. 5), the electrophoretic migration of the CYP1A1 mRNA was the same at the two doses, suggesting that a full-length message is synthesized at the high (suppressive)

dose. Furthermore, it was reported previously [8] that hepatic CYP1A1 mRNA isolated from scup treated with 5 mg TCB/kg was competent for *in vitro* translation. These findings argue against hypothesis (a), reduced CYP1A1 mRNA translation, to explain the degree of CYP1A1 suppression, although assays of translational efficiency would be needed to confirm this.

The data also are inconsistent with hypothesis (b), reduced heme availability, as an explanation for the suppressed CYP1A1 content at the high dose of TCB. TCB exposure has been reported to cause uroporphyria in chick embryo hepatocytes [54, 55], and in fish hepatoma cells [56], which could reflect an inhibition of heme synthesis [54, 55]. CYP1A1 was the only microsomal heme protein examined which showed a substantial decline in content at the high dose of TCB. The content of holo-cytochrome b_5 was similarly elevated at the two doses of TCB. We cannot rule out the possibility of a suppressive effect of TCB on other P450s, but any such effect would appear to be weak compared with the effect on CYP1A1. The half-life of heme in teleost CYP1A1 has been estimated at 100 hr, longer than the 28 hr in rat [57], although the CYP1A1 protein half-lives are similar, about 38 hr. If half-lives of other P450s and of b_5 in scup are similar to those in rat [58, 59], then a deficiency in heme availability sufficient to produce a decline in CYP1A content could be expected over the course of 12 days to result in a decline in the content of other heme proteins as well.

Several lines of evidence support hypothesis (c), that inactivation of CYP1A1 protein and possibly enhanced degradation could account for the decline in CYP1A1 protein content *in vivo* at the high dose. First, the finding that suppression was specific to CYP1A1 is consistent with a mechanism involving a direct interaction between TCB and CYP1A1 *in vivo*. Second, prior work indicates that TCB is a competitive inhibitor of CYP1A1, implying that it binds in the CYP1A1 active site [8]. Third, microsomal incubations (Fig. 8) demonstrated that CYP1A1 is inactivated by a time-dependent process in the presence of TCB and cofactor, whereas other CYP are not inactivated. The requirement for both TCB and NADPH in the inactivation suggests that both substrate binding and electron transfer to the CYP1A1/TCB complex, i.e. the initial steps in the catalytic cycle, are involved. TCB is metabolized very slowly by scup,* and the data here imply that protein-binding, i.e. suicide inactivation by TCB, is slight. However, TCB could uncouple the monooxygenation reaction. Reactive oxygen species (ROS) may be generated under those circumstances [60], and ROS could be involved in the inactivation of scup CYP1A1. Studies to elucidate further the possible mechanisms of CYP1A1 inactivation by interaction with TCB are currently underway. Enzyme inactivation like that observed *in vitro*, were it to occur *in vivo*, could result in protein degradation, and thus account for the suppressed levels of hepatic CYP1A1 protein in animals with high hepatic TCB content. However, we cannot rule out the possibility that reduced transcription and/or translation also contribute to CYP1A1 suppression at the high dose of TCB.

In summary, TCB at concentrations of 0.1 μg/g liver wet weight or more appears to simultaneously cause transcriptional induction of CYP1A1, catalytic inhibition, and post-transcriptional suppression of CYP1A1 protein content in fish (scup) liver. The content of TCB in fish from some contaminated environments [11] suggests that CYP1A1 suppression could occur under some conditions of environmental exposure. Whether this might be a component of the resistance of fish from highly contaminated regions to toxicity of PCBs or polychlorinated dibenzo-*p*-dioxins [61] is not known. Suppression by PCB could cause a diminished CYP1A1 inducibility and thus diminished CYP1A1 catalytic capacity. The loss of this adaptive pathway could then limit the capacity to metabolize and depurate, or to activate, other CYP1A1 substrates such as carcinogenic polynuclear aromatic hydrocarbons. Alternately, the possible generation of active oxygen due to a TCB–CYP1A1 interaction could be involved in a variety of other effects, leading to toxicity of TCB.

The authors would like to thank Dr. Neal Cornell, Dr. Hilary Morrison, and Grace Bruning (Marine Biological Laboratory, Woods Hole, MA) for providing nucleic acid probes; Dr. Michael Moore (WHOI) for assistance with treatment of the fish; and Dr. Mark Hahn (WHOI) for commenting on the manuscript. This research was supported, in part, by NIH Grant P30ES02109; EPA Grant R817988; and Grant NA-90-AA-D-SG480 from the Coastal Ocean Program of the National Oceanic and Atmospheric Administration to the Woods Hole Oceanographic Institution Sea Grant Program. Contribution No. 9043 of the Woods Hole Oceanographic Institution.

References

1. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW, P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6:** 1–42, 1996.
2. Nebert DW and Gonzalez FJ, P450 genes: Structure, evolution, and regulation. *Annu Rev Biochem* **56:** 945–993, 1987.
3. Eliasson E, Johansson I and Ingelman-Sundberg M, Substrate-, hormone-, and cAMP-regulated cytochrome P450 degradation. *Proc Natl Acad Sci USA* **87:** 3225–3229, 1990.
4. Eliasson E, Mkrtchian S, Halpert JR and Ingelman-Sundberg M, Substrate-regulated, cAMP-dependent phosphorylation, denaturation, and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1. *J Biol Chem* **269:** 18378–18383, 1994.
5. Hahn ME, Poland A, Glover E and Stegeman JJ, Photoaffinity labeling of the Ah receptor: Phylogenetic survey of diverse vertebrate and invertebrate species. *Arch Biochem Biophys* **310:** 218–228, 1994.
6. Parkinson A, Robertson L, Safe L and Safe S, Polychlorinated biphenyls as inducers of hepatic microsomal enzymes: Structure–activity rules. *Chem Biol Interact* **30:** 271–285, 1980.
7. Parkinson A, Safe SH, Robertson LW, Thomas PE, Ryan DE, Reik LM and Levin W, Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver mi-

* White *et al.*, manuscript submitted for publication.

crosomes from polychlorinated or polybrominated biphenyl-treated rats. *J Biol Chem* **258:** 5967–5976, 1983.
8. Gooch JW, Elskus AA, Kloepper-Sams PJ, Hahn ME and Stegeman JJ, Effects of *ortho* and non-*ortho* substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotomus chrysops*). *Toxicol Appl Pharmacol* **98:** 422–433, 1989.
9. Melancon MJ and Lech JJ, Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquat. Toxicol* **4:** 51–61, 1983.
10. Miranda CL, Wang J-L, Chang H-S and Buhler DR, Multiple effects of 3,4,5,3',4',5'-hexachlorobiphenyl administration on hepatic cytochrome P450 isozymes and associated mixed-function oxidase activities in rainbow trout. *Biochem Pharmacol* **40:** 387–390, 1990.
11. Monosson E and Stegeman JJ, Cytochrome P450E (P450IA) induction and inhibition in winter flounder by 3,3',4,4'-tetrachlorobiphenyl: Comparison of response in fish from Georges Bank and Narragansett Bay. *Environ Toxicol Chem* **10:** 765–774, 1991.
12. Lindstrom-Seppa P, Korytko PJ, Hahn ME and Stegeman JJ, Uptake of waterborne 3,3',4,4'-tetrachlorobiphenyl and organ and cell-specific induction of cytochrome P4501A in adult and larval fathead minnow *Pimephales promelas*. *Aquat Toxicol* **28:** 147–167, 1994.
13. Shimada T and Sawabe Y, Activation of 3,4,3',4'-tetrachlorobiphenyl to protein bound metabolites by the rat liver cytochrome P-448-containing monooxygenase system. *Toxicol Appl Pharmacol* **70:** 486–493, 1983.
14. Mills RA, Millis CD, Dannan GA, Guengerich FP and Aust SD, Studies on the structure–activity relationships for the metabolism of polybrominated biphenyls by rat liver microsomes. *Toxicol Appl Pharmacol* **78:** 96–104, 1985.
15. Ishida C, Koga N, Hanioka N, Saeki HK and Yoshimura H, Metabolism *in vitro* of 3,4,3',4'- and 2,5,2',5'-tetrachlorobiphenyl by rat liver microsomes and highly purified cytochrome P450. *J Pharmacobiodyn* **14:** 276–284, 1991.
16. Sawyer T and Safe S, PCB isomers and congeners: Induction of aryl hydrocarbon hydroxylase and ethoxyresorufin O-deethylase enzyme activities in rat hepatoma cells. *Toxicol Lett* **13:** 87–94, 1982.
17. Hahn ME, Lamb TM, Schultz ME, Smolowitz RM and Stegeman JJ, Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). *Aquat Toxicol* **26:** 185–208, 1993.
18. Kennedy SW, Lorenzen A, James CA and Collins BT, Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multi-well plate reader. *Anal Biochem* **211:** 102–112, 1993.
19. Rodman LE, Shedlofsky SI, Swim AT and Robertson LW, Effects of polychlorinated biphenyls on cytochrome P450 induction in the chick embryo hepatocyte culture. *Arch Biochem Biophys* **275:** 252–262, 1989.
20. Voorman R and Aust SD, Inducers of cytochrome P-450d: Influence on microsomal catalytic activities and differential regulation by enzyme stabilization. *Arch Biochem Biophys* **262:** 76–84, 1988.
21. De Matteis F, Trenti T, Gibbs AH and Greig JB, Inducible bilirubin-degrading system in the microsomal fraction of rat liver. *Mol Pharmacol* **35:** 831–838, 1989.
22. Lambrecht RW, Sinclair PR, Bement WJ and Sinclair JF, Uroporphyrin accumulation in cultured chick embryo hepatocytes: Comparison of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3,4,3',4'-tetrachlorobiphenyl. *Toxicol Appl Pharmacol* **96:** 507–516, 1988.
23. Sinclair PR, Bement WJ, Bonkovsky HL and Sinclair JF, Inhibition of uroporphyrinogen decarboxylase by halogenated biphenyls in chick hepatocyte cultures. Essential role for induction of cytochrome P-448. *Biochem J* **222:** 737–748, 1984.
24. Sinclair P, Frezza J, Sinclair J, Bement W, Haugen S, Healey J and Bonkovsky H, Immunochemical detection of different isoenzymes of cytochrome P-450 induced in chick hepatocyte cultures. *Biochem J* **258:** 237–245, 1989.
25. Stegeman JJ, Hahn ME, Weisbrod R, Woodin BR, Joy JS, Najibi S and Cohen RA, Induction of cytochrome P4501A1 by Ah-receptor agonists in porcine aorta endothelial cells in culture, and CYP1A1 activity in intact cells. *Mol Pharmacol* **47:** 296–306, 1995.
26. Wirgin I, Kreamer GL, Grunwald C, Squibb K, Garte SJ and Courtenay S, Effects of prior exposure history on cytochrome P4501A mRNA induction by PCB congener 77 in Atlantic tomcod. *Marine Environ Res* **34:** 103–108, 1992.
27. Celander M and Forlin L, Decreased responsiveness of the hepatic cytochrome P4501A1 system in rainbow trout (*Oncorhynchus mykiss*) after prolonged exposure to PCB. *Aquat Toxicol* **34:** 141–153, 1995.
28. Hahn ME and Stegeman JJ, Regulation of cytochrome P4501A1 in teleosts: Sustained induction of CYP1A1 mRNA, protein, and catalytic activity by 2,3,7,8-tetrachlorodibenzofuran in the marine fish *Stenotomus chrysops*. *Toxicol Appl Pharmacol* **127:** 187–198, 1994.
29. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
30. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239:** 2370–2378, 1964.
31. Stegeman JJ and Binder RL, High benzo[*a*]pyrene hydroxylase activity in the marine fish *Stenotomus versicolor*. *Biochem Pharmacol* **28:** 1686–1688, 1979.
32. Kloepper-Sams PJ, Park SS, Gelboin HV and Stegeman JJ, Specificity and cross-reactivity of monoclonal and polyclonal antibodies against cytochrome P450E of the marine fish scup. *Arch Biochem Biophys* **253:** 268–278, 1987.
33. Stegeman JJ, Smolowitz RM and Hahn ME, Immunohistochemical localization of environmentally induced cytochrome P450IA1 in multiple organs of the marine teleost *Stenotomus chrysops* (scup). *Toxicol Appl Pharmacol* **110:** 486–504, 1991.
34. Park SS, Miller H, Klotz AV, Kloepper-Sams PJ, Stegeman JJ and Gelboin HV, Monoclonal antibodies to liver microsomal cytochrome P-450E of the marine fish *Stenotomus chrysops* (scup): Cross reactivity with 3-methylcholanthrene induced rat cytochrome P-450. *Arch Biochem Biophys* **249:** 339–350, 1986.
35. Morrison HG, Oleksiak MF, Cornell NW, Sogin ML and Stegeman JJ, Identification of cytochrome P-450 1A (CYP1A) genes from two teleost fish, toadfish (*Opsanus tau*) and scup (*Stenotomus chrysops*), and phylogenetic analysis of CYP1A genes. *Biochem J* **308:** 97–104, 1995.
36. Stegeman JJ, Woodin BR and Waxman DJ, Structural relatedness of mammalian cytochromes P450 IIB and cytochrome P450B from the marine fish scup (*Stenotomus chrysops*). *FASEB J* **4:** A739, 1990.
37. Stegeman JJ, Woodin BR and Smolowitz RM, Structure, function and regulation of cytochrome P-450 forms in fish. *Biochem Soc Trans* **18:** 19–21, 1990.
38. Celander M, Buhler DR, Forlin L, Goksoyr A, Miranda CL, Woodin BR and Stegeman JJ, Immunochemical relationships of cytochrome P4503A-like proteins in teleost fish. *Fish Physiol Biochem* **15:** 323–332, 1996.
39. Hahn ME, Woodward BL, Stegeman JJ and Kennedy SW, Rapid assessment of induced cytochrome P4501A (CYP1A)

protein and catalytic activity in fish hepatoma cells grown in multi-well plates. *Environ Toxicol Chem* **15:** 582–591, 1996.
40. Stegeman JJ, Binder RL and Orren A, Hepatic and extrahepatic microsomal electron transport components and mixed-function oxygenases in the marine fish *Stenotomus versicolor*. *Biochem Pharmacol* **28:** 3431–3439, 1979.
41. Andersson T, Pesonen M and Johansson C, Differential induction of cytochrome P-450-dependent monooxygenase, epoxide hydrolase, glutathione transferase, and UDP glucuronyltransferase activities in the liver of the rainbow trout by β-naphthoflavone or Clophen A50. *Biochem Pharmacol* **34:** 3309–3314, 1985.
42. Clarke DJ, Burchell B and George SG, Functional and immunological comparison of hepatic UDP-glucuronosyltransferases in a piscine and a mammalian species. *Comp Biochem Physiol* **102B:** 425–432, 1992.
43. Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18:** 5294–5299, 1979.
44. Clemens MJ, Extraction of RNA by the guanidium thiocyanate procedure. In: *Transcription and Translation: A Practical Approach* (Eds. Hames BD and Higgins SJ), p. 217. IRL Press, Oxford, 1986.
45. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, Ed, Vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
46. Hollander MC and Fornace AJ, Estimation of relative mRNA content by filter hybridization to a polythymidylate probe. *Biotechniques* **9:** 174–179, 1990.
47. Helrich K, In: *Official Methods of Analysis of the Association of Official Analytical Chemists* (Ed. Helrich K), 15th Edn, pp. 283–284. Association of Official Analytical Chemists, Arlington, VA, 1990.
48. Manly BFJ, *Randomization and Monte Carlo Methods in Biology*. Chapman & Hall, London, 1991.
49. Klotz AV, Stegeman JJ and Walsh C, An alternative 7-ethoxyresorufin O-deethylase activity assay: A continuous visible spectrophotometric method for measurement of cytochrome P-450 monoxygenase activity. *Anal Biochem* **140:** 138–145, 1984.
50. Shimada T and Sato R, Covalent binding of polychlorinated biphenyls to rat liver microsomes *in vitro*: Nature of reactive metabolites and target macromolecules. *Toxicol Appl Pharmacol* **55:** 490–500, 1980.
51. Klotz AV, Stegeman JJ and Walsh C, An aryl hydrocarbon hydroxylating hepatic cytochrome P-450 from the marine fish *Stenotomus chrysops*. *Arch Biochem Biophys* **226:** 578–592, 1983.
52. Kloepper-Sams PJ and Stegeman JJ, The temporal relationships between cytochrome P-450E protein content, catalytic activity and mRNA levels in the teleost *Fundulus heteroclitus* following treatment with β-naphthoflavone. *Arch Biochem Biophys* **268:** 525–535, 1989.
53. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34:** 3337–3345, 1985.
54. Sassa S, Sugita O, Ohnuma N, Imajo S, Okumura T, Noguchi T and Kappas A, Studies of the influence of chlorosubstituent sites and conformational energy in polychlorinated biphenyls on uroporphyrin formation in chick-embryo liver cell cultures. *Biochem J* **235:** 291–296, 1986.
55. Sinclair PR, Bement WJ, Bonkovsky HL, Lambrecht RW, Frezza JE, Sinclair JF, Urquhart AJ and Elder GH, Uroporphyrin accumulation produced by halogenated biphenyls in chick embryo hepatocytes: Reversal of the accumulation by piperonyl butoxide. *Biochem J* **237:** 63–71, 1986.
56. Hahn ME and Chandran K, 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. *Arch Biochem Biophys* **329:** 163–174, 1996.
57. Kloepper-Sams PJ and Stegeman JJ, Turnover of hepatic microsomal cytochrome P4501A protein and heme in β-naphthoflavone-induced *Fundulus heteroclitus*. *Mol Mar Biol Biotech* **3:** 171–183, 1994.
58. Greim H, Schenkman JB, Klotzbucher M and Remmer H, The influence of phenobarbital on the turnover of hepatic microsomal cytochrome b_5 and cytochrome P450 hemes in the rat. *Biochim Biophys Acta* **201:** 20–25, 1970.
59. Kuriyama Y, Omura T, Siekevitz P and Palade GE, Effects of phenobarbital on the synthesis and degradation of the protein components of rat liver microsomal membranes. *J Biol Chem* **244:** 2017–2026, 1969.
60. Karuzina II and Archakov AI, The oxidative inactivation of cytochrome P450 in monooxygenase reactions. *Free Radic Biol Med* **16:** 73–97, 1994.
61. Prince R, Comparisons of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on chemically impacted and nonimpacted subpopulations of *Fundulus heteroclitus*. Ph.D. Thesis, Rutgers/Robert Wood Johnson Medical School, Piscataway, NJ, 1993.