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ELEVATION OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD) POLYCHLORINATED BIPHENYLS

STRUCTURE–ACTIVITY RELATIONSHIPS

MARY ANNE DENOMME,* BRIAN LEECE,* ANGELA LI,* RHEAL TOWNER* and STEPHEN SAFE†‡

* Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ontario, Canada, N1G 2W1; and † Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX 77843, U.S.A.

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Abstract—Administration of the commercial polychlorinated biphenyl (PCB) Aroclor 1254 to immature male Wistar rats resulted in increased levels (80–110%) of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) hepatic cytosolic receptor protein which remained elevated for 14 days. The effects of structure on the activity of individual PCB congeners to modulate hepatic cytosolic receptor levels were compared to the structure–activity relationships (SARs) which have been developed previously for PCBs as inducers of hepatic microsomal monooxygenases. 3,3',4,4'-Tetra- and 3,3',4,4',5-pentachlorobiphenyl induced the cytochrome P-448-dependent monooxygenase, ethoxyresorufin *O*-deethylase (EROD), and resembled 3-methylcholanthrene in their mode of monooxygenase enzyme induction. These congeners also bound to the receptor protein; however, neither compound increased hepatic cytosolic receptor protein levels. Several PCB congeners which exhibit low binding affinities for the cytosolic receptor protein resembled phenobarbitone (PB) in their mode of monooxygenase enzyme induction and, like PB, elevated cytosolic receptor protein levels. Nevertheless, a comparison of the time course of monooxygenase enzyme induction and receptor protein elevation by 2,2',4,4',5,5'-hexachlorobiphenyl and PB illustrated significant differences in their activities. PB-mediated elevation of receptor levels was maximized 24 hr after the last dose, and 48 hr later the receptor levels decreased to control values. In contrast, 5 days after administration of a single dose of 2,2',4,4',5,5'-hexachlorobiphenyl (300 μmoles/kg) the receptor levels were elevated significantly, and these increased levels (205–127% increases over control) persisted for 14 days. There was no correlation between increased levels of hepatic receptor protein and the induction of the cytochrome P-450-dependent monooxygenases, aldrin epoxidase or 4-dimethylaminoantipyrine *N*-demethylase. Two PCBs, 2,3,3',4,4',5- and 2,2',3,4,4',5-hexachlorobiphenyl, which resembled Aroclor 1254 in their mode of monooxygenase enzyme induction, also elevated hepatic receptor protein levels but were less active than the PB-type inducers. Thus, the SARs developed for PCBs which elevate cytosolic receptor levels demonstrate that the most active compounds exhibit the lowest affinity for the receptor protein and do not induce EROD. In contrast, the more toxic PCB congeners which are approximate isostereomers of 2,3,7,8-TCDD both induced EROD and bound with high affinity to the receptor protein but did not increase hepatic cytosolic receptor protein levels.

Genetic studies with inbred strains of mice have demonstrated that some strains, typified by C57BL/6 mice, are responsive to the monooxygenase enzyme (e.g. aryl hydrocarbon hydroxylase, AHH) induction effects of 3-methylcholanthrene (MC) and related aryl hydrocarbons. In contrast, other strains, typified by DBA/2 mice, are non-responsive to the induction effects of these compounds [1–6]. Comparable differences in responsiveness to aryl hydrocarbons have also been observed using primary and fetal mammalian cells in culture [7, 8]. Responsiveness has been associated with the Ah locus, and genetic inbreeding studies between C57BL/6 and DBA/2 mice indicate autosomal dominant expression of responsiveness in the offspring [1–6]. Like the aryl hydrocarbons, the activities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related toxic halogenated aryl hydrocarbons also segregrate with the Ah locus [9–13]. However, in contrast to MC, 2,3,7,8-TCDD also induces AHH in the non-responsive mice at dose levels ten to twenty times higher than the dose required for the responsive mice.

The discovery of the potent AHH inducer, 2,3,7,8-TCDD, and the subsequent preparation of [^{3}H]-2,3,7,8-TCDD, have lead to the identification of the Ah regulatory gene product, namely the murine Ah receptor protein [14–22]. This receptor protein has been identified in the hepatic and extrahepatic tissues of several animal species and in mammalian cells in culture, and the levels are variable but generally less than 80 fmoles/mg cytosolic protein. Recent studies [23] have demonstrated that radiolabeled MC, benzo[*a*]pyrene and dibenzo[*a*,*h*]anthracene also bind to the receptor protein, and competition experiments with radiolabeled and cold ligands suggest that 2,3,7,8-TCDD and the aryl hydrocarbons compete for the same protein binding site. A recent paper [24] reported that administration of phenobarbitone (PB) to Sprague–Dawley rats and C57BL/6J mice significantly increases hepatic cytosolic receptor levels, whereas administration of PB does not

‡ Author to whom all correspondence should be addressed.

increase hepatic receptor levels in non-responsive DBA/2J mice. Previous studies in several laboratories have demonstrated the remarkable effects of structure on the biologic and toxic effects of polychlorinated biphenyl (PCB) isomers and congeners [13, 25–29]. This report investigates the activity of PCBs as modulators of hepatic cytosolic receptor protein levels in male Wistar rats and assesses any possible structure–activity relationships (SARs) for this type of activity.

MATERIALS AND METHODS

Chemicals and biochemicals. Aroclor 1254 was obtained from the Monsanto Chemical Co., St. Louis, MO. The synthesis of 3,3′,4,4′-tetrachlorobiphenyl (TCB), 3,3′,4,4′,5-pentachlorobiphenyl (PeCB), 2,2′,4,4′,5,5′-, 2,3′,4,4′,5′,6-, 2,3,3′,4,4′,5-, 2,2′,3,4,4′,5-, 2,2′,4,4′,6,6′- and 2,2′,4,4′,5,6′-hexachlorobiphenyl (HCB), and 2,2′,3,4,4′,6,6′-heptachlorobiphenyl (HpCB) have been described previously [24–31]; the purity of all the congeners was > 98% as determined by gas chromatographic analysis using electron-capture detection. NADP, glucose-6-phosphate, NADPH and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co., St. Louis, MO; 4-dimethylaminoantipyrine was purchased from the Aldrich Chemical Co., Milwaukee, WI; ethoxyresorufin was synthesized by T. Sawyer. [^{3}H]-2,3,7,8-TCDD (sp. act. 50 Ci/mmole) was purchased from KOR Isotopes, Cambridge, MA, and was > 95% pure after clean up by thin-layer chromatography.

Animal treatment and isolation of microsomes. Immature male Wistar rats (average weight, 100 g) were housed in wire cages and allowed free access to Purina certified rodent chow, No. 5002, and water. PB (500 μmoles/kg) was administered as the sodium salt by intraperitoneal injection on days 1, 2 and 3; the PCB congeners, in corn oil (0.5 ml), were administered on day 1 to the rats at the dose levels indicated in Table 1. Preliminary experiments indicated that there was considerable variation in the (corn oil) control levels of hepatic cytosolic receptor protein, and each set of experiments with the PCB congeners and PB at the different time points was accompanied by a corn oil treated group (N = 4) of rats. Animals were fasted for 24 hr prior to being killed by cervical dislocation. The livers were immediately perfused by the hepatic portal vein with ice-cold isotonic saline (25 ml) supplemented with EDTA (0.1 mM). Equal portions of the liver from each animal were used to prepare microsomes and a cytosolic fraction as described [32].

Assays. Protein concentrations and microsomal dimethylaminoantipyrene (DMAP) *N*-demethylase, aldrin epoxidase and ethoxyresorufin *O*-deethylase

Table 1. Effects of PB, Aroclor 1254 and several polychlorinated biphenyl congeners on hepatic cytosolic receptor levels and microsomal monooxygenase enzymes

Treatment	Day	Receptor levels (% of control)	DMAP *N*-demethylase (nmoles product/mg protein/min)	Aldrin epoxidase (nmoles product/mg protein/min)	EROD (nmoles product/mg protein/min)
Control		100 ± 14.9	8.08 ± 1.04	0.67 ± 0.05	0.36 ± 0.07
PB*	4	264.3 ± 56.7¶	16.8 ± 1.45¶	13.1 ± 0.59¶	0.82 ± 0.72
	6	154.3 ± 21.2**	13.8 ± 0.68¶	9.51 ± 1.36¶	0.80 ± 0.10
	8	128.8 ± 36.6	7.79 ± 1.22	4.74 ± 0.34¶	0.60 ± 0.10
2,2′,4,4′,5,5′-HCB†	4	162.0 ± 41.2¶	15.3 ± 1.39¶	6.22 ± 0.92¶	0.82 ± 0.15
	6	279.7 ± 104.0¶	15.7 ± 1.36¶	12.2 ± 1.09¶	1.03 ± 0.08¶
	8	305.4 ± 36.2¶	15.4 ± 1.16¶	19.8 ± 1.65¶	0.53 ± 0.34
	14	227.4 ± 52.5¶	17.0 ± 1.19¶	22.0 ± 2.67¶	0.65 ± 0.08
Aroclor 1254‡	3	156.9 ± 27.5¶	15.5 ± 1.0¶	1.87 ± 0.25	17.0 ± 0.63¶
	5	210.9 ± 58.8¶	15.1 ± 0.82¶	1.30 ± 0.40	17.7 ± 0.85¶
	7	190.2 ± 49.0¶	11.0 ± 0.48¶	2.07 ± 0.34	20.4 ± 0.64¶
	14	179.8 ± 22.2¶	15.9 ± 0.82¶	16.2 ± 1.58¶	41.2 ± 4.75¶
2,3′,4,4′,5′,6-HCB†	3	100.8 ± 7.7	9.74 ± 0.25**	4.62 ± 0.79¶	3.00 ± 0.56¶
	5	97.9 ± 20.9	11.1 ± 0.97¶	3.33 ± 0.25¶	1.50 ± 0.45
	7	114.1 ± 13.6	10.8 ± 0.76¶	6.87 ± 0.45¶	6.34 ± 0.78¶
2,3,3′,4,4′,5-HCB†	3	252.6 ± 18.6¶	11.5 ± 0.72¶	3.27 ± 0.57¶	40.8 ± 1.79¶
	5	136.7 ± 35.2**	13.6 ± 0.89¶	5.42 ± 0.64¶	61.8 ± 2.94¶
	7	130.2 ± 26.7	12.0 ± 1.01¶	3.00 ± 0.82¶	48.8 ± 12.1¶
3,3′,4,4′,5-PeCB§	3	142.5 ± 39.7	8.66 ± 0.86	1.52 ± 0.05¶	31.4 ± 5.34¶
	5	107.6 ± 17.3	9.22 ± 0.46	0.82 ± 0.14	38.5 ± 3.09¶
	7	154.6 ± 32.7**	8.03 ± 1.26	0.56 ± 0.13	42.6 ± 2.06¶
2,2′,3,4,4′,5-HCB†	7	252.9 ± 35.7¶	15.6 ± 0.67¶	17.6 ± 2.27¶	39.03 ± 11.1¶
2,2′,4,4′,6,6′-HCB†	7	192.2 ± 23.4¶	11.7 ± 0.79¶	2.58 ± 0.35	4.25 ± 0.34¶
2,2′,4,4′,5,6′-HCB†	7	192.2 ± 28.0¶	10.4 ± 1.33¶	4.32 ± 1.52¶	3.93 ± 0.75¶
2,2′,3,4,4′,6,6′-HpCB†	7	146.5 ± 12.9¶	11.8 ± 0.64¶	3.54 ± 0.34**	3.22 ± 0.58¶
3,3′,4,4′-TCB‖	7	89.5 ± 16.3	8.5 ± 0.73	1.775 ± 0.69¶	10.05 ± 0.09¶

*–‖ Values are means ± S.D.; there were four animals per treatment group. Doses: * 500 μmoles/kg; † 300 μmoles/kg; ‡ 600 μmoles/kg; § 0.5 μmoles/kg; and ‖ 250 μmoles/kg.

¶, ** Different from the controls: ¶$P < 0.01$ and **$P < 0.05$.

(EROD) were determined as described [32]. The cytosol receptor binding assay was performed as previously described [33]; 1 ml of hepatic cytosol (5–6 mg protein) was incubated for 1 hr at 0° with a large excess of [^{3}H]-2,3,7,8-TCDD (10 nM solution). The excess unbound material was removed by dextran/charcoal, and the bound [^{3}H]-2,3,7,8-TCDD was further fractionated by sucrose-density gradient centrifugation as previously described [33]. A parallel experiment in which the incubation mixture also contained 100 μM unlabeled 2,3,7,8-TCDD was carried out. The amount of specifically bound receptor–ligand complex was determined by the difference in the total amount of radioactivity in the binding peak (fractions 16–22) after incubation only with [^{3}H]-2,3,7,8-TCDD minus the amount of radioactivity which occurs in this peak after competition with excess cold 2,3,7,8-TCDD [33]. K_D values were determined using the hydroxylapatite receptor assay as described [34]; the only major change in the assay was the use of Triton X-100 as the detergent. The K_D values were obtained from the slopes of the lines derived from plotting the saturation isotherm using various concentrations of [^{3}H]-2,3,7,8-TCDD and cytosolic protein from untreated and 2,2',4,4',5,5'-hexachlorobiphenyl-treated rats (7 days after the initial exposure to the PCB).

The statistical differences between the means of the experimental and the control data were determined using the method described by Dunnett [35].

RESULTS

Effects of PB and PCB congeners on cytosolic receptor protein elevation. Table 1 and Fig. 1 summarize the effects of PB and several PCB congeners as modulators of cytosolic receptor protein levels and as inducers of cytochrome P-450-dependent monooxygenases. The doses of PB and the PCB congeners were derived from previous studies and for most compounds were sufficient to induce hepatic microsomal monooxygenase enzymes. After administration of PB (500 μmoles/kg) on 3 consecutive days (days 1–3 inclusive) the elevation of hepatic cytosolic receptor protein levels was determined on days 4, 6 and 8. There was a significant increase in receptor levels 24 hr after administration of the third dose of PB, and these levels rapidly decreased with time (Fig. 1). Moreover, the decrease in receptor levels was paralleled by a decrease in the hepatic microsomal DMAP *N*-demethylase and aldrin epoxidase enzyme activities. Figure 1 also illustrates the time-dependent elevation of hepatic cytosolic receptor levels by 2,2',4,4',5,5'-hexachlorobiphenyl, Aroclor 1254, 2,3',4,4',5',6-HCB, 2,3,3',4,4',5-HCB and 3,3',4,4',5-PeCB. Aroclor 1254 and 2,3,3',4,4',5-HCB both moderately elevated hepatic receptor protein levels, and the commercial mixture was the more potent of the two PCBs. Both compounds also induced DMAP *N*-demethylase, EROD, and aldrin epoxidase, and the enzyme induction results were consistent with a mixed-type induction pattern as previously reported [13, 28–30]. It was also evident that there was no apparent correlation between enzyme induction and the elevation of receptor protein levels. Administration of 2,2',3,4,4',5-HCB, a diortho-substituted PCB also elevated monooxygenase enzyme and receptor levels. 2,2',4,4',5,5'-HCB elevated cytosolic receptor levels in a time-dependent fashion with the maximum level (205% increase over control) observed 7 days after administration of the compound (Fig. 2). The time course of 2,2',4,4',5,5'-HCB monooxygenase enzyme induction did not par-

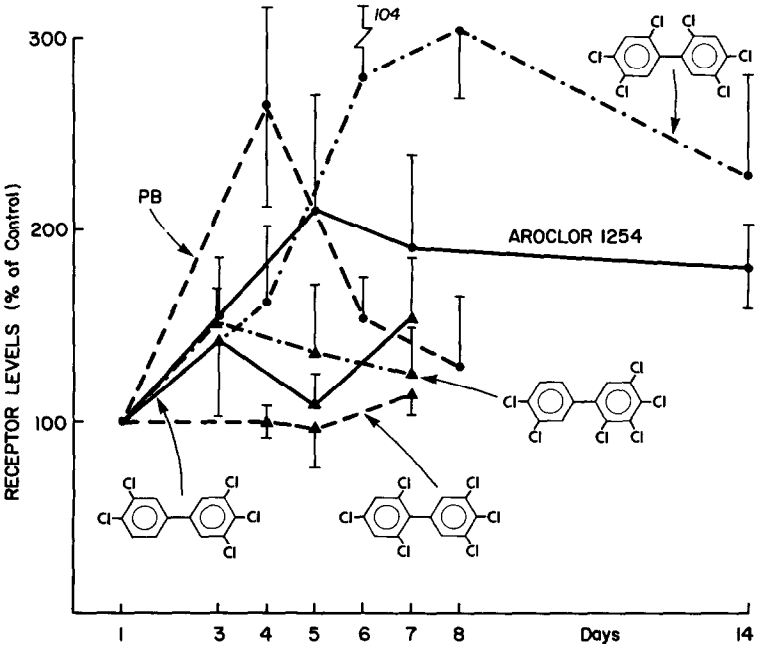

Fig. 1. Time-course modulation of hepatic cytosolic receptor levels by PB, Aroclor 1254 and several PCB congeners.

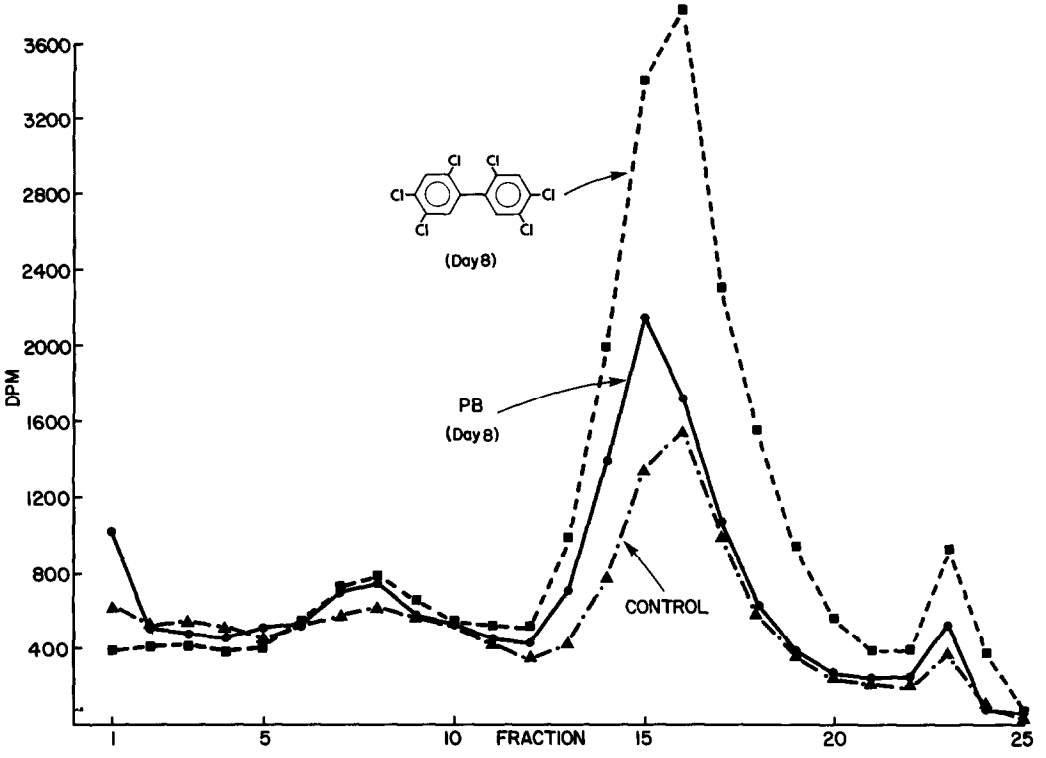

Fig. 2. Fractionation of [^{3}H]-2,2,7,8-TCDD : hepatic cytosolic receptor protein complexes after sucrose density gradient separation of the proteins. The cytosolic fractions were obtained from immature male Wistar rats 8 days after pretreatment with a single dose of corn oil (0.5 ml) and 2,2',4,4',5,5'-hexachlorobiphenyl (300 μmoles/kg) and 5 days after administration of three daily doses of PB (500 μmoles/kg). Note: the increase in the hepatic cytosolic receptor levels in the PB-treated rats was not significant.

allel the receptor elevation data; DMAP *N*-demethylase was maximally induced from days 4 to 14, aldrin epoxidase increased throughout the day 4–14 time period, and EROD was not induced by this compound. Several other PCB congeners (i.e. 2,2',4,4',6,6'-HCB, 2,2',4,4',5,6-HCB and 2,2',3,4,4',6,6'-HpCB) which resemble PB and 2,2',4,4',5,5'-HCB in their mode of microsomal monooxygenase enzyme induction [13, 25–30, 32] also elevated (50–100% increase over control) the cytosolic receptor protein levels 7 days after treating the animals. Neither 3,3',4,4'-TCB nor 3,3',4,4',5,5'-HCB, two ligands with high affinity for the receptor protein [33], significantly elevated cytosolic receptor protein levels. Administration of 300 μmoles/kg of 2,3',4,4',5',6-HCB resulted in the induction of aldrin epoxidase and small increases in DMAP *N*-demethylase and EROD but did not increase cytosolic receptor levels 3, 5 or 7 days after treatment with this chemical. Scatchard plot analysis of cytosol from untreated and 2,2',4,4',5,5'-HCB-treated rats gave K_D values of 0.72 and 0.64 nM respectively.

DISCUSSION

Okey and Vella [24] have reported that administration of PB (25–100 mg/kg) for 3 consecutive days results in the induction of the hepatic cytosolic receptor protein in Sprague–Dawley rats and C57BL/6J mice using both [^{3}H]-MC and [^{3}H]-2,3,7,8-TCDD as radioligands. This effect was also observed in the male Wistar rat, and a time–course study (Fig. 1) clearly demonstrated the rapid decline of the elevated receptor levels between 24 and 48 hr after PB treatment was terminated. This was also accompanied by a parallel decrease in microsomal DMAP *N*-demethylase and aldrin epoxidase enzyme activities. These observations are consistent with the rapid metabolically-mediated clearance of PB from the liver.

Several studies have demonstrated the SARs for PCBs as inducers of hepatic microsomal monooxygenases and cytochrome P-450 isozymes in rats [13, 25–30, 32]; PCBs substituted in both *para* and two or more *meta* positions resembled MC or 2,3,7,8-TCDD in their mode of induction of monooxygenase enzymes and cytochrome P-450 isozymes; a second group of PCB congeners and many of the commercial mixtures have been classified as mixed-type inducers and resemble PB plus MC (coadministered) in their monooxygenase enzyme and cytochrome P-450 isozyme induction characteristics. Several other PCBs resemble PB in their mode of monooxygenase enzyme induction or are inactive as inducers of this enzyme system [13]. The results summarized in Table 1 and Fig. 1 are consistent with these monooxygenase enzyme induction SARs and also illustrate a structure-dependent elevation of the cytosolic receptor

protein by PCB isomers and congeners. 3,3',4,4',5-PeCB and 3,3',4,4'-TCB are both ligands for the cytosolic receptor protein and induced the cytochrome P-448-dependent monooxygenase enzyme, EROD. However, these congeners did not elevate cytosolic receptor protein levels. Aroclor 1254 and 2,3,3',4,4',5-HCB induced DMAP *N*-demethylase, aldrin epoxidase and EROD 3, 5 and 7 days after administration of a single dose of these compounds. Although both PCBs induced the same monooxygenase enzymes, there were significant differences in the time–course and extent of induction of these enzymes. Time–course differences were also noted for the elevation of hepatic levels; treatment of the rats with Aroclor 1254 resulted in a modest (210–179% of control) but persistent elevation of hepatic receptor protein levels; however, since Aroclor 1254 is a mixture of PCB isomers and congeners, it is not possible to determine which group of PCBs present in the mixture were responsible for modulating receptor levels. 2,3,3',4,4',5-HCB has also been characterized previously as a mixed-type inducer [28–30]; however, in contrast to Aroclor 1254, this compound elevated hepatic cytosolic receptor protein levels on day 3 but these levels rapidly returned to control values. This result cannot be due to pharmacokinetic factors since the monooxygenases remained induced for 7 days; moreover, 2,3,3',4,4',5-HCB is a highly tissue-persistent PCB congener [27]. 2,2',3,4,4',5-HCB, a diortho-substituted congener which exhibits mixed-type monooxygenase enzyme induction activity [35] also induced the hepatic levels of the receptor protein.

Table 1 and Fig. 1 summarize the time-dependent elevation of hepatic cytosolic receptor protein levels 4, 6, 8 and 14 days after administration of a single dose (300 μmoles/kg) of 2,2',4,4',5,5'-HCB. There was a peak of receptor protein elevation on day 7 (> 200% above control rat levels); however, significantly increased receptor levels were also detected on day 14. DMAP *N*-demethylase was fully induced throughout the 14-day period, whereas aldrin epoxidase activity gradually increased throughout the duration of the time–course study. At dose levels of 300 μmoles/kg, several other PB-type monooxygenase enzyme inducers including 2,2',4,4',6,6'-HCB, 2,2',4,4',5,6'-HCB and 2,2',3,4,4',6,6'-HpCB also elevated hepatic cytosolic receptor protein levels. 2,3',4,4',5',6-HCB, a relatively poor monooxygenase enzyme inducer [28], did not significantly alter hepatic receptor protein levels.

These studies confirm that administration of PCB mixtures and individual PCB congeners elevates cytosolic receptor protein levels in immature male Wistar rats. The 3,3',4,4'- and 3,3',4,4',5-substituted congeners are approximate isostereomers of 2,3,7,8-TCDD, bind with high affinity to the receptor protein in competitive binding assays [33], induce EROD, but do not increase hepatic receptor protein concentrations. In contrast, several PCBs which exhibit mixed or PB-type monooxygenase enzyme induction characteristics elevated hepatic receptor protein levels. Although PB and all the active PCB modulators of the receptor concentration were all inducers of the cytochrome P-450-dependent monooxygenases, DMAP *N*-demethylase and aldrin epoxidase, there was no apparent correlation between enzyme induction and increases in hepatic receptor protein levels. This was particularly evident in the time–course studies. The SARs for PCBs demonstrated that 2,2',4,4',5,5'-HCB, a major component of Aroclor 1254, and several other PB-type monooxygenase enzyme inducers increased hepatic cytosolic receptor protein levels, and these compounds probably contributed to the persistent elevation of the receptor by Aroclor 1254. It is also important to note that the K_D values obtained from control and 2,2',4,4',5,5'-HCB-treated animals were similar (0.72 and 0.64 nM respectively).

The biologic and toxic significance of the modulation of cytosolic receptor protein levels is not known. Previous studies in which MC or 3,3',4,4'-tetrachlorobiphenyl has been coadministered with PB or 2,2',4,4'-tetrachlorobiphenyl (note: this compound also enhances cytosolic receptor protein levels) do not indicate that the elevation of cytosolic receptor levels influences the extent of cytochrome P-448-dependent monooxygenase enzyme induction. However, most of these experiments have been carried out by administering chemical dosages which elicited maximum enzyme induction activities. The interactive effects of PCBs and other toxic halogenated aryl hydrocarbons at submaximal dosage levels are unknown and are currently being investigated in our laboratory.

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