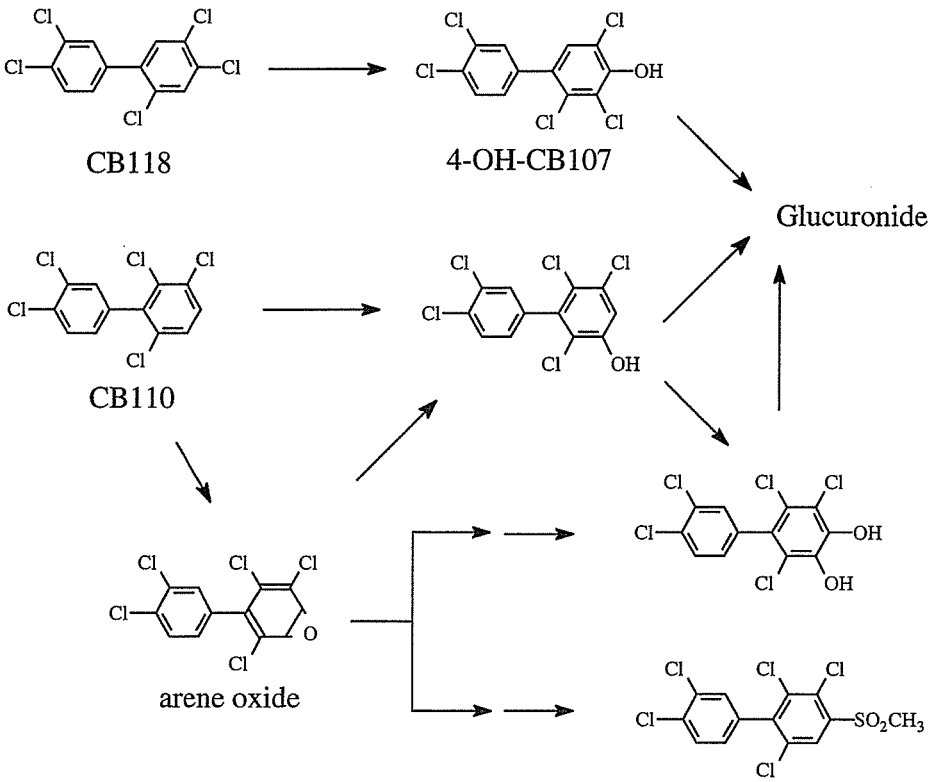
Scheme 1. Possible Formation Pathways of Hydroxylated or Catechol Metabolites from CB118 and CB110



be altered by the activities of UGT1A or UGT2B rather than other such phase II enzyme activities. Despite the absence of glucuronidation of bilirubin as an excretory pathway, Gunn rats excrete bilirubin by alternative routes, probably as the thioether glutathione conjugates (*40*). The decreased $MeSO_2$-CB levels in Gunn rats may be due to the depletion of glutathione, which could hardly lead PCBs to the glutathione conjugation pathway for $MeSO_2$-CBs.

A number of phenolic PCBs that persist in mammals and human blood have a hydroxy group in the *meta*- or *para*-position flanked with one or two chlorine atoms (*41*). The precursors of hydroxylated PCBs characterized in the present study are likely to be 2,4,2′,4′,5′-pentaCB (CB99), CB118, and 2,3,4,2′,4′,5′-hexaCB (CB138), all of which are major components in KC500 and have 2,4,5-chlorine substitution in the molecule. The major metabolite 4-OH-CB107, which was expected from CB105 and/or CB118 via NIH chlorine shift, was retained selectively and to a similar extent in the serum of both rat strains (Table 3), indicating that serum retention of 4-OH-CB107 would not be affected by UGT activities. This finding may support the observation that some persistent phenolic PCBs are poor substrates for UGTs and resist conjugation (*4*). The blood affinity of 4-OH-CB107 could be explained by the structural similarity of the metabolite with T4 to bind to TTR with high affinity (*41*). Although the retention mechanism of catechols in Gunn rats is not obvious, we hypothesize that the catechol is hardly glucuronidated because of poor UGT activities in Gunn rats and alternately bind to TTR (*14*) or to estrogen receptors with high affinity (*42*). In addition, KC500-treated Gunn rats showed reduced serum T4 levels without induction of UGTs (*25*), suggesting that thyroid hormone alterations may be associated with persistence of phenolic PCBs including catechols. In both rat strains, on the other hand, *meta*-$MeSO_2$-CBs were formed from 2,5-chlorinated homologues, whereas *para*-$MeSO_2$-CBs were formed selectively from 2,3,6-chlorinated homologues (*38*). Although the distribution levels of $MeSO_2$-CBs were largely different between both strains, the congener profiles seem not to be altered by UGTs.

Recently, Garner et al. (*18*) have demonstrated that the catechols from lower chlorinated biphenyls have estrogenic activity in vitro, and the activity of 3,4-catechols increases with the degree of chlorination. They also reported that further oxidative metabolism of estrogenic phenolic PCBs would not necessarily result in lowering the total estrogenic burden of a PCB-exposed organism. As catecol PCBs are known to be potent inhibitors of catechol estrogen-O-methylation (*42*), the catechol PCBs detected in the present study may also alter the metabolism of catechol estrogen.

In conclusion, UGT deficient Gunn rats effectively metabolized PCBs with 2,5- or 2,5,6-chlorine substitution to the 3,4-catechols, but less formed $MeSO_2$-CBs in the liver. The higher concentration of the catechols in Gunn rats could be due to reduced elimination of the 3,4-catechol and suggests that conjugation of catechol PCBs may be restricted by UGT deficiency in Gunn rats. In addition, the catechol PCBs are likely to have blood affinity similar to 4-OH-CB107. The persistence of catechol PCBs in UGT deficient organisms may have implications for increased toxicity resulting from exposure to PCBs.

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A Possible Mechanism for Decrease in Serum Thyroxine Level by Polychlorinated Biphenyls in Wistar and Gunn Rats

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We have previously demonstrated that in mice, the decrease in serum thyroxine (T_4) level by polychlorinated biphenyls (PCBs) occurs without an increase in the UDP-glucuronosyltransferase (T_4-UDP-GT) for T_4 glucuronidation, although the PCB-induced decrease in rats is generally thought to occur through induction of T_4-UDP-GT, UGT1A1, and UGT1A6. In the present study, to further clarify the relationship between the decrease in serum T_4 level and the increase in UGT1A activity by PCB in rats, we examined the relationship using Wistar rats and Gunn rats, a mutant strain of Wistar rats deficient in UGT1A isoforms. The serum total T_4 level was markedly decreased not only in the Wistar rats but also in the Gunn rats 4 days after treatment with a PCB, Kanechlor-500 (KC500, 100 mg/kg) or 2,2′,4,5,5′-pentachlorobiphenyl (PentaCB, 112 mg/kg), and there was no significant difference in magnitude of the decrease between the two rat strains. At the same time, the level and activity of T_4-UDP-GT were significantly increased by treatment with either KC500 or PentaCB in Wistar rats but not in Gunn rats. In addition, no significant change in the level of serum total triiodothyronine (T_3) and thyroid-stimulating hormone by the KC500 treatment was observed in either Wistar or Gunn rats. Furthermore, significant decrease in the activity of hepatic type-I deiodinase, which mediates the deiodization of T_4 and T_3, by treatment with KC500 or PentaCB was observed in both Wistar and Gunn rats. From the serum of KC500- or PentaCB-treated Wistar and Gunn rats, mono- and di-hydroxylated PCB metabolites, which would bind to T_4 binding serum protein (transthyretin), were detected. In conclusion, the present results suggest that the decrease in serum total T_4 level by either KC500 or PentaCB in Gunn rats was not dependent on the increase in hepatic T_4-UDP-GT activity. The findings further suggest that the PCB-mediated decrease in serum T_4 level might occur, at least in part, through formation of the hydroxylated PCB metabolites. Furthermore, even in Wistar rats, the PCB-mediated decrease in serum T_4 level might occur not only through the increase in hepatic T_4-UDP-GT but also via formation of hydroxylated PCB metabolites.

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Key Words: polychlorinated biphenyls; Kanechlor-500; thyroid hormones; UDP-glucuronosyltransferases; Wistar rats; Gunn rats

INTRODUCTION

Most polychlorinated biphenyl (PCB) congeners are known to decrease the levels of serum thyroid hormone and to increase the activities of hepatic drug-metabolizing enzymes in rats (Craft *et al.*, 2002; Li *et al.*, 2001; Ness *et al.*, 1993; Van Birgelen *et al.*, 1995). Among the possible mechanisms for the PCB-mediated decrease in level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PCBs and displacement of the hormone from serum transport proteins [transthyretin (TTR)] are considered (Barter and Klaassen, 1992, 1994; Brouwer *et al.*, 1998). In particular, the decrease in the level of serum thyroxine (T_4) by 3,3′,4,4′,5-pentachlorobiphenyl, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats is thought to occur mainly through the induction of the UDP-glucuronosyltransferase (T_4-UDP-GT) responsible for glucuronidation of T_4 (Barter and Klaassen, 1994; Schuur *et al.*, 1997; Van Birgelen *et al.*, 1995). This hypothesis is supported by previous reports that a number of T_4-UDP-GT inducers, such as phenobarbital, 3-methylcholanthrene, and pregnenolone-16α-carbonitrile, show ability to decrease serum thyroid hormone (Barter and Klaassen, 1994; De Sandro *et al.*, 1992; Saito *et al.*, 1991). However, the magnitude of decrease in the level of serum total T_4 is not necessarily correlated with that of increase in T_4-UDP-GT activity (Craft *et al.*, 2002; De Sandro *et al.*, 1992; Hood *et al.*, 2003). Recently, we have found that treatment with Kanechlor-500 (KC500) resulted in a significant decrease in the serum T_4 level in both rats and mice, although a significant increase in T_4-UDP-GT activity occurred only in rats but not in mice (Kato *et al.*, 2003).

In the present study, therefore, we examined a relationship between the decrease in serum total T_4 level and the increase in

the hepatic T_4-UDP-GT (UGT1A1 and UGT1A6) by PCB using Wistar and UGT1A-deficient Wistar rats (Gunn rats). In this way, we demonstrated that the PCB-mediated decrease in serum total T_4 level in rats was not necessarily dependent on the increase in hepatic T_4-glucuronidation.

MATERIALS AND METHODS

Chemicals. 2,2′,4,5,5′-Pentachlorobiphenyl (PentaCB) was synthesized by using the Cadogan coupling reactions (Cadogan, 1962). Panacete 810 (medium-chain triglycerides) was purchased from Nippon Oils and Fats Co. Ltd. (Tokyo, Japan). The [^{125}I]-reverse T_3 and [^{125}I]T_4, radiolabeled at the 5′ position of the outer ring, was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA). All other chemicals were obtained commercially in appropriate grades of purity.

Animal treatments. Male Wistar rats (160–200 g) and UGT1A-deficient Wistar rats (Gunn rats, 190–260 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Male Wistar and Gunn rats were housed three or four per cage with free access to commercial chow and tap water, and were maintained on a 12-h dark/light cycle (8:00 a.m.–8:00 p.m. light) in an air-controlled room (temperature: 24.5 ± 1°C, humidity: 55 ± 5%), and were handled with humane care under the guidelines of the University of Shizuoka (Shizuoka, Japan). Treatments of rats with KC500 (100 mg/kg) and PentaCB (112 mg/kg) were performed according to the method of Kato *et al.* (2001, 2003). Briefly, the rats received a single ip injection of KC500 (100 mg/kg) or PentaCB (112 mg/kg) dissolved in Panacete 810 (5 ml/kg). Control animals were treated with vehicle alone (5 mg/kg).

Analysis of serum hormones. All rats were killed by decapitation on day 4 after the dosing, and the liver was removed. Blood was collected from each animal between 10:30 and 11:30 a.m. After clotting at room temperature, serum was separated by centrifugation and stored at −50°C until used. The levels of total T_4, total triiodothyronine (T_3), free T_4 and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using the T-4· and T-3· RIABEAD (DAINABOT Co., Ltd, Tokyo, Japan), free T_4 (Diagnostic Products Corporation; Los Angels, CA), and Biotrak rTSH [^{125}I] assay system (Amersham Life Science Ltd.; Little Chalfont, UK), respectively.

Hepatic microsomal UDP-GT and deiodinase assays. Hepatic microsomes were prepared according to the method of Kato *et al.* (1995). The amount of protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The activities of microsomal UDP-GT toward T_4 and chloramphenicol were determined by the methods of Barter and Klaassen (1992) and Ishii *et al.* (1994), respectively. All UDP-GT activities were measured after activation of the UDP-GTs by 0.05% Brij 58. The activity of hepatic microsomal type I outer-ring deiodinase was determined by the method of Hood and Klaassen (2000).

Western blot analysis. Polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and a specific antibody against UGT1A1, UGT1A6, or UGT2B1 were used (Ikushiro *et al.* 1995, 1997). Western analyses for microsomal UGT isoforms were performed by the method of Luquita *et al.* (2001). The detection of protein was performed using a chemical luminescence (ECL detection kit, Amersham Pharmacia Biotech), and the band intensity was quantified densitometrically with LAS-1000 (FUJIFILM, Japan).

Determination of hydroxylated PCB metabolites in the serum. The extraction and sample clean-up procedures for serum PCB metabolites were preformed by the method of Haraguchi *et al.* (1998). The identification of hydroxylated PCB metabolites was carried out on a GC/MS system (GC-17A, QP-5000, Shimadzu, Japan) with a DB-5 capillary column (60 m × 0.25 mm, i.d.). The temperature program was as follows: 100°C, 2 min, 100–250°C at 20°C/min, 250–280°C at 2°C/min (Mimura *et al.*, 1999). Quantification of the hydroxylated PCB metabolites was performed on GC/ECD (GC-14A, Shimadzu, Japan) by comparison with an internal standard of 2,2′,3,4′,5,5′,6-heptachloro-4-[^{13}C]biphenylol. The major hydroxylated PCB metabolites (>5 ng/g liver) were analyzed.

Statistics. The data obtained were statistically analyzed according to Dunnett's test after the analysis of variance (ANOVA).

RESULTS

Serum Hormone Levels

Serum constitutive levels of total T_4, free T_4, total T_3, and TSH were more than 1.5-fold higher in Gunn rats than in Wistar rats. The effects of KC500 and PentaCB on the concentration of serum thyroid hormones were next examined in Wistar and Gunn rats (Fig. 1). In both Wistar and Gunn rats, levels of serum total T_4 and free T_4 were significantly decreased by treatment with either KC500 or PentaCB, and the magnitude of the decrease was almost the same in the both rats. In contrast, no significant change in the level of serum total T_3 and TSH was observed in either Wistar or Gunn rats, with the exception of the slight decrease of serum total T_3 in PentaCB-treated Gunn rats.

Hepatic UDP-GT and Type-I Deiodinase Activities

It has been reported that T_4 glucuronidation is primarily mediated by the UGT1A enzymes UGT1A1 and UGT1A6 in the rat liver (Visser, 1996). Therefore, we examined the effects of KC500 and PentaCB on the hepatic T_4-UDP-GT activity in Wistar and Gunn rats. In addition, we examined whether Gunn rats show the response for the PCB-mediated induction of another UGT isoform, UGT2B1. Constitutive activity of the T_4-UDP-GT was more than 2.1-fold higher in Wistar rats than in Gunn rats. The activity of T_4-UDP-GT (UGT1A1 and UGT1A6) was significantly increased by either KC500 or PentaCB in Wistar rats but not in Gunn rats (Fig. 2). In contrast, treatment with each PCB resulted in a significant increase in the activity of UDP-GT (UGT2B1) toward chloramphenicol in both rats, although the increased level was more than 2.5-fold higher in Wistar rats than in Gunn rats.

Hepatic type-I T_4-deiodinase activity in Wistar rats was significantly decreased by KC500 but not by PentaCB, although in Gunn rats, it was significantly decreased by either PentaCB or KC500 (Fig. 3).

Immunoblot Analysis for UGT1As

The intensities of immunoreactive bands for hepatic UGT1A isoforms, such as UGT1A1 and UGT1A6, were increased by either KC500 or PentaCB in Wistar rats but not in Gunn rats (Figs. 4 and 5). In addition, no constitutive expression of the UGT1A isoforms was confirmed in Gunn rats. In contrast, the level of UGT2B1 was increased by either KC500 or PentaCB in both Wistar and Gunn rats, and the increased level was higher in Wistar rats than in Gunn rats (Figs. 4 and 5).

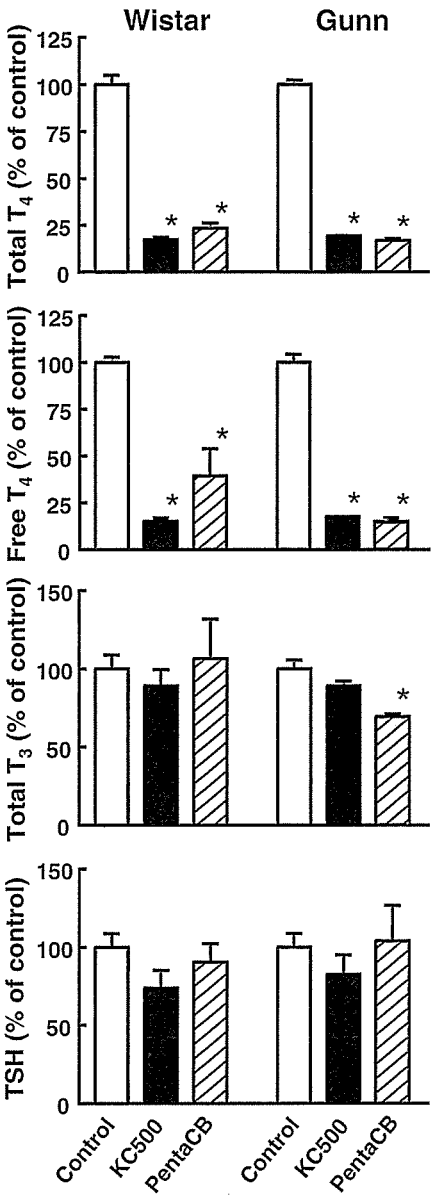

FIG. 1. Effects of KC500 and PentaCB on levels of serum total thyroxine (T_4), free T_4, total triiodothyronine (T_3), and thyroid-stimulating hormone (TSH) in Wistar and Gunn rats. Animals were killed 4 days after the administration of KC500 (100 mg/kg, ip) or PentaCB (112 mg/kg, ip), and levels of serum thyroid hormones were measured as described in Materials and Methods. Constitutive levels: total T_4 (μg/dl), 2.95 ± 0.15(Wistar) and 8.59 ± 0.21(Gunn); free T_4 (ng/dl), 1.47 ± 0.11(Wistar) and 2.26 ± 0.14(Gunn); total T_3 (ng/ml), 0.34 ± 0.03(Wistar) and 0.96 ± 0.05 (Gunn); TSH (ng/ml), 9.14 ± 0.79 (Wistar) and 20.85 ± 1.79(Gunn). Each column represents the mean ± SE (vertical bars) for three to eight animals. *$p < 0.01$, significantly different from each control.

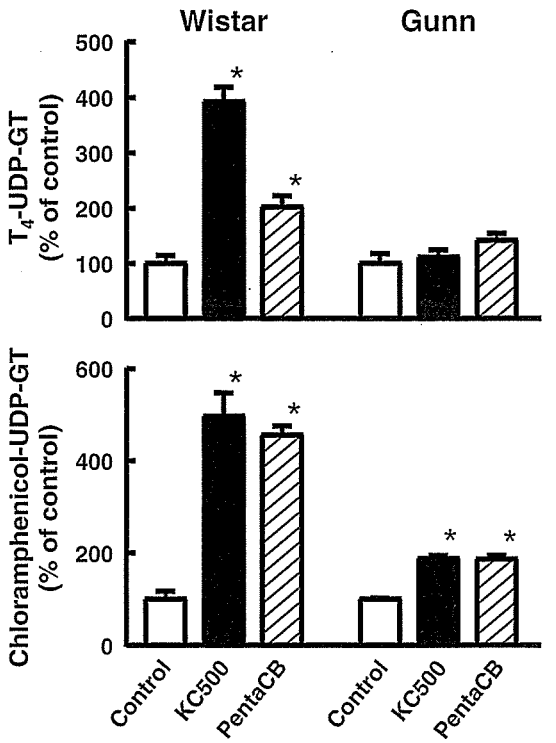

FIG. 2. Effects of KC500 and PentaCB on hepatic microsomal UDP-glucuronyltransferase activities in Wistar and Gunn rats. Each column represents the mean ± SE (vertical bars) for four to six animals. Constitutive levels: T_4-UDP-GT (pmol/mg protein/min), 12.60 ± 0.69 (Wistar) and 5.95 ± 1.06 (Gunn); chloramphenicol-UDP-GT (nmol/mg protein/min), 0.74 ± 0.13 (Wistar) and 0.76 ± 0.02 (Gunn). *$p < 0.05$, significantly different from each control.

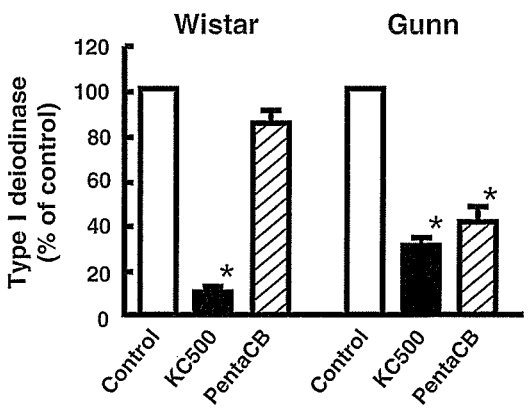

FIG. 3. Effects of KC500 and PentaCB on hepatic microsomal type-I deiodinase activity in Wistar and Gunn rats. Each column represents the mean ± SE (vertical bars) for four to six animals. *$p < 0.001$, significantly different from each control.

Hydroxylated PCB Metabolites in Serum

KC500 or PentaCB was administered to Wistar and Gunn rats, and 4 days after administration, hydroxylated PCB metabolites in each serum were analyzed (Table 1). In KC500-treated Wistar and Gunn rats, three mono-hydroxylated metabolites (3-OH-2,2′,4,4′,5-pentachlorobiphenyl, 4-OH-2,3,3′,4′,5-pentachlorobiphenyl, and 3′-OH-2,2′,3,4,4′,5′-hexachlorobiphenyl) and three dihydroxylated metabolites (3,4-$(OH)_2$-2,3′,4′,5-tetrachlorobiphenyl, 3′,4′-$(OH)_2$-PentaCB, and

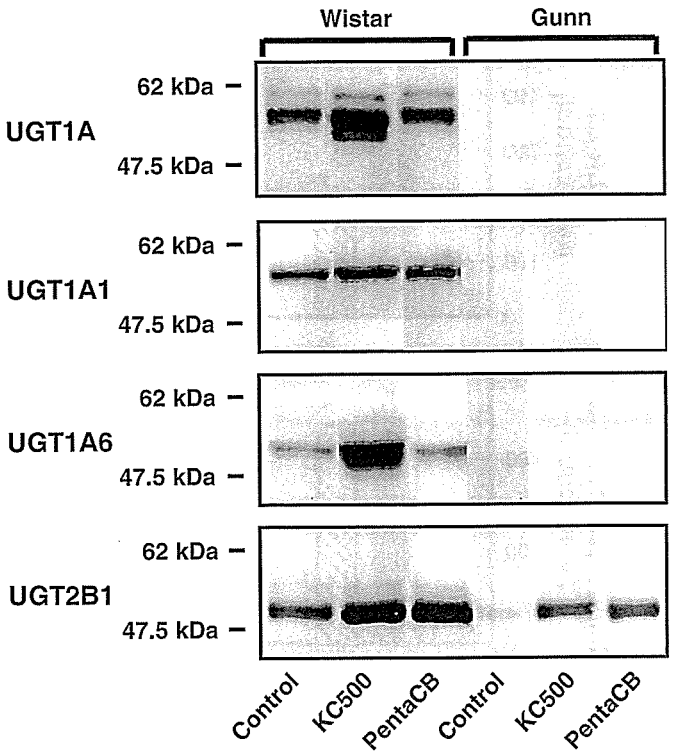

FIG. 4. Representative immunoblot patterns for hepatic microsomal UGT isoforms in KC500-treated or PentaCB-treated Wistar and Gunn rats.

3,4-$(OH)_2$-2,3′,4′, 5,6-pentachlorobiphenyl) were detected. 4-OH-2,3,3′,4′,5-pentachlorobiphenyl was a main hydroxylated metabolite, and the amounts in Wistar and Gunn rats were 89% and 56%, respectively, of the total hydroxylated metabolites detected. The sum of dihydroxylated metabolites, 3,4-$(OH)_2$-2,3′,4′,5-tetrachlorobiphenyl, 3′,4′-$(OH)_2$-PentaCB, and 3,4-$(OH)_2$-2,3′,4′,5,6-pentachlorobiphenyl was 37% of the total hydroxylated PCB metabolites detected in KC500-treated Gunn rats, whereas in KC500-treated Wistar rats, these dihydroxylated metabolites were hardly detected (Table 1). In addition, total amounts of the hydroxylated metabolites in Wistar and Gunn rats were almost the same.

In PentaCB-treated Wistar and Gunn rats, three monohydroxylated metabolites (3-OH-PentaCB, 3′-OH-PentaCB, and 4′-OH-PentaCB) and one dihydroxylated metabolite, 3′,4′-$(OH)_2$-PentaCB, were detected in the serum (Table 1). The relative levels of 3′,4′-$(OH)_2$-PentaCB to the total hydroxylated metabolites of PentaCB detected in Wistar and Gunn rats were almost the same (about 83% of total hydroxylated metabolites in the corresponding rats), although the absolute level of the dihydroxylated metabolite was 2.6-fold higher in Gunn rats than in Wistar rats. In addition, serum concentrations of PentaCB, which were determined according to the method for hydroxylated PCB metabolites, were 91.6 and 127.8 ng/ml in PentaCB-treated Wistar and Gunn rats, respectively. Namely, the serum concentrations of total OH-PentaCBs in PentaCB-treated Wistar

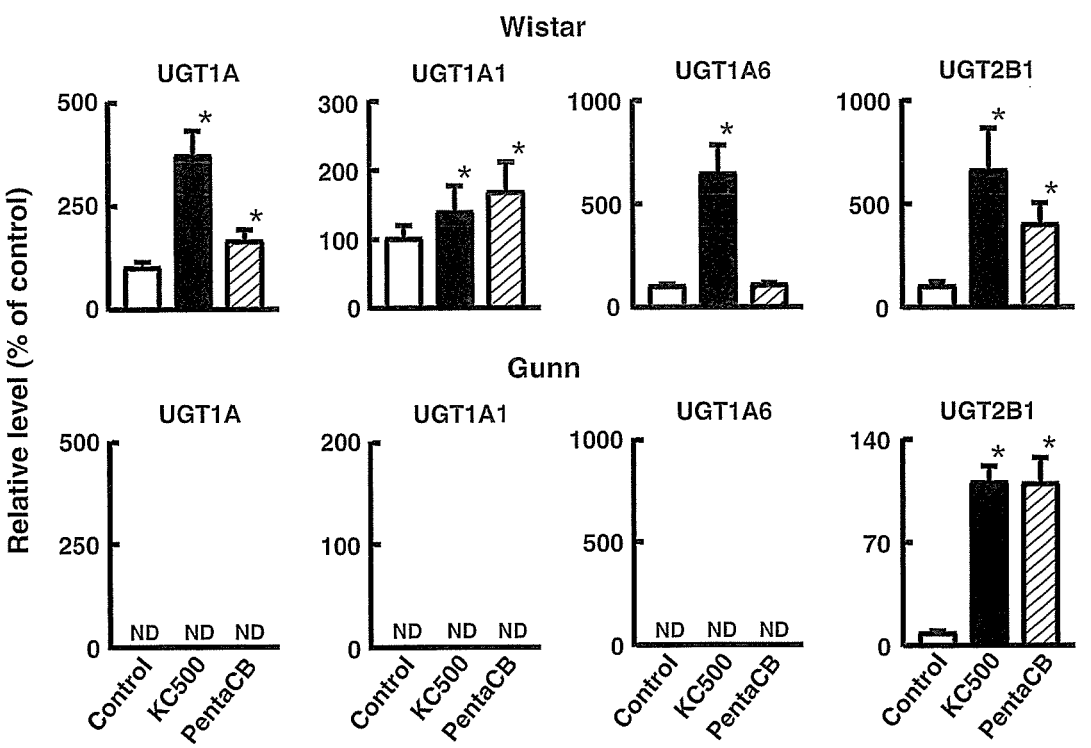

FIG. 5. Effects of KC500 and PentaCB on the level of hepatic microsomal UGT isoforms in Wistar and Gunn rats. After the immnoblot as shown in Figure 4, the isolated bands responsible for UGT isoforms were densitometrically quantified as described in Materials and Methods. The data are represented as the mean ± SE (vertical bars) for five to ten animals. *$p < 0.01$, significantly different from each control. ND: not detectable.

TABLE 1

Serum Concentrations of Hydroxylated PCB Metabolites After the Administration of KC500 or PentaCB to Wistar and Gunn Rats

Treatment	Metabolite	OH-PCB concentration (ng/ml serum)	
		Wistar	Gunn
KC500	3-OH-2,2′,4,4′,5-pentachlorobiphenyl	98.1 ± 12.0	13.9 ± 3.8
	4-OH-2,3,3′,4′,5-pentachlorobiphenyl	1206.3 ± 131.6	796.5 ± 34.9
	3′-OH-2,2′,3,4,4′,5′-hexachlorobiphenyl	45.3 ± 3.0	82.0 ± 6.6
	3,4-$(OH)_2$-2,3′,4′,5-tetrachlorobiphenyl	<5	109.1 ± 25.9
	3′,4′-$(OH)_2$-PentaCB	<5	108.9 ± 33.9
	3,4-$(OH)_2$-2,3′,4′,5,6-pentachlorobiphenyl	<5	313.3 ± 70.6
	Sum of OH-PCBs	1349.8 ± 145.9	1423.7 ± 147.5
PentaCB	3-OH-PentaCB	28.6 ± 9.5	17.1 ± 3.4
	3′-OH-PentaCB	10.9 ± 2.0	90.1 ± 17.5
	4′-OH-PentaCB	12.7 ± 4.4	12.1 ± 2.0
	3′,4′-$(OH)_2$-PentaCB	229.3 ± 20.3	611.8 ± 60.1
	Sum of OH-PentaCBs	281.5 ± 23.1	731.1 ± 68.8

Note.—The experimental conditions were the same as described in Figure 1. Results are expressed as mean ± SE for 3–6 animals.

and Gunn rats were 3.1 time higher and 5.7 times higher, respectively, than those of PentaCB in the corresponding rats.

DISCUSSION

In the present study, we found that treatment with either KC500 or PentaCB resulted in a drastic decrease in serum total T_4 and free T_4 levels in both Wistar and Gunn rats, although a significant increase in the activity of T_4-UDP-GT occurred only in Wistar rats. The present findings demonstrate that in Gunn rats, the PCB-mediated decrease in level of serum T_4 does not occur through induction hepatic T_4 glucuronidation enzymes. Although decreases in serum T_4 level by Aroclor 1254 in Gunn rats has been reported (Collins and Capen, 1980), the biochemical mechanism for the PCB-mediated decrease in serum T_4 has remained unclear. In addition, the decrease in serum T_4 level without any increase in T_4-UDP-GT activity has been reported in clofibrate-treated Gunn rats (Visser *et al.*, 1993).

In general, PCBs, including 3,3′,4,4′,5-pentachlorobiphenyl, 2,2′,4,4′,5,5′- hexachlorobiphenyl, and Aroclor 1254 have been thought to decrease the level of serum T_4 through increase in the activity of hepatic T_4-UDP-GT (Barter and Klaassen, 1994; Schuur *et al.*, 1997; Van Birgelen *et al.*, 1995). However, it has been reported that the difference between rats and mice in magnitude of decrease in level of serum total T_4 by 2,2′,4,4′,5,5′-hexachlorobiphenyl is not well correlated with that of increase in activity of T_4-UDP-GT (Craft *et al.*, 2002). Furthermore, we have found that KC500 resulted in a significant decrease in the serum T_4 level in both rats and mice, although a significant increase in T_4-UDP-GT activity occurred only in rats but not in mice (Kato *et al.*, 2003). In addition, a decrease in the serum level of total T_4 by PentaCB or 2,2′,3,3′,4,6′-hexachlorobiphenyl occurred in both rats and mice, although a significant change in activity of UDP-GT, specially UGT1A6, was hardly observed in the both species (Kato *et al.*, 2001). These previous reports strongly support the finding that the decrease in serum total T_4 level by PCB does not occur only through an increase in hepatic T_4-UDP-GT activity.

As possible mechanisms for the PCB-mediated decrease in serum T_4, changes in type-I deiodinase activity and serum TSH level might also be considered. However, no increase (significant decrease) in hepatic activity of microsomal type-I deiodinase, which mediates the deiodization of T_4 and T_3, was observed in either Wistar or Gunn rats. Similar results have been reported in previous study using Aroclor 1254-treated Sprague-Dawley rats (Hood and Klassen, 2000). Accordingly, a PCB-mediated decrease in serum T_4 level is thought to occur through a type-I deiodinase-independent pathway. Furthermore, the level of serum TSH in both Wistar and Gunn rats was not significantly changed by either KC500 or PentaCB, indicating that TSH is not related to the PCB-mediated decrease in the serum T_4 level. In addition, it had been reported that the serum TSH level was little affected by PCB (Hallgren *et al.*, 2001; Hood *et al.*, 1999; Liu *et al.*, 1995; Kato *et al.*, 2003).

As another possible mechanism, binding of hydroxylated PCBs to TTR, a major T_4-transporting protein, might be considered, (1) because hydroxylated PCB metabolites show the binding affinity for TTR (Brouwer *et al.*, 1998; Lans *et al.*, 1993) and (2) because the binding affinity of 4-OH-2,3,3′,4′,5-pentachlorobiphenyl, which was detected as a main hydroxylated metabolite in KC500-treated rats in the present experiments, is 3.3-fold higher than that of the natural ligand T_4 (Meerts *et al.*, 2002). The present findings and previous reports suggest that the decrease in the level of serum T_4 in either

KC500-treated or PentaCB-treated Wistar and Gunn rats might occur, at least in part, through a TTR-associated pathway. Furthermore, dihydroxylated PCBs have been reported to show a several fold higher affinity for TTR than monohydroxylated PCBs (Lans *et al.*, 1993). In KC500-treated Gunn rats, the sum of three dihydroxylated PCB metabolites was 37% of the total hydroxylated PCB metabolites detected, although in the PCB-treated Wistar rats, the dihydroxylated metabolite was hardly detected. In addition, in PentaCB-treated Wistar and Gunn rats, the amount of 3′,4′-$(OH)_2$-PentaCB was more than 80% of the total hydroxylated PCB metabolites detected in the serum. Furthermore, PentaCB, which shows a weaker affinity for TTR than natural T_4 (Chauhan *et al.*, 2000), was also detected in the serum at a low level, as compared with the total hydroxylated metabolites. Accordingly, the binding of dihydroxylated PCB metabolites and PentaCB to TTR might also be attributed, in part, to a decrease in the level of serum T_4 in either KC500-treated or PentaCB-treated rats. However, an increase in the serum free T_4 level did not occur in any rats treated with either KC500 or PentaCB, although Pedraza and colleagues (1996) have shown that the synthetic flavinoid EM-21388, which displaces T_4 from TTR, increases the serum free T_4 level. Considering the hydroxylated metabolites of the PCBs examined, the decrease in serum total T_4 level by KC500 or PentaCB seems to occur, at least in part, through a TTR-associated pathway, although the reason that the serum level of free T_4 was decreased remains unclear. Furthermore, two other factors might be considered as possible mechanisms for the PCB-mediated decrease in the level of serum T_4: (1) the change in the performance of the hypothalamo-pituitary-thyroid-axis (Khan *et al.*, 2002; Khan and Hansen, 2003) and (2) the increase in estrogen sulfotransferase, which efficiently catalyzes the sulfation of iodothyronines (Kester *et al.*, 1999). However, the exact mechanisms for the PCB-mediated decrease in the serum T_4 level remains unclear.

In conclusion, the present findings demonstrate that the decrease in serum total T_4 level by PCB in Gunn rats occurs without an increase in hepatic T_4-UDP-GT activity; they further suggest that in rats, especially Gunn rats, the PCB-mediated decrease might occur, at least in part, through formation of the hydroxylated PCB metabolites. In Wistar rats, however, the PCB-mediated induction of T_4-UDP-GT might also contribute to the decrease. Further studies are necessary for understanding the susceptibility toward a PCB-mediated decrease in serum T_4 level in animals, including humans.

ACKNOWLEDGMENTS

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Polychlorinated Biphenyls (PCBs) Exert Thyroid Hormone-like Effects in the Fetal Rat Brain but Do Not Bind to Thyroid Hormone Receptors

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Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants routinely found in human and animal tissues. Developmental exposure to PCBs is associated with neuropsychologic deficits, which may be related to effects on thyroid hormone (TH) signaling in the developing brain. However, PCBs may interfere with TH signaling solely by reducing circulating levels of TH, or they may exert direct effects on TH receptors (TRs). Therefore, we tested whether maternal exposure to a commercial PCB mixture, Aroclor 1254 (A1254), exerts effects in the fetal brain by one or both of these mechanisms. Dams were dosed daily with 0, 1, or 4 mg/kg A1254 from gestational day 6 (GD6) until they were sacrificed on GD16. A1254 significantly reduced circulating levels of triiodothyronine (T_3) and thyroxine (T_4) in pregnant rats but increased the expression of several TH-responsive genes in the fetal cortex, including neuroendocrine-specific protein A (*NSP-A*), *RC3*/neurogranin, and *Oct-1*. These findings are consistent with a direct action of PCBs on TRs. However, we did not identify parent PCB congeners or metabolites that bound to rat TRs isolated from hepatic nuclei. These findings indicate that PCBs can interfere with TH signaling in the fetal brain by direct actions on the fetus rather than by producing maternal hypothyroidism. *Key words:* brain development, endocrine disruption, NSP-A, NSP-C, Oct-1, PCBs, RC3/neurogranin, thyroid, thyroid hormone. *Environ Health Perspect* 112:516–523 (2004). doi:10.1289/ehp.6672 available via *http://dx.doi.org/* [Online 22 December 2003]

Polychlorinated biphenyls (PCBs) are industrial chemicals consisting of paired phenyl rings with various degrees of chlorination (Chana et al. 2002). Although the production of PCBs was banned in the mid-1970s, these contaminants are routinely detected in the environment (Breivik et al. 2002). The chemical stability and lipophilicity of these compounds allow them to bioaccumulate through the food chain, and they are found in high concentrations in samples of human tissues (Fisher 1999). A number of epidemiologic studies have indicated that children developmentally exposed to PCBs suffer from neuropsychologic deficits such as a lower full-scale IQ, reduced visual recognition memory, attention deficits, and motor deficits (Ayotte et al. 2003; Huisman et al. 1995; Jackson et al. 1997; Korrick and Altshul 1998; Osius et al. 1999; Walkowiak et al. 2001). Both postnatal and prenatal exposure to PCBs contributes to these deficits, although some authors argue that prenatal exposure is more strongly associated with neurologic deficits, which indicates that fetal neurodevelopment is particularly vulnerable to PCB exposure (Jacobson and Jacobson 2002).

The specific neuropsychologic domains affected by developmental exposure to PCBs overlap with those affected by maternal thyroid hormone (TH) insufficiency, including lower IQ, visual memory deficits, and motor function and attention deficits (Haddow et al. 1999; Morreale de Escobar et al. 2000; Pop et al. 1999). Therefore, several investigators have speculated that PCBs may affect brain development by interfering with TH signaling (McKinney and Waller 1998; Porterfield 2000; Porterfield and Hendry 1998). This hypothesis is supported by the observation that the concentrations of PCBs, or of specific PCB congeners, in maternal and cord blood are associated with lower TH levels in both the mother and infant (Koopman-Esseboom et al. 1994; Schantz et al. 2003). Although several epidemiologic studies have failed to identify an association between TH and PCB body burden (Hagmar et al. 2001; Longnecker et al. 2000; Matsuura and Konishi 1990; Sala et al. 2001; Steuerwald et al. 2000), experimental studies consistently find that PCB exposure decreases circulating levels of thyroxine (T_4) in rats (Bastomsky 1974; Bastomsky et al. 1976; Brouwer et al. 1998). Therefore, it is possible that PCB body burden is negatively associated with serum TH levels in humans but that the variability inherent in human populations makes this association difficult to reveal.

Despite the finding that PCBs uniformly reduce circulating levels of TH in experimental animals, PCBs do not exert effects in experimental animals that are fully consistent with experimentally produced hypothyroidism using goitrogens such as propylthiouracil (PTU). For example, developmental exposure to PCBs in experimental animals induces hearing loss (Crofton et al. 2000a, 2000b; Goldey et al. 1995a, 1995b), a reduction in choline acetyltransferase in the cerebral cortex (Juarez de Ku et al. 1994), and an increase in testicular growth, all consistent to some degree with effects produced by PTU. Moreover, T_4 replacement can at least partially ameliorate these effects (Goldey and Crofton 1998; Juarez de Ku et al. 1994), indicating that PCBs can influence brain development in part by causing a reduction in serum TH. However, developmental hypothyroidism induced by PTU exposure causes a significant increase in serum concentrations of thyroid-stimulating hormone (thyrotropin; TSH) (Connors and Hedge 1981), reduced body and brain weight as well as reduced brain size of rat pups (Schwartz 1983), and a delay in eye opening and tooth eruption (Varma et al. 1978). In contrast, PCB exposure at doses that lower serum TH does not always produce these effects (Goldey et al. 1995a; Hood and Klaassen 2000; Kolaja and Klaassen 1998; Zoeller et al. 2000). Therefore, there is a discrepancy between the ability of PCBs to reduce circulating levels of TH and their ability to produce symptoms of hypothyroidism.

Some authors have proposed that this discrepancy may be attributable to PCBs acting as imperfect agonists/antagonists on TH receptors (TRs) (McKinney and Waller 1994). TRs are members of the steroid/thyroid superfamily of ligand-dependent transcription factors (Mangelsdorf et al. 1995). They are encoded by two genes, designated α and β *c-erbA* (encoding TRα and TRβ) (Sap et al. 1986; Weinberger et al. 1986). Both genes are alternatively spliced in a tissue-specific manner,

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producing a variety of functional TR isoforms (TRβ1, TRβ2, TRβ3, TRα1) (Flamant and Samarut 2003). TRα1 and TRβ1 are the predominant isoforms that are expressed throughout brain development (Bradley et al. 1989, 1992, 1994) and in other tissues such as liver, intestine, and heart (Brent 2000). However, only one report has addressed this proposal directly (Cheek et al. 1999), finding that two hydroxylated PCB congeners (4′-OH-PCB-14 and 4′-OH-PCB-106) exhibit a relatively low affinity for human TRβ1 (K_i = 32 μM).

Considering these findings, the present studies were initiated for two reasons. First, we tested the hypothesis that maternal PCB exposure could affect the fetal cerebral cortex by reducing the availability of TH to the fetus. We previously showed that low maternal TH, produced experimentally by goitrogen treatment, can alter gene expression in the fetal cortex before the onset of fetal thyroid function (Dowling et al. 2000, 2001; Dowling and Zoeller 2000). Therefore, if PCBs reduce circulating levels of maternal TH, then gene expression in the fetal cortex should respond in a manner consistent with hypothyroidism. Second, we tested the hypothesis that individual PCBs or their metabolites could bind to the TR. To test this, we used rat hepatic nuclei as a source of both TRα1 and TRβ1.

Materials and Methods

Chemicals. Aroclor 1254 (A1254; lot no. A8110048) and individual PCB congeners (PCBs 77, 105, 118, 126, 138, and 153) were purchased from AccuStandard, Inc. (New Haven, CT). Methylsulfonyl-PCBs ($MeSO_2$-PCBs) were synthesized according to Haraguchi et al. (1987). The purity of these compounds was > 99% as determined by gas chromatography. The hydroxylated PCBs were synthesized using the Suzuki coupling of chlorobenzene boronic acids with bromochloro anisoles followed by demethylation with boron tribromide. The characterization and purity of these compounds have been described previously (Bauer et al. 1995; Lehmler and Robertson 2001).

Animals. All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996) and were approved by the University of Massachusetts-Amherst Institutional Animal Care and Use Committee before initiating these studies. Timed-pregnant Sprague-Dawley rats (n = 18; Zivic-Miller, Inc., Pittsburgh, PA) arrived in our animal facility 2 days after insemination [gestational day (GD) 2]. The animals were individually housed in plastic cages with food and water provided continuously and were maintained on a 12:12-hr light cycle (0600 hr to 1800 hr). All dams were provided daily with a Keebler Golden Vanilla Wafer (The Kellog Company, Battlecreek, MI) dosed with A1254 on GD6 through GD16. The wafers were calibrated to provide doses of 0, 1, and 4 mg/kg (n = 6/group); details of this method have been described previously (Zoeller et al. 2000).

On GD16, dams were euthanized with carbon dioxide, and trunk blood was collected for measurement of serum total T_4 by radioimmunoassay (RIA). The pregnant uterine horns were removed and immediately placed on ice. Fetuses were dissected from the uterus, frozen on pulverized dry ice, and stored at –80°C until cryosectioned.

In situ *hybridization.* The NSP-A cDNA construct and NSP-C oligonucleotide probes have been described previously (Dowling et al. 2001), and the RC3/neurogranin construct (pPRC/CMV-RC3) was kindly provided by J. Bernal (Madrid, Spain; Iniguez et al. 1996). A fragment of rat Oct-1 transcript was cloned by standard polymerase chain reaction methods using primers designed to amplify a 1.2-kb region of the gene (246–1481, GenBank accession number U17013; National Center for Biotechnology Information, Bethesda, MD). The forward (5′-GCACCCAACCAC-CAACTTGC-3′) and reverse (5′-GGTGC-CATCAGGCCTGGATT-3′) primers were synthesized by Custom Primers (Invitrogen, Inc., Carlsbad, CA). The 1.2-kb fragment was then ligated into the pCRII TOPO vector using the Topo TA cloning kit according to the manufacturer's instructions (Invitrogen), and its authenticity was confirmed by sequence analysis.

Probes were generated *in vitro* by linearization with the restriction enzymes and transcription with the RNA polymerases specified for each construct (Table 1). Transcription reactions and *in situ* hybridization procedures have been described previously (Zoeller et al. 1997). After *in situ* hybridization, slides were arranged in X-ray cassettes and apposed to BioMax film (Eastman Kodak Co., Rochester, NY); the duration of exposure was dependent on the specific activity of the probe and the abundance of the target mRNA (2 weeks for the RC3/neurogranin cRNA probe, 1 week for the Oct-1 and NSP-A cRNA probes, and 2 days for the NSP-C oligonucleotide probe). To verify that the films were not overexposed, ^{14}C-labeled standards (American Radiolabeled Chemicals Inc., St. Louis, MO) were simultaneously apposed to all films. The hybridization signal was analyzed as follows. First, a 5× magnified image was captured using a Scion AG-5 capture board interfaced with the public domain software NIH-Image 1.61/ppc (W. Rasband, National Institute of Mental Health, Bethesda, MD) run on a Macintosh G4 computer (Apple Computer Inc., Cupertino, CA). The optical system consisted of a Dage-MTI72 series video camera equipped with a Nikon macro lens mounted onto a bellows system over a light box. Measurements of relative mRNA levels were taken as the area of the signal over the cortex of GD16 fetal brains multiplied by the relative density of the film (Figure 1). The resulting values were averaged

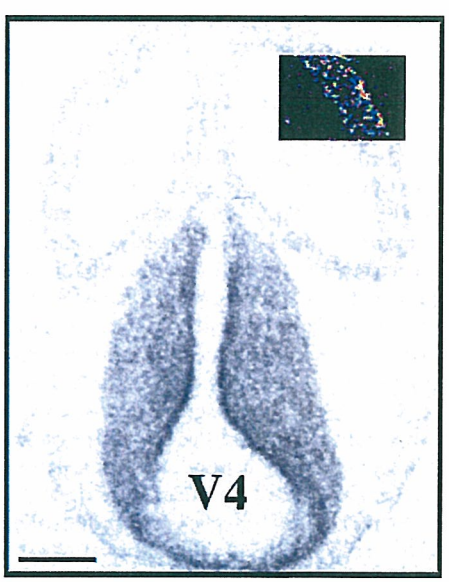

Figure 1. Film autoradiographic image of NSP-A expression in the GD16 fetal brain illustrating the region of the cortex in which gene expression was measured. Fetal brain tissue was collected from the cortex by taking 12-μm horizontal sections. The top of the image is rostral, the lower portion of the image is caudal, and the fourth ventricle is labeled for orientation (V4). Using imaging software, the signal within the boxed region was delineated, and the area and density of this region of interest were then measured for each of eight sections taken from a single brain. Inset: Pseudocolor (signal intensity: red > yellow > blue > black) illustrates the mRNA expression pattern specific to each TH-responsive gene. Bar = 1.0 mm.

Table 1. Characteristics of plasmids used to prepare cRNA probes for *in situ* hybridization.

Target mRNA	Plasmid	Strand	Enzyme for linearization	Promoter	Transcript size	Gene target region	Reference
RC3	PRC/CMV	Antisense	*Hind* III	Sp6	337	253–486	Iniguez et al. 1996
		Sense	*Apa* I	T7	344		
NSP-A	PCR-II	Antisense	*BamH*1	T7	202	1946–2147	Dowling et al. 2001
		Sense	*EcoR* V	Sp6	210		
Oct-1	PCR-II	Antisense	*Hind* III	T7	1226	246–1481	Gauger et al. 2002
		Sense	*Xho* I	Sp6	1234		
NSP-C	Synthetic oligo	Antisense	NA	NA	NA	230–183	Dowling et al. 2001

NA, not applicable.

across eight sections for each fetus, with one fetus per litter and six litters per treatment group.

Radioimmunoassay. Total T_4 was measured in 5 µL of rat serum using a barbital buffer system. Briefly, each assay tube contained 100 µL barbital buffer (0.11 M barbital, pH 8.6; 0.1% wt/vol 8-anilino-1-naphthalenesulfonic acid ammonium salt; 15% bovine γ-globulin Cohn fraction II; 0.1% gelatin), 100 µL anti-T_4 (rabbit, diluted to provide a final concentration of 1:30,000; Sigma, St. Louis, MO), and 100 µL ^{125}I-labeled T_4 (Perkin-Elmer/NEN; Boston, MA). Standards were prepared from T_4 (Sigma) measured using a Cahn electrobalance; standards were run in triplicate, whereas samples were run in duplicate. Standards were calibrated to measure serum T_4 levels from 0.4 µg/dL to 25.6 µg/dL. Tubes were incubated at 37°C for 30 min and then chilled on ice for 30 min. Bound counts were precipitated by adding 300 µL ice-cold polyethylene glycol 8000 (20% wt/wt; Sigma). Tubes were centrifuged at 1800 × *g* for 20 min at 4°C; the supernatant was then aspirated and the pellet counted in a gamma counter (Packard Cobra II; Global Medical Instrumentation, Inc., Albertville, MN). The assay was run at 40–50% binding; nonspecific binding was generally < 8%. The assay was validated for rat serum by demonstrating parallelism between the standard curve and a dilution series of rat serum. The two slopes did not vary significantly as evaluated by *t*-test for two slopes (data not shown). The variability within the assay was determined by running 10 replicates of three different standards that represent a low, medium, and high value on the standard curve. The coefficients of variance were 0.9% for 0 ng/mL; 4.7% for 3.2 µg/dL, and 3.8% for 25.6 µg/dL. All experimental samples were evaluated in a single assay.

Total triiodothyronine (T_3) was measured according to the manufacturer's instructions using a T_3 RIA kit (ICN Diagnostics, Costa Mesa, CA). This assay was performed at 49% binding with detection limits of 50–800 ng/dL. All samples were evaluated in a single assay.

Isolation of hepatic nuclei. Adult male Sprague-Dawley rats were euthanized with carbon dioxide and perfused with ice-cold saline through the aorta until the liver was cleared of blood. Twenty grams of liver was then washed in 3 mM $MgCl_2$ and 0.14 M NaCl, minced, and homogenized in 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.32 M sucrose using a motor-driven Teflon mortar and glass pestle. The homogenate was centrifuged at 600 × *g* for 10 min, and the crude nuclear pellet was resuspended in 3 mM $MgCl_2$, 1 mM DTT, and 1.8 M sucrose. After centrifugation at 53,000 × *g* for 45 min, the nuclei were resuspended in binding buffer (3 mM $MgCl_2$; 1 mM DTT; 20 mM Tris HCl, pH 7.6; 0.32 M sucrose; and 0.3% bovine serum albumin) and stored at –80°C.

TH binding assay. For saturation analysis, nuclear isolates frozen in binding buffer were thawed on ice, and triplicate aliquots (~0.1 g of the original liver) were incubated with increasing concentrations of ^{125}I-T_3 (1×10^{-10} to 8×10^{-9} M; 3,300 µCi/µg; NEN, Boston, MA) for 30 min at 37°C. Nonspecific binding was determined at each concentration of ^{125}I-T_3 (and each competitor concentration) by performing the assay as described in a parallel set of tubes that included the addition of 10,000-fold excess cold T_3 (final concentration 1×10^{-6} M). The reaction was terminated by placing samples on ice and by adding binding buffer/1% Triton X-100. Samples were centrifuged at 13,000 × *g* for 10 min, the supernatant was discarded, and the nuclear pellet was washed in 1 mL binding buffer. The bottom of the microfuge tube

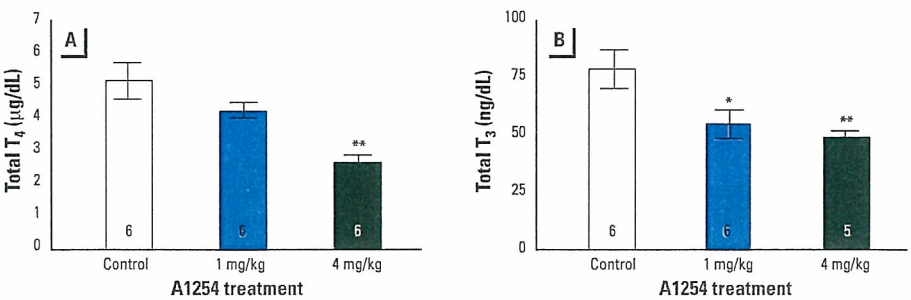

Figure 2. Effect of A1254 treatment on serum concentrations of total T_4 (*A*) and total T_3 (*B*) in dams at the time they were sacrificed on GD16. Bars represent mean ± SEM; number of animals in each group is shown within each bar. See Materials and Methods for treatment details.
*$p < 0.05$; **$p < 0.01$ (significantly different from control group using Bonferroni's *t*-test after one-way ANOVA).

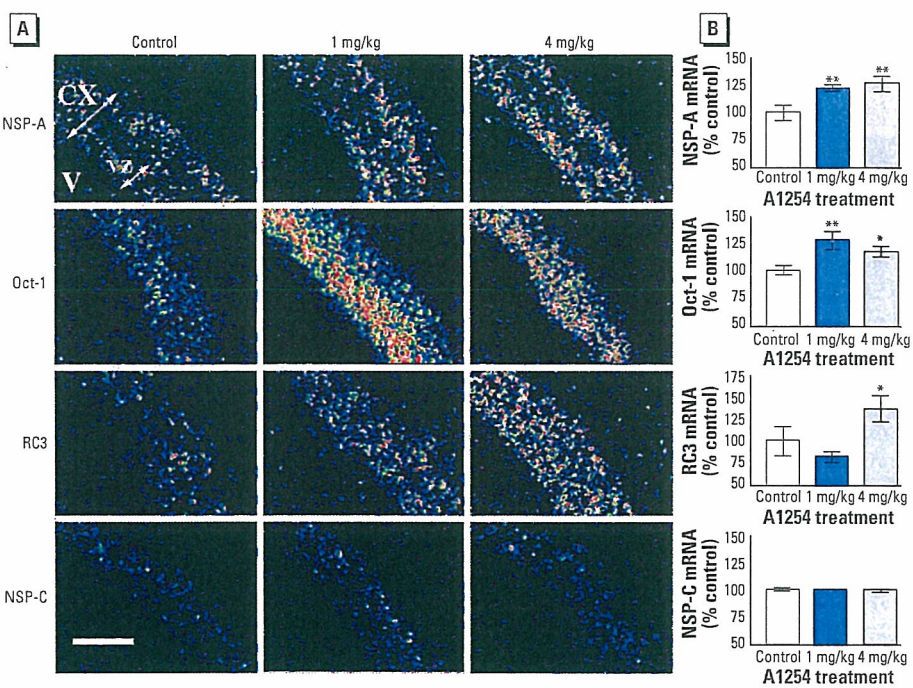

Figure 3. Effect of A1254 treatment on the levels and patterns of NSP-A, Oct-1, RC3/neurogranin, and NSP-C mRNA expression in the GD16 fetal cortex. (*A*) Representative pseudocolor autoradiograms obtained after *in situ* hybridization (pseudocolor signal intensity: red > yellow > blue > black). (*B*) Bar graphs showing the relative abundance of mRNA expression as reflected by the mean ± SEM film density (Oct-1 and NSP-C), area (NSP-A), or integrated density (RC3) and are presented as percent control for the purpose of illustration. Abbreviations: CX, cortex; V, lateral ventricle; VZ, ventricular zone. Measurements were taken from the VZ for NSP-A and revealed that the relative area of mRNA expression was significantly elevated in the 1 mg/kg and 4 mg/kg treatment groups. The pattern of Oct-1 expression was not specific to the VZ, and measurements taken in the CX showed a relative increase in the density of Oct-1 mRNA in both the 1 mg/kg and 4 mg/kg treatment groups. Measurements taken for RC3/neurogranin were also taken in the CX, and when density measurements were normalized with respect to area (integrated density), an increase in the level of mRNA expression was detected in the 4 mg/kg A1254 treatment group. All CX measurements revealed that NSP-C mRNA levels did not differ among treatment groups. Bar = 50 µm.
*$p < 0.05$. **$p < 0.01$ (significantly different from control using Bonferroni's *t*-test after one-way ANOVA).

containing the pellet was cut off and placed in a 14 × 70 mm test tube, which was counted in a gamma counter (Packard Cobra II). For competitive binding assays, isolated nuclei were incubated with a final concentration of 1×10^{-10} M ^{125}I-T_3 and increasing concentrations of competitors (TR agonists: T_3, T_4, Triac (triiodothyroacetic acid), and Tetrac (tetraiodothyroacetic acid, 1×10^{-12} M to 1×10^{-9} M; PCBs, 1×10^{-9} M to 1×10^{-3} M). For noncompetitive binding assays, isolated nuclei were incubated with 1×10^{-10} M ^{125}I-T_3 and increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 alone or increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 in the presence of 2×10^{-6} M of competitors. Nonspecific binding was established as described above.

Statistical analysis. Results were analyzed using a one-factor analysis of variance (ANOVA), and post hoc tests, where appropriate, were performed by Bonferroni's *t*-test, where the mean squared error term in the ANOVA table was used as the point estimate of the pooled variance (SuperAnova Software; Abacus Concepts, Inc., Berkeley, CA). A test for outliers was performed on all data; none were identified. Some samples were lost in processes; therefore, there are some unequal cell sizes.

Results

Dams. Exposure to A1254 significantly reduced circulating levels of total T_4 and total T_3 in dams (T_4: $F_{(2,15)} = 11.031$, $p = 0.0011$; T_3: $F_{(2,14)} = 5.772$, $p = 0.0142$; Figure 2). Post hoc analysis using Bonferroni's *t*-test revealed that dams treated daily with 4 mg/kg A1254 exhibited T_4 levels that were significantly lower than those of control animals (Figure 2A). There was a trend in animals treated with 1 mg/kg A1254 to exhibit lower circulating levels of T_4, but this did not reach statistical significance (Figure 2A). Moreover, animals treated with either 1 or 4 mg/kg A1254 exhibited significantly lower levels of circulating T_3 compared with control animals (Figure 2B).

Fetal brains. Quantitative analysis of film autoradiograms after *in situ* hybridization revealed that PCB exposure selectively affected TH-responsive genes in the fetal cortex (Figure 3). We focused on four different genes. RC3/neurogranin expression was significantly higher in the cortex of fetuses derived from dams treated with 4 mg/kg A1254 ($F_{(2,15)} = 5.423$, $p = 0.0169$). NSP-A expression was significantly elevated in fetuses derived from dams treated with either 1 or 4 mg/kg A1254 ($F_{(2,13)} = 8.212$, $p = 0.0049$),

Table 2. TH receptor agonists, parent PCB congeners, $MeSO_2$-PCB metabolites, and hydroxylated PCB metabolites tested in competitive TR binding assays.

Competitor	Abbreviation	IUPAC nomenclature	K_i	± CV
Triiodothyronine	T_3	L-3,3′,5-Triiodothyronine	8.37×10^{-10} M	12.70%
Thyroxine	T_4	L-3,3′,5,5′-Tetraiodothyronine	3.74×10^{-9} M	14.40%
Triiodothyroacetic acid	Triac	3,3′,3-Triiodothyroacetic acid	1.29×10^{-9} M	17.30%
Tetraiodothyroacetic acid	Tetrac	3,3′,5,5′-Tetraiodothyroacetic acid	2.37×10^{-7} M	18.40%
Parent PCBs	PCB-77	3,3′,4,4′-TetraCB	ND	NA
	PCB-105	2,3,3′,4,4′-PentaCB	ND	NA
	PCB-118	2,3′,4,4′,5-PentaCB	ND	NA
	PCB-126	3,3′, 4,4′,5-PentaCB	ND	NA
	PCB-138	2,2′,3,4′,4′,5′-HexaCB	ND	NA
	PCB-153	2,2′,4,4′,5,5′-HexaCB	ND	NA
$MeSo_2$-PCBs	3-$MeSO_2$-PCB-49	3-$MeSO_2$-2,2′,4′,5-tetraCB	ND	NA
	4-$MeSO_2$-PCB-49	4-$MeSO_2$-2,2′,4′,5-tetraCB	ND	NA
	3-$MeSO_2$-PCB-70	3-$MeSO_2$-2,3′,4′,5-tetraCB	ND	NA
	4-$MeSO_2$-PCB-70	4-$MeSO_2$-2,3′,4′,5-tetraCB	ND	NA
	3-$MeSO_2$-PCB-87	3-$MeSO_2$-2,2′,3′,4′,5-pentaCB	ND	NA
	3-$MeSO_2$-PCB-101	3-$MeSO_2$-2,2′,4,5,5′-pentaCB	ND	NA
	4-$MeSO_2$-PCB-101	4-$MeSO_2$-2,2′,4,5,5′-pentaCB	ND	NA
	3-$MeSO_2$-PCB-132	3-$MeSO_2$-2,2′,3,4′,5,5-hexaCB	ND	NA
	3-$MeSO_2$-PCB-141	3-$MeSO_2$-2,2′,3′,4′,5,5′-hexaCB	ND	NA
	3-$MeSO_2$-PCB-149	3-$MeSO_2$-2,2′,4′,5,5,6-hexaCB	ND	NA
	4-$MeSO_2$-PCB-149	4-$MeSO_2$-2,2′,4′,5,5,6-hexaCB	ND	NA
	4-$MeSO_2$-PCB-52	4-$MeSO_2$-2,2′,4,4′-tetraCB	ND	NA
	3-$MeSO_2$-PCB-77	3-$MeSO_2$–3′,4,4′,5-tetraCB	ND	NA
	3-$MeSO_2$-PCB-105	3-$MeSO_2$-2′,3′,4,4′,5-pentaCB	ND	NA
	3-$MeSO_2$-PCB-118	3-$MeSO_2$-2′,4,4′,5,5′-pentaCB	ND	NA
	3-$MeSO_2$-PCB-156	3-$MeSO_2$-2′,3′4,4′,5,5′-hexaCB	ND	NA
Hydroxylated PCBs	4′-OH-PCB-3	4′-OH-4-monoCB	ND	NA
	4′-OH-PCB-9	4′-OH-2,5-diCB	ND	NA
	4-OH-PCB-14	4′-OH-3′,5′-diCB	ND	NA
	4′-OH-PCB-12	4′-OH-3,4-diCB	ND	NA
	4-OH-PCB-30	4′-OH-2,4,6-triCB	ND	NA
	4′-OH-PCB-34	4′-OH-2,3′,5′-triCB	ND	NA
	4′-OH-PCB-36	4′-OH-3,3′,5-triCB	ND	NA
	4′-OH-PCB-20	4′-OH-2,3,3′-triCB	ND	NA
	4′-OH-PCB-35	4′-OH-3,3′,4-triCB	ND	NA
	4-OH-PCB-39	4-OH-3,4′,5-triCB	ND	NA
	4′-OH-PCB-58	4′-OH-2,3,3′,5′-tetraCB	ND	NA
	4′-OH-PCB-72	4′-OH-2,3′,5,5′-tetraCB	ND	NA
	4′-OH-PCB-106	4′-OH-2,3,3′,4,5-pentaCB	ND	NA
	4′-OH-PCB-112	4′-OH-2,3,3′,5,6-pentaCB	ND	NA
	4′-OH-PCB-159	4′-OH-2,3,3′,4,5,5′-hexaCB	ND	NA
	4′-OH-PCB-165	4′-OH-2,3,3′,5,5′,6-hexaCB	ND	NA
	3′,4′-(di)OH-PCB-12	3′,4′-OH-3,4-diDB	ND	NA
	3′,4′-(di)OH-PCB-3	3′,4′-OH-4-monoCB	ND	NA
	3′-OH-PCB-3	3′-OH-4-monoCB	ND	NA
	2′-OH-PCB-3	2′-OH-4-monoCB	ND	NA

Abbreviations: CB, chlorinated biphenyl; IUPAC, International Union of Pure and Applied Chemistry; NA, not applicable; ND, no detectable binding.

as was Oct-1 ($F_{(2,14)} = 5.399$, $p = 0.0183$). In contrast, NSP-C expression in the GD16 cortex was not affected by PCB exposure ($F_{(2,15)} = 0.202$, $p = 0.819$; not significant).

TH receptor binding. To test the hypothesis that individual PCB congeners may bind to TRs to produce the observed effects on gene expression *in vivo*, we tested a number of parent PCBs and metabolites for their ability to bind to the TR (Table 2). To validate the assay, we first performed a saturation analysis and established that T_3 bound to TRs in isolated hepatic nuclei with a K_d of $9.7 \times 10^{-10} \pm 2.02 \times 10^{-10}$ M (Figure 4). We then tested the ability of T_3 and other TR agonists (T_4, Triac, and Tetrac) to displace ^{125}I-T_3 from TRs in hepatic nuclei (Figure 5A); using the K_d obtained from saturation analyses, a specific K_i was calculated for each compound (Table 2). However, none of the tested parent PCB congeners, hydroxylated metabolites, or $MeSO_2$ metabolites significantly displaced ^{125}I-T_3 in this assay (Figure 5B). Similarly, noncompetitive binding analysis revealed that parent PCB congeners, grouped according to their *ortho*-substitution pattern, did not alter the affinity of T_3 for TRs in isolated nuclei (Figure 6).

Discussion

The present study demonstrates that the commercial PCB mixture A1254 significantly reduces serum TH levels, T_4, and T_3, in pregnant rats on GD16. This developmental time occurs before the onset of thyroid function in the fetus (Fisher et al. 1977); therefore, it is reasonable to propose that this PCB-induced decrement in maternal TH would cause a reduction in the expression of genes positively regulated by TH. However, we found that PCB exposure up-regulated the expression of genes that are positively regulated by TH. These findings indicate that PCBs can activate TRs, perhaps directly, and the implication is important because inappropriate activation of TRs in the developing brain may produce adverse consequences on brain development (Kopelman 1983; Rastogi and Singhal 1976, 1979; Zoeller 2003).

Our finding that A1254 decreased circulating levels of TH in pregnant rats is consistent with previous studies showing that exposure to A1254, or specific PCB congeners, causes a reduction in circulating levels of total T_4 in pregnant rats (Meerts et al. 2002; Morse et al. 1993, 1996). Additionally, we found that serum total T_3 was also reduced in the dams by A1254, indicating the degree to which serum T_4 was reduced, because nearly 80% of circulating T_3 is derived from peripheral deiodination of T_4 (Taurog 2000). Although PCB exposure reduced serum T_4 in the dams, several descriptive measures of hypothyroidism were not altered. For example, maternal body weight, weight gain, litter size, and pup weight were all unaffected by PCB treatment in this experiment (data not shown), similar to results of our previous study (Zoeller et al. 2000). Therefore, like others, we observed a discrepancy between the ability of PCB exposure to lower serum TH levels and its ability to produce symptoms of hypothyroidism.

The present finding that fetuses derived from A1254-treated dams exhibited a significant increase in RC3/neurogranin and Oct-1 expression represents strong evidence that PCBs can produce TH-like effects in the fetal brain because maternal TH increases the expression of these genes in the fetal brain (Dowling et al. 2000; Dowling and Zoeller 2000). Considering that A1254 exposure produced a significant reduction in circulating T_4 and T_3 in the dam, this finding is fully consistent with the hypothesis that PCBs can directly activate TRs in the fetal brain. Moreover, A1254 exerted selective effects on the expression of TH-responsive genes because NSP-C, which is not influenced by TH in the fetus (Dowling et al. 2000, 2001), was not affected by A1254. Furthermore, previous work in our laboratory has demonstrated that A1254 increases the expression of myelin basic protein, a known TH-responsive gene (Farsetti et al. 1991; Marta et al. 1998; Rodriguez-Pena et al. 1993), in the cerebellum and RC3/neurogranin in the forebrain of postnatal rats, despite the finding that these pups exhibited a

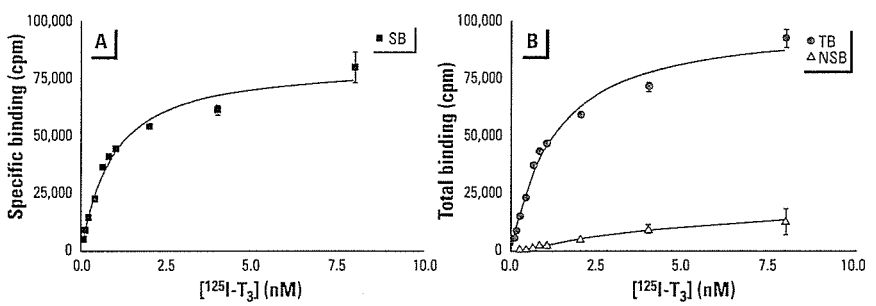

Figure 4. (*A*) Saturation analysis of ^{125}I-T_3 specific binding (SB) to TRs in nuclei isolated from rat liver tissue, and (*B*) total binding (TB) obtained by incubating hepatic nuclei with increasing concentrations (1×10^{-10} to 8×10^{-9}) ^{125}I-T_3 ($n = 3$; see "Materials and Methods" for assay conditions). NSB, nonspecific binding.

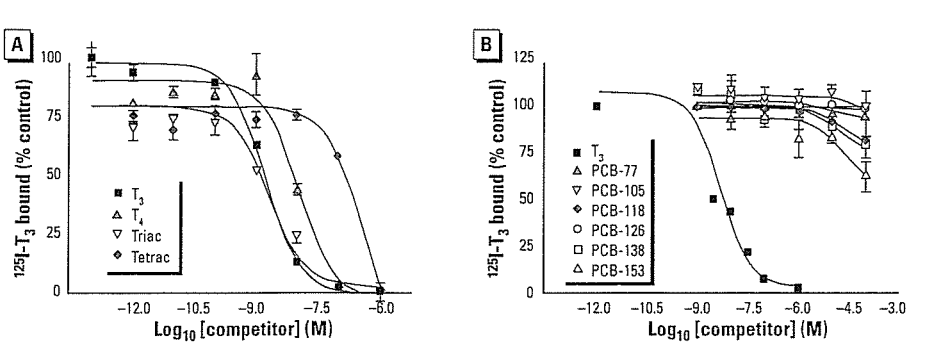

Figure 5. Competitive binding of known TH receptor agonists (*A*) and parent PCB congeners (*B*) to isolated hepatic nuclei. In (*A*), rat liver nuclear extracts were incubated with 1.0 nM ^{125}I-T_3 and increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3, T_4, Triac, or Tetrac; TR agonists displayed an expected order of binding affinity to isolated nuclei ($T_3 \approx$ Triac > T_4 >> Tetrac). In (*B*), isolated nuclei were incubated with 1.0 nM ^{125}I-T_3 and increasing concentrations (1×10^{-9} M to 1×10^{-3} M) of one of the parent PCB congeners; no parent PCB congener exhibited significant binding to isolated nuclei. (*B*) is representative of results obtained from assays that tested hydroxylated and $MeSO_2$-PCB metabolites for their ability to displace ^{125}I-T_3 from TRs in hepatic nuclei. These compounds also showed no significant binding to nuclear extracts. All curves are were obtained from results of a single experiment and are representative of three separate experiments.

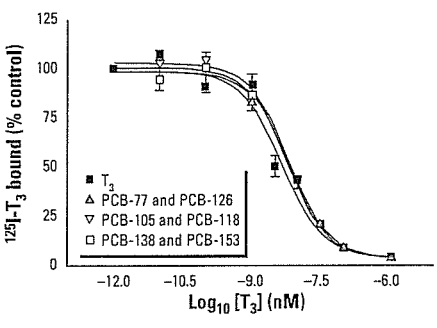

Figure 6. Noncompetitive binding analysis of parent PCB congeners binding to isolated hepatic nuclei. Assays were performed to determine whether PCBs present in A1254 were able to alter the affinity TRs for T_3. Isolated nuclei were incubated with 1.0 nM ^{125}I-T_3, and increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 alone or with increasing concentrations (1×10^{-12} M to 1×10^{-6} M) of T_3 in the presence of 20 μM of the PCB competitor. These PCBs were unable to alter the established K_i of T_3 for TRs in hepatic nuclei.

severe reduction in the circulating levels of TH (Zoeller et al. 2000). Taken together, these data indicate that A1254 can exert agonistic effects on a variety of positively regulated TH-responsive genes in different brain regions at different developmental times.

Although A1254 produced TH-like effects on the expression of RC3/neurogranin and on Oct-1 in the fetal cortex in the present study, it did not exert these effects on NSP-A expression. We previously found that NSP-A expression is significantly increased in the brain of fetal rats derived from hypothyroid dams (Dowling et al. 2000, 2001). Therefore, the present finding that NSP-A expression is increased in the cortex of fetuses exposed transplacentally to A1254 suggests that the expression of this gene is responding to low maternal T_4 in PCB-treated dams, not to agonistic actions of PCBs on TRs. Previously, we showed that T_4 treatment of hypothyroid dams did not restore NSP-A expression in the fetal cortex (Dowling et al. 2001). Because T_4 was provided for only a short time, the interpretation was that the duration of T_4 treatment was not sufficient to produce a significant reduction in cellular levels of NSP-A mRNA. However, Chan et al. (2003) have recently reported that NSP-A expression is not directly sensitive to TH in N-Tera-2 cells, indicating that NSP-A may not be directly regulated by TH. If maternal hypothyroidism increases NSP-A expression indirectly, and NSP-A is not directly regulated by TH, then our present results indicate that PCBs produce effects on the fetal brain by exerting direct TH-like effects as well as by inducing low maternal TH.

Considering these findings, we tested a number of PCB congeners and specific metabolites for their ability to bind to TRs using a well-established binding assay (DeGroot and Torresani 1975). We found that neither the parent PCB congeners nor the hydroxylated or $MeSO_2$ metabolites significantly displace T_3 from rat hepatic nuclei. It is not likely that these observations are false negatives because the observed K_i for several control ligands, including T_3, T_4, Tetrac, and Triac, were all within the published range (Evans and Braverman 1986; Goslings et al. 1976; Ichikawa and DeGroot 1987). In addition, we demonstrated in preliminary studies that the PCB diluent (dimethyl sulfoxide) does not interfere with the assay; moreover, we used different diluents in initial studies and obtained results that did not differ from those reported here. Thus, our finding that individual PCB congeners or their metabolites do not displace T_3 from its receptor indicates that these compounds do not interact with the TR in a competitive manner.

We employed hepatic nuclei to test whether individual PCB congeners could bind to rat TRs because the TR isoforms expressed in hepatocytes are also the predominant isoforms expressed in the fetal cortex (Bradley et al. 1992; Nakai et al. 1988). Therefore, the observation that individual PCB congeners did not displace T_3 from liver nuclei suggests that they also do not displace T_3 from TRs in the fetal cortex. However, Cheek et al. (1999) demonstrated that several hydroxylated PCB metabolites bind to the human TRβ1, although the affinity for the TR was reported to be 10,000-fold lower than that of T_3. We evaluated two of these metabolites, 4′-OH-PCB-14 and 4′-OH-PCB-106, but did not find significant binding to the TRs in rat hepatic nuclei. These two studies may differ in their findings because we used TR isoforms from a different species, or because both TRα1 and TRβ1 are expressed in hepatocytes (Nakai et al. 1988). Specifically, it is possible that we may not have observed significant T_3 displacement if a PCB congener binds to only one of the two TRs with low affinity.

There are two major implications of the present study. First, the observation that PCB exposure selectively alters gene expression in the fetal cortex strongly suggests that PCBs can exert deleterious effects on fetal brain development regardless of the mechanism by which this effect is mediated. Because we used TH-responsive genes as end points for this study, it is likely that the observed effects reflect the ability of PCBs to disrupt TH action in the fetal brain. It will be important to determine whether PCB exposure can interfere with neurodevelopmental events by interfering with TH action.

The second major implication of our present results is that PCBs do not appear to bind to TRs in a competitive manner. We were surprised to find no individual PCB congeners or metabolites that exhibited strong binding to TRs, especially considering previous speculation about this (McKinney et al. 1987; McKinney and Waller 1998; Porterfield 1994, 2000; Porterfield and Hendry 1998). However, there is evidence that PCB congeners can affect TR activation without displacing T_3. Specifically Iwasaki et al. (2002) showed that 4′-OH-PCB-106 suppressed T_3-induced transactivation by TR in various cell lines. This appeared to be specific to the TR because it did not suppress glucocorticoid receptor–mediated transactivation. In addition, they showed that this PCB congener suppressed the ability of the TR to recruit the coactivator SRC-1. Because we found that this hydroxylated PCB did not displace T_3 from rat TRs in the present study, these observations strongly suggest that PCBs can directly alter TR action by a mechanism that is not well understood.

Our failure to identify individual PCBs that can bind with high affinity to the rat TRs requires alternate explanations for their effects on TH-responsive genes and developmental events. Individual PCB congeners may alter TH metabolism by tissue deiodinases, thereby changing the amount of hormone available to the TR. Previous studies indicate that PCBs can increase type-2 deiodinase activity in the adult (Hood and Klaassen 2000) and fetal (Meerts et al. 2002; Morse et al. 1996) rat brain. Moreover, PCBs are also known to affect second messenger signaling in the brain by affecting calcium homeostasis, receptor-mediated inositol phosphate production, and translocation of protein kinase C (Kodavanti et al. 1993, 1994). In addition, PCBs can produce toxic effects by binding either to the aryl hydrocarbon receptor (Safe 1990) or the ryanodine receptor (Schantz et al. 1997; Wong et al. 1997). These studies demonstrate that PCBs, especially as a mixture such as A1254, clearly produce multiple effects. However, there is no evidence that these other mechanisms of PCB action can exert specific effects on TH-responsive genes.

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PCB類による甲状腺ホルモンかく乱作用とその作用機構：動物種差

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Species differences among mice, hamsters, rats and guinea pigs in PCB-induced alteration of serum thyroid hormone level

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Summary

In the present study, we investigated the mechanism for the decrease in levels of serum thyroid hormones, especially thyroxine (T_4), by polychlorinated biphenyls (PCBs) such as Kanechlor-500 (KC500), 2,2',4',5,5'-pentachlorobiphenyl (PentaCB), and 2,2',3',4',5,6-hexachlorobiphenyl (HexaCB), and studied species differences among mice, hamsters, rats, and guinea pigs in the PCB effect. Significant decrease in serum total T_4 level by KC500 was observed in all four species. On the other hand, there were differences in the level of decrease of serum total T_4 level by PentaCB and HexaCB. Differences in the level of hepatic methylsulfonyl-PCB metabolites of KC500, PentaCB and HexaCB, which were thought to be associated with the PCB-toxic effects, did not necessarily correlate with the magnitude of decrease in serum total T_4 level. Likewise, the induction of UDP-glucuronosyltransferases (T_4-UDP-GT) toward T_4 by PCB did not necessarily correlate with the decrease in serum T_4 level in the animals used. Further studies on transthyretin (TTR) and serum T_4-transporter suggested that decrease in serum total T_4 level induced by PCB occurred not only by induction of T_4-UDP-GT but also by the alteration of levels of T_4-TTR binding and hepatic T_4-transporter. In addition, species difference in the decrease of serum total T_4 was associated with various PCB-induced total effects, including induction of T_4-UDP-GT, decrease in T_4-TTR binding level, the increase of hepatic thyroid hormone transporter, and other thyroid function correlates.

Keywords: species differences, polychlorinated biphenyls, thyroid hormones, UDP-glucuronosyltransferases, transthyretin

緒　　言

Polychlorinated biphenyl (PCB) は，多くの野生生物の組織のみならず，ヒトの血液，母乳，肝臓，脂肪組織などにも見出され，生体に対する影響が懸念されている (ATSDR, 2000; Hansen, 1999)．ラットでは，すでに，PCB投与により血中T_4濃度が低下することが報告され (Table 1)，ヒトにおいてもPCB曝露により甲状腺ホルモン（サイロキシン (T_4)，3,5,3'-トリヨードサイロニン (T_3)）の撹乱が引き起こされている可能性が指摘されている (Koopman-Esseboom et al., 1994; Jacobson et al., 1990)．

一般に，PCBによるラットでの血中T_4濃度の低下は，肝臓のUDP-グルクロン酸転移酵素 (UDP-GT) が誘導

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Table 1 Effects of PCB and TCDD on serum thyroid hormone levels

Treatment	Animals	T_4	T_3	TSH	Refarences
2,4,4'-trichlorobiphenyl	Sprague-Dawley rats	→	→	n.t.	Ness et al., 1993
2,3',4,4',5-pentachlorobiphenyl	"	↓	→	n.t.	"
2,2',4,4',5,5'-hexachlorobiphenyl	"	↓	→	n.t.	"
3,3',4,4'-tetrachlorobiphenyl	"	↓	→	→	Seo et al., 1995
3,3',4,4',5-pentachlorobiphenyl	"	→	→	→	"
3,3',4,4',5-pentachlorobiphenyl	"	↓	n.t.	n.t.	van Birgelen et al., 1995
2,3,3',4,4',5-hexachlorobiphenyl	"	↓	n.t.	n.t.	"
TCDD	"	↓	n.t.	n.t.	"
Aroclor 1254	"	↓	↓	↑	Barter and Klaassen, 1994
"	"	↓	↓	↑	Liu et al., 1995
3,3',4,4',5-pentachlorobiphenyl	"	↓	↓	→	Desaulniers et al., 1999
2,2',4,4',5,5'-hexachlorobiphenyl	"	↑	n.t.	→	"
3,3',4,4',5-pentachlorobiphenyl	Long-Evans rats	↓	n.t.	n.t.	Craft et al., 2002
"	C57BL/6J mice	→	n.t.	n.t.	"
2,2',4,4',5,5'-hexachlorobiphenyl	Long-Evans rats	↓	n.t.	n.t.	"
"	C57BL/6J mice	↓	n.t.	n.t.	"

PCB: polychlorinated biphenyl, TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, n.t.: not tested.

Table 2 Effects of KC500, PentaCB and HexaCB on serum total T_4, free T_4, total T_3 and TSH levels in mice, hamsters, rats and guinea pigs

Treatment	Animals	Total T_4	Free T_4	Total T_3	TSH
KC500	Mice	43% ↓	44% ↓	→	→
	Hamsters	71% ↓	57% ↓	→	→
	Rats	77% ↓	76% ↓	→	→
	Guinea pigs	74% ↓	76% ↓	60% ↓	→
PentaCB	Mice	42% ↓	n.t.	42% ↓	→
	Hamsters	34% ↓	n.t.	→	→
	Rats	46% ↓	n.t.	→	→
	Guinea pigs	→	n.t.	→	→
HexaCB	Mice	39% ↓	n.t.	→	→
	Hamsters	→	n.t.	→	→
	Rats	→	n.t.	→	→
	Guinea pigs	→	n.t.	→	→

Animals were killed at 4 days after the administration of KC500 (37.5 mg/kg, ip), PentaCB (11 mg/kg, ip) or HexaCB (19 mg/kg, ip). Results are expressed as the mean for 5-6 animals. n.t.: not tested.

され，T_4のグルクロン酸抱合化が促進，そして，胆汁排泄が高まることによると考えられている（Barter and Klaassen, 1994; Schuur et al., 1997; van Birgelen et al., 1995）．しかし，PCBによる血中T_4濃度の低下と肝臓でのT_4-UDP-GT活性の増加には必ずしも定量的関連性は見られない（Craft et al., 2002; Kato et al., 2003a）など不明な点もある．また，PCBの血中甲状腺ホルモンへの影響に関する研究は，ほとんどラットを用いて行なわれたものであり，その低下作用における動物種差については，ほとんど知られられていない．

そこで，著者らはPCB投与による血中T_4濃度低下における動物種差やその発現機序を明らかにする目的で，まずはじめに，種々動物種におけるPCBの血中T_4濃度への影響を検討し，次いで各動物におけるPCB代謝やT_4-UDP-GT誘導性の相違を追求した．本稿では，これまでに得られた成果を紹介する．

1．PCBの血中甲状腺ホルモン量への影響：動物種差

種々の用量のKanechlor-500（KC500，PCB製品であるPCB混合物），2,2',4',5,5'-pentachlorobiphenyl（PentaCB）あるいは2,2',3',4',5,6-hexachlorobiphenyl（HexaCB）をマウスに投与し，血中total T_4濃度を50%低下させる各PCBの用量を求めた．次いで，各PCBを本用量でddY系マウス，Syrian系ハムスター，Wistar系ラットあるいはHartley系モルモットに処理し，血中甲状腺ホルモン濃度への影響を調べた．

その結果，マウス，ハムスター，ラットおよびモルモットに，KC500を投与すると，用いたいずれの動物種でも血中total T_4およびfree T_4濃度の低下が認められた