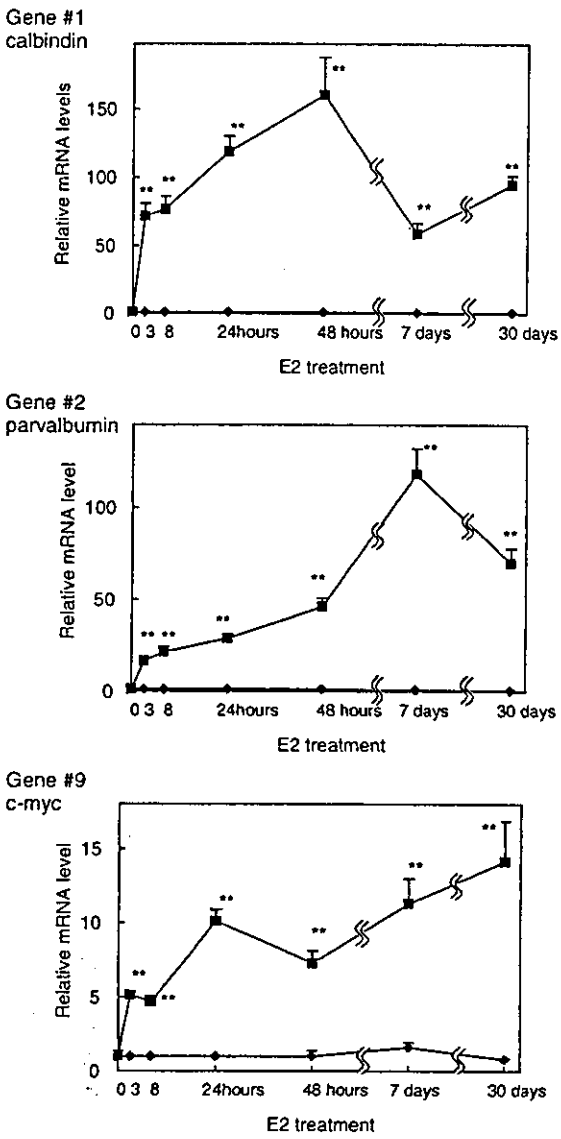

Fig. 4. Time-dependent analysis of three estrogen-responsive genes in the pituitary in vivo. Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 3, 8, 24, and 48 h and 7 and 30 days. Gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and fold changes were calculated based on the mRNA level in the ovariectomized controls at time zero. All mRNA contents were normalized with reference to G3PDH mRNA. Each point and bar represent mean ± S.E.M. ($n = 5$), *,** Indicates significant differences from the control values at 0.05 and 0.01, respectively.

regulated by estrogen with confirmation in most cases by quantitative real-time PCR. Suppression by ICI of E2-induced gene expression was also confirmed. The degrees of change were similar with real-time PCR analysis and GeneChip data and although we selected up-regulated genes after 24 h of estrogen exposure, some genes proved to be rapidly regulated (Fig. 3(B)) including these for the progesterone receptor and c-*myc*. Estrogen-responsive induction of progesterone receptor is well documented for the primary target, the uterus, as well as in the anterior pituitary gland [26,27]. Estrogen activation of c-*myc* also has been reported in the anterior pituitary gland and breast cancer cells [28,29]. A total of seven genes could be listed as down-regulated but they were not analyzed further, since all of them displayed relatively small degrees of change to 0.34–0.48 of the control values. Other known estrogen-responsive genes in the pituitary gland, such as prolactin and TGFα were not on the array used in the present study.

Interestingly, the in vivo expression of two genes, calbindin-D9k and parvalbumin, was found to be highly induced by E2 both in the short and longer term, which may suggest that hypothalamus or other indirect endocrine pathways would be involved in regulating genes in addition to the direct transcriptional activation. Calbindin-D9k is a vitamin D-dependent intestinal calcium-binding protein that is detectable in the duodenum, uterus and placenta [30–32]. Another vitamin D-dependent calcium-binding protein, calbindin-D28k, expressed in kidney and brain has no homology with calbindin-D9k either at the nucleotide or at the transcript levels [33]. The calbindin-D9k gene has been reported to contain a 15-base-pair imperfect palindrome with high homology to the estrogen- and glucocorticoid-responsive elements (ERE and GRE) [34]. Although there is no evidence that this protein is regulated by estrogen in the intestine through this motif, it is possible that the imperfect ERE is functional for the hormone-dependent transcription in the pituitary gland. Parvalbumin is another calcium-binding protein that belongs to the EF-hand calcium-binding protein like calbindin-D9k [35]. It is abundant in fast contracting/relaxing muscle fibers, where it plays a role as a calcium buffer and is also found in neurons as well as in endocrine glands including pituitary, thyroid, adrenals, testes and ovaries [36]. It has been postulated that parvalbumin can prevent cell death due to calcium overload in neurons. Although its expression is developmentally regulated in muscle, brain and other tissues, no evidence indicating hormonal regulation has been reported [37,38]. The 5′ flanking region of the gene seems to function as the promoter but it does not contain any motifs for estrogen-dependent transcription [39,40].

Since RNA was extracted from whole anterior pituitary tissue in the present study, it is not clear which types of cell actually contributed to the increase in mRNA levels. Chronic treatment of rats with E2 is known to result in the development of lactotrophic tumors [5]. The F344 strain is the most sensitive to E2 and somatolactotrophs of the pituitary become hyperplastic after exposure for a week and steadily proliferate thereafter. In the present study, major response of GH3 cells was cell proliferation so that some of the identified genes might be expected to be mitosis-related and involved in estrogen-induced pituitary hyperplasia/tumorigenesis. Although up-regulation of the calbindin-D9k and parvalbumin gene are evident on long-term treatment of E2, there was no obvious correlation with the time period for pituitary hyperplasia in contrast to the c-*myc* expression which steadily increase.

In conclusion, the present microarray analysis allowed identification of a number of estrogen-responsive genes in GH3 cells whose regulation appears biologically relevant in the pituitary gland in vivo. The actual significance of two calcium-binding proteins discovered to be prominently induced by E2 remains to be explored in the future.

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Metabolism of Polychlorinated Biphenyls by Gunn Rats: Identification and Serum Retention of Catechol Metabolites

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The tissue distributions of persistent metabolites of polychlorinated biphenyls (PCBs) in Wistar rats and homozygous uridine diphosphate glucuronosyltransferase (UGT) deficient Gunn rats exposed to 2,4,5,2′,5′-pentachlorobiphenyl (CB101) and the commercial PCB mixture, Kanechlor-500 (KC500), were investigated. After exposure to CB101, four hydroxy and two methylsulfonyl ($MeSO_2$) metabolites were detected in liver, lung, kidney, blood, and adipose tissues. One was identified as 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCB, which was retained selectively in the serum of Gunn rats. Comparative analysis of the metabolite profiles in both rat strains after exposure to KC500 showed higher formation ratios of several dihydroxy PCB metabolites in the liver of Gunn rats; major metabolites are the catechols from 2,5,3′,4′-tetraCB, CB101, 2,3,6,3′,4′-pentaCB, and 2,3,6,2′,4′,5′-pentaCB. Thus, Gunn rats effectively metabolized PCBs with 2,5- or 2,5,6-chlorine substitution to the 3,4-catechol, but less formed $MeSO_2$ metabolites in the liver. Although both rat strains retained 4-OH-2,3,5,3′,4′-pentaCB in serum, Gunn rats also retained the catechol PCBs, accounting for about 52% of the total phenolic PCBs. These results suggest that a lack of UGTs markedly alters the formation ratios and retention profiles of catechols and $MeSO_2$ metabolites of PCBs.

Introduction

Polychlorinated biphenyls (PCBs)[1] are ubiquitous environmental contaminants that exhibit specific toxicity in different animals (*1*). PCBs undergo biotransformation by cytochrome P450 (CYP) to hydroxylated metabolites (*2*). Further metabolism involves the formation of catechol and quinone products (*3*) or glucuronidation (*4*), conjugation of arene oxide intermediates with glutathione, and subsequent formation of methylsulfonyl PCBs ($MeSO_2$-CBs) (*5*).

Some of the phenolic PCBs and $MeSO_2$-CBs have been shown to persist in blood or tissues of laboratory animals (*6*) as well as in humans (*7*−*9*). Recent concerns have focused on the action of these metabolites as endocrine disrupters. The hormone disruption may be due to the induction or inhibition of enzymes responsible for the metabolism of PCBs or PCB metabolites (*10*, *11*). Phenolic PCBs with one or more chlorine atoms adjacent to the hydroxyl group bind competitively to the thyroxine (T4) transporting protein, transthyretin (TTR), with high affinity (*12*) and alter thyroid hormone metabolism (*13*). Some hydroxylated PCBs are known to interact with estrogen receptors (*14*) or with sulfotransferases that sulfate estrogen (*11*). Similarly, several $MeSO_2$-CB metabolites reduce thyroid hormone levels in rats (*15*) and also exhibit antiestrogenicity in vitro (*16*).

Catechol PCB metabolites have been reported to be formed in rodents exposed to PCBs (*3*, *17*). Garner et al. (*18*) demonstrated that in vitro estrogenicity of catechol PCB metabolites was within the range of effects observed for phenolic PCBs. In addition, catechols can potentially be metabolized by peroxidases to quinones that are responsible for the formation of DNA adducts and exhibit cytotoxicity (*19*, *20*).

Generally, phenolic PCBs undergo detoxification by glucuronidation catalyzed by the uridine diphosphate glucuronosyltransferases (UGTs). A number of PCBs and PCB metabolites have been shown to induce hepatic UGTs (*21*−*23*) to facilitate excretion of PCBs. Although the increased glucuronidation resulted in the decreased serum T4 levels because of the increased clearance (*24*), a decrease in serum T4 levels also occurred in PCB-exposed Gunn rats, a mutant strain of Wistar rats

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[1] Abbreviations: PCBs, polychlorinated biphenyl; CYP, cytochrome P450; $MeSO_2$-CBs, methylsulfonyl PCBs; T4, thyroxine; TTR, transthyretin; UGT, uridine diphosphate glucuronosyltransferase; CB101, 2,4,5,2′,5′-pentachlorobiphenyl; KC500, Kanechlor-500; CB, chlorobiphenyl; SIM, selected ion monitoring; ECD, electron capture detector; diOH-CB101, 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentachlorobiphenyl; diOH-CB70, 3,4-$(OH)_2$-2,5,3′,4′-tetrachlorobiphenyl; diOH-CB87, 3′,4′-$(OH)_2$-2,3,4,2′,5′-pentachlorobiphenyl; diOH-CB110, 4,5-$(OH)_2$-2,3,6,3′,4′-pentachlorobiphenyl; diOH-CB132, 4′,5′-$(OH)_2$-2,3,4,2′,3′,6′-hexachlorobiphenyl; 3-OH-CB99, 3-OH-2,4,5,2′,4′-pentachlorobiphenyl; diOH-CB149, 4,5-$(OH)_2$-2,3,6,2′,4′,5′-hexachlorobiphenyl; 4-OH-CB107, 4-OH-2,3,5,3′,4′-pentachlorobiphenyl; 3′-OH-CB138, 3′-OH-2,3,4,2′,4′,5′-hexachlorobiphenyl; CB105, 2,3,4,3′,4′-pentachlorobiphenyl; CB118, 2,4,5,3′,4′-pentachlorobiphenyl; CB138, 2,3,4,2′,4′,5′-hexachlorobiphenyl; CB146, 2,3,5,2′,4′,5′-hexachlorobiphenyl; CB153, 2,4,5,2′,4′,5′-hexachlorobiphenyl; CB156, 2,3,4,5,3′,4′-hexachlorobiphenyl; CB167, 2,4,5,3′,4′,5′-hexachlorobiphenyl; CB170, 2,3,4,5,2′,3′,4′-heptachlorobiphenyl; CB180, 2,3,4,5,2′,4′,5′-heptachlorobiphenyl; CB187, 2,3,5,6,2′,4′,5′-heptachlorobiphenyl.

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deficient in several forms of UGTs (*25*). Tampal et al. (*4*) demonstrated that phenolic PCB metabolites that persist in the body are poor substrates for hepatic UGTs and resist conjugation. Therefore, it would be of interest to determine the persistent PCB metabolite profiles in relation to UGT deficiency.

The present study was performed to investigate whether Gunn rats (UGT1A deficient Wistar rats) exposed to PCBs show altered formation ratios of phenolic PCB metabolites as compared to Wistar controls. Several catechol PCB metabolites were isolated from Gunn rats dosed with PCBs. This paper describes the identification and characterization of three kinds of persistent metabolites (monohydroxy, dihydroxy, and methylsulfonyl) in the liver and blood of Gunn and Wistar rats exposed to a single dose of 2,4,5,2′,5′-pentachlorobiphenyl (CB101) and the commercial PCB mixture, Kanechlor-500 (KC500).

Materials and Methods

Caution: *Synthetic PCBs and their metabolites should be considered potentially toxic and hazardous and therefore should be handled in an appropriate manner.*

Chemicals. CB101 was synthesized using the Cadogan coupling reaction (*26*). Methoxy PCBs were synthesized according to the method reported by Bergman et al. (*27*). $MeSO_2$-CBs were synthesized as described previously (*28*). Methylated derivatives of catechols (veratrole PCBs) were prepared and characterized as follows. 4-Amino veratrole was treated with chlorobenzene to yield veratrole chlorobiphenyls (CBs), which were further chlorinated by sodium chlorate in hydrochloric acid. The products were separated on an ODS semipreparative column, eluted with acetonitrile/water (8:2, v/v). The purities of isomers were determined to be >99% by gas chromatography. The mass spectral and ^{1}H NMR data were characterized as follows.

3,4-Dimethoxy-2,5,3′,4′-tetrachlorobiphenyl (diOH-CB70 Derivative). ^{1}H NMR (500 MHz, chloroform-*d*): δ 3.95 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 7.12 (s, 1H, 6-H), 7.25 (dd, J = 2.0, 8.6 Hz, 1H, 6′-H), 7.28 (d, J = 2.0, 1H, 2′-H), 7.50 (d, J = 8.6 Hz, 1H, 5′-H). MS (EI) *m/z* (relative intensity): 350 (87) [M$^+$], 352 (100) [M$^+$ + 2], 374 (50) [M$^+$ + 4], 335 (45) [M$^+$ − CH_3], 307 (20) [M$^+$ − $COCH_3$], 292 (20) [M$^+$ − $COCH_3$ − CH_3], 272 (25) [M$^-$ − $COCH_3Cl$], 194 (25) [M$^+$ − $(COCH_3Cl)_2$].

3′,4′-Dimethoxy-2,3,4,2′,5′-pentachlorobiphenyl (diOH-CB87 Derivative). ^{1}H NMR (500 MHz, chloroform-*d*): δ 3.99 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 7.12 (s, 1H, 6-H), 7.47 (d, J = 8.1 Hz, 1H, 5′-H), 7.47 (d, J = 8.1 Hz). MS (EI) *m/z* (relative intensity): 384 (68) [M$^+$], 386 (100) [M$^+$ + 2], 388 (65) [M$^+$ + 4], 369 (25) [M$^+$ − CH_3], 341 (10) [M$^+$ − $COCH_3$], 326 (10) [M$^+$ − $COCH_3$ − CH_3], 306 (20) [M$^-$ − $COCH_3Cl$], 228 (15) [M$^+$ − $(COCH_3Cl)_2$].

3′,4′-Dimethoxy-2,4,5,2′,5′-pentachlorobiphenyl (diOH-CB101 Derivative). ^{1}H NMR (500 MHz, chloroform-*d*): δ 3.96 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 7.05 (s, 1H, 6-H), 7.35 (s, 1H, 6′-H), 7.59 (s, 1H, 3′-H). MS (EI) *m/z* (relative intensity): 384 (70) [M$^+$], 386 (100) [M$^+$ + 2], 388 (70) [M$^+$ + 4], 369 (20) [M$^-$ − CH_3], 341 (18) [M$^+$ − $COCH_3$], 326 (15) [M$^+$ − $COCH_3$ − CH_3], 306 (22) [M$^+$ − $COCH_3Cl$], 228 (20) [M$^+$ − $(COCH_3Cl)_2$].

4,5-Dimethoxy-2,3,6,3′,4′-pentachlorobiphenyl (diOH-CB110 Derivative). ^{1}H NMR (500 MHz, chloroform-*d*): δ 3.94 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 7.07 (dd, J = 8.3, 2.0 Hz, 1H, 6′-H), 7.33 (d, J = 2.0 Hz, 1H, 2′-H), 7.55 (d, J = 8.3 Hz, 1H, 5′-H). MS (EI) *m/z* (relative intensity): 384 (70) [M$^+$], 386 (100) [M$^+$ + 2], 388 (68) [M$^+$ + 4], 369 (25) [M$^+$ − CH_3], 341 (20) [M$^+$ − $COCH_3$], 326 (18) [M$^+$ − $COCH_3$ − CH_3], 306 (15) [M$^-$ − $COCH_3Cl$], 228 (22) [M$^+$ − $(COCH_3Cl)_2$].

4′,5′-Dimethoxy-2,3,4,2′,3′,6′-hexachlorobiphenyl (diOH-CB132 Derivative). ^{1}H NMR (500 MHz, chloroform-*d*): δ 3.95 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 7.04 (d, J = 8.4 Hz, 1H, 6′-H), 7.50 (d, J = 8.4 Hz, 1H, 5′-H). MS (EI) *m/z* (relative intensity): 418 (50) [M$^+$], 420 (100) [M$^+$ + 2], 422 (80) [M$^+$ + 4], 403 (20) [M$^+$ − CH_3], 375 (15) [M$^+$ − $COCH_3$], 360 (15) [M$^+$ − $COCH_3$ − CH_3], 340 (18) [M$^+$ − $COCH_3Cl$], 262 (22) [M$^+$ − $(COCH_3Cl)_2$].

4,5-Dimethoxy-2,3,6,2′,4′,5′-hexachlorobiphenyl (diOH-CB149 Derivative). ^{1}H NMR (500 MHz, chloroform-*d*): δ 3.95 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 7.30 (s, 1H, 6′-H), 7.63 (s, 1H, 3′-H). MS (EI) *m/z* (relative intensity): 418 (50) [M$^+$], 420 (100) [M$^+$ + 2], 422 (82) [M$^+$ + 4], 403 (25) [M$^+$ − CH_3], 375 (12) [M$^+$ − $COCH_3$], 360 (20) [M$^+$ − $COCH_3$ − CH_3], 340 (22) [M$^+$ − $COCH_3Cl$], 262 (35) [M$^+$ − $(COCH_3Cl)_2$].

Animal Treatments. Male Wistar rats (160−200 g) and homozygous Gunn rats (190−260 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Animals were housed three or four per cage with free access to commercial chow and tap water and maintained under a 12 h dark/light cycle (lights on, 08:00−20:00) in an air-controlled room (temperature, 24.5 ± 1 °C; humidity, 55 ± 5%). Groups of six rats received an intraperitoneal injection of CB101 (112 mg/kg) and KC500 (100 mg/kg each) dissolved in Panacete 810, a mixture of glycerides of medium chain fatty acids (Nippon Oils and Fats Co., Tokyo) (5 mL/kg). All rats were killed by decapitation on day 4 after the dosing, and the liver, kidney, brain, lung, blood, and adipose tissues were removed and weighed. After the blood clotted at room temperature, serum was separated by centrifugation and stored at −20 °C prior to analysis of PCB metabolites.

Isolation of Metabolites. Sample cleanup and quantification were carried out according to our methods reported previously (*29*). Briefly, tissue samples were homogenized with acetone/*n*-hexane (2:1, v/v). Three internal standards (2,3,4,5,6,3′,4′,5′-octaCB, 70 ng; 4-hydroxy-2,3,5,6,2′,3′,4′,5′-octachloro[^{13}C]-biphenyl, 50 ng; and 3-methylsulfonyl-4-methyl-5,2′,3′,4′,5′-pentaCB, 28 ng) were added to each extract, which was subjected to a gel permeation column packed with Bio-Beads S-X3 (50 g, Bio-Rad Laboratories, Hercules, CA). Dichloromethane/*n*-hexane (1:1, v/v) was used as a mobile phase at a flow rate of 4 mL/min. The metabolite fraction (120−200 mL) was partitioned between *n*-hexane and a 1 M KOH/ethanol (5:2, v/v) solution. The aqueous solution was acidified with HCl and then extracted with *n*-hexane/diethyl ether (9:1) for acidic metabolites, which were subsequently methylated by an excess of diazomethane. The neutral fraction was applied to a silica gel mini-column (1 g, Wakogel S-1, Wako Pure, Co. Ltd., Japan), eluted with *n*-hexane (10 mL) for PCBs and successively with dichloromethane (10 mL) for $MeSO_2$-CBs. Mean recoveries (n = 3) of three internal standards spiked into control liver tissues at 50 ng each were 91% for PCBs, 85% for phenolic PCBs, and 88% for $MeSO_2$-CBs.

Identification and Quantification. For CB101 metabolites, quantification was performed on a GC-14A (Shimadzu Co., Kyoto, Japan) instrument equipped with a ^{63}Ni electron capture detector (ECD) and a DB-5 capillary column (60 m × 0.25 mm, i.d. J&W Scientific, United States). Injection was carried out in the splitless mode. Temperature program: 100 °C (2 min), 100−250 °C at 20 °C/min, 250−280 °C at 2 °C/min. Composition analyses of KC500 and its metabolites were carried out on a GC/MS system (AOC-17, GC-17A, QP-5000, Shimadzu, Co., Ltd.) in electron ionization−selected ion monitoring (SIM) mode, with column conditions similar to those described above. The monols and catechol PCBs were monitored at molecular ion (M$^+$) and M$^+$ + 2 for methylated derivatives of tetra-, penta-, and hexachlorinated congeners. Individual metabolites were quantified by a standard curve for GC peak area ratios relative to the internal satandard vs the concentration of metabolites.

Results

CB101 Metabolism. Figure 1 shows the GC/ECD chromatograms of combined fractions of neutral and phenolic PCBs (after methylation by diazomethane) in liver and serum of Gunn rats 4 days after exposure to CB101. On GC and GC/MS analyses, four hydroxylated

Table 1. Concentrations of Unchanged PCB, Phenolic, and Methylsulfonyl Metabolites in the Liver and Serum of Gunn and Wistar Rats Dosed with CB101 (112 mg/kg, ip)[a]

	concentration (ng/g wet)			
	Gunn rat		Wistar rat	
congener	liver	serum	liver	serum
unchanged CB101	2428 ± 405*	128 ± 9	1254 ± 207	109 ± 23
3-OH-CB101 (M-1)	56 ± 27	17 ± 4	27 ± 10	33 ± 8
3′-OH-CB101 (M-2)	238 ± 50	90 ± 21	223 ± 70	87 ± 26
4′-OH-CB101 (M-3)	22 ± 7	12 ± 2	94 ± 42	13 ± 3
diOH-CB101(M-4)	328 ± 60**	612 ± 73**	91 ± 41	273 ± 65
sum of (monols + diol)	644 ± 102	731 ± 84	434 ± 153	406 ± 105
3′-$MeSO_2$-CB101 (M-5)	49 ± 17**	17 ± 1**	337 ± 63	90 ± 2
4′-$MeSO_2$-CB101 (M-6)	70 ± 15**	14 ± 4**	589 ± 109	52 ± 10
sum of $MeSO_2$-CBs	119 ± 31**	31 ± 3**	926 ± 171	142 ± 12
		ratios		
diol/monols	1.04	5.14	0.26	2.05
diol/CB101	0.14	4.78	0.07	2.50
3′-/4′-$MeSO_2$-CBs	0.70	1.21	0.57	0.17
$MeSO_2$-CBs/CB101	0.05	0.24	0.74	0.56

[a] Values represent means ± standard errors for $n = 4-5$. The statistical difference of changes were analyzed by Student's t-test, $p < 0.05$ (*) and $p < 0.001$ (**), as compared with Wistar controls.

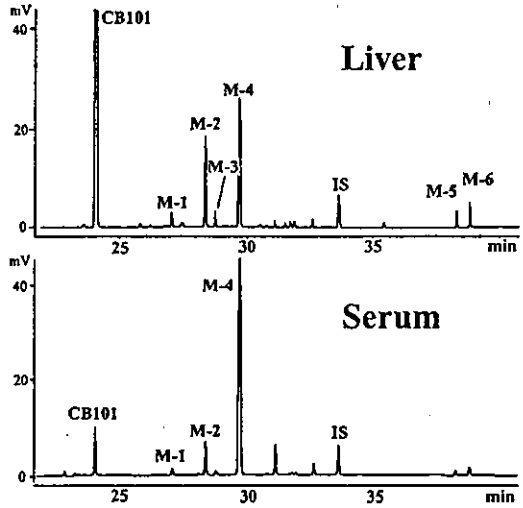


Figure 1. GC/ECD profiles of unchanged PCB and PCB metabolites in liver and serum of Gunn rats after exposure to CB101. The neutral and phenolic fractions were combined and methylated by diazomethane.

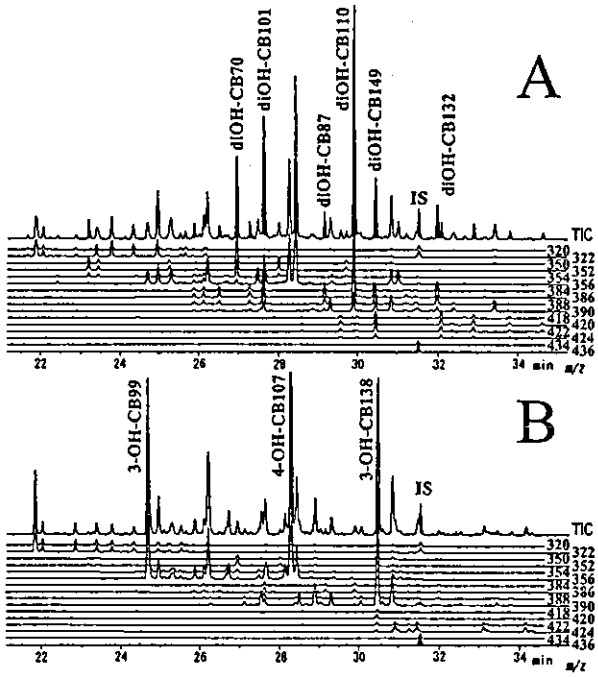


Figure 2. GC/MS/SIM profiles of phenolic PCBs (as methyl derivatives of monols and diols) in liver of Gunn (A) and Wistar (B) rats after exposure to KC500. Monitoring ions are M^+ and $M^+ + 2$ for methylated derivatives of monols and diols from tetra-, penta-, and hexaCBs.

metabolites (M-1 to M-4) and two $MeSO_2$ metabolites (M-5 and M-6) were detected in Gunn rats. The metabolite structures were identified as 3-OH-2,4,5,2′,5′-pentaCB (M-1), 3′-OH-2,4,5,2′,5′-pentaCB (M-2), 4′-OH-2,4,5,2′,5′-pentaCB (M-3), 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCB (diOH-CB101, M-4), 3′-$MeSO_2$-2,4,5,2′,5′-pentaCB (M-5), and 4′-$MeSO_2$-2,4,5,2′,5′-pentaCB (M-6) by GC/MS comparison with authentic standards. The levels of unchanged CB101, phenolic PCBs, and $MeSO_2$-CBs in liver and serum of both strains are shown in Table 1. More than 90% of the dose was eliminated from the body of both strains at 4 days after exposure to CB101. Unchanged CB101 was abundant in adipose tissue (approximately 7.5% of the dose), whereas OH-PCBs and $MeSO_2$-CBs were distributed in lung, kidney, and brain (data not shown) as well as liver and serum.

The hepatic concentration of residual CB101 in Gunn rats was significantly higher ($p < 0.05$) than that in Wistar rats. For hydroxylated PCBs, M-2 was most abundant in the liver of Wistar rats, whereas the catechol (M-4) was dominant in the serum of Gunn rats. The concentration ratios of catechol/monols in Gunn and Wistar rats were 1.04 and 0.26, whereas the ratios of catechol/parent CB101 were 0.14 and 0.07, respectively. In contrast, hepatic $MeSO_2$-CB levels in Gunn rats were 7.8-fold lower than those in Wistar controls. Thus, the concentration ratios of $MeSO_2$-CBs/CB101 in livers of Gunn and Wistar rats were 0.05 and 0.74, respectively.

KC500 Metabolism. Figure 2 shows the GC/MS/SIM profiles for monols and diols of tetra- to hexachlorinated congeners in the phenolic PCB fraction (methylated) of the livers of Wistar and Gunn rats after exposure to KC500. Phenolic PCB metabolites in Wistar rats were dominated by three monols, 3-OH-2,4,5,2′,4′-pentaCB (3-OH-CB99), 4-OH-2,3,5,3′,4′-pentaCB (4-OH-CB107), and 3-OH-2,4,5,2′,3′,4′-hexaCB (3-OH-CB138). On the other hand, phenolic PCB metabolites in Gunn rats were

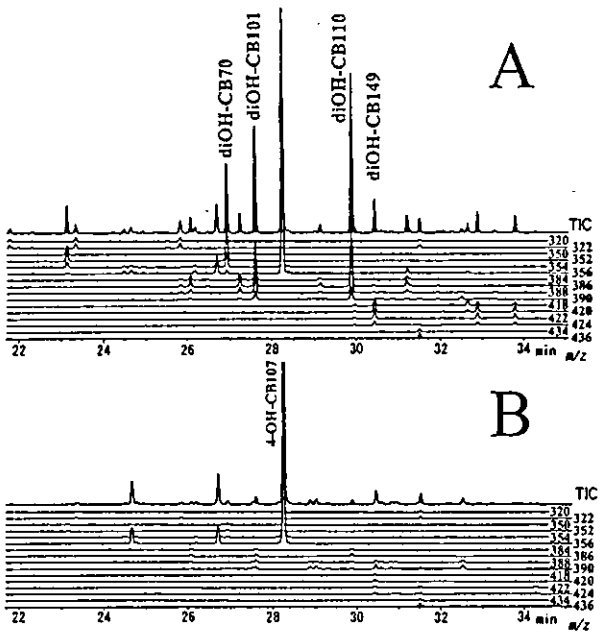


Figure 3. GC/MS/SIM profiles of phenolic PCB metabolites (as methyl derivatives of monols and diols) in serum of Gunn (A) and Wistar (B) rats after exposure to KC500. Monitoring ions are M^+ and $M^+ + 2$ for methylated derivatives of monols and diols from tetra-, penta-, and hexaCBs.

dominated by diols derived from tetraCBs (m/z = 350, M^+ for methylated derivatives), pentaCBs (m/z = 384), and hexaCBs (m/z = 418), some of which were identified as diOH-CB70, diOH-CB87, diOH-CB101, diOH-CB110, diOH-CB132, and 4,5-$(OH)_2$-2,3,6,2′,4′,5′-hexaCB (diOH-CB149) by comparison of mass spectra and relative GC retention times with reference standards.

Figure 3 shows the GC/MS/SIM profiles of phenolic PCB fraction (as methylated derivatives) for monols and diols of tetra- to hexaCB congeners in the serum of Wistar and Gunn rats after exposure to KC500. Metabolite profiles in the serum of Wistar rats were dominated by 4-OH-CB107. On the other hand, the metabolite profiles in serum of Gunn rats were dominated by four catechols, diOH-CB70, diOH-CB101, diOH-CB110, and diOH-CB149, in addition to 4-OH-CB107.

The levels of unchanged PCBs and phenolic PCB metabolites (monols and diols) in liver and serum of both rat strains are shown in Table 2. The total levels of major residual PCBs in liver of Gunn rats were 2.2-fold lower, but the levels of CB101 and CB149 were 2.5- and 6.0-fold higher, respectively, as compared to Wistar rats. For monol metabolites, 4-OH-CB107 was present at the highest level in all tissues of both strains. Thus, the concentration ratios of 4-OH-CB107 relative to the possible parent CBs, 2,3,4,3′,4′-pentachlorobiphenyl (CB105) and 2,4,5,3′,4′-pentachlorobiphenyl (CB118), in Gunn and Wistar rats were 0.12 and 0.08 in liver, whereas they were 9.2 and 6.4 in serum, respectively. For catechol metabolites, diOH-CB110 was present at the highest levels in serum of Gunn rats, whereas the level in Wistar rats was less than one-tenth. Thus, the levels of six catechol PCBs accounted for about 52% of total phenolic PCBs. The ratios of diOH-CB101/CB101 and diOH-CB149/CB149 were higher in serum than in liver for both rat strains.

Table 2. Congener Specific Determination of Unchanged and Phenolic PCBs in the Liver and Serum of Gunn and Wistar Rats Dosed with KC500 (100 mg/kg, ip)[a]

	concentration (ng/g wet)			
	Gunn rat		Wistar rat	
congener	liver	serum	liver	serum
	PCB			
CB99	313 ± 82*	21 ± 5*	1018 ± 14	47 ± 5
CB101	300 ± 61*	14 ± 3	121 ± 3	13 ± 2
CB105	383 ± 78*	25 ± 5*	664 ± 24	56 ± 4
CB118	680 ± 135*	47 ± 10*	1532 ± 26	95 ± 6
CB138	750 ± 169*	48 ± 10*	1571 ± 52	151 ± 9.2
CB146	139 ± 40*	11 ± 2*	289 ± 17	33 ± 3
CB149	155 ± 52*	3.6 ± 0.9	26 ± 2	4.1 ± 0.8
CB153	438 ± 114	38 ± 9*	1095 ± 57	129 ± 7.8
CB156	199 ± 48*	29 ± 12*	753 ± 30	84 ± 17
CB167	234 ± 59*	14 ± 3*	307 ± 17	27 ± 2
CB170	122 ± 37*	6.4 ± 1.4*	464 ± 25	49 ± 3
CB180	184 ± 55*	11 ± 2*	659 ± 50	84 ± 5
CB187	66 ± 21*	4.6 ± 1.2*	219 ± 16	25 ± 2
sum of PCBs	3964 ± 792*	270 ± 65*	8715 ± 283	798 ± 58
	phenolic PCB			
3-OH-CB99	32 ± 6*	14 ± 4	84 ± 7	14 ± 5
4-OH-CB107	127 ± 34	567 ± 42*	186 ± 13	905 ± 121
3-OH-CB138	28 ± 3*	82 ± 8*	141 ± 10	45 ± 8
sum of monols	186 ± 32*	663 ± 40	411 ± 22	964 ± 104
diOH-CB70	37 ± 14*	109 ± 31*	ND	5 ± 1
diOH-CB87	22 ± 5*	69 ± 25*	ND	ND
diOH-CB101	65 ± 19*	109 ± 41*	6 ± 2	6 ± 1
diOH-CB110	98 ± 32*	313 ± 87*	8 ± 2	10 ± 1
diOH-CB132	11 ± 3*	31 ± 9*	ND	ND
diOH-CB149	33 ± 5*	98 ± 24*	4 ± 2	5 ± 2
sum of diols	267 ± 43*	729 ± 141*	18 ± 2	26 ± 3
	ratio			
4-OH-CB107/(CB105 + CB118)	0.12	9.2	0.08	6.4
diOH-CB101/CB101	0.22	7.8	0.05	0.5
diOH-CB149/CB149	0.21	55	0.15	1.2

[a] Values represent means ± standard errors for n = 4–5. *Significantly different from Wistar controls (Student's t-test, $p < 0.001$); ND = not detected (<0.1 ng/g).

The hepatic concentrations of $MeSO_2$-CBs in both rat strains are shown in Table 3. Eighteen $MeSO_2$-CBs congeners were identified in the liver, consisting of *meta*- and *para*-$MeSO_2$-CBs derived from nine parent CB congeners with 2,5- or 2,3,6-chlorine substitution. When classified according to the chlorination pattern, $MeSO_2$-CBs with 2,5-chlorine substitution were dominated by *meta*-$MeSO_2$-CBs (*meta*/*para* concentration ratios = 0.64–11.3), whereas $MeSO_2$-CBs with 2,3,6-chlorine substitution were dominated by *para*-$MeSO_2$-CBs (the ratios = 0.07–0.25). Although there were no differences in the congener profiles of $MeSO_2$-CBs between both rat strains, the levels of each congener in Gunn rats were significantly low ($p < 0.001$) as compared to Wistar rats.

The levels of unchanged PCBs, phenolic PCBs, and $MeSO_2$-CB metabolites in the liver of both strains exposed to KC500 are summarized in Table 4. In comparison with Wistar rats, Gunn rats exhibited lower levels of total $MeSO_2$-CBs, but higher levels of catechol PCBs in the liver (statistical significance; Student's t-test, $p < 0.001$).

Discussion

To our knowledge, this is the first report of the identification and tissue retention of catechol PCB metabolites of tetra- to hexachlorinated biphenyls in rats exposed to KC500. To date, the formation of catechol PCBs has been demonstrated only from selected CB

Table 3. Concentrations of $MeSO_2$-CBs in the Liver of Gunn and Wistar Rats Dosed with KC500 (100 mg/kg, ip)[a]

	concentration (ng/g wet) and ratio			
congener	Gunn rat	*meta*/*para* ratio	Wistar rat	*meta*/*para* ratio
2,5-chlorine substitution				
3-$MeSO_2$-2,5,4′-triCB	9.2 ± 2.2*	11.3	32 ± 6	11.2
4-$MeSO_2$-2,5,4′-triCB	0.8 ± 0.2*		2.7 ± 0.3	
3-$MeSO_2$-2,5,2′,4′-tetraCB	10 ± 2*	7.76	59 ± 5	7.55
4-$MeSO_2$-2,5,2′,4′-tetraCB	1.3 ± 0.4*		7.7 ± 0.5	
3-$MeSO_2$-2,5,3′,4′-tetraCB	18 ± 4*	0.97	81 ± 8	1.54
4-$MeSO_2$-2,5,3′,4′-tetraCB	20 ± 3*		53 ± 5	
3′-$MeSO_2$-2,3,4,2′,5′-pentaCB	6.4 ± 4.3*	1.05	22 ± 2	1.09
4′-$MeSO_2$-2,3,4,2′,5′-pentaCB	6.1 ± 4.2*		20 ± 2	
3′-$MeSO_2$-2,4,5,2′,5′-pentaCB	19 ± 7*	0.64	129 ± 15	1.17
4′-$MeSO_2$-2,4,5,2′,5′-pentaCB	29 ± 8*		110 ± 6	
sum	119 ± 26*		515 ± 34	
2,3,6-chlorine substitution				
5-$MeSO_2$-2,3,6,2′,4′-pentaCB	3.1 ± 0.6*	0.25	40 ± 7	0.28
4-$MeSO_2$-2,3,6,2′,4′-pentaCB	12 ± 2*		123 ± 20	
5-$MeSO_2$-2,3,6,3′,4′-pentaCB	1.7 ± 0.5	0.07	4.6 ± 2.1	0.09
4-$MeSO_2$-2,3,6,3′,4′-pentaCB	24 ± 6		53 ± 6	
5′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCB	1.3 ± 0.6*	0.21	9.1 ± 2.0	0.98
4′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCB	6.1 ± 2.7		9.4 ± 2.0	
5-$MeSO_2$-2,3,6,2′,4′,5′-hexaCB	0.8 ± 0.5	0.16	2.0 ± 0.9	0.21
4-$MeSO_2$-2,3,6,2′,4′,5′-hexaCB	5.2 ± 2.2*		9.8 ± 2.1	
sum	55 ± 9.3*		256 ± 28	
sum of *meta*-$MeSO_2$-CBs	70 ± 16*	0.67	401 ± 20	1.08
sum of *para*-$MeSO_2$-CBs	104 ± 27*		370 ± 32	
total $MeSO_2$-CBs	174 ± 39*		771 ± 40	

[a] Values represent means ± standard errors for n = 4–5. *Significantly different from Wistar controls (Student's t-test, p < 0.001).

Table 4. Concentrations of PCBs and Their Metabolites in the Liver of Wistar and Gunn Rats Exposed to KC500 (100 mg/kg, ip)[a]

	concentration (ng/g wet weight)		ratio
congener	Gunn rat	Wistar rat	Gunn/Wistar
PCBs	3964 ± 884*	8715 ± 283	0.45
OH-PCBs	186 ± 32*	411 ± 22	0.45
diOH-PCBs	267 ± 43*	18 ± 2	14.8
$MeSO_2$-PCBs	174 ± 39*	771 ± 40	0.23
	ratio		
OH-PCBs/PCBs	0.05	0.05	1.0
diOH-PCBs/PCBs	0.07	0.002	35
$MeSO_2$-CBs/PCBs	0.04	0.09	0.4
PCB metabolites/PCBs	0.16	0.14	1.1

[a] Values represent means ± standard errors for n = 4–5. *Significantly different from Wistar controls (Student's t-test, p < 0.001).

congeners that are not relevant in the environment except for CB101 (*17*). The present study demonstrated that catechols could be readily formed from CBs with 2,5- or 2,3,6-chlorine substitution in UGT1A deficient Gunn rats treated with KC500.

In CB101-treated Gunn rats, we isolated three monol and one diol PCB metabolites (as methylated derivatives) from the phenolic fraction, as well as two $MeSO_2$ metabolites from the neutral fraction of tissues analyzed, where a catechol PCB and two $MeSO_2$-CBs were distributed at higher levels than monol metabolites, especially in serum. The metabolic pathway of CB101 also included 3-hydroxylation on the 2,4,5-trichlorinated phenyl ring. Such hydroxylation has been observed in minks exposed to CB101 (*30*).

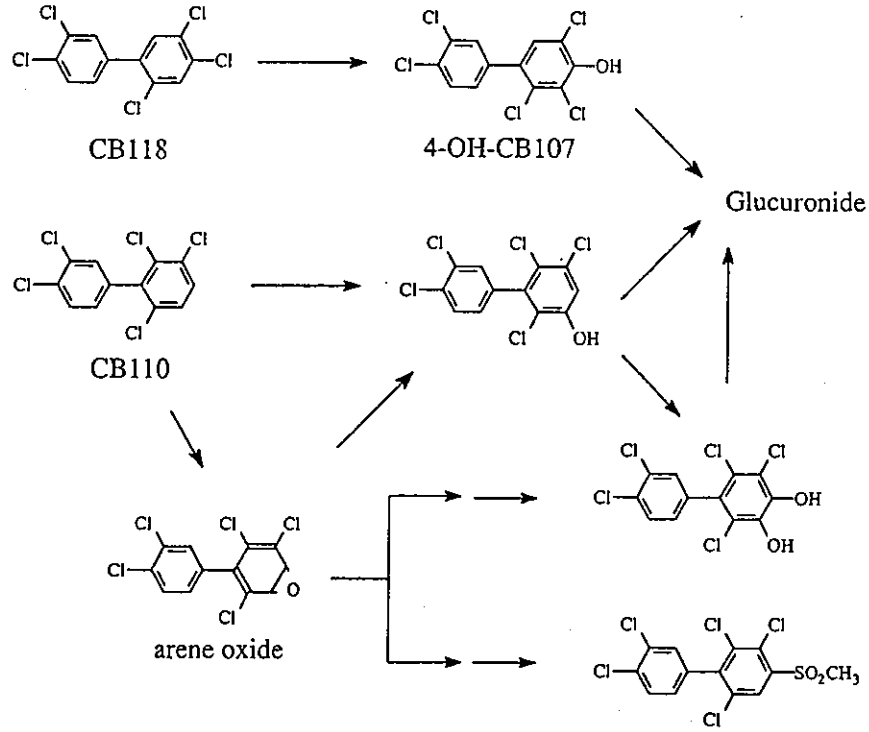
In KC500-treated Gunn rats, we identified six catechols derived from CB70, CB87, CB101, CB110, CB132, and CB149 in all tissues and blood. These parent CBs are the major components (38% in total) of KC500 that have chlorine atoms at the 2,5- or 2,3,6-position of one ring and at the 3,4-, 2,3,4-, or 2,4,5-position of the other (*31*). The percentage compositions of catechol PCBs were estimated to be more than 60% in total of phenolic PCBs detected in the liver. Although we could not identify catechols from 2,5,2′,5′-tetraCB or 2,3,6,2′,5′-pentaCB (15% in total of KC500) due to lack of authentic standards, we hypothesized that all PCB components with 2,5- or 2,5,6-chlorine substitution were likely to be substrates for 3,4-catechol formation in Gunn rats. The possibility of the formation of 2,3-catechols from CBs with *meta*- and *para*-substituted chlorine atoms should not be excluded, as previous reports indicated that 3,4,3′,4′-tetraCB could be metabolized to the 2,3-catechol in rats (*32*) and 2,4,5,2′,4′,5′-hexaCB could be metabolized via 2,3-epoxide in guinea pigs (*33*). In some cases, the catechol may be formed through dechlorination from an NIH shift metabolite, such as 4-OH-CB107 in rats (*29*).

P450-catalyzed hydroxylation of biphenyl rings has been observed in vitro by use of bioactivation systems based on hepatic microsomes from rodents treated with various enzyme inducers (*34*) and human hepatic microsomes (*35*). It is therefore hypothesized that treatment of Gunn rats with KC500 may activate an alternative pathway of PCB metabolism involving the induced form of CYP. The possible metabolic pathways for monol or catechol formation from CB118 and CB110 (major components in KC500) are illustrated in Scheme 1. Metabolism of CB118 may involve 4-hydroxylation via 3,4-epoxide and NIH shift to form 4-OH-CB107. Aromatic hydroxylation of CBs with 2,3,6-chlorine substitution such as CB110 in Gunn rats is likely to involve the direct insertion of a hydroxyl group on the *meta*-position and/or isomerization or hydration of an arene oxide intermediate to form monol or dihydrodiol, followed by further oxidation of monols or dehydration of a dihydrodiol (*3*, *18*, *36*).

The present study also demonstrated marked differences in the tissue levels of parent PCBs, catechol PCBs, and $MeSO_2$-CBs between Gunn and Wistar rats (Table 4). CB101 metabolism by Gunn rats resulted in higher levels of residual CB101 as compared to Wistar controls (Table 1). Similarly, the concentration ratios of both CB101 and CB149 relative to other residual PCBs were higher in KC500-treated Gunn rats (Table 2). This may be explained by the observation that Gunn rats are less susceptible to induction of CYP2B1/2 that catalyzes the metabolism of CB101 and CB149 as compared to Wistar rats (*37*). On the other hand, the levels of catechols were higher in Gunn rats, whereas the levels of $MeSO_2$-CBs were lower in Gunn rats as compared to Wistar rats. Thus, the hepatic concentration ratio of ΣdiOH-PCB/Σ$MeSO_2$-CB was 1.5 in Gunn rats, while it was 0.02 in Wistar rats (Table 4). These results suggest that a lack of UGTs markedly alters the formation ratios of catechol PCBs and $MeSO_2$-CBs.

Marniemi et al. (*39*) have reported that although the activities of bilirubin UGTs and UDP-glucosyltransferase are significantly low in Gunn rats as compared to Wistar rats, microsomal epoxide hydrase and soluble gluthatione-epoxide transferase activities are normal. In our recent study (*25*), UGT deficient Gunn rats exhibited no activity of UGT1A and a lower activity of UGT2B as compared to Wistar rats. These observations suggest that the formation ratios of catechol and $MeSO_2$ metabolites may

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).

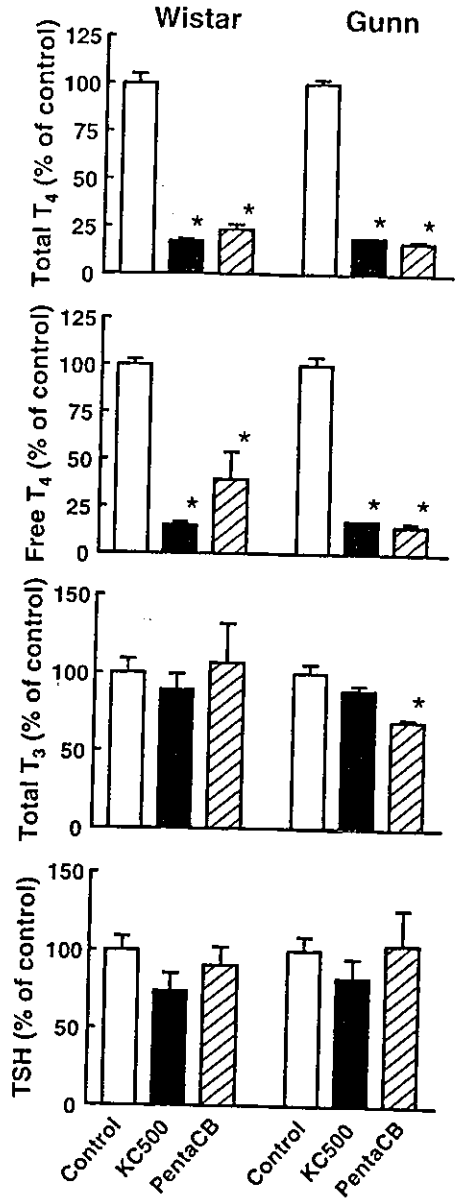

FIG. 1. Effects of KC500 and PentaCB on levels of serum total thyroxine (T_4), free T_4, total triiodothyronine (T_3), and thyroid-stimulating hormone (TSH) in Wistar and Gunn rats. Animals were killed 4 days after the administration of KC500 (100 mg/kg, ip) or PentaCB (112 mg/kg, ip), and levels of 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.

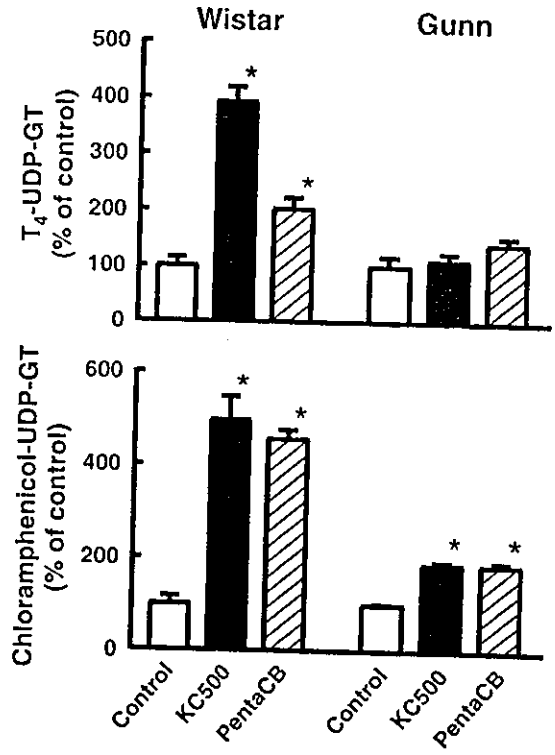

FIG. 2. Effects of KC500 and PentaCB on hepatic microsomal UDP-glucuronyltransferase activities in Wistar and Gunn rats. Each column represents the mean ± SE (vertical bars) for four to six animals. Constitutive levels: T_4-UDP-GT (pmol/mg protein/min), 12.60 ± 0.69 (Wistar) and 5.95 ± 1.06 (Gunn); chloramphenicol-UDP-GT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

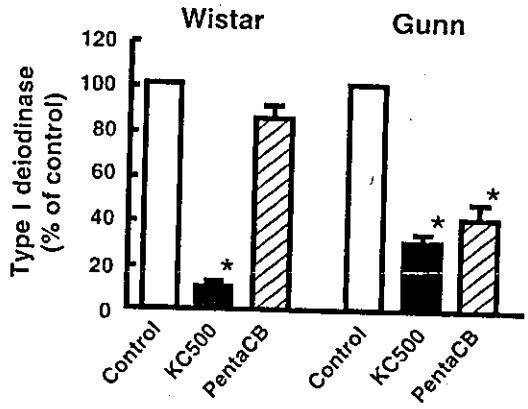

FIG. 3. Effects of KC500 and PentaCB on hepatic microsomal type-I deiodinase activity in Wistar and Gunn rats. Each column represents the mean ± SE (vertical bars) for four to six animals. *$p < 0.001$, significantly different from each control.

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

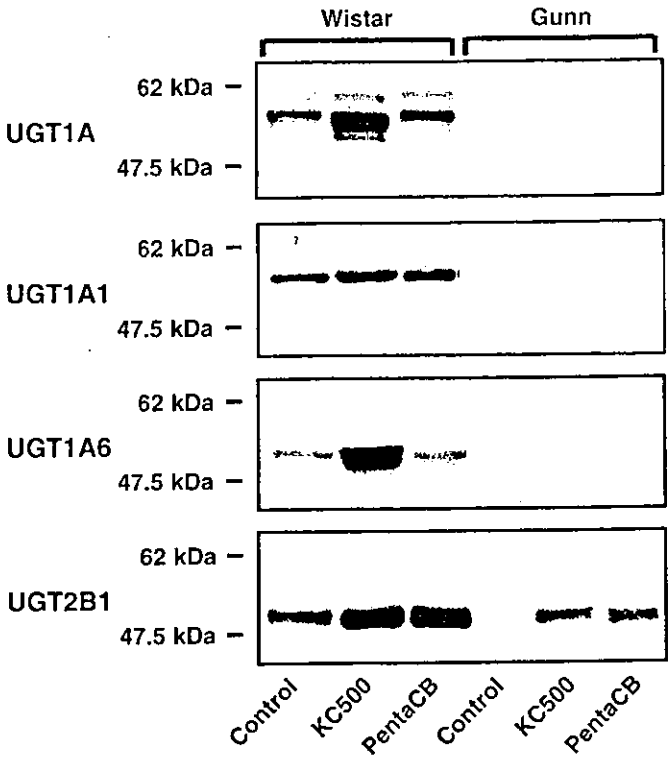

FIG. 4. Representative immunoblot patterns for hepatic microsomal UGT isoforms in KC500-treated or PentaCB-treated Wistar and Gunn rats.

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

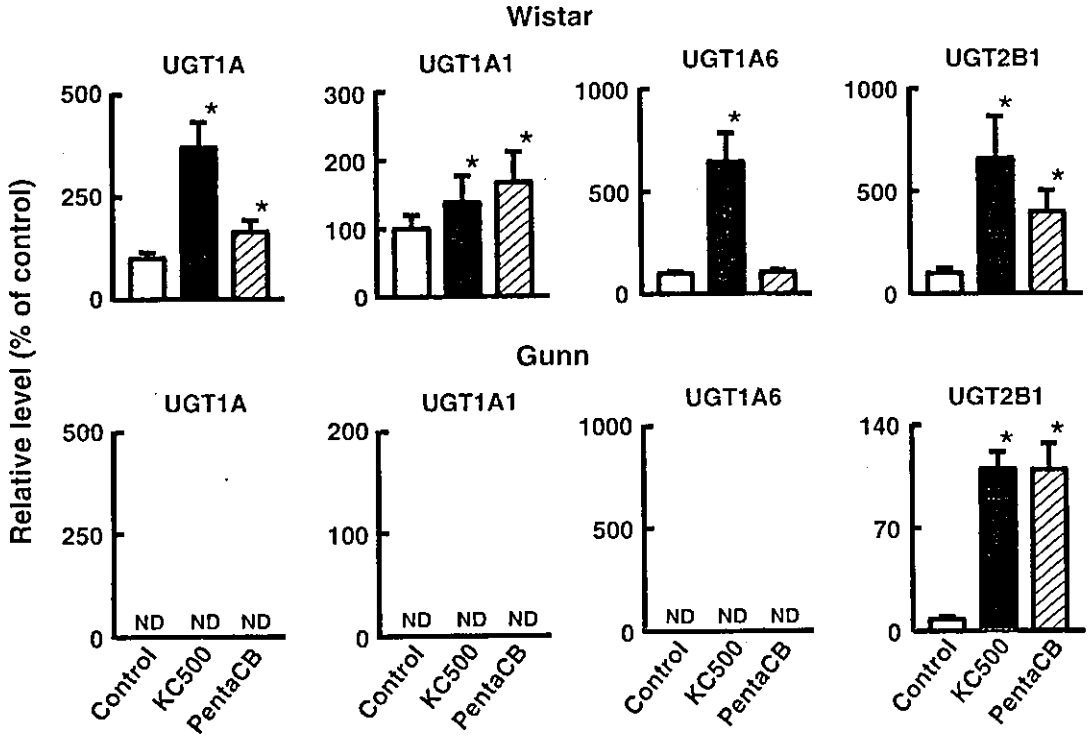

FIG. 5. Effects of KC500 and PentaCB on the level of hepatic microsomal UGT isoforms in Wistar and Gunn rats. After the immnoblot as shown in Figure 4, the isolated bands responsible for UGT isoforms were densitometrically quantified as described in Materials and Methods. The data are represented as 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.

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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 sofrware 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

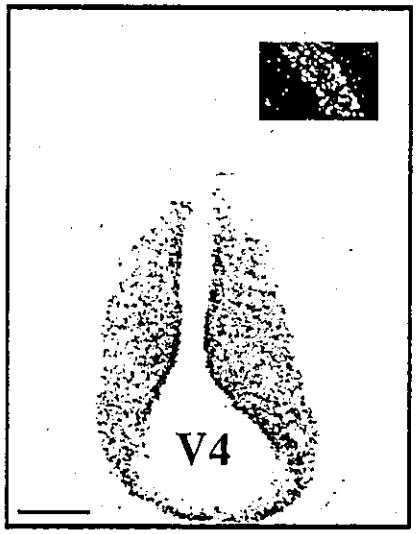

Figure 1. Film autoradiographic image of NSP-A expression in the GD16 fetal brain illustrating the region of the cortex in which gene expression was measured. Fetal brain tissue was collected from the cortex by taking 12-μm horizontal sections. The top of the image is rostral, the lower portion of the 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*I	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*1	Sp6	1234		
NSP-C	Synthetic oligo	Antisense	NA	NA	NA	230–183	Dowling et al. 2001

NA, not applicable.