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In vitro metabolism of 2,2′,3,4′,5,5′,6-heptachlorobiphenyl (CB187) by liver microsomes from rats, hamsters and guinea pigs

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Abstract
The metabolism of 2,2′,3,4′,5,5′,6-heptachlorobiphenyl (heptaCB) (CB187) was studied using liver microsomes of rats, hamsters and guinea pigs, and the effect of cytochrome P450 (CYP) inducers, phenobarbital (PB) and 3-methylcholanthrene (MC), was also investigated. In untreated animals, guinea pig liver microsomes formed three metabolites which were deduced to be 4′-hydroxy-2,2′,3,5,5′,6-hexachlorobiphenyl (M-1), 4′-hydroxy-2,2′,3,3′,5,5′,6-heptaCB (M-2) and 4-OH-CB187 (M-3) from the comparison of GC/MS data with some synthetic authentic samples. The formation rate of M-1, M-2 and M-3 was 18.1, 36.6, 14.7 pmol h^{-1} mg $protein^{-1}$, respectively. Liver microsomes of untreated rats and hamsters did not form CB187 metabolites. In guinea pigs, PB-treatment increased M-1 and M-2 significantly to 1.9- and 3.4-fold of untreated animals but did not affect the formation of M-3. In rats, PB-treatment resulted in the appearance of M-2 and M-3 with formation rates of 87.1 and 13.7 pmol h^{-1} mg $protein^{-1}$, respectively, but M-1 was not observed. In hamsters, PB-treatment formed only M-2 at a rate of 29.4 pmol h^{-1} mg $protein^{-1}$. On the other hand, MC-treatment of guinea pigs decreased the formation of M-1 and M-2 to less than 50% of untreated animals. MC-microsomes of rats and hamsters produced no metabolites. Preincubation of antiserum (300 μl) against guinea pig CYP2B18 with liver microsomes of PB-treated guinea pigs produced 80% inhibition of M-1 and the complete inhibition of M-2 and M-3. These results suggest that PB-inducible CYP forms, especially guinea pig CYP2B18, rat CYP2B1 and hamster CYP2B, are important in CB187 metabolism and that CB187 metabolism in guinea pigs may proceed via the formation of 3,4- or 3′,4′-oxide and subsequent NIH-shift or dechlorination.

Keywords: *Heptachlorobiphenyl, CB187, cytochrome P450, guinea pig, rat, metabolism*

Introduction

PCB congeners, worldwide environmental pollutants, possess an extremely high lipophilicity and biological stability, and as a result they are not easily eliminated from the body

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(Safe 1989). In particular, not only 2,4,5-trichloro-substituted but also six or more chlorine-substituted PCBs such as 2,2′,3,4,4′,5′-hexachlorobiphenyl (hexaCB) (CB138), 2,2′,4,4′,5,5′-hexaCB (CB153), 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (heptaCB) (CB180) and 2,2′,3,4′,5,5′,6-heptaCB (CB187) have been detected in blood and adipose tissues of mammals (Sonzogni et al. 1991, Haraguchi et al. 1992, Bergman et al. 1994, Kannan et al. 1994, Roots and Talvari 1997, Mimura et al. 1999b, Humphrey et al. 2000) and human mother's milk (Franchi and Focardi 1991, Prachar et al. 1994, Mimura et al. 1999b).

Generally, PCB congeners are mainly metabolized by liver microsomal cytochrome P450s (CYP) belonging to two subfamilies, CYP1A and CYP2B (Koga and Yoshimura 1996), and the hydroxyl (OH) metabolites are excreted via bile into the feces (Yoshimura et al. 1987, Koga et al. 1990). Highly chlorinated biphenyls with a 2,4,5-trichloro-substituted benzene ring resist oxygenation by CYP (Schnellmann et al. 1985). However, it has been reported that dogs and guinea pigs can readily metabolize such PCB congeners in animals tested so far and that dog CYP2B11 and guinea pig CYP2B18 hydroxylate 2,4,5-trichloro-substituted PCB congeners (Duignan et al. 1987, Ariyoshi et al. 1992, 1995, 1997, Koga et al. 2001).

Certain 4-hydroxy (OH)-PCB metabolites have been detected in animal blood at relatively high concentrations (Bergman et al. 1994, Sandau et al. 2000, Fängström et al. 2002, Sandala et al. 2004) and have been shown to have a high affinity to transthyretin, which is a major plasma thyroid hormone-binding protein forming the plasma transport system of vitamin A together with retinol-binding protein. As a result, 4-OH metabolites perturb thyroid hormone and vitamin A homeostasis in animal blood (Brouwer and van den Berg 1986, Brouwer et al. 1990, 1998). In addition, some OH metabolites have been found to be estrogenic or antiestrogenic (Korach et al. 1988, Jansen et al. 1993, Connor et al. 1997, Kramer et al. 1997) and can also inhibit estrogen sulfotransferase (Kester et al. 2002). Recently, among 4-OH metabolites derived from PCB congeners, 4-OH-CB187 have been shown to be present at high concentrations in the blood of fish (Campbell et al. 2003), bear (Sandala et al. 2004) and human (Bergman et al. 1994, Sandau et al. 2000, Fängström et al. 2002). Although CB187 is one of the minor components in commercial PCB preparations such as Clophen, Aroclor and Kanechlor (Ballschmiter et al. 1989), the toxic equivalency factor (TEF) determined for dioxin-like PCB congeners such as coplanar- and some mono-*ortho*-PCBs to assess the potency of the toxicity has not been investigated for CB187 in addition to CB138, CB153 and CB180. Thus, 4-OH-CB187 is toxicologically more important than the parent CB187 because it acts as a mimic of thyroids and other steroidal hormones. However, there is little information available on the biotransformation of CB187 in animals. Therefore, we examined CB187 metabolism by liver microsomes of rats, hamsters and guinea pigs and also the effect of the CYP inducers, phenobarbital (PB) and 3-methylcholanthrene (MC) on CB187 metabolism.

Materials and methods

Chemicals

CB187 was synthesized by the method of Cadogan (1962) using 1,2,4,5-tetrachlorobenzene and 2,4,5-trichloroaniline as starting materials, both of which were purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo) and Wako Pure Chemical Industries, Ltd (Osaka, Japan), respectively. 4-Methoxy (MeO)-CB187 and 4′-MeO-2,2′,3,5,5′,6-hexaCB (CB146) were also synthesized by the method of Cadogan (1962). 3′-MeO-CB187 and

4′-MeO-2,2′,3,3′,5,5′,6-heptaCB (CB178) were obtained by the diazo-coupling reaction with 2,3,5,6-tetrachloroaniline (Aldrich, St. Louis, MO) and 2,3,6-trichlorophenol (Lancaster Synthesis, Morecambe, UK) (Hutzinger et al. 1971) and subsequent methylation with dimethylsulfate. Finally, they were purified by silica gel 60 (0.040–0.063 mm, Merck) column chromatography, TLC (silica gel $60F_{254}$, Merck) and reverse-phase HPLC. The HPLC conditions were as follows; column, ODS (20 × 250 mm, 5 μm, YMC); mobile phase, acetonitrile; flow rate, 4 ml min^{-1}; detection wavelength, 250 nm. PB (Na salt) and MC were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Nicotine adenine dinucleotide phosphate ($NADP^+$) and glucose-6-phosphate (G-6-P) were purchased from Oriental Yeast Co., Ltd (Tokyo). G-6-P dehydrogenase and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest quality commercially available.

4-MeO-CB187 (M-3). ^{1}H-NMR (500 MHz, chloroform-*d*) δ 3.990 (3H, s, 4-CH_3O), 7.287 (1H, s, 3′-H), 7.646 (1H, s, 6′-H); MS(EI) *m/z* (relative intensity) 422(100) [M^+], 424(227) [M^++2], 426(214) [M^++4], 428(118) [M^++6], 396(27) [M^++8], 407(15) [M^+-CH_3], 379(46) [M^+-$COCH_3$], 309(54) [M^+-$COCH_3Cl_2$].

3′-MeO-CB187. ^{1}H-NMR (500 MHz, chloroform-*d*) δ 3.967 (3H, s, 3′-CH_3O), 7.106 (1H, s, 6′-H), 7.700 (1H, s, 4-H); MS(EI) *m/z* (relative intensity) 422(100) [M^+], 424(223) [M^++2], 426(214) [M^++4], 428(113) [M^++6], 430(36) [M^++8], 407(13) [M^+-CH_3], 379(40) [M^+-$COCH_3$], 372(12) [M^+-CH_3Cl_2], 309(56) [M^+-$COCH_3Cl_2$].

4′-MeO-CB178 (M-2). ^{1}H-NMR (500 MHz, chloroform-*d*) δ 4.004 (3H, s, 4′-CH_3O), 7.155 (1H, s, 6′-H), 7.697 (1H, s, 4-H); MS(EI) *m/z* (relative intensity) 422(100) [M^+], 424(218) [M^++2], 426(206) [M^++4], 428(106) [M^++6], 430(33) [M^++8], 407(28) [M^+-CH_3], 379(41) [M^+-$COCH_3$], 309(74) [M^+-$COCH_3Cl_2$].

2′-MeO-2,3,5,6,3′,5′-hexaCB (CB165). MS(EI) *m/z* (relative intensity) 388(100) [M^+], 390(189) [M^++2], 392(153) [M^++4], 394(66) [M^++6], 396(15) [M^++8], 338(154) [M^+-CH_3Cl], 318(22) [M^+-Cl_2], 275(40) [M^+-$COCH_3Cl_2$].

3′-MeO-2,3,5,6,2′,5′-hexaCB (CB151). MS(EI) *m/z* (relative intensity) 388(100) [M^+], 390(194) [M^++2], 392(155) [M^++4], 394(67) [M^++6], 396(9) [M^++8], 345(24) [M^+-$COCH_3$], 318(9) [M^+-Cl_2], 275(46) [M^+-$COCH_3Cl_2$].

4′-MeO-CB151 (M-1). ^{1}H-NMR (500 MHz, chloroform-*d*) δ 5.778 (1H, s, 4′-OH), 7.149 (1H, s, 3′-H), 7.218 (1H, s, 6′-H), 7.675 (1H, s, 4-H); MS(EI) *m/z* (relative intensity) 388(100) [M^+], 390(190) [M^++2], 392(152) [M^++4], 394(66) [M^++6], 396(16) [M^++8], 373(5) [M^+-CH_3], 345(34) [M^+-$COCH_3$], 318(7) [M^+-Cl_2], 275(43) [M^+-$COCH_3Cl_2$].

5′-MeO-2,3,5,6,2′,4′-hexaCB (CB147). MS(EI) *m/z* (relative intensity) 388(100) [M^+], 390(183) [M^++2], 392(147) [M^++4], 394(63) [M^++6], 396(15) [M^++8], 373(20) [M^+-CH_3], 345(27) [M^+-$COCH_3$], 318(7) [M^+-Cl_2], 275(50) [M^+-$COCH_3Cl_2$].

6′-MeO-CB151. MS(EI) *m/z* (relative intensity) 388(100) [M^+], 390(192) [M^++2], 392(154) [M^++4], 394(66) [M^++6], 396(16) [M^++8], 338(86) [M^+-CH_3Cl], 318(43) [M^+-Cl_2], 275(36) [M^+-$COCH_3Cl_2$].

6-MeO-CB146. ^{1}H-NMR (500 MHz, chloroform-*d*) δ 3.605 (3H, s, 6-CH_3O), 7.327 (1H, s, 4-H), 7.609 (1H, s, 3′-H), 7.643 (1H, s, 6′-H); MS(EI) *m/z* (relative intensity) 388(100)

$[M^+]$, 390(193) $[M^++2]$, 392(156) $[M^++4]$, 394(86) $[M^++6]$, 396(16) $[M^++8]$, 338(134) $[M^+-CH_3Cl]$, 318(29) $[M^+-Cl_2]$, 275(39) $[M^+-COCH_3Cl_2]$.

5-MeO-2,3,6,2′,4′,5′-hexaCB (CB149). ^{1}H-NMR (500 MHz, chloroform-*d*) δ 3.951 (3H, s, 5-CH_3O), 7.127 (1H, s, 4-H), 7.284 (1H, s, 3′-H), 7.634 (1H, s, 6′-H); MS(EI) *m/z* (relative intensity) 388(100) $[M^+]$, 390(190) $[M^++2]$, 392(154) $[M^++4]$, 394(65) $[M^++6]$, 396(16) $[M^++8]$, 373(4) $[M^+-CH_3]$, 345(30) $[M^+-COCH_3]$, 338(16) $[M^+-CH_3Cl]$, 318(6) $[M^+-Cl_2]$, 275(42) $[M^+-COCH_3Cl_2]$.

Animal treatment

Nine male Wistar rats (body weight about 200 g), nine male Golden Syrian hamsters (body weight about 90 g) and nine male Hartley guinea pigs (body weight about 300 g) were used and divided into three groups, untreated, PB-treated and MC-treated groups. PB and MC were dissolved with saline and corn oil and injected i.p. to animals at a dose of 80 and 20 mg kg^{-1} day^{-1} for 3 days, respectively. Animals were killed 24 h after the last injection of each inducer, and liver microsomes were prepared as described elsewhere (Koga et al. 1990).

Quantification of metabolites

In the *in vitro* study, 40 µM CB187 was incubated at 37°C with 1 mg of protein from animal liver microsomes, NADPH-generating system (0.33 mM $NADP^+$, 5 mM G-6-P, 1 unit of G-6-P dehydrogenase), 6 mM $MgCl_2$ and 100 mM HEPES buffer (pH 7.4) in a total volume of 1 ml. After incubation for 60 min, unchanged CB187 and its metabolites were extracted three times with chloroform–methanol (2:1, v/v) and *n*-hexane. The pooled organic layer was evaporated to dryness, methylated with diazomethane and applied to a gas chromatograph HP5890 Series II equipped with ECD under the following conditions: DB-1 capillary column (30 m × 0.25 mm, 0.25 µm thickness); carrier gas, N_2 (1 ml min^{-1}); column temperature, 230°C; injection port temperature, 250°C; detector temperature, 250°C. The CB187 metabolites were quantified by a standard calibration curve for GC peak area. Analysis of CB187 and its metabolites by GC/MS was performed using Agilent 5973 inert MSD under the following conditions: HP-5 fused capillary column (60 m × 0.25 mm, 0.25 µm thickness); carrier gas, He (1 ml min^{-1}); column temperature, 70°C (1.5 min)–20°C min^{-1}–230°C (0.5 min)–4°C min^{-1}–280°C (5 min); injection port temperature, 250°C; detector temperature, 230°C.

Inhibition study with antiserum against guinea pig CYP2B18

Rabbit antiserum against guinea pig CYP2B18 was prepared as reported previously (Koga et al. 1998a). Antiserum (100, 200 and 300 µl each) was preincubated with liver microsomes (1 mg of protein) of PB-treated guinea pigs at 4°C for 16 h in 100 mM HEPES buffer (pH 7.4). After the addition of 6 mM $MgCl_2$, 40 µM CB187 and NADPH-generating system (0.33 mM $NADP^+$, 5 mM G-6-P, 1 unit of G-6-P dehydrogenase), the incubation was performed at 37°C for 60 min. Analysis of metabolites was done in a same manner as described above.

Other methods

Protein measurement was made by the method of Lowry et al. (1951) using BSA as a standard.

Results

Identification of CB187 metabolites

When CB187 was incubated for 60 min with guinea pig liver microsomes, three metabolites were observed at retention times of 17.57 min, 21.49 min and 22.51 min in GC/ECD and named tentatively M-1, M-2 and M-3, respectively, as shown in Figure 1. To obtain some information on the chemical structure of the metabolites, a large-scale incubation (100 ml) with liver microsomes of PB-treated guinea pigs was conducted at 37°C for 60 min, and the metabolites were extracted with organic solvents, methylated by diazomethane and applied to GC/MS. Table I shows the mass spectral data and retention times of three methylated metabolites. The methylated derivatives of M-2 and M-3 gave the same molecular weight of 422 and the fragment ion of M^{+}-15 (*m/z* 407) indicating monomethoxylated compounds at the 4- or 4′-positions. Moreover, their retention times in GC/MS were completely identical with synthetic 4′-MeO-CB178 and 4-MeO-CB187, indicating that M-2 and M-3 were 4′-OH-CB178 and 4-OH-CB187, respectively. On the other hand, methylated M-1 had a molecular weight of 388, which suggested that M-1 is a monohydroxylated and dechlorinated metabolite. From a comparison of the retention time and mass fragmentation

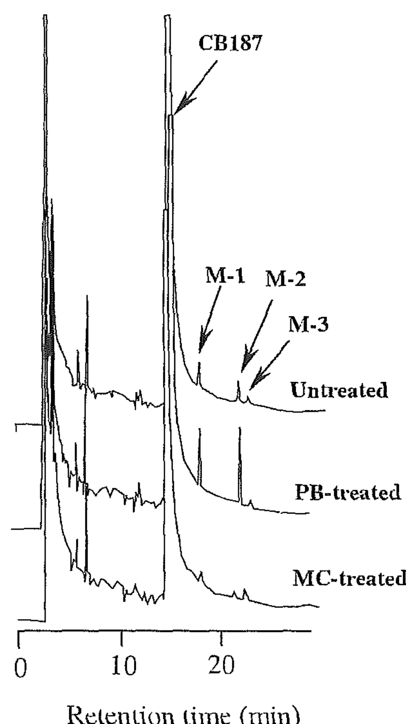

Figure 1. Gas chromatograms of the methylated derivatives of CB187 metabolites formed by guinea pig liver microsomes.

Table I. Mass spectral data and retention times of methylated derivatives of three CB187 metabolites and synthetic compounds.

Compound	Molecular weight	Mass spectral data							Retention time (min)
		[M^+]	[M^+-15]	[M^+-35]	[M^+-43]	[M^+-50]	[M^+-70]	[M^+-113]	
CB187	392	100	–	26	–	–	84	–	14.57
M-1	388	100	5	–	36	–	8	43	15.67
M-2	422	100	26	–	31	–	–	55	16.63
M-3	422	100	26	–	40	–	–	58	16.88
2′-MeO-hexaCB (CB165)	388	100	–	–	–	154	22	40	14.19
6-MeO-hexaCB (CB146)	388	100	–	–	–	134	29	39	14.35
5′-MeO-hexaCB (CB147)	388	100	20	–	27	–	7	50	15.29
3′-MeO-hexaCB (CB151)	388	100	–	–	24	–	9	46	15.34
6′-MeO-hexaCB (CB151)	388	100	–	–	–	86	43	36	15.62
4′-MeO-hexaCB (CB151)	388	100	5	–	34	–	7	43	15.67
5-MeO-hexaCB (CB146)	388	100	4	–	30	16	6	42	15.72
3′-MeO-heptaCB (CB187)	422	100	13	–	40	12	–	6	16.45
4′-MeO-heptaCB (CB178)	422	100	28	–	41	–	–	74	16.63
4-MeO-heptaCB (CB187)	422	100	15	–	46	–	–	54	16.88

pattern in GC/MS with synthetic monomethoxylated hexaCBs, M-1 was determined to be 4′-OH-CB151 (Table I).

Effect of CYP inducers on CB187 metabolism

The metabolic profile of CB187 was compared between liver microsomes of guinea pigs, rats and hamsters, and the metabolites were analyzed by GC/ECD. The formation rates of M-1, M-2 and M-3 were 18.1, 36.6 and 14.7 $pmol\,h^{-1}\,mg\,protein^{-1}$ in untreated guinea pigs, whereas in untreated rats and hamsters, no metabolite was observed (Figure 2). The formation of M-1 and M-2 in guinea pigs increased by 1.9- and 3.4-fold of the control value by PB-treatment, respectively, but decreased to less than 50% following MC-treatment. In contrast, the formation of M-3 was not affected by PB- or MC-treatment. In rats, M-2 and M-3 was formed only by PB-treated liver microsomes at rates of 87.1 and 13.8 $pmol\,h^{-1}\,mg\,protein^{-1}$, respectively, whereas M-1 was not found in any of the microsomes tested. In hamsters, PB-treated microsomes formed only M-2 but at a slower rate than those of guinea pigs and rats, but untreated and MC-treated microsomes formed no metabolite.

Inhibitory effect of antiserum against guinea pig CYP2B18

To elucidate the possible involvement of guinea pig CYP2B18 in CB187 metabolism, an inhibition study using antiserum against CYP2B18 was performed (Figure 3). When anti-CYP2B18 antiserum (300 µl) was preincubated with liver microsomes from PB-treated guinea pigs at 4°C for 16 h, the formation of M-1 was inhibited up to 80% of the control serum values, and the formation of both M-2 and M-3 was completely inhibited. These results suggested that guinea pig CYP2B18 is involved in CB187 metabolism to a major extent.

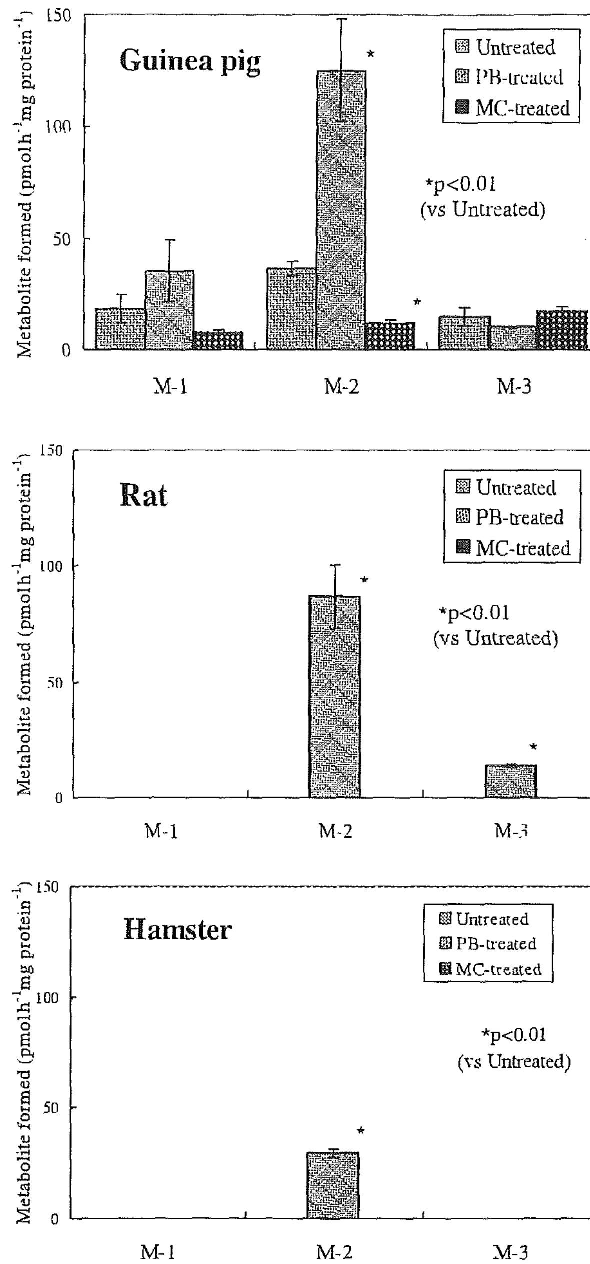

Figure 2. Effects of CYP inducers on CB187 metabolism with liver microsomes of guinea pigs, rats and hamsters. Each column represents the mean ± SD (vertical bars) of three animals. *Significantly different from untreated animals ($p < 0.01$).

Discussion

In this study, we first found that CB187 was metabolized to three metabolites by guinea pig liver microsomes, and these corresponded to a monohydroxy-dechlorinated metabolite (M-1) and two monohydroxy-heptaCB (M-2 and M-3) from the GC/MS data.

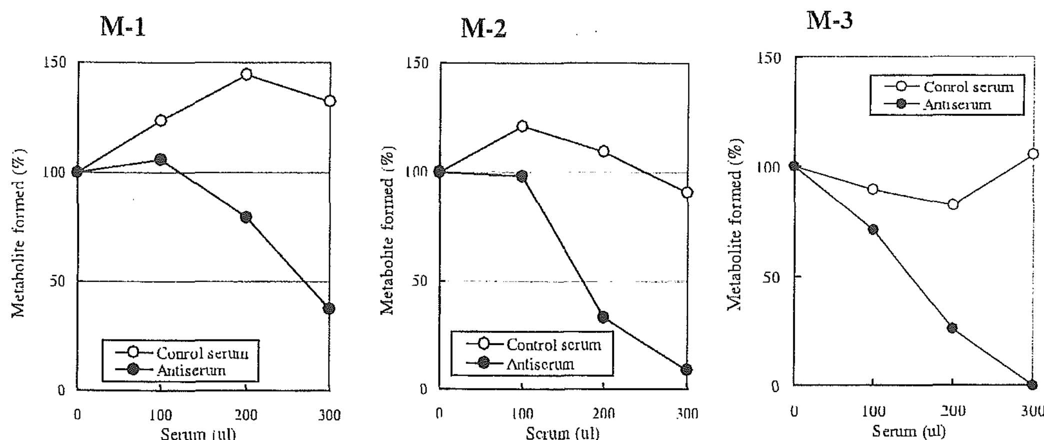

Figure 3. Inhibitory effects of anti-CYP2B18 antiserum on CB187 metabolism. Each point represents the mean of two determinations.

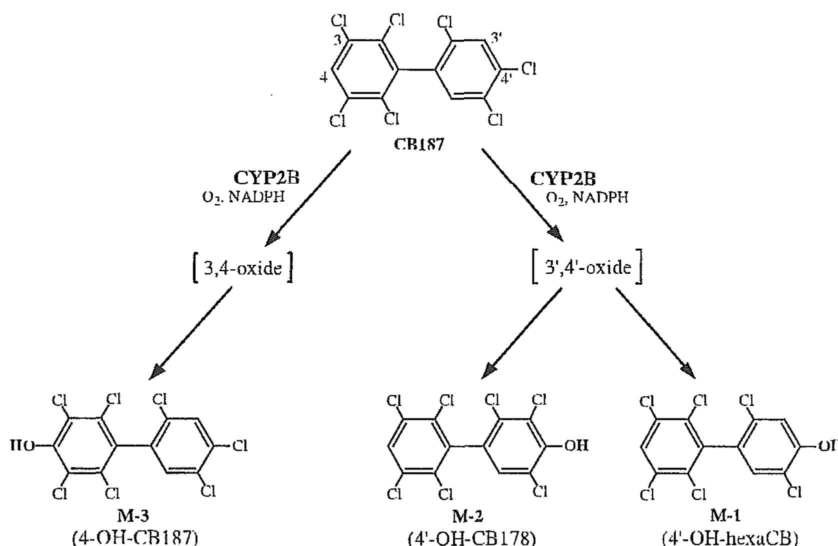

Figure 4. Postulated pathways of CB187 in laboratory animals.

By comparison with synthetic standards, M-1, M-2 and M-3 were determined to be 4′-OH-CB151, 4′-OH-CB178 and 4-OH-CB187, respectively, and the postulated metabolic pathways are shown in Figure 4. Our recent studies have demonstrated that 2,4,5-trichloro-substituted PCB congeners such as CB118, CB138 and CB153 are metabolized to their 2-OH metabolites as a major metabolite in dogs and guinea pigs and that 2-hydroxylation was catalyzed by PB-inducible CYP enzymes, dog CYP2B11 and guinea pig CYP2B18 (Ariyoshi et al. 1995, 1997, Koga et al. 2001, 2002). These facts suggest that the metabolism of 2,4,5-trichloro-substituted PCB congeners proceed mainly via a 2,3-oxide formation and subsequent NIH-shift of a chlorine at the 2-position to the 3-position on the 2,4,5-trichloro-substituted benzene ring. However, we observed the production of two 4′-OH metabolites,

M-1 and M-2, during CB187 metabolism, indicating that CB187 metabolism proceeds via the formation of 3′,4′-oxide as an intermediate and subsequent NIH-shift of a chlorine at the 4′-position to the 3′-position or dechlorination on the 2,4,5-trichloro-substituted benzene ring. In addition, it is possible that M-3 may be produced by the formation of the 3,4-oxide as an intermediate or by direct hydroxylation (Preston et al. 1983). The reason why the metabolic profile of CB187 is different from that of CB118, CB138 and CB153 is unclear at present but the fact that CB187 is a tri-*ortho*-PCB and may be more bulky than mono- or di-*ortho*-PCB such as CB118, CB138 and CB153, needs to be considered.

CB187 metabolism was also increased by PB treatment in all three species examined herein, which suggests the involvement of PB-inducible CYP enzymes such as guinea pig CYP2B18 (Oguri et al. 1991, Narimatsu et al. 1992, Koga et al. 1998a, b), rat CYP2B1/2B2 (Kaminsky et al. 1981, Ishida et al. 1991) and hamster CYP2B (HPB-1) (Koga et al. 1995). In fact, addition of antiserum against CYP2B18 showed almost complete inhibition of M-1, M-2 and M-3 formed by liver microsomes of PB-treated guinea pigs. Unexpectedly, as mentioned above, the metabolite formed by PB-treated animals was not a 2-OH metabolite but a 4- or 4′-OH metabolite. From these results, it is suggested that the substrate specificity of the CYP2B enzyme for 2,4,5-trichloro-substituted PCB congeners could be influenced by the degree of the angle where two phenyl rings in a PCB molecule rotate relative to each other.

The dechlorinated monohydroxy-metabolites have often been observed in the metabolism of PCB congeners such as CB77 (Yoshimura et al. 1987, Koga et al. 1989, Wehler et al. 1989), CB138 (Koga et al. 2001), CB153 (Kato et al. 1980, Ariyoshi et al. 1992) and CB155 (2,2′,4,4′,6,6′-hexaCB) (Ariyoshi et al. 1993). However, the formation mechanism still remains obscure at present although the oxidation by CYPs is required as an initial event. In the current study, guinea pig liver microsomes produced the dechlorinated monohydroxy-metabolite, M-1, at a relatively high rate, and the formation was increased by PB treatment. Thus, the enzyme system present in guinea pig liver microsomes may be useful to elucidate the reaction mechanism.

As described above, 4-OH-CB187 is the most abundant metabolite of all hydroxylated metabolites in human blood, whereas it was not a major metabolite formed by animal liver microsomes used in the present study. Recently, Malmberg et al. (2004) compared the half-lives of 4-OH-2,3,3′,4′,5-pentaCB (CB107), a main metabolite of CB118, and 4-OH-CB187 in rat blood after an intravenous injection of both compounds and demonstrated that the half-life of 4-OH-CB187 and 4-OH-CB107 was 15.4 and 4.3 days, respectively. These fact indicate that even if the amount of 4-OH-CB187 formed in animal liver is very small, it would be distributed preferably to blood and retained there selectively during the lifespan of the animals. On the other hand, 4-OH-CB187 could be produced from 2,2′,3,4,4′,5′,6-heptaCB (CB183), which has also been detected as a minor component in PCB preparations (Ballschmiter et al. 1989, Mimura et al. 1999a). Further studies on CB183 metabolism are needed to elucidate the toxicological significance of 4-OH-CB187.

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DECREASE IN SERUM THYROXINE LEVEL BY PHENOBARBITAL IN RATS IS NOT NECESSARILY DEPENDENT ON INCREASE IN HEPATIC UDP-GLUCURONOSYLTRANSFERASE

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ABSTRACT:

We have previously reported that there is a poor correlation between increase in the levels of UDP-glucuronosyltransferases, UGT1A1 and UGT1A6, and decrease in the levels of serum total thyroxine (T_4) and free T_4 in phenobarbital (PB)-treated rats, although the PB-induced decrease in rats is generally thought to occur through induction of the UDP-glucuronosyltransferase (T_4-UDP-GT: UGT1A1 and UGT1A6). In the present study, to clarify a relationship between the decrease in serum T_4 level and the increase in the T_4-UDP-GT activity by PB in rats, we examined the relationship using Gunn rats, a mutant strain of Wistar rats deficient in UGT1A isoforms. Levels of serum total T_4, free T_4, and total triiodothyronine (T_3) were markedly decreased not only in Wistar rats but also in Gunn rats 1 day after the final administration of PB (80 mg/kg i.p., once daily for 4 days), and no significant difference in magnitude of the decrease between Wistar and Gunn rats was observed. On the other hand, the level and activity of T_4-UDP-GT were significantly increased by treatment with PB in Wistar rats but not in Gunn rats. Furthermore, significant decrease in the activity of hepatic type I iodothyronine deiodinase, which mediates the deiodination of T_4 and T_3, by PB treatment was observed in both Wistar and Gunn rats. In addition, no significant change in the level of serum thyroid-stimulating hormone, the activity of hepatic sulfotransferase, and the binding of $[^{125}I]T_4$ to serum transthyretin and albumin by PB treatment was observed in either Wistar or Gunn rats. In conclusion, the present results demonstrate that the decrease in serum total T_4 level by PB in Gunn rats is not dependent on the increase in hepatic T_4-UDP-GT activity and suggest that even in Wistar rats, the PB-induced decrease in serum T_4 level does not occur only through increase in hepatic T_4-UDP-GT.

Phenobarbital (PB) is well known to decrease the level of serum thyroid hormone and to increase the activities of hepatic drug-metabolizing enzymes in rats and mice (O'Connor et al., 1999; Hood et al., 2003). Furthermore, PB increases levels of serum thyroid-stimulating hormone (TSH) and thyroid gland growth in rats (Hood et al., 1999). As a possible mechanism for the PB-induced decrease in the level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PB is considered (McClain, 1989; Capen, 1997). Especially, the decrease in the level of serum thyroxine (T_4) by PB in rats is thought to occur mainly through the induction of T_4-UDP-glucuronosyltransferase (T_4-UDP-GT), responsible for glucuronidation of T_4 (Barter and Klaassen, 1992a; Liu et al., 1995). This hypothesis is supported by the previous reports that a number of T_4-UDP-GT inducers, such as polychlorinated biphenyl (PCB), 3-methylcholanthrene, and pregnenolone-16α-carbonitrile, show the ability to decrease serum thyroid hormone (Saito et al., 1991; De Sandro et al., 1992; Barter and Klaassen, 1994). However, the magnitude of decrease in the level of serum total T_4 by PB is not necessarily correlated with that of increase in T_4-UDP-GT activity (Saito et al., 1991; Hood et al., 2003). Likewise, our preliminary study (Suzuki et al., 2004) has indicated that there is a poor correlation between increase in the levels of UGT1A1 and UGT1A6 and decrease in the levels of serum total T_4 and free T_4 in PB-treated rats.

In the present study, therefore, we examined a relationship between the decrease in serum total T_4 level and the increase in hepatic T_4-UDP-GT (UGT1A1 and UGT1A6) by PB using UGT1A-deficient Wistar rats (Gunn rats) and demonstrated that the PB-induced decrease in serum total T_4 level in rats was not necessarily dependent on the increase in hepatic T_4-UDP-GT activity.

Materials and Methods

Chemicals. PB was purchased from Nakakita Yakuhin Co., Ltd. (Aichi, Japan). The ^{125}I-reverse triiodothyronine (T_3) and $[^{125}I]T_4$, radiolabeled at the 5′-position of the outer ring, was obtained from PerkinElmer Life and Analytical Sciences (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.

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ABBREVIATIONS: PB, phenobarbital; ID-I, iodothyronine deiodinase; PCB, polychlorinated biphenyl; T_3, triiodothyronine; T_4, thyroxine; TTR, transthyretin; TSH, thyroid-stimulating hormone; UDP-GT, UDP-glucuronosyltransferase.

(Shizuoka, Japan). Male Wistar and Gunn rats were housed three or four per cage with free access to commercial chow and tap water, maintained on a 12-h dark/light cycle (8:00 AM-8:00 PM light) in an air-controlled room (temperature, 24.5 ± 1°C; humidity, 55 ± 5%), and handled with humane care under the guidelines of the University of Shizuoka (Shizuoka, Japan). Rats received four consecutive intraperitoneal injections of PB (80 mg/kg) dissolved in 0.9% saline (5 ml/kg). Control animals were treated with a vehicle alone (5 ml/kg). All rats were killed by decapitation 1 day after the final administration. The liver was removed, and hepatic microsomes and cytosols were prepared according to the method of Kato et al. (1995) and stored at −85°C until used. Blood was collected from each animal between 10:30 and 11:30 AM. After clotting at room temperature, serum was separated by centrifugation and stored at −50°C until used.

Analysis of Serum Hormones. Levels of serum total T_4, free T_4, total T_3, and TSH were measured by radioimmunoassay using the Total T4 and Free T4 kit (Diagnostic Products Corporation, Los Angels, CA), the T-3 · RIABEAD (Abbott Japan Co., Ltd., Tokyo, Japan), and the rTSH [^{125}I] Biotrak assay system (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), respectively.

Hepatic T_4-Metabolizing Enzyme Assays. Amounts of proteins of hepatic subcellular fractions, microsomes and cytosols, were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The activity of microsomal UDP-GT toward T_4 was determined by the method of Barter and Klaassen (1992b). The activity of microsomal type I iodothyronine deiodinase (ID-I) was determined by the method of Hood and Klaassen (2000). The activity of cytosolic sulfotransferase toward T_4 was determined by the method of Kaptein et al. (1997).

Western Blot Analysis. Polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and specific antibodies against UGT1A1, UGT1A6, and UGT2B1 were used (Ikushiro et al., 1995, 1997). Western blot analyses for microsomal UGT isoforms were performed by the method of Luquita et al. (2001). Isolated proteins corresponding to UGT1A1, UGT1A6, and UGT2B1 were detected using chemical luminescence (ECL detection kit; Amersham Biosciences Inc., Piscataway, NJ), and the level of each protein was determined densitometrically with LAS-1000 (Fuji Photo Film Co., Ltd., Toyko, Japan).

Analysis of [^{125}I]T_4 Binding to Serum Proteins. At 1 day after a consecutive 4-day treatment with PB, rats were anesthetized with 50 mg/ml sodium pentobarbital combined 1:1 with 1 mg/ml potassium iodide at 2 mg/ml. The femoral artery was cannulated (polyethylene tube SP31; Natume Inc., Tokyo, Japan) and primed with heparinized saline (33 units/ml). Fifteen minutes later, rats were given 1 ml of [^{125}I]T_4 i.v., at 15 μCi/ml in 10 mM NaOH saline + 1% normal rat serum. After the administration of [^{125}I]T_4, a portion (0.3 ml) of blood was sampled from the artery at the indicated time, and serum was collected and stored at −50°C for assay. Serum was diluted in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, and 30% glycerol, and subjected to electrophoresis on 4 to 20% gradient native polyacrylamide gels PAG Mid "Daiichi" 4/20 (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), using 0.025 M Tris (pH 8.4) containing 0.192 M glycine as running buffer, for 11 h at 20 mA at 4°C. Human albumin and transthyretin (TTR), which were incubated with [^{125}I]T_4, were also applied on the gel as templates. After the electrophoresis, a gel was dried and radioautographed for 20 h at room temperature using Imaging Plate 2040 (Fuji Photo Film Co., Ltd). The levels of [^{125}I]T_4-albumin and [^{125}I]T_4-TTR complexes were determined by counting the gel fractions identified from a Bio Imaging Analyzer (BAS-2000II IP Reader; Fuji Photo Film Co., Ltd.).

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

Results

Serum Hormone Levels. Constitutive levels of serum total T_4, free T_4, total T_3, and TSH were more than 1.8-fold higher in Gunn rats than in Wistar rats. Effects of PB on levels of serum thyroid hormones were next examined in Wistar and Gunn rats (Fig. 1). In both Wistar and Gunn rats, PB treatment resulted in decreases of serum total T_4, free T_4, and total T_3, and the magnitude of the decrease in each serum thyroid hormone was almost the same in both rats. On the other hand, no significant change in the level of serum TSH by PB treatment was observed in either Wistar or Gunn rats.

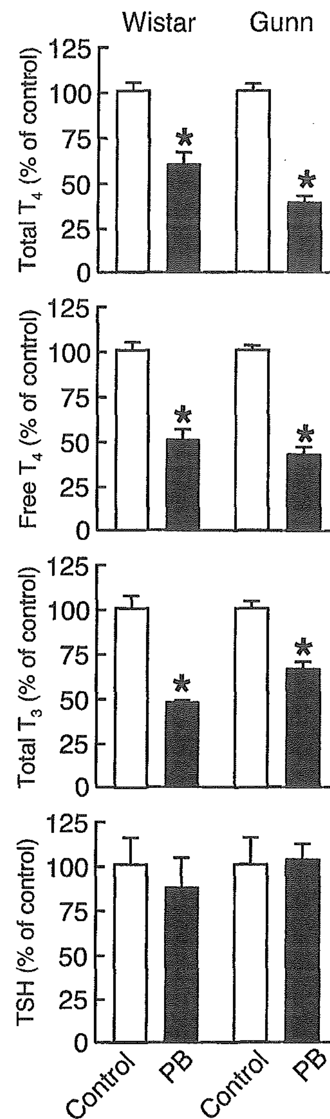

FIG. 1. Effect of PB on levels of serum total thyroxine (T_4), free T_4, total triiodothyronine (T_3), and TSH. Animals were killed 1 day after the final administration of PB (80 mg/kg i.p., once daily for 4 days), and levels of serum thyroid hormones were measured as described under *Materials and Methods*. Constitutive levels: total T_4, 3.32 ± 0.21 (Wistar, $N = 7$) and 7.99 ± 0.30 μg/dl (Gunn, $N = 4$); free T_4, 1.54 ± 0.07 (Wistar, $N = 7$) and 2.77 ± 0.07 ng/dl (Gunn, $N = 4$); total T_3, 0.34 ± 0.03 (Wistar, $N = 6$) and 0.96 ± 0.05 ng/ml (Gunn, $N = 4$); TSH, 8.37 ± 1.25 (Wistar, $N = 6$) and 20.85 ± 1.79 ng/ml (Gunn, $N = 4$). Each column represents the mean ± S.E. (vertical bars) for four to seven animals. *, $P < 0.01$. significantly different from each control.

Hepatic T_4-Metabolizing Enzyme Activities. It has been reported that T_4 glucuronidation is primarily mediated by UGT1A enzymes, UGT1A1 and UGT1A6, in the rat liver (Visser, 1996). Therefore, we examined effects of PB on hepatic microsomal T_4-UDP-GT activity in Wistar and Gunn rats. Constitutive activity of T_4-UDP-GT was more than 2.1-fold higher in Wistar rats than in Gunn rats. PB treatment resulted in a significant increase of T_4-UDP-GT activity in Wistar rats but not in Gunn rats (Fig. 2).

Hepatic microsomal ID-I activity in both Wistar and Gunn rats was significantly decreased by PB (Fig. 3). On the other hand, no significant change in activity of hepatic sulfotransferase by PB treatment was observed in either PB-treated Wistar or Gunn rats (data not shown).

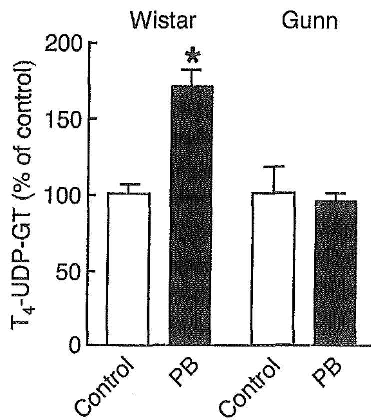

FIG. 2. Effect of PB on the activity of hepatic microsomal UDP-glucuronyltransferase. Each column represents the mean ± S.E. (vertical bars) for four animals. Constitutive levels: T_4-UDP-GT, 12.60 ± 0.69 (Wistar) and 5.95 ± 1.06 pmol/mg protein/min (Gunn). *, $P < 0.01$, significantly different from each control.

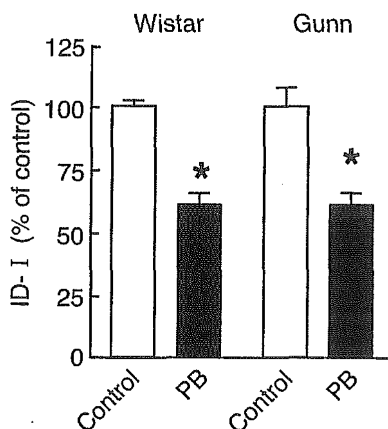

FIG. 3. Effect of PB on the activity of hepatic microsomal type I iodothyronine deiodinase (ID-I). Each column represents the mean ± S.E. (vertical bars) for four animals. Constitutive levels: ID-I, 161.8 ± 3.1 (Wistar) and 126.3 ± 9.5 pmol/mg protein/min (Gunn). *, $P < 0.01$, significantly different from each control.

Immunoblot Analysis for UGT1As. Levels of immunoreactive proteins responsible for UGT1A isoforms, UGT1A1 and UGT1A6, were increased by PB 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. On the other hand, the level of UGT2B1 was significantly increased by PB in both Wistar and Gunn rats, and the magnitude of the increase was higher in Gunn rats than in Wistar rats (Figs. 4 and 5).

Serum Protein Binding of [^{125}I]T_4. The effect of PB on the binding of [^{125}I]T_4 to serum proteins, TTR and albumin, was examined in Wistar rats. No significant change in the binding level of [^{125}I]T_4 to each serum protein by PB treatment was observed with the exception of a decrease in the level of [^{125}I]T_4-TTR complex at 120 min after [^{125}I]T_4 administration (Fig. 6).

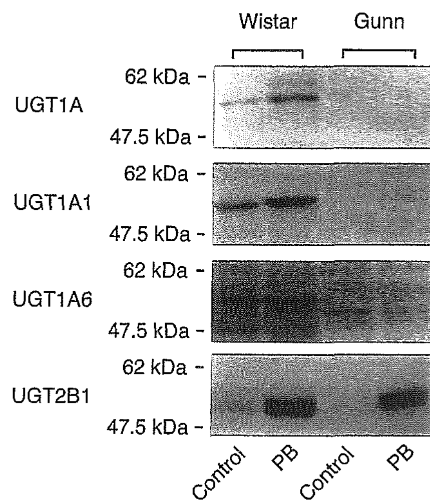

FIG. 4. Representive Western blot patterns for hepatic microsomal UGT isoforms.

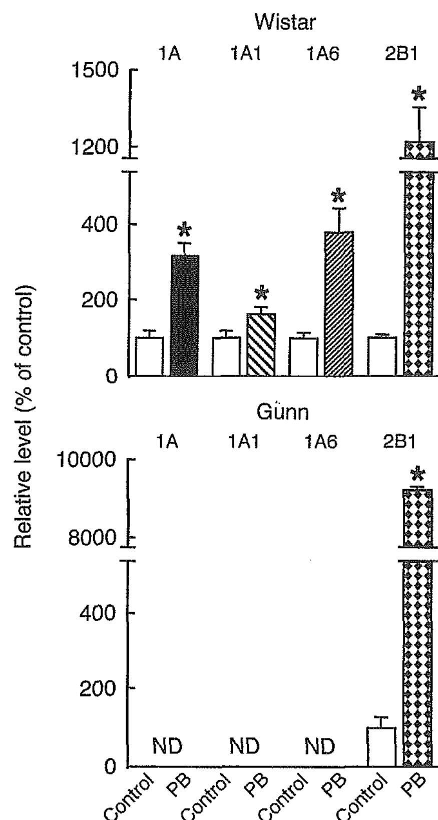

FIG. 5. Effect of PB on levels of hepatic microsomal UGT isoforms. After Western blot as shown in Fig. 4, the isolated bands responsible for UGT isoforms were densitometrically quantified as described under *Materials and Methods*. The data are represented as the mean ± S.E. (vertical bars) for four animals. *, $P < 0.05$, significantly different from each control. ND, not detectable.

Discussion

In the present study, we found that treatment with PB resulted in a drastic decrease in serum total T_4 and free T_4 levels in both Wistar and Gunn rats, although significant increase in the activity of T_4-UDP-GT occurred only in Wistar rats and not in Gunn rats. The present findings demonstrate that in Gunn rats, PB-induced decrease in the level of serum T_4 occurs in a hepatic T_4-UDP-GT-independent fashion. In addition, constitutive levels of serum total T_4 and T_3 were more than 1.8-fold higher in Gunn rats than in Wistar rats, suggesting that the deficit of T_4 glucuronidation results in higher T_4 serum levels. Similar results and suggestions have been obtained by Benathan et al. (1983).

In general, T_4-UDP-GT inducers, including PB, clobazam, PCB, 3-methylcholanthrene, and pregnenolone-16α-carbonitrile, have been considered to decrease a level of serum T_4 through an increase in hepatic T_4-UDP-GT (Barter and Klaassen, 1994; Van Birgelen et al., 1995; Miyawaki et al., 2003). However, it has been reported that the difference between rats and mice in the magnitude of decrease in the level of serum total T_4 by an inducer of T_4-UDP-GT is not well

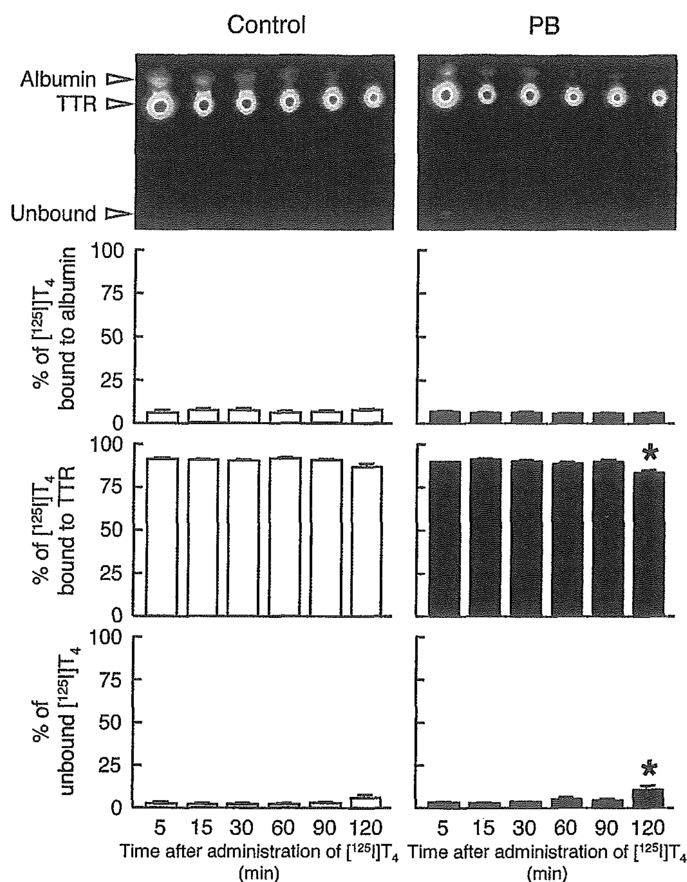

FIG. 6. Effect of PB on the binding of $[^{125}I]T_4$ to the serum proteins in Wistar rats. The binding of $[^{125}I]T_4$ to the serum proteins was assessed by nondenaturing polyacrylamide gel electrophoresis, as described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for three animals. *, $P < 0.05$, significantly different from each control.

correlated with that of the increase in activity of T_4-UDP-GT (Craft et al., 2002; Hood et al., 2003). Furthermore, we have reported previously that the PCB-induced decrease in serum T_4 level might occur not only through the increase in hepatic T_4-UDP-GT activity but also via formation of hydroxylated PCB metabolites in rats (Kato et al., 2004) and that in PB-treated rats, there was a poor correlation between the increase in the levels of UGT1A1 and UGT1A6 and the decrease in the levels of serum total T_4 and free T_4 (Suzuki et al., 2004). These previous reports strongly support a possibility that the decrease in serum total T_4 level by PB does not occur only through an increase in hepatic T_4-UDP-GT activity.

As possible mechanisms for the PB-induced decrease in serum T_4 level, changes in hepatic ID-I activity and serum TSH level might be considered. However, a significant decrease in activity of hepatic ID-I, which mediates the deiodination of T_4 and T_3, by PB treatment was observed in both Wistar and Gunn rats. Similar results have been reported in PB-treated Sprague-Dawley rats (O'Connor et al., 1999; Hood and Klaassen, 2000). Accordingly, the PB-induced decrease in serum T_4 level is thought to occur through an ID-I-independent pathway. Furthermore, in the present study, levels of serum TSH in both Wistar and Gunn rats were not significantly changed by PB, indicating that TSH is not related to the PB-induced decrease in serum T_4 level, although a significant increase in serum TSH level has been reported to occur in PB-treated rats (Hood et al., 1999; O'Connor et al., 1999). The difference between the previous results and our present results might be attributed to the difference in the dose examined.

Although it has been reported that the binding of PCB and its hydroxylated metabolites to TTR, a major T_4-transporting protein, might be attributed, in part, to a decrease in the level of serum T_4 in PCB-treated rats (Lans et al., 1993; Brouwer et al., 1998; Kato et al., 2004), displacement of T_4 from serum TTR by PB did not occur in PB-treated rats, with the exception of the slight displacement in Wistar rats at 120 min after PB treatment. Accordingly, the decrease in the level of serum T_4 in PB-treated rats occurs in a TTR-independent pathway. In addition, no change in activity of hepatic sulfotransferase, which efficiently catalyzes the sulfation of iodothyronines (Kester et al., 1999), by PB treatment was observed in either Wistar or Gunn rats.

In conclusion, the present findings demonstrate that the decrease in serum total T_4 level by PB in Gunn rats occurs without increases in hepatic T_4-metabolizing enzymes (T_4-UDP-GT, ID-I, and sulfotransferase), the binding of PB to serum TTR, and the level of serum TSH, although an exact mechanism for the PB-induced decrease remains unclear. In Wistar rats, PB-induced T_4-UDP-GT might also contribute, in part, to the decrease in serum T_4 level. To clarify the exact mechanisms for the PB-induced decrease in serum thyroid hormones, further studies involving T_4 transporters, nonhepatic T_4-UDP-GT, and exchangeable thyroid hormone pools in nonhepatic tissues would be necessary.

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THE DECREASE IN LEVEL OF SERUM THYROXINE BY 2,2′,4,5,5′-PENTACHLOROBIPHENYL IN RATS AND MICE: NO CORRELATION WITH FORMATION OF METHYLSULFONYL METABOLITES

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ABSTRACT:

A relationship between formation of methylsulfonyl ($MeSO_2$) metabolites of 2,2′,4,5,5′-pentachlorobiphenyl (PentaCB) and decrease in serum thyroxine (T_4) level was examined in the rats and mice after a single i.p. injection of PentaCB (342 μmol/kg body weight). In either rats or mice, levels of the 3- and 4-$MeSO_2$ metabolites of PentaCB in the liver and feces increased in a time-dependent fashion up to 8 days after PentaCB-treatment. However, there was a marked difference between rats and mice in the amount of the metabolites formed, and the cumulative amount of the either $MeSO_2$ metabolite for 8 days after PentaCB treatment in the liver was 4 to 15 times higher in mice than in rats. On the other hand, a 40 to 60% decrease in level of serum total T_4 occurred in both rats and mice at 1 day after PentaCB treatment, and the decrease was retained up to 8 days after PentaCB treatment. Thus, there was a marked difference between rats and mice in the formation of $MeSO_2$ metabolites from PentaCB but not a significant difference between rats and mice in PentaCB-induced decrease in the level of serum total T_4, indicating that PentaCB-induced decrease in the level of serum total T_4 is not necessarily dependent on the $MeSO_2$ metabolites formed.

Polychlorinated biphenyls (PCBs) are environmental pollutants that accumulate in the food chain due to their high lipophilicity and low biotransformation rate (Safe, 1993). Their bioaccumulation has been detected in the environment (Olafsson et al., 1987; Kannan et al., 1989) and in human serum, adipose tissue, and milk (Safe et al., 1985; Dewailly et al., 1991; Fängström et al., 2002).

A number of methylsulfonyl ($MeSO_2$) metabolites of PCBs have been found in several species of animals in Canada, Sweden, and East Greenland (Haraguchi et al., 1992; Bergman et al., 1994; Letcher et al., 1995; Chu et al., 2003; Sandala et al., 2004) and in both healthy humans and Yusho patients in Japan (Haraguchi et al., 1986, 1989). Additionally, $MeSO_2$-PCBs were identified in human milk, blood, liver, and adipose tissue (Norén et al., 1996; Weistrand and Norén, 1997; Weistrand et al., 1997; Guvenius et al., 2002). The main $MeSO_2$-PCBs found are 3- and 4-$MeSO_2$ derivatives of nonplanar PCBs (Haraguchi et al., 1992; Bergman et al., 1994; Letcher et al., 1995; Norén et al., 1996; Guvenius et al., 2002; Sandala et al., 2004).

The 3-$MeSO_2$-PCBs derived from nonplanar PCBs, including 2,2′,4,5,5′-pentachlorobiphenyl (PentaCB) 2,3′,4′,5-tetrachlorobiphenyl, and 2,2′,3′,4′,5-pentachlorobiphenyl, show much greater activities for inducing hepatic drug-metabolizing enzymes than the corresponding parent PCBs (Kato et al., 1995, 1999b), whereas the 4-$MeSO_2$-PCBs have no such capacity (Kato et al., 1995). More recently, we have found that some of $MeSO_2$ metabolites, including 3-$MeSO_2$- and 4-$MeSO_2$-PentaCBs (Fig. 1), were able to reduce level of serum thyroxine (T_4) and/or to increase level of serum thyroid-stimulating hormone (TSH) in rats (Kato et al., 1998, 1999a, 2000b). Furthermore, the $MeSO_2$ metabolite-induced decrease in serum T_4 level was suggested to occur, at least in part, through increase in hepatic UDP-glucuronosyltransferases (UDP-GTs) UGT1A1 and UGT1A6, responsible for glucuronidation of T_4 (Kato et al., 2000a). Thus, $MeSO_2$ metabolites of nonplanar PCBs seem to play important roles in PCB-induced toxicities.

There is a species difference in the response to PCB-induced toxicities, including induction of drug-metabolizing enzymes, endocrine disruption, carcinogenicity, and impairment of immune system (Safe, 1994). As one of reasons for the species difference, difference in a PCB metabolism would be considered.

In the present study, we examined a species difference in the in vivo metabolism of PentaCB between rats and mice and a relationship between formation of $MeSO_2$ metabolites and decrease in level of

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ABBREVIATIONS: PCB, polychlorinated biphenyl; $MeSO_2$, methylsulfonyl; PentaCB, 2,2′,4,5,5′-pentachlorobiphenyl; T_4, thyroxine; TSH, thyroid-stimulating hormone; UDP-GT, UDP-glucuronosyltransferase; AUC, area under the curve.