Bull. Environ. Contam. Toxicol. (1994) 53:583–590

Environmental Contamination and Toxicology

Multiple Dose Toxicokinetic Influence on the Estrogenicity of 2,2′,4,4′,5,5′-Hexachlorobiphenyl

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Received: 25 October 1993/Accepted: 1 March 1994

Accurate assessment of polychlorinated biphenyl (PCB) hazards remains a challenge because of multiple effects and multiple components of PCB mixtures (Hansen, 1979; 1987). A given chlorobiphenyl (CB) in an environmental residue may antagonize and/or potentiate the effects of other CBs in the mixture; furthermore, PCBs can induce biotransformation enzymes such as ethoxyresorufin *O*-deethylase (EROD, P450IA1) and pentoxyresorufin *O*-dependentylase (PROD, P450IIB1) which can qualitatively as well as quantitatively alter the action(s) of individual CBs. Dose:response relationships of even single CBs may well be nonlinear due to these changes. Therefore, important effects may not be detected depending on the dose of the CB.

There is growing public health concern regarding the potential risk from exposure to environmental estrogens. In a recent epidemiological study a significant positive association was reported between levels of 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE) in blood and risk of breast cancer; a positive, but nonsignificant association, was also observed for PCBs (Wolff et al. 1993). Estrogenic activity has been ascribed to some lower-chlorinated Aroclor mixtures (Bitman and Cecil 1970; Ecobichon and Mackenzie 1974; Jansen et al. 1993); however, coplanar CBs (Jansen et al. 1993; Krishnan and Safe 1993) as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Astroff and Safe 1988) have been shown to be antiestrogenic. The antiestrogenic potency appears to be related to *Ah* receptor binding affinity. On the other hand, some putative PCB metabolites were found to have relatively high estrogen receptor binding affinities *in vitro* and to stimulate uterine weight increases *in vivo* (Korach et al. 1988; Jansen et al. 1993). These studies clearly suggest that the estrogenicity of PCBs is not related to potency as *Ah* receptor agonists. Therefore, it is important to investigate the estrogenicity of environmentally abundant *ortho*-chlorinated CB congeners in order to more accurately predict the hazards from PCB residues in the environment.

Because the effects of PCBs are multiple and can be either dependent on or independent of the *Ah* receptor mediated mechanism, it is important to examine

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alternative effects simultaneously and to investigate the possible interactions or relationships among these effects. Only a few congener-specific effects on thyroxine (T4) level have been reported, but it appears that coplanar CBs are the most effective (Brouwer 1989; Morse et al. 1992; Ness et al. 1993). Therefore, both TCDD-like effects (EROD induction and thyrotoxic effect) and non-TCDD-like effects (PROD induction and estrogenic activity) of a persistent di-*ortho* conformationally restricted CB congener (2,2',4,4',5,5'-hexachlorobiphenyl, CB 153) and a commercial PCB mixture, Aroclor 1242, were examined in immature female Sprague-Dawley rats.

MATERIALS AND METHODS

Corn oil was purchased at a local grocery. All solvents were analytical grade (Burdick and Jackson, Muskegon, MI). All chemicals and reagents used in enzyme assays were obtained from Sigma Chemical Co. (St. Louis, MO). Aroclor 1242 was a gift from Monsanto Chemical Co. (St. Louis, MO), and 17-ß estradiol was purchased from Sigma. Radioimmunoassay kits (Coat-A-Count TT4) for determining serum total T4 (TT4) concentration were purchased from Diagnostic Products Corp. (Los Angeles, CA). CB 153 was provided from previously synthesized stock (Hansen, 1979). Alumina (80-200 mesh) was from Fisher Scientific (Springfield, NJ), and AX-21 charcoal was from Anderson (Adrian, MI). CB 153 was repurified before use by column chromatography (3 g of 2% deactivated alumina mixed with 1.5% AX-21 activated charcoal; elution with hexane). The concentration of CB 153 in the corn oil solution was determined by a Hewlett Packard 5790 gas liquid chromatograph (GLC) with electron capture detection on a 60 m DB-5 capillary column. The actual dose of CB 153 was then calculated from the total amount of CB 153 injected divided by the actual average weight of rats in the same treatment group (Table 1).

Table 1. Quantitation of actual CB 153 doses used in this study.

CB 153 concentration in corn oil[a] (mg/mL)	Actual Dose[b] (mg/kg/day)
3.4 ± 0.1	8
4.4 ± 0.1	11
12.2 ± 0.1	25
20.3 ± 1.0	51
25.0 ± 0.7	59

[a] Mean ± SE (N=3) determined by GLC.

[b] Actual Dose = (amount of CB 153 administered) / (mean body wt).

Sprague-Dawley breeder rats were obtained from SASCO (Lincoln, NE). Pups were culled to 10 animals per litter on the day of birth (d 0) and were weaned at

20 days of age. Female pups (40.0 ± 0.9 g at d 20 and 44.4 ± 1.3 g at d 22) were injected ip with CB 153 or Aroclor 1242 dissolved in 0.1 mL corn oil or corn oil alone between 1:00 and 2:00 p.m. on d 20 and d 21. Estradiol (0.02 mg/kg/day) was used as a positive control. Rats were killed by decapitation between 9:00 and 11:00 a.m. on d 22 and their uteri were excised, trimmed of fat and connective tissue, and weighed. The uterotropic effects were determined by comparing (uterine wet wt in mg) / (body wt in g) ratios to the ratios found in control animals. Blood was collected immediately after decapitation and allowed to clot. Serum was separated by centrifugation and stored at -20°C until analyzed for TT4 by RIA and CB 153 residues by GLC.

Liver microsomes were prepared by standard centrifugation procedures and stored in liquid nitrogen or at -80°C until assayed. EROD and PROD were determined by a modification of the method of Pohl and Fouts (1980). In brief, the reaction mixture contained 5 mM $MgCl_2$, 1 mg bovine serum albumin, microsomal suspension (200 to 500 µg of microsomal protein for EROD and 400 to 800 µg for PROD), 2.5 µM 7-ethoxyresorufin or 10 µM 7-pentoxyresorufin in 0.05 M Tris-HCl (pH 7.5) and a NADPH-generating system (0.8 mg $NADP^+$, 1.5 mg glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase). The reaction was initiated by adding the NADPH-generating system and stopped by the addition of 2 mL of methanol. The reaction was carried out 4 min for EROD and 10 min for PROD at 37°C. The formation of resorufin was determined by measuring sample fluorescence relative to a known amount of resorufin with excitation at 550 nm and emission at 585 nm in a Perkin-Elmer 203 Fluorescence Spectrophotometer. Microsomal protein was determined by the method of Lowry (1951) using bovine serum albumin as a standard. All samples were run in duplicate.

The serum concentrations of CB 153 in animals following treatment were analyzed by GLC as above. In brief, serum (100 µL) was extracted by adding 1.0 mL of acetone followed by 1.0 mL of hexane; after thorough mixing, enough sodium sulfate was added in small amounts to adsorb all the water. Then 0.2 mL of the CB 153 treated serum extract was added to 0.8 mL of hexane containing 50 ng/mL mirex as an internal standard. The extraction efficiency was 99% ± 3.4% (mean ± SE). Extract (1 µL) was injected onto the column and the oven temperature was programmed for 175°C to 266°C at 3°C per min. The temperature of the injection port and electron capture detector were 250°C and 325°C, respectively. Hydrogen was used as carrier gas and argon/methane was used for make-up gas. Quantification was accomplished by comparing to known amounts of CB 153 standards.

The data were first analyzed by F test to determine the homogeneity of variance between treated and control groups. If variances were homogeneous, Student's t test was used to compare the difference between treated and control groups. The Mann-Whitney U test was used when variances were not homogeneous. Differences were considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Aroclor 1242 at 400 mg/kg produced significant effects on all the endpoints used in this study. It caused a 200% increase in uterine wet wt relative to controls (Table 2), increased EROD (12-fold) and PROD (6-fold) activities (Table 3), and decreased serum TT4 concentration to less than 33% of control levels (Table 4). On the other hand, 17ß-estradiol at 0.04 mg/kg nearly tripled the uterine wt without any effect on P450 enzyme induction or serum TT4 concentration.

Uterine wet wt increased significantly relative to control rats in those rats receiving CB 153 at both 50 mg/kg and 102 mg/kg but not at 118 mg/kg (Table 2). CB 153 significantly increased PROD activity at all doses tested but not EROD activity (Table 3). CB 153 did not significantly reduce serum TT4 (Table 4). Serum CB 153 concentrations peaked at 102 mg/kg, interestingly the same dose which induced maximal PROD activity in this study (Figure 1). Although the patterns are similar, there was no absolute association between PROD induction and serum CB 153 concentration. The correlation coefficient between serum CB 153 concentration and PROD activity among individuals from CB 153 treatments was only 0.379.

Table 2. Uterotropic effect of PCBs administered on d 20 and 21 to immature Sprague-Dawley female rats.

Treatment	Total Dose (mg/kg)	n	% (UWW mg)/(g BW)[a]	% Control
Control		16	45.2 ± 1.6	100
17ß-Estradiol	0.04	7	121.6 ± 9.7**	283
Aroclor 1242	400	5	90.5 ± 6.5**	200
CB 153	16	4	44.7 ± 2.8	99
	22	4	51.7 ± 1.9	114
	50	5	73.5 ± 5.9**	163
	102	4	65.9 ± 4.2**	146
	118	4	45.2 ± 3.5	100

[a] % Uterine wet wt mg/g body wt expressed as Mean ± SE.
** Significantly different from control; P≤0.05.

CB 153 only exhibited estrogenic activity within the narrow dose range that also produced maximal PROD induction and CB 153 serum concentrations. To interpret these results, one must reflect on the fact that estrogenicity is not controlled solely by estrogen receptor-binding affinities. Metabolism of foreign or endogenous compounds can lead to deactivation, elimination, or activation which may alter the biological activity of a parent compound dramatically. For

Table 3. Liver microsomal enzyme activities in immature female rats administered PCBs.

Treatment	Total Dose (mg/kg)	n	Microsomal enzyme activities[a] EROD[b]	PROD[b]
Control		6	142.2 ± 26.2	3.1 ± 0.6
17ß-Estradiol	0.04	4	157.0 ± 20.0	2.2 ± 0.2
Aroclor 1242	400	5	1650.5 ± 236.6**	17.3 ± 3.7**
CB 153	16	4	183.7 ± 34.0	10.0 ± 2.3**
	22	4	193.8 ± 15.3	10.7 ± 2.7**
	50	5	316.2 ± 69.8	11.8 ± 2.7**
	102	4	251.1 ± 13.3	26.8 ± 3.3**
	118	4	245.0 ± 15.5	12.9 ± 2.1**

[a] Mean ± SE.
[b] Activity expressed as pmol resorufin formed/min/mg protein.
** Significantly different from control; P≤0.05.

Table 4. Serum TT4 concentrations in immature PCB-treated female rats.

Treatment	Total Dose (mg/kg)	n	TT4 (µg/dL)[a]	% control
Control		6	3.17 ± 0.37	100.0
Estradiol	0.04	4	2.88 ± 0.32	90.9
Aroclor 1242	400	5	1.04 ± 0.11**	32.8
CB 153	16	3	3.13 ± 0.09	98.7
	22	4	3.44 ± 0.30	108.5
	50	5	2.91 ± 0.15	91.8
	102	4	2.30 ± 0.28	72.6
	118	4	2.35 ± 0.22	74.1

[a] Mean ± SE.
** Significantly different from control; P<0.05.

example, methoxychlor *per se* is not active as an estrogen until it is metabolized into phenolic derivatives which appear to be potent estrogens (Kupfer and Bulger 1982). Phenolic metabolites of some PCBs have also been found to have greater estrogenic activity than the parent compound(s) (Korach et al. 1988; Jansen et al. 1993). There is evidence that phenobarbital(PB)-induced cytochrome P450 isozymes (P450IIB) preferentially metabolize di-*ortho*-chloro substituted biphenyls (Kaminsky et al. 1981). In addition, PB-induced rats have 2-fold higher specific activity towards CB 153 than untreated rats (Sipes & Schnellmann, 1987). Therefore, induction of PROD activity by CB 153, an increase of the isozyme

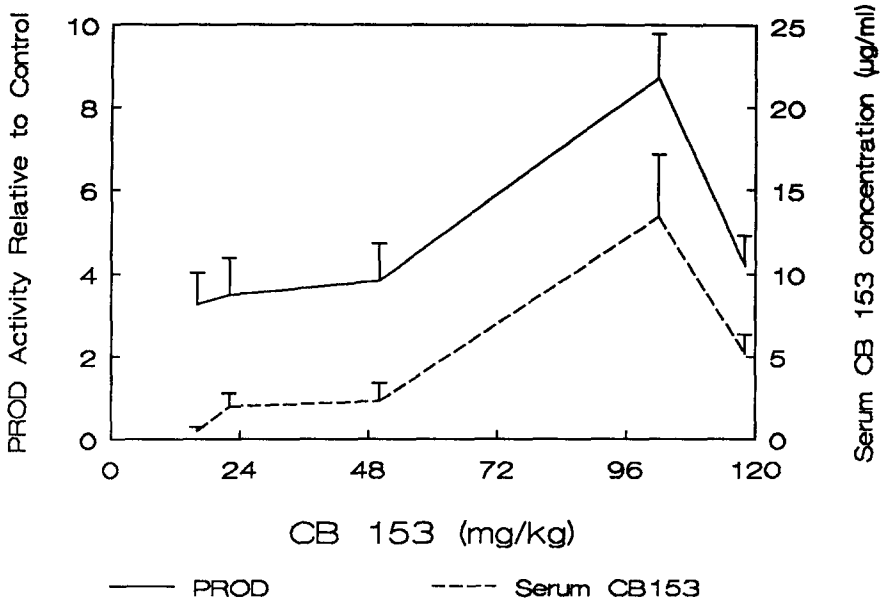

Figure 1. Dose:response relationship of PROD activity and CB 153 serum concentrations in immature female Sprague-Dawley rats.

levels of the P450IIB family, would be expected to oxidize the slowly metabolized CB 153 to hydroxylated products in the present study. Many CBs and PCB mixtures require very high single doses for a uterotropic response (Ecobichon and MacKenzie, 1974), but the effective dose may be decreased nearly 100-fold by separating it into two consecutive doses 24-hr apart (Jansen et al., 1993). This suggests that the first dose of CB 153 may initiate monooxygenase induction which would enhance the activation of the second dose to phenolic metabolites with estrogenic activity.

At the highest dose (118 mg/kg), the 48-hr body burden of CB 153, as measured by serum concentrations, was lower and apparent monooxygenase induction was attenuated. The first dose apparently accelerated phase 1 monooxygenase metabolism and elimination to a greater extent in order to significantly reduce body burdens following the second dose. In addition, the rate of elimination of some active metabolites via phase 2 conjugation may be more effective at higher doses than at moderate doses. Therefore, enhanced elimination of CB 153 and metabolites would be expected to result in attenuation of many effects, including estrogenicity.

The demonstration that one of the least readily metabolized CBs, CB 153, is

estrogenic indicates that direct interaction of parent CB with estrogen receptors may be an important mechanism for estrogenicity. On the other hand, biotransformation activity of CB 153 may also contribute to the narrow window for its estrogenicity. PROD induction peaked in the same dosage range where marked estrogenicity of CB 153 was observed and the correlation may have been strengthened by increased production of active metabolites. At the highest dose, however, further increases in phase 1 metabolism and/or increased activity of phase 2 conjugation enzymes reduced the amount and effect of CB 153. The present study indicates that biotransformation activity of hepatic cytochrome P450 plays a significant role in altering the toxicokinetics as well as modifying the toxicity of CBs. However, the exact relationship between these factors still needs to be determined.

As demonstrated in this study, there are multiple dosage relationships for different manifestations of PCB exposure, further suggesting the need for a more comprehensive approach to hazard and risk assessment for environmental PCBs rather than a simple toxic equivalency approach.

Acknowledgments. The authors are grateful to Drs. Paul S. Cooke, Aslam S. Hassan and Patricia G. Weston for their assistance in this study and to Carol Davis for the TT4 analyses. We also thank John Porcelli, Ursala Kief, and Peggy Staderman for their assistance in animal maintenance and husbandry. Ms. Li was supported by an Environmental Toxicology Scholarship from Institute for Environmental Studies, University of Illinois at Champaign-Urbana.

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