Biochemical Pharmacology, Vol. 43, No. 2, pp. 153–157, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

LACTATIONAL TRANSFER OF 3,3′,4,4′-TETRACHLORO- AND 2,2′,4,4′,5,5′-HEXACHLOROBIPHENYL INDUCES CYTOCHROME P450IVA1 IN NEONATES

EVIDENCE FOR A POTENTIAL SYNERGISTIC MECHANISM

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(*Received* 20 *June* 1991; *accepted* 27 *September* 1991)

Abstract—On the first day of lactation, material rats were treated with a single low dose of 5 mg/kg body weight of 3,3′,4,4′-tetrachlorobiphenyl (TCB) or 2,2′,4,4′,5,5′-hexachlorobiphenyl (HCB) or with a combination of both congeners. Lactational transfer of these polychlorinated biphenyls (PCBs) was found in neonates and significant increases in microsomal cytochrome P450, cytochrome b_5 and in glutathione-*S*-transferase activity were observed. Treatment with HCB did not increase neonatal ethoxyresorufin-O-de-ethylation (EROD) activities whereas a more than 26-fold increase in EROD activity was noted in response to exposure to TCB. However, EROD activities were increased more than 65-fold in response to the combined exposure to TCB and HCB. Exposure via milk to TCB caused a significant reduction in the N-demethylation of aminopyrine, but the combined exposure to TCB and HCB produced a significant reduction in the N-demethylation of dimethylnitrosamine. Lactational transfer of either TCB or HCB reduced marginally peroxisomal enzyme activities; however, exposure to a combination of TCB and HCB resulted in the highly significant reduction in KCN-insensitive palmitoyl-CoA oxidation and acetyl-CoA oxidation. Contrary to the reduction of these enzyme activities, the specific concentrations of CYP4A1 were significantly increased when neonates were exposed to either TCB or HCB. The largest induction, however, was observed in response to the combined exposure to both PCBs. Evidence is presented to suggest an induction of CYP4A1 which may be independent of the molecular substitution pattern of the two PCBs used in our studies but on a possible mode of synergistic interaction.

Polychlorinated biphenyls (PCBs¶) were frequently prepared as oil-like fluids some of which contained a complex mixture of more than 80 different PCB isomers and congeners. Only a few of these commercial preparations, including products termed as Aroclors or Clophens, have been, to some extent, characterized with respect to their PCB isomeric and congeneric composition. The desirable physicochemical properties of PCBs have led to their widespread use as industrial fluids, flame retardants, hydraulic fluids and heat conducting media for capacitors and transformers. Careless disposal of PCB contaminated waste has resulted in global pollution with PCBs and there have been numerous reports detailing the dynamics of pollution with PCBs [1]. It is now evident that PCBs are detected routinely in basically all compartments within the ecosphere and their accumulation in food chains is documented as being present in the fatty tissues of animals, including humans at the apex of food chains [1]. The biochemical toxicology by PCBs has been well documented and they have been shown to promote carcinogenicity in mammalian species as judged by the formation of hepatocellular changes, e.g. trabecular carcinoma, adenocarcinoma, neoplastic nodules, as well as the occurrence of intestinal metaplasia, adenofibrosis, thymoma (i.e. tumor of the thymus) and bile duct hyperplasia [2]. PCBs are inducers of hepatic drug metabolism and the detailed studies by Parkinson *et al.* [3] provide comprehensive evidence of the induction of individual cytochrome P450 isoenzymes in response to treatment of rats with individual PCB isomers and congeners. Oxidative biotransformation of PCBs via cytochrome P450-dependent monooxygenases has been shown to be crucial in the metabolic disposal of these ubiquitously distributed pollutants.

We have recently reported evidence that cytochrome P450IVA1-catalysed reactions were induced significantly when rats were treated with a single dose of 600 μmol/kg body weight of Aroclor 1254, as judged by the observed increase in the 12-hydroxylation of lauric acid [4]. This reaction is thought to be relatively specific for the assessment

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¶ Abbreviations: PCB, polychlorinated biphenyl; HCB, 2,2′,4,4′,5,5′-hexachlorobiphenyl; TCB, 3,3′,4,4′-tetrachlorobiphenyl; EROD, ethoxyresofurin-O-de-ethylation.

of the catalytic function of the cytochrome P450IVA subfamily of haemoproteins [5].

At present, it is unknown whether the induction of cytochrome P450IVA1 requires a specific molecular substitution pattern of PCBs, in view of the complex studies by Parkinson *et al.* [3] which have documented that the type of molecular substitution pattern of a single PCB molecule determines the induction of a specific isoform of cytochrome P450-dependent monooxygenase. For example, poly-*ortho* substituted PCBs (i.e. HCB) induce distinctively gene family II, e.g., CYP2B1, where as non-*ortho*, so called coplanar PCBs (i.e. TCB) induce gene family I, e.g. CYP1A1. It is, however, not known whether the induction of CYP4A1 requires a similar specificity in the substitution pattern with chlorine atoms to achieve the induction of the enzyme protein encoded by the gene for CYP4A1.

In addition, the well established knowledge that PCBs are transferred in humans via milk [6], raises the important question, whether low, i.e. environmentally realistic concentrations of PCBs, would result in the induction of cytochrome P450-dependent monooxygenases in neonates.

We therefore assessed the induction of CYP4A1 in neonates by treating maternal rats with a low dose of either the di-*ortho*-substituted HCB, which induces CYP2B1 or with the non-*ortho*-substituted TCB which induces CYP1A1 or with a combination of both congeners to induce CYP families I and II simultaneously. The results are discussed in relation to the lactational transfer of these chemicals to the newborn.

MATERIALS AND METHODS

Synthesis of TCB and HCB. TCB was synthesized by the Cadogan coupling of 3,4-dichloroaniline with 1,2-dichlorobenzene using a 10-fold molar excess of the latter. HCB was synthesized by the diazo (Cadogan) coupling reaction using 2,2',5,5'-tetrachlorobenzidine as the precursor [7].

The identity of the synthetic products was established by proton nuclear magnetic resonance spectroscopy (PNMR) and by direct probe mass spectroscopy (MS). It was found that the PNMR and MS analysis were fully consistent with the proposed structure (data not shown). The synthesis was carried out by Dr L. W. Robertson, Graduate Center of Toxicology, University of Lexington, KY 40506-0054, U.S.A.

Treatment of maternal rats with TCB and HCB. First day lactating rats were given a single i.p. injection of 5 mg/kg body weight of TCB (N = 4) or HCB (N = 4) or a combination (N = 4) of both, i.e. 25 mg of TCB and of HCB to obtain a final dose of 5 mg/kg body weight, respectively. Controls received a single i.p. injection of corn oil only. The neonates were weaned 21 days later and killed immediately thereafter.

Preparation of hepatic microsomes and assessment of microsomal and cytosolic enzyme activities. Hepatic microsomes were prepared as detailed by Borlakoglu *et al.* [8]. Cytochromes P450 and b_5 were measured by the methods of Omura and Sato [9] and Gibson and Skett [10], respectively, and the concentration of protein in microsomal suspensions by the method of Lowry *et al.* [11] using bovine serum albumin as the standard. EROD activity was determined by the method of Burke *et al.* [12] and the N-demethylation of aminopyrine and dimethylnitrosamine were measured essentially as described by Anderson *et al.* [13].

Peroxisomal enzyme activities. Tissue whole homogenates were assayed for activity of KCN-insensitive palmitoyl-CoA oxidation as described previously [14], but with the addition of 10 μM FAD to each cuvette. Acyl-CoA oxidase was assayed as palmitoyl-CoA-dependent H_2O_2-forming activity [15]. Catalase activity was measured in fresh liver homogenates using the method of Lück [16].

Enzyme-linked immunosorbent assay (ELISA) for cytochrome P450IVA1. The specific content of cytochrome P450IVA1 was determined by ELISA using an experimental protocol detailed by Sharma *et al.* [17]. It should be noted that the polyclonal antibody raised to electrophoretically homogeneous rat liver cytochrome P450IVA1 is not monospecific. Western blot analyses of both control and clofibrate-induced rat liver microsomes using this antibody reveal two immunoreactive bands, the major one of which is definitely cytochrome P450IVA1. In addition, a second and weaker band of slightly higher monomeric molecular weight (52 vs 51.5 kDa) is additionally recognized by the antibody. Although the precise identity of this weaker immunoreactive band is not known, it is probably a closely related member of the cytochrome P450IVA sub-family, as described in detail elsewhere [18].

RESULTS AND DISCUSSION

Microsomal enzyme activities and microsomal cytochrome P450 and b_5

Treatment of maternal rats with a low dose of 5 mg/kg body weight of TCB or HCB and its lactational transfer to neonates resulted in a significant induction of cytochrome P450, cytochrome b_5 and of cytochrome P450 catalysed reactions. The induction of neonatal cytochrome P450 was similar, when maternal rats were treated with either TCB or HCB, however, treatment with a combined TCB and HCB corn oil solution resulted in a lesser induction of total microsomal cytochrome P450 (see Table 1). The N-demethylation of aminopyrine was significantly reduced when maternal rats were treated with TCB. The results reported in Table 1 show a similar reduction ($P < 0.05$) in the N-demethylation of dimethylnitrosamine, in response to treatment with a combination of HCB and TCB. These findings suggest that treatment with the non-*ortho*-substituted TCB congener may result in a reduced expression of CYP2B1 and CYP2E1, as treatment with the poly-*ortho*-substituted HCB congener did not produce this effect. It is noteworthy to point out that only the combined treatment resulted in a significantly reduced N-demethylation of dimethylnitrosamine, thus, suggesting a potential synergistic interaction of HCB and TCB in suppressing CYP2E1-catalysed reactions. Neonatal cytochrome b_5 was marginally induced when mothers were treated with HCB, but significant increases were observed when

Table 1. Neonatal hepatic drug metabolizing enzyme activities in response to lactational transfer of two congeneric PCBs

	Cytochrome P450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)	N-Demethylation		EROD (pmol/mg protein/min)	Glutathione-*S*-transferase (μmol/mg protein/min)
			Aminopyrine (nmol/mg protein/min)	Dimethylnitrosamine		
Control	0.50 ± 0.16	0.32 ± 0.05	6.9 ± 2.4	2.3 ± 1.0	< 10	0.08 ± 0.03
HCB	1.07 ± 0.12‡	0.52 ± 0.09	6.2 ± 2.2	3.4 ± 0.8	< 10	0.13 ± 0.03
TCB	1.06 ± 0.09‡	0.63 ± 0.07*	3.5 ± 0.7‡§	1.7 ± 0.2§	260 ± 90	0.13 ± 0.03
Combined	0.86 ± 0.03†¶**	0.62 ± 0.03†	7.8 ± 2.7	1.2 ± 0.7‖	650 ± 140‡¶	0.13 ± 0.03*

* $P < 0.05$, † $P < 0.02$, ‡ $P < 0.01$ when compared to control.
§ HCB vs TCB, $P < 0.05$.
‖ HCB vs combined treatment, $P < 0.01$.
¶ TCB vs combined treatment, $P < 0.01$.
** HCB vs combined treatment, $P < 0.02$.
Values are mean ± SD with N = 7 for controls, N = 4 for HCB, N = 4 for TCB and N = 4 for the combined treatment group.

maternal rats received either TCB or a combination of TCB and HCB (see Table 1).

As expected, treatment with HCB did not result in the induction of CYP1A1, however, treatment with TCB resulted in a > 26-fold increase in EROD activity. The combined treatment with TCB and HCB produced a more than 65-fold increase in EROD activity and was significantly greater ($P < 0.01$), when compared with the TCB treatment alone. This finding was unexpected, as treatment with HCB does not produce an increase in EROD activity (see Table 1) and the combined treatment of maternal rats with TCB and HCB reduces the TCB dose injected by 50%. A statistical comparison of the combined treatment group versus the TCB treatment group yielded a significant *t*-value ($P < 0.01$) thus suggesting a non-additive and potentially synergistic interaction of both PCB congeners in causing a further increase in CYP1A1 induction. For comparison, the activity of the Phase II drug metabolizing enzyme glutathione-*S*-transferase was measured, and the results detailed in Table 1 suggest a similar increase ($P < 0.05$) within all treatment groups.

Peroxisomal enzyme activities

The activities of the peroxisomal enzyme markers catalase, peroxisomal β-oxidation and fatty acid CoA oxidation are detailed in Table 2. The results show that treatment with either HCB or TCB caused a general reduction in these enzyme activities, however, highly significant ($P < 0.01$) reductions were only observed when neonates were exposed to a combination of HCB and TCB (i.e. combined treatment group). These findings indicate that the molecular substitution pattern of TCB and HCB, i.e. non-*ortho* versus di-*ortho* chlorine substitution did not cause a differential treatment effect, but maximal reduced activities in β-oxidation and fatty acid-CoA oxidation were only observed when neonates were exposed to a combination of TCB and HCB. The observed reduction in peroxisomal enzyme activities within the combined TCB/HCB treatment group provides additional evidence for a potential synergistic interaction of HCB and TCB.

Induction of CYP4A1: evidence for a potential synergistic interaction

Table 2 details the cytochrome P450IVA1 content, and the results show that treatments with TCB or HCB were equipotent in producing a significant induction of cytochrome P450IVA1. However, this inductive effect in neonates was more pronounced when maternal rats were given a combination of TCB and HCB. The results presented in Table 2 may indicate a synergistic interaction of TCB and HCB by causing a highly significant induction of cytochrome P450IVA1.

We have reported that treatment of rats with a commercial mixture of PCBs (i.e. 600 μmol/kg body weight) resulted in a 5-fold increase in the 12-hydroxylation of lauric acid [4]. The measurement of lauric acid ω-hydroxylation enables the induction of CYP4A1 and related sub-family members to be assessed. The present study confirms, with immunological methods, the induction of CYP4A1

Table 2. Neonatal hepatic enzyme activities associated with peroxisomal proliferation in response to lactational transfer of two congeneric PCBs

	Catalase (U/mg)	β-Oxidation (nmol/mg/min)	Fatty acid-CoA oxidation (nmol/mg/min)	Specific content of P450IVA1 (pmol/mg protein)
Control	305.1 ± 84.1	1.85 ± 0.55	1.79 ± 0.44	26.34 ± 8.22
HCB	286.7 ± 65.4	1.35 ± 0.39	1.15 ± 0.33*	41.84 ± 1.55‡
TCB	290.7 ± 36.2	1.43 ± 0.59	1.26 ± 0.54	41.28 ± 2.85‡
Combined	338.8 ± 79.6	0.88 ± 0.36†	0.87 ± 0.20‡	64.31 ± 0.48‡§‖

* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.
§ Comparison of HCB versus combined treatment group, $P < 0.01$.
‖ Comparison of TCB versus combined treatment group, $P < 0.01$.
Values are mean ± SD with N = 7 for controls, N = 4 for HCB, N = 4 for TCB and N = 4 for the combined treatment group.

and related isozymes and provides evidence for a potential non-additive, synergistic mechanism. Moreover, our results led us to suggest that the induction of CYP4A1 appears to be independent of the molecular substitution pattern of PCBs, since treatment with the non-*ortho*- or poly-*ortho*-substituted PCBs produced a similar induction (see Table 2). However, it must be pointed out that the PCB isomers used in our study may have different dose–response curves with respect to enzyme induction, and it is just fortuitous that the induction is similar at the same dose level. We consider this possibility unlikely but it clearly requires further experimentation. In addition, it is conceivable that HCB interferes in TCB metabolism thus increasing the effective concentration of TCB. For comparison, Mills *et al.* [19] have shown that 3,3′,4,4′,5,5′-hexabromobiphenyl reduces the metabolism of 3,3′,4,4′-tetrabromobiphenyl by approximately 90% at a substrate concentration of 3.3 μM of 3,3′,4,4′,5,5′-hexabromobiphenyl. Nevertheless, the present study reports the induction of CYP4A1 that appears to be independent of the molecular structure of HCB and TCB which contrasts the induction reported for other CYP families by PCBs, especially families I and II. The detailed immunological studies of Parkinson *et al.* [3] have elucidated precise structure–activity relationships of PCBs as treatment of rats with the non-*ortho*-substituted TCB causes the specific induction of CYP1A1 whereas the di-*ortho*-substituted PCB congener HCB induces specifically CYP2B1. Studies detailed by Nebert and Gonzalez [20] have shown that high affinity binding of certain chemical agents to a cytosolic receptor to cause an Ah-receptor mediated mechanism of induction of CYP1A1, but the mechanism of induction of CYP2B1 remains uncertain.

Peroxisomal proliferators are potent inducers of CYP4A1 [5] and a recent report [21] suggests a mechanism of action that involves binding of these chemicals to a member of the steroid hormone receptor superfamily. The data summarized in Table 2 indicate the absence of specific molecular requirements of PCBs in inducting CYP4A1, but treatment with a combination of TCB and HCB may result in a synergistic mechanism of action. Nevertheless, the possibility exists of multiple and interactive PCB-responsive elements in the regulatory 5′ flanking region of the P450IVA1 gene.

The results of the present study suggest that the receptor mediated induction of CYP4A1 differs fundamentally when compared with the mechanism of CYP1A1 induction, as rigid molecular substitution patterns of PCBs are needed to bind with high affinity to the Ah-receptor. A detailed account of the complex structure–activity relationships among PCBs in inducing CYP isoenzymes has been summarized by Safe [1].

The highly significant reduction of peroxisomal β-oxidation and fatty acid-CoA oxidation indicates fundamental differences in the ways by which PCBs induce cytochrome P450IVA1 when compared with the results obtained by treating rats with hypolipidaemic drugs such as clofibrate. It is well known that treatment of rodents with hypolipidaemic drugs results in concomitant increases of peroxisomal enzyme activities [5] and in the induction of cytochrome P450IVA1. Electronmicroscopical investigations have shown that increases in peroxisomal enzyme activities in response to treatment of rats with hypolipidaemic drugs were coincident with proliferation of this organelle. However, the data in Table 2 details a reduction in these enzyme activities and it is suggested that HCB and TCB induced CYP4A1 without the well known increase in peroxisomal enzyme activities. The reason for these differences in inducer responsiveness are presently unknown.

Lactational transfer and synergistic interactions of xenobiotics

The complexity of the relationship between the chemical structures of PCBs and the toxic responses they produce has been highlighted in a recent report which points out that low concentrations of certain individual PCBs can act synergistically to produce chromosomal damage to human lymphocytes in culture [22]. This could have far-reaching implications. It suggests that some of the toxic effects observed may be the result of individual PCBs interacting in cells to produce synergistic genotoxic effects.

A recent review by Gallenberg and Vodicaik [23] summarizes the current knowledge on the transfer

of persistent chemicals in milk and reports cited in this review suggest occupational and accidental exposure of humans to certain chemicals in excess of 70,000-fold of the concentration that was considered to be harmless. Although chemicals identified in human milk have rarely been shown to be acutely toxic to the nursing infant, it is notoriously difficult to predict from acute toxicity data the effects of long-term exposure to low levels of chemical contamination. In addition, the increased sensitivity of the neonate to chemical toxicants do not permit a risk assessment in the infant on the basis of toxicity data obtained from adult animals to be made. A recent study by Schantz [24] has shown that lactational transfer of an extreme low dose of PCBs resulted in a highly significant reduction in the cognitive abilities of monkeys. In routine toxicity studies such tests are rarely conducted but it is clear that the results reported by this author have far-reaching implications. It is self-evident that intensified investigations are required to take developmental aspects and synergistic interactions of chemicals into account to obtain more reliable information on the toxicity of chemicals and its effects on the wellbeing of neonates. The identification of highly sensitive markers are urgently needed to predict perturbations in the development of the young in response to exposure to low levels of chemicals, at early stages.

In conclusion, lactational transfer of HCB and/or TCB resulted in the induction of CYP4A1 at a low maternal treatment dose. This induction was not associated with the usually observed increase in peroxisomal enzyme activities. The molecular mechanism of CYP4A1 induction by PCBs appears to be independent of chlorine substitution and thus differs to the Ah-receptor-mediated mechanism of induction of CYP1A1 or to the mechanism of induction of CYP2B1.

Acknowledgements—The support by the Cancer Research Campaign of the United Kingdom (to J. T. Borlakoglu and R. R. Dils) is gratefully acknowledged. We are indebted to Dr. L. W. Robertson for his kind gift of TCB and HCB. We also thank the British Council for a research studentship to S. Huang and the Medical Research Council and Wellcome Trust for project grants to G. G. Gibson.

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