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Lactational transfer of 2,4,5,2′,4′,5′-hexachlorobiphenyl but not 3,4,3′,4′-tetrachlorobiphenyl, induces neonatal CYP4A1

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Abstract—In order to study the lactational transfer of polychlorinated biphenyls, lactating rats were treated with a low dose of either 3,4,3′,4′-tetrachlorobiphenyl (TCB), 2,4,5,2′,4′,5′-hexachlorobiphenyl (HCB) or a combination of TCB and HCB. For comparison, animals were also treated with Aroclor 1254, Lactational transfer of these chemicals resulted in the induction of neonatal hepatic CYP4A haemoproteins, the isozymes induced being dependent on the compound used. CYP4A1 was not detected in control, TCB or HCB/TCB-treated animals, but was induced in neonates when mothers were treated with HCB or Aroclor 1254. In the case of Aroclor 1254, the magnitude of the effect appeared to be dependent on the dose used. CYP4A2 and CYP4A3 were induced in the neonates when mothers were treated with Aroclor 1254 but not with the other agents used. It appears that TCB induces novel members of the CYP4A gene family. The present study provides immunochemical evidence for the ability of congeneric polychlorinated biphenyls to modulate differentially the expression of CYP4A isozymes in lactating mothers and their suckling offspring. These findings further support the potential hazards induced by lactational transfer of inert lipophilic chemicals and exemplify the complexity of the regulation of genes within this gene family.

Polychlorinated biphenyls (PCBs*) are a group of chemicals noted for their chemical stability and industrial utility. Most commercial preparations (e.g. Aroclor 1254) are oil-like fluids of which some may contain more than 70 different isomers and congeners [1]. Careless disposal of PCB-contaminated waste has resulted in ubiquitous pollution of the earth. Individual PCB isomers and congeners accumulate in biological systems and their biomagnification at the apex of food chains has been reported [2–4]. For example, we have routinely identified PCB isomers and congeners in human milk and human omental tissue samples [5, 6].

It is well documented that PCBs modulate and disturb normal cellular functions and several reviews are available which detail information on all aspects of the toxic properties of PCBs including their ability to promote and perhaps to initiate malignant tumour formation [7, 8]. Furthermore, PCBs are powerful inducers of hepatic monooxygenases, but the induction of individual P450 isoenzymes was found to be highly dependent upon the structure of individual PCB isomers and congeners, as shown previously [9, 10].

Recently, the induction of rat CYP4A1 in response to treatment with Aroclor 1254 was reported [11]. Furthermore, treatment of first day lactating rats with a low dose of 5 mg/kg body weight of either 3,4,3′,4′-tetrachloro- or 2,4,5,2′,4′,5′-hexachlorobiphenyl (TCB or HCB) produced significant induction of neonatal CYP4A haemoproteins, but the antibody used in this study was not monospecific and western blot analysis revealed two bands with approximate molecular masses of 51.5 and 52 kDa [12].

To investigate further the induction of rat neonatal CYP4A haemoproteins by PCBs, western blot analysis was carried out using monospecific antibodies to CYP4A isoenzymes. Consequently, first day lactating rats were treated with a low dose of either TCB or HCB or with a combination of both. Previous studies have shown that TCB is a potent inducer of CYP1A proteins, whereas HCB strongly induces members of the CYP2B sub-family [9, 10]. For comparison, rats were also treated with either 100 or 250 mg/kg body weight Aroclor 1254 to assess the induction of CYP4A isoenzymes in the mother and offspring.

Materials and Methods

Synthesis of TCB and HCB. The synthesis of TCB and HCB was carried out by the di-azo coupling of the corresponding chlorinated aniline with excess of chlorobenzene as described previously [13]. The products were purified by alumina/Florisil column chromatography, followed by repeated recrystallization from methanol or methylene chloride and flash chromatography. The structures were confirmed by proton nuclear magnetic resonance spectrometry (Jeol FX 90Q, 90 MHz) in deuterated methylene chloride and by capillary gas chromatography–mass spectrometry using a Joel JMS-DX double focusing mass spectrometer and electron impact ionization [13]. The synthetic products were of more than 99% purity and were fully consistent with the proposed structures. All other chemicals were obtained from commercial sources and were of highest purity.

Animals. Adult female Sprague–Dawley rats (body weight ranged from 175 to 200 g) were obtained from Charles River (Margate, U.K.). Food and water were provided *ad lib*. First day lactating rats (N = 4 animals per group) were treated with a single i.p. injection of either TCB (5 mg/kg body weight) or HCB (5 mg/kg body weight) or a combination of TCB and HCB (2.5 mg TCB and 2.5 mg HCB/kg body weight) dissolved in corn oil. In addition, a separate group of first day lactating rats received either 100 mg/kg body weight (N = 4) or 250 mg/kg body weight (N = 4) Aroclor 1254 dissolved in corn oil. Controls were injected with an equal amount of corn oil only. Twenty-one days later, the hepatic microsomal fraction was prepared as described previously [12].

Western immunoblot analysis of P450 isoenzymes. SDS–PAGE with 3 μg of microsomal protein was carried out according to the method of Laemmli [14], using 9% separating gels. Furthermore, the intensity of staining of SDS–PAGE-separated proteins was compared to ensure a uniform loading. Immunoblotting was performed essentially as described by Towbin *et al.* [15] with modifications according to Henderson and Wolf [16]. After separation, proteins were transferred electrophoretically to nitrocellulose, and probed with various antisera to rat liver

* Abbreviations: PCB, polychlorinated biphenyl; TCB, 3,4,3′,4′-tetrachlorobiphenyl; HCB, 2,4,5,2′,4′,5′-hexachlorobiphenyl; Aroclor 1254, a complex mixture of individual PCB isomers and congeners; CYP4A, CYP4A1, CYP4A2 and CYP4A3, cytochrome P450 isoenzymes.

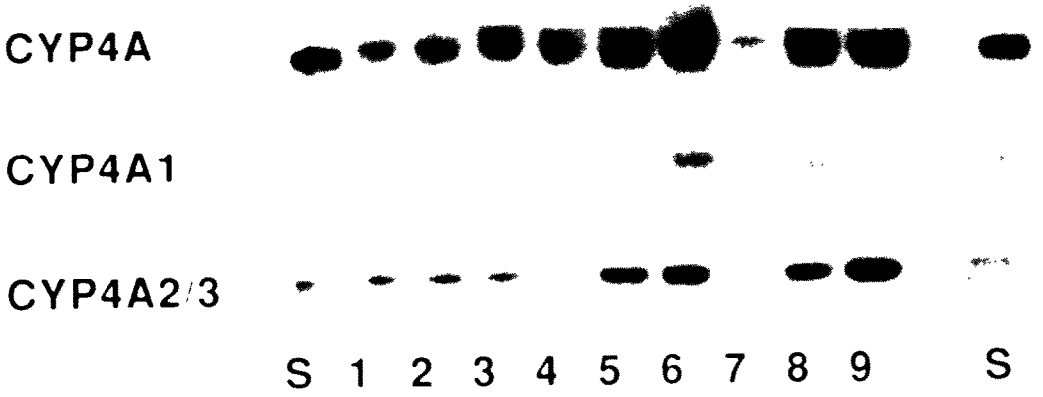

Fig. 1. Western blot analysis of maternal and neonatal microsomal P450A proteins. Microsomal proteins were separated on SDS–PAGE, transferred to nitro-cellulose and probed with anti-P450 antisera, as detailed in Materials and Methods. Lane 1, neonatal control; lane 2, neonates, HCB treatment; lane 3, neonates, TCB treatment; lane 4, neonates, TCB/HCB treatment; lanes 5 and 6, neonates, 100 and 250 mg/kg body weight Aroclor 1254 treatment, respectively; lane 7, maternal control; lanes 8 and 9, mothers, 100 and 250 mg/kg body weight Aroclor 1254, respectively. S represents standards.

cytochrome P450s. CYP4A1 was isolated according to the method of Tamburini *et al.* [17] and was of high purity, running as a single band on SDS–PAGE. A polyclonal antibody raised to CYP4A as well as monoclonal antibodies raised to CYP4A1 (51.5 kDa) and CYP4A2/3 (51.1 and 52 kDa) were used. After visualization of the immunoreactive polypeptides using horseradish peroxidase-labelled second antibody, the signal was enhanced with 1251-protein A (Amersham International plc) and subsequent autoradiography (Kodak X-Omat AR5 X-ray film) at −70°. Differing exposure times were used in order to optimize the autoradiographic signal.

Results and Discussion

Western blot analysis of hepatic CYP4A expression using a polyclonal antibody to CYP4A1. The western blot analysis is shown in Fig. 1. The polyclonal antibody used cross-reacted with CYP4A1, 4A2 and 4A3 and probably other members of the gene family. This antibody cross-reacted predominantly with a single 52 kDa microsomal protein in control and HCB-treated rats, whereas for the other treatment groups at least one additional band with a higher apparent molecular mass was also seen, both in neonatal and adult samples. A significant induction of CYP4A proteins was observed with TCB or Aroclor treatment. Aroclor 1254 induced CYP4A expression in lactating (see lanes 8 and 9) and neonatal (see lanes 5 and 6) rats in a dose-dependent fashion and with the 52 kDa protein being expressed more strongly.

Western blot analysis using monoclonal antibodies raised to CYP4A1 and CYP4A2/3. CYP4A1 was not detected in controls and TCB- and HCB/TCB-treated rats, but was induced following HCB and Aroclor 1254 treatment (Fig. 1). Neonatal (lanes 5 and 6) and lactating rats (lanes 8 and 9) differed in their response: a dose-dependent increase in immunoreactive protein was seen in the neonatal rats, but not in the mothers in response to Aroclor 1254.

CYP4A2/3-related proteins were expressed in control and in all treatment groups, albeit at different levels. Treatment of lactating mothers with TCB or HCB did not induce these proteins in the neonate. Indeed, a reduction in expression was observed when mothers were treated with a HCB/TCB mixture (lane 4). This reduction in CYP4A2/3 is not understood, but a similar reduction in cytochrome P450 proteins in response to PCB treatment has been reported [10]. It is noteworthy that treatment with the complex PCB mixture Aroclor 1254 produced strong induction in both neonates and mothers in an apparently dose-dependent fashion. It is important to note that a protein detected by the polyclonal CYP4A1 antibody, which was highly inducible by TCB (lane 3), was not detected by the monoclonal antibodies to either CYP4A1 or CYP4A2/3, indicating the induction of novel CYP4A protein.

The present study demonstrates the induction of CYP4A isoenzymes in response to PCB treatment. This response was, however, dependent upon the molecular structure of PCBs. For instance, the expression of CYP4A1 was not modulated by TCB, but treatment of lactating rats with HCB or Aroclor 1254 resulted in significant induction. HCB is a di-ortho-substituted congener which resembles phenobarbital in its mode of cytochrome P450 induction, whereas TCB is a non-ortho-substituted congener which has similar inducing properties to 3-methylcholanthrene. It is intriguing that HCB modulates the expression of CYP2B1 and CYP4A1 concomitantly, particularly since induction of CYP4A1 is reported to be via the peroxisomal proliferation activation receptor [18]. Whether HCB acts directly or indirectly through this receptor remains to be determined. It is, however, tempting to speculate that there is a common mechanism of CYP2B1 and CYP4A1 induction.

CYP4A2/3 was highly induced by Aroclor 1254 treatment. However, when lactating rats were treated with either TCB or HCB or a combination of both, no increase in immunoreactive protein was observed. This finding indicates that there are further novel P450-inducing agents in Aroclor 1254 which are distinct from the prototypic TCB and HCB. It is noteworthy that the induction of CYP4A isoenzymes is not linked to an increase in peroxisomal enzyme activities [12]. This contrasts the mode of action of clofibrate and other chemical agents, which concomitantly induce CYP4A isozymes and peroxisomal proliferation. Recently, Huang and Gibson [19] reported a species-dependent and congener-specific induction of CYP4A haemoproteins. In particular, it was reported that treatment with the highly toxic 3,4,5,3′,4′-pentachlorobiphenyl significantly induced the 12-hydroxylation of lauric acid in the guinea pig, but not in the rat. Indeed, this treatment suppressed rat lauric acid hydroxylase activity. In contrast,

treatment with TCB enhanced lauric acid hydroxylase activities in the rat, but not in the guinea pig. These species differences could be linked to differences in the PCB toxicity observed in rat and guinea pig [2]. Furthermore, it is conceivable that 3,4,5,3′,4′-pentachlorobiphenyl interferes with the metabolism of lauric acid in the rat, possibly by binding to the catalytic site of CYP4A and thereby suppressing the metabolism of lauric acid [20].

In conclusion, congeneric PCBs differentially modulate the expression of CYP4A isoenzymes in lactating mothers and their offspring. However, the extent to which the induction of CYP4A isozymes is deleterious to the nursing infant requires further study.

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