Biochemical Pharmacology, Vol. 28, pp. 2511–2514
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0006-2952/79/0815–2511 $02.00/0

SHORT COMMUNICATIONS

Altered ontogeny of hepatic steroid-metabolizing enzymes by pure polychlorinated biphenyl congeners

(*Received* 26 *October* 1978; *accepted* 25 *January* 1979)

The polychlorinated biphenyls (PCBs) are major environmental pollutants which have been used extensively since the 1930s. The PCBs are produced commercially as a mixture of many different isomers and most of the developmental toxicology studies on the PCBs have been performed with these mixtures.

The PCBs interact with the microsomal steroid-metabolizing system in adult mice, as evidenced by induction of the *in vivo* biotransformation of testosterone in the male mouse following PCB exposure [1]. Similarly, treatment of birds with PCBs results in an increased microsomal metabolism of sex steroids *in vitro* [2]. These effects are apparently a function of increased enzyme synthesis due to PCB interaction with enzyme synthetic processes.

In addition to possible direct effects on enzyme activity, a second type of modulation may occur via neonatal "imprinting", or programming, of enzyme activity. Programming is defined as the ability of hormones or hormonally active compounds, administered during a critical period of early development (i.e. just prior to or after birth), to predetermine the level of enzyme activities in the resultant adult animal. For example, adult sexual differentiation of brain development [3, 4] and of certain hepatic enzyme activities [5] is thought to require neonatal sex steroids. These programming actions are for the most part irreversible and are only manifested in postpubertal animals.

The PCBs have been reported to exhibit a relatively low level of estrogenicity, perhaps via their *in vivo* biotransformation to hydroxylated metabolites [6] which could bind to cytosolic estrogen receptors. Since it has been theorized that imprinting of sexual differentiation is initiated neonatally by hormone–receptor interactions in the developing brain [7], the hormonal activity of the PCBs [6, 8] might result in alterations of the normal programming of hepatic metabolism.

Because the pharmacokinetics of pure PCB congeners differ markedly as a function of chlorine position, these studies have utilized isomers which represent a wide spectrum of pharmacological characteristics. The specific isomers used are 4-chlorobiphenyl (1-CB), 3,4-3′4′-tetrachlorobiphenyl (4-CB) and 2,4,5-2′,4′,5′-hexachlorobiphenyl (6-CB). The availability of two adjacent unsubstituted carbon atoms on the biphenyl ring enhances the biotransformation of the PCB molecule [9]. Accordingly, the metabolic rate of 6-CB is 200 times greater than that of 1-CB [9]. 4-CB exhibits unique teratogenic activity not seen with the 1-CB and 6-CB isomers [10].

These studies were designed to determine the ability of selected PCBs to induce or repress the activities of hepatic steroid-metabolizing enzymes following pre- and/or postnatal exposure. In addition, these studies attempt to determine the ability of the PCBs to alter the normal programming of the activities of hepatic enzymes following neonatal exposure. In order to use enzymes with different sexual differentiation characteristics, these studies examine 16α-hydroxylase (activity higher in adult male rats than in female rats) and 5α-reductase (activity much higher in adult female rats than in males).

The compounds NADPH and EGTA* were purchased from the Sigma Chemical Co. (St. Louis, MO). The radioactive substrates testosterone[4-^{14}C] and androst-4-ene-3,17-dione[4-^{14}C] were obtained from New England Nuclear (Boston, MA). Corresponding unlabeled steroids used in achieving desired substrate concentrations for *in vitro* incubations and for metabolite identification were purchased from Steraloids, Inc. (Wilton, NH). Thin-layer chromatography plates were obtained from EM Laboratories, Inc. (Elmsford, NY). 1-CB was provided by the Aldrich Chemical Co. (Milwaukee, WI), while the 4-CB and 6-CB congeners were synthesized by Mr. Michael P. Walker (NIEHS Chemistry Department, Research Triangle Park, NC). All other reagents were obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ).

Pregnant rats were treated with five oral doses of vehicle (0.6 ml cotton seed oil) or PCB in vehicle from days 8 to 18 of gestation (days 8, 11, 13, 15 and 18). 1-CB and 6-CB were administered at doses of 10 mg/kg or 30 mg/kg; 4-CB was administered at doses of 3 mg/kg or 10 mg/kg. Offspring of these animals were killed at designated stages of development. Since PCBs are secreted in milk, the newborn received lactational exposures to the PCBs following transplacental exposure during their development. After killing the rats, their livers were rapidly excised and placed on ice. Initial studies using whole homogenates were performed by preparing 1:90 (w/v) liver homogenates in ice-cold KCl (1.15%) using a glass–glass-Duall tissue grinder (Kontes, Vineland, NJ). For subsequent studies of hepatic microsomal enzyme activities, a 20% liver homogenate was prepared in ice-cold TMK buffer (10 mM Tris, 14 mM $MgCl_2$ and 0.6 M KCl, pH 7.2). Homogenates were centrifuged at 20,000 *g* for 10 min; the supernatant fraction was decanted and spun at 105,000 *g* for 60 min. The microsomal pellet was resuspended in Tris buffer (150 mM, pH 7.4) so that the suspension contained the equivalent of 25 mg fresh liver (approximately 0.8 mg of microsomal protein) per ml of homogenate. PCB-mediated effects on enzyme activity were similar using microsomes or whole homogenates.

Aliquots of suspension containing approximately 50 μg protein (whole liver homogenates unless otherwise specified) were placed into tubes containing the incubation medium. Incubation mixtures consisted of 0.5 mM NADPH, 0.5 mM EDTA, 3.4 μM radioactive substrate (i.e. testosterone[4-^{14}C] or androst-4-ene-3,17-dione[4-^{14}C]) and potassium phosphate buffer (0.1 M, pH 7.4) to make a total volume of 0.2 ml. In addition, unlabeled substrate was added to provide a total substrate concentration of 107.6 μM which yielded maximal specific enzyme activities.

Incubations were conducted, and radiosteroids were extracted and analyzed on thin-layer plates as described previously [11]. 5α-Reductase activity was determined by the amount of 5α-dihydrotestosterone[^{14}C] formed from testosterone[^{14}C]; 16α-hydroxylase activity was calculated by the amount of 16α-hydroxyandrostenedione[^{14}C] formed from [^{14}C]androstenedione. All results are expressed as specific enzyme activities (pmoles/min/mg of protein).

Offspring of pregnant rats which had received the PCB congeners exhibited normal body wt at all postnatal ages

* EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

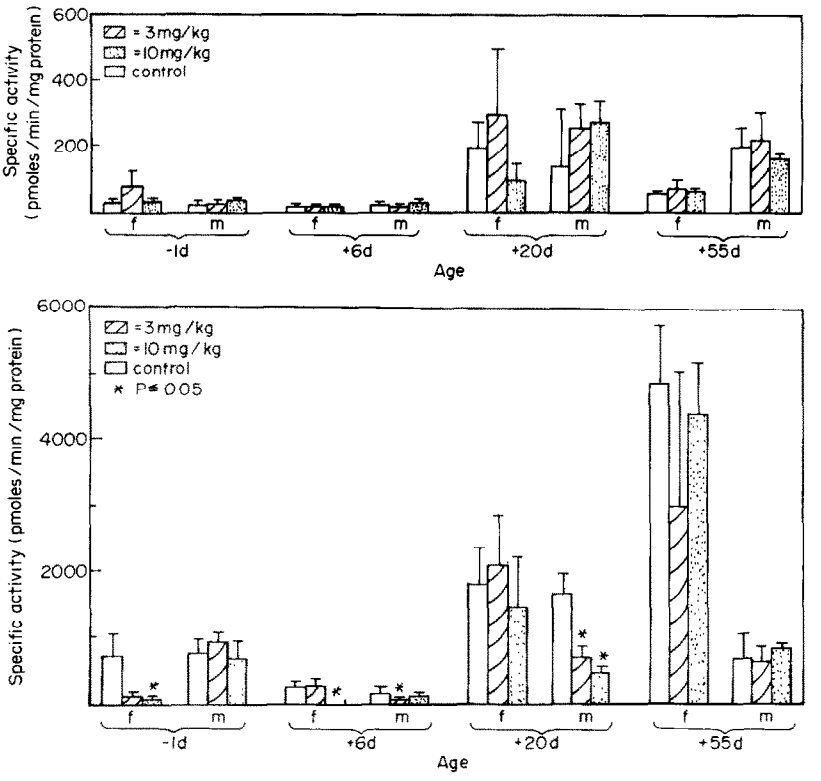

Fig. 1. Effects of tetrachlorobiphenyl (4-CB) on hepatic 16α-hydroxylase (panel A) and 5α-reductase (panel B) activities. Animals were treated with 4-CB prenatally (days 8, 11, 13, 15 and 18 of gestation), and 16α-hydroxylase activity was determined on whole homogenates at various ages. Enzyme activity is expressed as specific activity (pmoles/min/mg of protein). Each bar represents the mean + S.D.; n = 6.

examined, including F_1 adults. Since 4-CB is more fetotoxic and teratogenic than the other congeners, this isomer was administered in lower doses for the developmental enzymology studies. The fetotoxicity of the PCB isomers was examined by counting the litter sizes at birth; the survival rate was measured by counting the litter sizes after 6 and 20 days of age. At the concentrations used, none of the isomers appear to be fetotoxic. However, the ability of the pups to survive to 20 days of age was diminished significantly by the 10 mg/kg dose of 4-CB, whereas 30 mg/kg of 1-CB or 6-CB had no effect on newborn survival.

In control animals, hepatic 16α-hydroxylase (16α-OH) activity was higher in the adult male rat than in the adult female rat, whereas no sex difference was evident from birth through puberty (Fig. 1A). In contrast to 16α-hydroxylase, hepatic 5α-reductase (5α-red) activity in adult rats was much higher in control females than in control males (Fig. 1B).

The 1-CB was examined as an example of a low toxicity compound which is readily hydroxylated, conjugated and excreted, having a short half-life of about 7 hr [12]. Neonatal exposure to this isomer produced no consistent alterations in hepatic steroid-metabolizing activities.

The 4-CB isomer has a greater degree of chlorination than the 1-CB, and has a slightly longer half-life [13]. Tissue distribution studies indicated that radioactivity was not detected in postpubertal offspring following treatment of the dam with radiolabeled 4-CB; offspring received both lactational and transplacental exposures [13, 14]. 4-CB induces adult hepatic cytochrome P-448 and, therefore, modulates the hepatic mono-oxygenase pathways and can be considered a 3-methylcholanthrene [3-MC] type inducer [15, 16]. Since 16α-hydroxylase is a microsomal-hydroxylating enzyme which utilizes the cytochrome P-450 system [17], the 4-CB isomer might not be expected to induce 16α-hydroxylase activity, a result which is in fact observed in our studies (Fig. 1). Both the adult and perinatal hepatic cytochrome P-450 systems, therefore, appear to be non-responsive to 3-MC type inducers. In contrast, 4-CB treatment appears to repress 5α-reductase activity in prepubertal rats; 5α-reductase returned to normal by adulthood (Fig. 1). Moreover, developmental toxicity studies on pure PCB congeners provide evidence for unique toxicological properties of 4-CB. Irreversible urogenital tract and CNS dysfunctions were demonstrated in offspring exposed prenatally to 4-CB but not to the other congeners tested [13–14]. The reason for the observed repressive effect of 4-CB on hepatic enzyme activity is not clear, but may be related to the high blood levels of 4-CB and its metabolites in fetal newborn and maternal blood [14].

Perinatal exposure to the 6-CB isomer resulted in dramatic increases in 16α-hydroxylase activity in both sexes after birth (Fig. 2A). The high dose produces a 4-fold increase at 6 days of age and a 4-fold increase at 20 days of age in the female rat, with slightly smaller increases in male 16α-hydroxylase activity at these ages. The low dose also increases enzyme activity. The stimulatory effect of 6-CB on this enzyme is somewhat diminished in the adult rats, but is still evident in both sexes.

6-CB does not contain two adjacent unsubstituted carbon atoms and represents a more persistent class of PCBs. This compound is readily stored in adipose tissue and has a negligible rate of metabolism, which results in a half-life exceeding the lifetime of the rat [12]. Unlike the 4-CB isomer, 6-CB induces the cytochrome P-450 system [15, 16]. In this regard, the dramatic increases in 16α-hydroxylase activities are not unexpected. In a parallel experiment using radiolabeled 6-CB (dose of 10 mg/kg to pregnant rats), 55-day-old offspring had approximately 10 ng of 6-CB/g of liver following transplacental and lactational exposures [18]. Therefore, induced levels of 16α-OH in 55-day-old rats following peri-

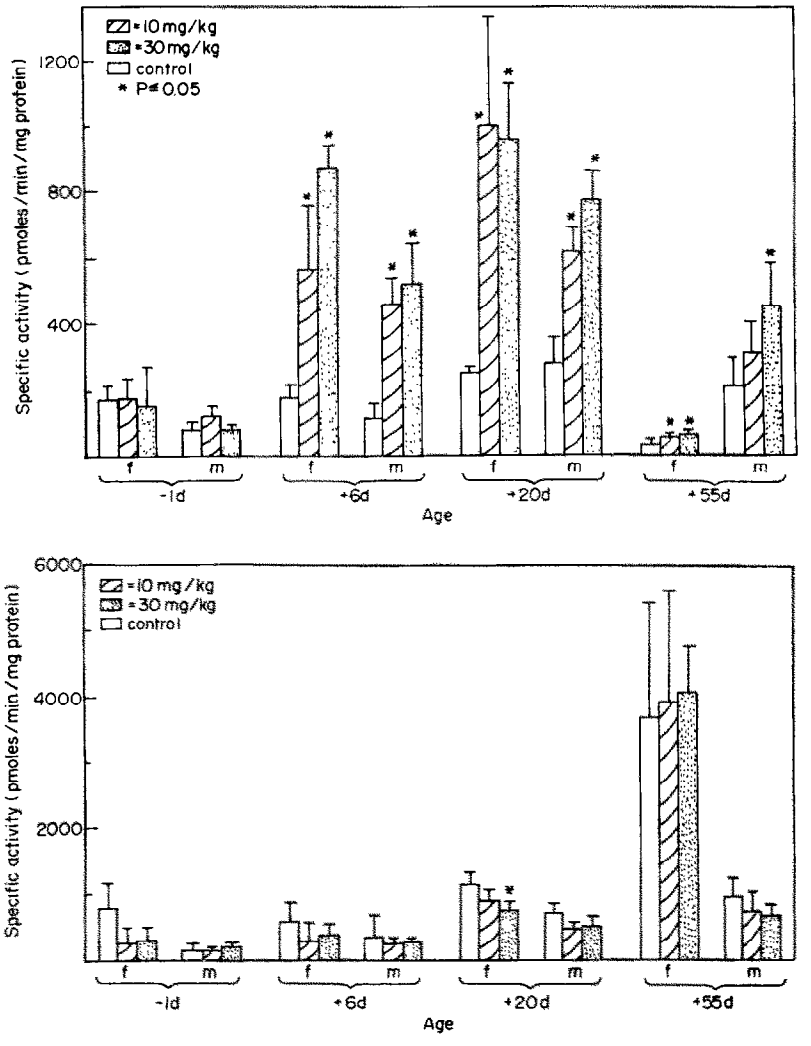

Fig. 2. Effects of hexachlorobiphenyl (6-CB) on hepatic 16α-hydroxylase (panel A) and 5α-reductase (panel B) activities. Animals were treated with 6-CB prenatally (days 8, 11, 13, 15 and 18 of gestation), and 16α-hydroxylase activity was determined on whole homogenates at various ages. Enzyme activity is expressed as specific activity (pmoles/min/mg of protein). Each bar represents the mean ± S.D.; n = 6.

natal exposure to 6-CB appear to reflect a high degree of sensitivity of this enzyme to 6-CB. The lack of effect of the 6-CB isomer on 5α-reductase (Fig. 2B) indicates that 5α-reductase responsiveness is not linked to the P-450 system.

Although the PCBs do possess hormonal activity [6, 8], neonatal exposure to the congeners tested did not alter the normal neonatal imprinting of the sexual differentiation of 5α-red and 16α-OH. In contrast, both 4-CB and 6-CB appear to feminize activity of microsomal UDP glucuronyltransferase in adult males exposed neonatally to these congeners [10].

In summary, three pure PCB congeners were examined for their ability to alter the normal development of two hepatic steroid-metabolizing enzymes in rats: 5α-red and 16α-OH. Congeners examined were: 1-CB, 4-CB and 6-CB. Perinatal administration of 1-CB had no significant effect on either 5α-red or 16α-OH development through adulthood. 4-CB treatment produced a repression of 5α-red in the prepubertal male rat with no effect on adult levels. 16α-OH was unaffected by 4-CB exposure. Perinatal exposure to 6-CB caused an induction of 16α-OH in the male and female rat at 6, 20 and 55 days of age. Conversely, 6-CB had little effect on 5α-red activity. These studies show that 16α-OH and 5α-red exhibit markedly differing responses to the PCBs, and that the degree and/or position of biphenyl chlorination qualitatively affects the perinatal response of hepatic steroid-metabolizing enzymes to chlorinated biphenyls. Moreover, hepatic 16α-OH activity appears to be an extremely sensitive marker to 6-CB body burdens during postnatal development.

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Biochemical Pharmacology, Vol. 28, pp. 2514–2515
0006-2952/79/0815–2514 $02.00/0
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2-Chloro-11-(1-piperazinyl)dibenz[b, f][1, 4]oxazepine (Amoxapine), an antidepressant with antipsychotic properties—A possible role for 7-hydroxyamoxapine

(*Received* 8 *November* 1978; *accepted* 2 *February* 1979)

Amoxapine, 2-chloro-11-(1-piperazinyl)dibenz[b,f][1,4]-oxazepine (Anamox), is the desmethyl derivative of loxapine, a potent neuroleptic of the dibenzoxazepine class [1–3]. Various clinical studies have shown that amoxapine, unlike loxapine, is a potent antidepressant [4–7]. Moreover, other investigators have reported that amoxapine possesses both antidepressant and neuroleptic activities in laboratory animals and in man [8, 9]. Since amoxapine is metabolized in mammals to 7-hydroxyamoxapine (7-OH-Amox) and to 8-hydroxyamoxapine (8-OH-Amox) [10], it was of interest to study not only amoxapine, but also its hydroxylated metabolites for their effects in neurochemical tests suggestive of antipsychotic and antidepressant actions. Three *in vitro* tests were chosen: (a) synaptosomal uptake of the labeled biogenic amines [^{3}H]-L-norepinephrine ([^{3}H]NE) and [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT); (b) dopamine-sensitive adenylate cyclase (DSAC); and (c) membrane [^{3}H]spiroperidol bindings.

Table 1. Calculated IC_{50} values for amoxapine and its analogs in blocking the accumulation of monoamines by crude synaptosomal fractions of rat diencephalon midbrain*

Drug	Inhibition of norepinephrine uptake (2×10^{-8} M)	Inhibition of serotonin uptake (2×10^{-8} M)
	IC_{50} (nM ± S.E.M.)	IC_{50} (nM ± S.E.M.)
Amoxapine	22.5 ± 2.0	566.5 ± 51.0
7-OH-Amox	16.6 ± 1.6	424.2 ± 14.8
8-OH-Amox	33.8 ± 7.5	323.2 ± 25.5
Imipramine	16.8 ± 2.3	55.8 ± 1.2

*Each drug was studied at four to five levels of concentration ranging from 0.005 to 1.0 μM. Each determination was done in duplicate and in three different tissue pools. The IC_{50} values were determined by log probit analysis of the data and represent the concentration of each drug required to inhibit the uptake of either [^{3}H]NE or [^{3}H]-5-HT at 2×10^{-8} M by 50 per cent.

Loxapine, amoxapine, 7-OH-Amox and 8-OH-Amox were synthesized by Dr. C. F. Howell of Lederle Laboratories (Pearl River, NY). Imipramine·HCl (Ciba-Geigy Corp., Summit, NJ) and spiroperidol (Janssen Pharmaceutical, Belgium) were generously donated to us. Radiolabeled [^{3}H]-L-norepinephrine, [^{3}H]-5-hydroxytryptamine and [^{3}H]spiroperidol were all purchased from New England Nuclear, Boston, MA. Brian tissues were from 200 to 300 g male Wistar rats obtained from Royalhart Farms (New Hampton, NY).

The preparations of synaptosomal fractions and the determinations of [^{3}H]NE and [^{3}H]-5-HT uptake activities were measured essentially by the method of Kuhar *et al.* [11], with the modifications described by Gal *et al.* [12].

Adenylate cyclase (AC) activity was assayed in striatal tissues by the method described by Kebabian *et al.* [13], in the presence or absence of dopamine (DA) and/or test compounds. The amount of cyclic adenosine-3′,5′-monophosphate (cAMP) formed was determined by the protein binding assay of Gilman [14]. The preparation of membrane fractions and the determinations of [^{3}H]spiroperidol binding were described in an earlier report [15]. Protein content in the tissue samples was determined by the method of Lowry *et al.* [16].

As can be seen in Table 1, amoxapine, 7-OH-Amox and 8-OH-Amox substantially inhibited the synaptosomal uptake of [^{3}H]NE, while having little effect on [^{3}H]-5-HT uptake. Imipramine, on the other hand, influenced the uptake of both monoamines to approximately the same degree. Amoxapine and 7-OH-Amox were equipotent with imipramine in inhibiting [^{3}H]NE uptake, whereas 8-OH-Amox was about half as potent as imipramine. No significant inhibitory effects were detected for amoxapine or 8-OH-Amox on the DA-induced stimulation of AC in striatal homogenates (Table 2). However, 7-OH-Amox and loxapine were very active in inhibiting the rise in cyclic adenosine-3′,5′-monophosphate formation induced by 30 μM DA. Moreover, 7-OH-Amox and loxapine