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Effect of prenatal exposure of lindane on alterations in the expression of cerebral cytochrome P450s and neurotransmitter receptors in brain regions

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ABSTRACT

Prenatal exposure to low doses (0.0625- or 0.125- or 0.25 mg/kg b. wt., orally) of lindane, an organochlorine insecticide, from gestation day (GD) 5–21 was found to produce a dose-dependent increase in the mRNA expression of cytochrome P450s (CYPs) and associated transcription factors in frontal cortex, cerebellum and corpus striatum isolated from the offsprings. Though the increase in the expression persisted up to postnatal day 60, the increase was significant at postnatal days 21-, and 45- in the offsprings exposed prenatally to relatively higher doses (0.125- or 0.25 mg/kg) of lindane and even up to postnatal day 60 in the offsprings exposed prenatally to the highest dose of lindane. A similar increase in the expression of dopamine D2, 5HT2A and $GABA_A$ receptors and associated neurotransmitter receptor binding was observed in the brain regions of the exposed offsprings. Scatchard analysis also suggested an increase in the levels of these neurotransmitter receptors in offsprings prenatally exposed to lindane. The data indicating similarities in the alterations of neurotransmitter receptors and CYPs in brain regions in prenatally exposed offsprings have suggested that neurotransmission processes and CYPs are closely linked that will eventually help in understanding the developmental neurotoxicity of lindane.

1. Introduction

Epidemiological studies have suggested that exposure of organochlorine pesticides during pregnancy is associated with poor cognitive, impaired motor functions and neurologic development and an increased risk of chronic diseases later in life (Siddiqui et al., 2003; Sagiv et al., 2007; Stillerman et al., 2008; Boucher et al., 2013). Likewise, exposure of polychlorinated biphenyls (PCBs), chlorinated hydrocarbons during pregnancy have been reported to influence birth weight, birth size of the infants and even reduce the gestation in humans (Kezios et al., 2012; Lignell et al., 2013; Lopez-Espinosa et al., 2011). Lindane (γ-hexachlorocyclohexane), an organochlorine pesticide, used in agriculture and public health, is known to persist in the environment (Alegria et al., 2006). It is thus likely that pregnant women and even children during early development could be exposed to lindane and other organochlorine pesticide. Lindane has been shown to accumulate in the fatty tissues and thus can be transferred through the placenta to the fetus and to the newborns through the mother's milk (Albertson et al., 1985).

Studies have suggested a link between exposure to lindane during pregnancy and fetal effects. Behavioral, neurochemical and even structural changes were reported in the brain isolated from developing rats treated with doses of lindane, which do not produce any effects in the adults (Rivera et al., 1998; Serrano et al., 1990). Lindane has been reported to induce the release of neurotransmitters by increasing the synaptosomal internal content of Ca^{+2} (Rivera et al., 1990). Though γ-amino butyric acid (GABA) receptor ionophore complex is the primary target of the pesticide, it has been shown that lindane causes neurobehavioral toxicity by several mechanisms, such as altering neurotransmitter levels and influencing the expression of cytochrome P450s (CYPs), involved in its metabolism and toxicity (Anand et al., 1998; Parmar et al., 2003a, 2003b; Rivera et al., 1998). Toxicokinetics of lindane play an important role in its neurobehavioral toxicity with DBA/2 mice, that lacks Ah receptor, reported to be more vulnerable to the convulsant effects of lindane (Liu and Morgan, 1986). The developing rats were found to be particularly sensitive to the neurotoxicity of the pesticide, with subconvulsant doses of lindane inducing an imbalance in the central monoaminergic systems (Rivera et al., 1998).

As the CYPs are not fully developed in the fetus and developing animals (Moscovitz and Aleksunes, 2013), any exposure of lindane

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occurring during gestation may not be detoxified readily resulting in the behavioral effects in the offsprings. Studies from our laboratory have shown that prenatal exposure to low doses of lindane alters the ontogeny of CYPs, involved in the neurobehavioral toxicity of lindane (Parmar et al., 2003a), in liver and brain of the offsprings at birth. The increase in the expression of CYPs in brain and liver of the offsprings was found to persist during postnatal development and were found to be associated with the neurobehavioral effects in the prenatally exposed offsprings (Johri et al., 2007, 2008a, 2008b).

Interestingly, CYPs have been reported to be closely associated with the various endogenous functions in the brain (Miksys and Tyndale, 2004; Parmar et al., 2003a, 2003b). The dopamine transporters have been shown to exhibit overlapping substrate specificity with the CYP2E1 (Wójcikowski et al., 2008). The role of CYP2D6 isoenzymes has been demonstrated in the conversion of tyramine to dopamine (Bromek et al., 2011). Ligands that interact with the $GABA_A$ receptor are also reported to increase the activity of CYP1A1, 2B1/2B2 and 3A1 isoenzymes in liver (Parmar et al., 2003b; Roberge et al., 2004). As animal studies have shown high vulnerability of the fetus to the neurotoxic effects of pesticides, the present study was initiated to investigate the effect of prenatal exposure to low doses of lindane on the expression of CYPs in brain regions, that catalyze specific neurotransmission processes, during postnatal development. Attempts were also made to study the prenatal effect of these doses on the expression of neurotransmitter activity in offsprings during postnatal development and to correlate these alterations in the neurotransmitter receptors with the cerebral CYPs.

2. Materials and methods

2.1. Chemicals

Lindane-technical grade was procured from Sigma-Aldrich, St. Louis, MO, U.S.A. Trizol reagent was obtained from Life Technologies, USA. The reagents for RT-PCR have been procured from ABI Biosystems U.S.A. All other chemicals used were of the highest purity commercially available and procured either from BDH (a subsidiary of E. Merck, India) or SISCO Research Laboratories Pvt. Ltd. (India). Phenobarbital sodium salt (PB) was a gift from Biodeal Laboratories (India).

2.2. Animals and treatment

Adult male (~12 weeks old) and female (~10 weeks old) Wistar rats of proven fertility were obtained from the Animal House facility of CSIR-Indian Institute of Toxicology Research (IITR), Lucknow. All the animals were maintained on a commercial pellet diet and water *ad libitum* in a temperature controlled room with a 12/12-h light/dark cycle and cared for in accordance to the policy laid down by Animal Care Committee of IITR, Lucknow. The animal experimentation was approved by the Ethical Committee of the Institute. Fifty-seven (57) female rats were allowed to mate with 19 adult males (3:1). On day 0 of pregnancy (confirmed by a positive vaginal smear), the pregnant rats (54 numbers) were randomly divided into 3 batches (one batch each for studying effects on expression of CYPs, neurotransmitter receptors and receptor binding) of four groups each. Animals in groups 1, 2 and 3 in each batch received 0.0625- or 0.125- or 0.25 mg/kg b. wt. of lindane, orally from gestation day 5 (GD5) to GD21. Animals in group 4 served as control and received corn oil in an identical manner. On the day of parturition, the average litter size was adjusted to eight per dam in all the groups with equal number of males and females as far as possible. The male offspring born to the control and treated dams were sacrificed on postnatal age of 21, 45 and 60 days.

Brains were immediately removed and dissected into regions (cerebellum, frontal cortex and corpus striatum) and processed for isolation of total RNA and membrane preparation for receptor binding. For isolating RNA, brain region tissues were snap frozen in liquid nitrogen and stored at –80 °C. The tissues were processed for isolation of RNA with Trizol (Life Technologies, USA) using the manufacturer's protocol.

2.3. Real time PCR (RT-PCR) analysis

qRT-PCR for the different CYP isoenzymes was carried out as described earlier (Shah et al., 2009). The sequences of primers used for CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1, CYP3A1, CYP2D1 and GAPDH have been described earlier (Baldwin et al., 2006; Yamaguchi et al., 2005). The threshold cycle value (Ct Values) of each sample was normalized with Ct values of endogenous control (GAPDH). Fold Change was calculated from ΔΔCt value of each sample, which was derived from ΔCt of treated – ΔCt of control. Similarly, RT-PCR for neuroreceptors such as $GABA_A$, DAD2 and 5HT2A was carried out using primers and conditions as described earlier (Andoh et al., 2006; Li et al., 2006; Ruddell et al., 2006). Likewise, RT-PCR for transcription factors such as aryl hydrocarbon receptor (AhR), aryl hydrocarbon nuclear transporter (Arnt), constitutive androstane receptor (CAR) and pregnane X-receptor (PXR) was carried out using primers and conditions as described earlier (Qin and Meng, 2006; Shirota et al., 2006; Vondracek et al., 2006; Wyde et al., 2005).

Table 1
Ligands and competitors used in receptor binding studies.

Receptor	Brain regions	Radioligands	Competitors
Dopamine (DAD2)	Corpus striatum	^{3}H-Spiperone (1×10^{-9} M)	Haloperidol (1×10^{-6} M)
Serotonin (^{5}HT2A)	Frontal cortex	^{3}H-Ketanserin (1×10^{-9} M)	Cinenserin (1×10^{-6} M)
$GABA_A$	Cerebellum	^{3}H- Muscimol (1×10^{-9} M)	GABA (1×10^{-6} M)

2.4. Receptor binding of dopamine-D2 (DAD2), serotonin-2A (5HT2A) and $GABA_A$ receptors in brain regions

Assay of DAD2 receptors in corpus striatum, 5HT2A receptor in frontal cortex and $GABA_A$ receptor in cerebellum was carried out by the radioligand binding assays following the standard procedure (Khanna et al., 1994). Briefly, crude synaptic membrane was prepared by homogenizing the brain regions in 19 volumes of Tris–HCl buffer (5 mM, pH 7.4) following centrifugation at $40,000\times g$ for 15 min at 4 °C. The sedimented pellet was washed twice and recentrifuged and pellet suspended in Tris–HCl buffer (40 mM, pH 7.4) for the binding assays. Protein concentration in membrane preparations were measured following the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a reference standard.

For receptor binding assays the details of radioligands, competitors and their conditions for the assay of specific receptors are mentioned in Table 1. In brief, the reaction mixture containing Tris–HCl buffer (40 mM, pH 7.4), together with membrane protein (300–400 μg) and appropriate radioligand were incubated for 15 min at 37 °C in the presence or absence of competitor to assess the non-specific and total binding respectively. The contents of the binding tubes were immediately filtered on glass fiber discs (25 mm diameter, 0.3 μ pore size, Whatman GF/B). Filteration on the glass fiber discs was carried out using Manifold Filteration assembly (Millipore, U.S.A.) under vacuum, washed twice and filters were dried and transferred into vials containing scintillation fluid. The scintillation cocktail used for radioactive counting was the mixture of 1, 4-bis (5-Phenyloxazole-2-y) benzene (POPOP), 2,5-diphenyloxazole (PPO), napthalene, toulene, methanol and 1–4-dioxane. The radioactivity was counted on scintillation counter (Packard, USA) at an efficiency of 30–40% for ^{3}H to determine the membrane bound radioactivity. Although the efficiency varies from instrument to instrument, 30–40% efficiency is appropriate to count the radioactivity. Specific binding was determined by subtracting the non-specific binding from the total binding and has been expressed as pmoles ligand bound/g protein.

Scatchard analysis was carried out at varying concentrations of radioligands (generally 1/10 to 10 times of the affinity) to ascertain whether change in the binding is due to alteration in the affinity (Kd) or number of receptor binding sites (Bmax). Scatchard analysis was carried out at different concentrations of radioligands (normally 1/10 to 10 times of the affinity of radioligand). Linear regression analysis using GraphPad Prism ver 3.0 was carried out to determine the affinity (Kd) and maximum number of receptor binding sites (Bmax). R value was close to 0.9.

2.5. Statistical analysis

All values are presented as mean ± SEM. Main effects of dose and duration of exposure of lindane on expression of individual CYP isoforms, their associated transcription factors and neurotransmitter receptors were ascertained using student's t-test to calculate the statistical significance between control and treated groups. $p < 0.05$ was considered to be significant when compared with the controls.

3. Results

3.1. Effect of prenatal exposure of lindane on mRNA expression of CYP isoforms in the different brain regions

Quantitative Real Time PCR (qRT-PCR) revealed that prenatal exposure of different doses of lindane increased the levels of CYP1A-, 2B-, 2D1, 2E1 and 3A1 isoenzymes and associated transcription factors in the brain regions of the offsprings during postnatal development (Figs. 1 and 2). Prior to relative quantification of CYPs, each sample was normalized with housekeeping gene (GAPDH), which served as an endogenous control. The expression of GAPDH