Biochemical Pharmacology, Vol 31, No 5, pp 647–651, 1982
Printed in Great Britain

0006-2952/82/050647-05 $03 00/0
Pergamon Press Ltd

ALTERED IMPRINTING OF RAT LIVER MONOAMINE OXIDASE BY *o,p'*-DDT AND METHOXYCHLOR

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(*Received* 10 *March* 1981; *accepted* 15 *July* 1981)

Abstract—Neonatal administration of *o,p'*-DDT [1,1,1-trichloro-2-(*o*-chlorophenyl-2-(*p*-chlorophenyl)ethane] or methoxychlor resulted in elevated levels of sex-differentiated hepatic monoamine oxidase activities in adult rats, but not in prepubertal animals. Exposure to these hormonally active xenobiotics may have changed the brain hormone environment during the critical period of development, resulting in endocrine alterations that were reflected by latent but permanent increases in hepatic monoamine oxidase activities, i.e. "altered imprinting". Hepatic glutathione *S*-transferases and cytochrome P-450 content also underwent sex differentiation, but neonatal treatment with *o,p'*-DDT or methoxychlor did not alter levels in adult rats. However, glutathione *S*-transferase activities and cytochrome P-450 content were higher in prepubertal animals treated neonatally with *o,p'*-DDT. In contrast to monoamine oxidase, effects on glutathione *S*-transferase activities and cytochrome P-450 content were attributed to induction by these xenobiotics.

From fertilization to maturity, the developing organism is exposed to hormones that trigger transcription, translation, and post-translational processes resulting in specific protein patterns which constitute and determine the endocrine biochemistry characteristic in mature differentiated tissue. In the rat, the perinatal period of development is recognized as a time during which critical organizational events are still taking place in the brain. Organizational effects are, in part, developmental alterations to nerve endings in the brain as a consequence of exposure to androgen during a limited critical period of fetal or early postnatal development. This results in the programming of a male-type of endocrine secretion [1], behaviour [2, 3], and hepatic enzyme metabolism [4, 5]. The permanent modification of the central nervous system by hormones clearly emphasizes and forms a useful model for the study of the capacity of xenobiotics to modify genomic expression. The developing organism is particularly susceptible to numerous environmental factors that can directly or indirectly influence brain development causing latent alterations in sexual differentiation.

Our laboratory has been investigating the effects of perinatal exposure to hormones and hormonally active xenobiotics on postnatal development. Diethylstilbestrol administered neonatally alters sexually dimorphic neurobehavioral and morphometric measurements [6], and the postnatal developmental course of hepatic enzymes in the resulting adult rat [7]. Exogenously administered testosterone or diethylstilbestrol to neonatal castrates prevents the rise in hepatic monoamine oxidase activity in the adult male following neonatal castration [5]. Gellert *et al.* [8] have reported that *o,p'*-DDT [1,1,1-trichloro - 2 - (*o*-chlorophenyl) - 2 - (*p*-chlorophenyl) ethane] administered neonatally produces precocious puberty and reduced feedback rise in serum gonadotrophin concentration in females. This action is thought to be a consequence of DDT estrogenicity [9, 10]. Lee and Visek [11] reported that administration of 3 mg *o,p'*-DDT to newborn male rats inhibited the binding of estradiol and testosterone to hypothalamic estrogen and androgen receptors.

Since DDT possesses hormone activity, we were interested in determining if this insecticide and its structurally related analog, methoxychlor (1,1,1-trichloro-2,2-bis[*p*-methoxyphenyl]ethone), could alter the postnatal developmental course of some sex-differentiated hepatic enzymes through a mechanism involving organizational effects.

MATERIALS AND METHODS

Experiments were carried out using Sprague–Dawley rats (Charles River Breeding Laboratories Inc., Wilmington, MA). Animals had free access to food (NIH Feed 31) and water. The animals were housed in a controlled environment (21°; 12 hr light–dark cycle), were weaned at 21–23 days of age, and were housed four animals per cage after weaning. Neonatal rats were treated subcutaneously with 1 mg or 3 mg/rat *o,p'*-DDT (99% pure) (Chem. Service, West Chester, PA) or methoxychlor (98% pure) (Sigma Chemical Co., St. Louis, MO) on days 2, 4, and 6 postpartum; controls received 20 μl of sesame oil.

Animals were decapitated and allowed to bleed, and their livers were rapidly removed and placed on

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ice. Twenty percent liver homogenates (w/v) in 20 mM potassium phosphate (pH 7.4) containing 0.25 M sucrose were prepared in a motor-driven glass Potter–Elvehjem homogenizer equipped with a Teflon pestle. Mitochondria were prepared by diluting 2.5 ml of this homogenate (0.5 g equivalent liver wt) with an equal volume of the same buffer and were centrifuged at 900 *g* for 15 min. The resulting supernatant fraction was recentrifuged at 12,000 *g* for 15 min to produce the mitochondrial pellet. This pellet was resuspended in 2.5 ml of 20 mM potassium phosphate buffer (pH 7.4) and then frozen at −76° for later assaying of monoamine oxidase activities. The remaining portion of the homogenate was centrifuged at 12,000 *g* for 10 min, and the supernatant fraction was decanted and centrifuged at 105,000 *g* for 60 min. The clear supernatant fraction was taken as the cytosolic portion and frozen at −76° for later assay of glutathione *S*-transferase activities. Previous experiments had demonstrated that there were no sex differences in the recovery of mitochondrial monoamine oxidase or cytosolic glutathione *S*-transferase activities from whole liver homogenates. We therefore assumed that recoveries would not differ between control groups and treated groups which exhibit altered sex differentiation of these enzymes. The resulting microsomal pellet was washed in 6 ml of 150 mM Tris buffer (pH 7.4) and recentrifuged at 105,000 *g* for 60 min. This microsomal preparation was resuspended in 5 ml of 150 mM Tris buffer (pH 7.4) and was used for measuring, on the same day, UDP-glucuronyltransferase activity and cytochrome P-450 content. Total hepatic monoamine oxidase activities (EC 1.4.3.4) are reported as the sum of monoamine oxidase A and B estimated spectrophotometrically using serotonin and phenylethylamine as substrates respectively [12]. Glutathione *S*-transferase (EC 2.5.1.13) activities were assayed by measuring the conjugation of glutathione with the substrates 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) [13]. UDP-glucuronyltransferase (EC 2.4.1.17) was assayed using *p*-nitrophenol as substrate [14]. Cytochrome P-450 content was determined by measuring the carbon monoxide difference spectra after reduction with dithionite [15]. Activity levels for monoamine oxidase, glutathione *S*-transferase and UDP-glucuronyltransferase are expressed as μmoles/mg protein while cytochrome P-450 content is expressed as nmoles/mg protein. In our experiments enzyme activities in prepubertal males and females were treated as individual samples, were found to behave similarly and are reported collectively.

Protein determinations were carried out by the method of Lowry *et al.* [16]. Statistical comparisons between groups were made using the Mann–Whitney U tests.

RESULTS

Hepatic glutathione *S*-transferase activities and cytochrome P-450 content were higher in 20-day-old rats treated neonatally with *o,p'*-DDT but at 63 days of age these variables were not significantly different from controls (Table 1). UDP-glucuronyltransferase activity was unchanged at all ages investigated.

Table 1. Effects of neonatal administration of *o,p'*-DDT on hepatic enzymes in the developing rat*

Enzyme marker	Prepubertal males and females	Adult	
		Males	Females
Monoamine oxidase			
Controls	0.75 ± 0.04	0.98 ± 0.04	1.57 ± 0.08
Low dose	0.80 ± 0.05	1.27 ± 0.10†	1.45 ± 0.10
High dose	0.84 ± 0.04	1.62 ± 0.08‡	1.71 ± 0.08
Glutathione *S*-transferase (CDNB)			
Controls	839 ± 41	1879 ± 110	1845 ± 64
Low dose	1015 ± 39†	2046 ± 110	1855 ± 101
High dose	1046 ± 40†	2032 ± 70	1850 ± 91
Glutathione *S*-transferase (DCNB)			
Controls	59 ± 3	127 ± 6	68 ± 3
Low dose	67 ± 3	130 ± 7	67 ± 4
High dose	68 ± 2†	121 ± 4	67 ± 4
UDP-glucuronyltransferase			
Controls	27.7 ± 1.7	31.7 ± 1.1	20.3 ± 0.9
Low dose	27.8 ± 0.4	32.7 ± 0.8	19.9 ± 0.8
High dose	28.8 ± 0.8	29.9 ± 0.8	19.8 ± 1.0
P-450 content			
Controls	0.49 ± 0.03	0.88 ± 0.06	0.56 ± 0.03
Low dose	0.66 ± 0.03†	0.71 ± 0.07	0.60 ± 0.02
High dose	0.62 ± 0.04†	0.83 ± 0.02	0.60 ± 0.03

* *o,p'*-DDT was administered subcutaneously on days 2, 4 and 6 post partum. Controls received 20 μl sesame oil. Low dose = 1 mg *o,p'*-DDT/animal; high dose = 3 mg *o,p'*-DDT/animal. Animals were killed on day 20 or 63. All values are means ± S.E.M.

† $P < 0.05$, when compared to respective control (N = 8).

‡ $P < 0.01$, when compared to respective control (N = 8).

Hepatic monoamine oxidase activities in treated animals did not differ significantly from controls at the prepubertal period, but activities were elevated in the resulting 63-day-old males (Table 1) and in 120-day-old males and females (data not shown).

Experiments were then carried out to determine whether methoxychlor, a structural non-biologically persistent analog of DDT, could exert a similar effect on these sex-differentiated hepatic enzymes. Neonatal administration of methoxychlor resulted in increases in monoamine oxidase activities in both adult males and females but not in prepubertal animals (Table 2). No changes were observed in the other enzyme makers. These results indicated that methoxychlor, like o,p'-DDT, alters sex differentiation of monoamine oxidase and is an inducer of drug-metabolizing enzymes.

We subsequently investigated the effect that an equivalent dose of o,p'-DDT or methoxychlor (111 and 333 mg/kg body wt) had on these enzymes when administered to adult or prepubertal animals later than the critical age when organizational effects are initiated [1, 17]. Adult male rats were treated on days 56, 58, and 60 and killed 18 hr after the last injection while prepubertal male rats were treated on days 15, 17, and 19 and killed either 18 hr after the last injection or 42 days later. Glutathione S-transferase activities and cytochrome P-450 levels were found to be higher in those prepubertal and adult male rats that were killed only 18 hr after o,p'-DDT treatment. The effect was not permanent, however, since adult rats treated prepubertally with o,p'-DDT had activity levels similar to the controls. This supports the data in Table 1 showing an increase in hepatic glutathione S-transferase activities and cytochrome P-450 content shortly after treatment and confirms previous work showing that DDT is an enzyme inducer of several drug-metabolizing systems [18]. Hepatic monoamine oxidase and UDP-glucuronyltransferase activities were unchanged in these DDT experiments. Adult male rats treated with equivalent doses of methoxychlor for a week did not have altered hepatic enzyme levels. However, methoxychlor treatment in prepubertal rats did cause a significant increase in glutathione S-transferase activities and in P-450 content in animals killed 18 hr after the last dose only.

Since testosterone is a negative modulator of hepatic monoamine oxidase [19], we investigated the effects of neonatal exposure to o,p'-DDT and methoxychlor on the male reproductive tract of prepubertal and adult males. No significant changes in testes, seminal vesicle, ventral prostate, and body weights were observed in these xenobiotic-treated animals versus the controls, indicating that androgen levels were normal in these animals.

DISCUSSION

The four hepatic enzyme systems that we selected for monitoring alterations in the expression of postnatal developmental patterns are characterized by sexual differentiation in adulthood. There is, however, an absence of sex differentiation prior to puberty. Glutathione S-transferase activities and cytochrome P-450 content in prepubertal rats treated neonatally with o,p'-DDT were elevated, but at 63 days of age these enzyme markers were not signifi-

Table 2. Effects of neonatal administration of methoxychlor on hepatic enzymes in the developing rat*

Enzyme marker	Prepubertal males and females	Adult	
		Males	Females
Monoamine oxidase			
Controls	0.84 ± 0.05	1.07 ± 0.04	1.77 ± 0.10
Low dose	0.74 ± 0.08	1.19 ± 0.06	2.00 ± 0.18
High dose	0.84 ± 0.08	1.99 ± 0.10†	2.58 ± 0.08†
Glutathione S-transferase (CDNB)			
Controls	580 ± 29	1855 ± 98	1818 ± 84
Low dose	470 ± 37	1777 ± 66	1597 ± 73
High dose	539 ± 26	2062 ± 83	1711 ± 47
Glutathione S-transferase (DCNB)			
Controls	46 ± 2	161 ± 10	76 ± 2
Low dose	38 ± 3	156 ± 6	68 ± 4
High dose	41 ± 2	170 ± 7	73 ± 4
UDP-glucuronyltransferase			
Controls	32.9 ± 1.1	33.1 ± 1.2	21.5 ± 1.6
Low dose	32.7 ± 0.9	35.4 ± 5.9	25.0 ± 1.0
High dose	32.3 ± 1.1	34.6 ± 3.8	18.7 ± 2.6
P-450 content			
Controls	0.62 ± 0.02	0.69 ± 0.03	0.48 ± 0.02
Low dose	0.56 ± 0.02	0.70 ± 0.04	0.42 ± 0.02
High dose	0.58 ± 0.02	0.71 ± 0.02	0.45 ± 0.05

* Methoxychlor was administered subcutaneously on days 2, 4 and 6 post partum. Controls received 20 μl sesame oil. Low dose = 1 mg methoxychlor/animal; high dose = 3 mg methoxychlor/animal. Animals were killed on day 20 or 63. All values are means ± S.E.M.

† $P < 0.01$, when compared to respective control ($N = 8$).

cantly different from controls. It appears that, in the presence of high concentrations of *o,p'*-DDT, the glutathione *S*-transferases and the cytochrome P-450 contents are subjected to activational effects, i.e. an increase in activity in the presence of the effector. These two enzyme systems have been found to be induced by organohalogens [18], and our induction experiments confirmed this. There were significant increases in glutathione *S*-transferase activities and P-450 content in prepubertal and adult male rats shortly after treatment (18 hr) with *o,p'*-DDT. These enzyme activities returned to normal levels (42 days later) presumably due to deactivation, excretion, redistribution to adipose tissue, and/or decreased tissue concentrations as a function of body growth.

The most significant finding, however, was the alteration in hepatic monoamine oxidase activities in adult animals following neonatal exposure to *o,p'*-DDT or methoxychlor. This is particularly interesting since prepubertal monoamine oxidase activities were unchanged, and this is, therefore, suggestive of programming effects [5]. Our attempts to induce monoamine oxidase activities in prepubertal and adult animals were not successful, suggesting that another mechanism must be responsible for these increases in monoamine oxidase in adult animals following neonatal exposure to these xenobiotics. It appears unlikely that lower concentrations of *o,p'*-DDT were having a direct effect on hepatic monoamine oxidase at day 63 but not at 20 days of age. Furthermore, methoxychlor has an estimated biological half-life in rats of approximately 24 hr compared to 10–15 years for DDT. The difference in clearance rates reflects the rapidly metabolized *p,p'*-methoxy substitutents on the phenyl rings of methoxychlor rather than the chlorines of *o,p'*-DDT.

Although the elevations in hepatic monoamine oxidase levels in these experiments were similar to results seen following castration of adult animals [19], neonatal administration of *o,p'*-DDT and methoxychlor caused no significant changes in reproductive organ weights in the affected animals. It, therefore, seems unlikely that these enzyme changes are due to gonadal dysgenesis in these adult animals. No attempt was made to quantitate the residual amounts of *o,p'*-DDT or methoxychlor in the adult animals since changes in monoamine oxidase do not appear to be due to the direct presence of the effector.

Gustafsson *et al.* [4] have proposed that the sex-dependent differentiation of certain hepatic steroid-metabolizing enzymes is imprinted in the male rat during the neonatal period by release of testicular androgens. McEwen *et al.* [17, 20] have proposed a mechanism for this imprinting whereby testosterone, converted to estradiol in the brain target cells, is the active imprinting metabolite that can bind the estrogen receptor and program for a male type of metabolism. The target cells are protected against the action of circulating natural estrogens by α-fetoprotein, present in the neonatal serum and cerebrospinal fluid, which binds estrogens and retards their entry into target cells. We have recently investigated the mechanism of sex differentiation of hepatic monoamine oxidase and demonstrated that neonatal androgen deprivation (castration) results in an increase in activity (feminization). The administration of testosterone or diethylstilbestrol (nonsteroidal estrogen) to neonatal castrates, on day 2 only, prevented the rise in monoamine oxidase activity observed in the adult male after neonatal castration [5].

Bulger *et al.* [21] have demonstrated that, while methoxychlor does not inhibit [^{3}H]estradiol binding to the 8S receptor, the di-demethylated metabolite [2,2-bis (*p*-hydroxyphenyl)-1,1,1-trichloroethane] markedly suppressed estrogen binding to the receptor. Furthermore, they have demonstrated that the *in vivo* di-demethylated derivative of methoxychlor is a better competitor for estrogen binding sites than *o,p'*-DDT [22]. These differences in binding affinity could cause the observed increase in monoamine oxidase activities in females and males at 63 days of age while *o,p'*-DDT affected only the males.

It appears that the ability of *o,p'*-DDT and methoxychlor to alter the sex differentiation of hepatic monoamine oxidase may be a consequence of changes in the hormonal milieu during a critical period of neonatal development. This increase in adult monoamine oxidase activities was demonstrated only following exposure to these xenobiotics during the first week after parturition and not following treatment during the third week or in adulthood. We suggest that these estrogenically active xenobiotics may reach the brain, bind hypothalamic estrogen receptors [11] and, consequently, interfere with normal maturational processes resulting in sex differentiation of the hypothalamus [5, 23]. These alterations are permanent and are expressed postpubertly presumably via the hypothalamic-pituitary axis [17, 19].

Acknowledgements—We gratefully acknowledge the excellent technical assistance provided by Pearlie Hudson, Brenda Smith, Tracy Sloop, and James Clark.

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