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Inhibitory effects of centrally acting drugs on the neonatal imprinting of sex differences in the hepatic metabolism of a dimethylated epoxide in the rat

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1 The effects of the neonatal administration of reserpine, chlorpromazine, phenobarbitone and morphine on the development of sex differences in hepatic drug metabolism in the rat have been investigated.

2 Treatment of neonatal male rats with reserpine or chlorpromazine for the first two weeks post-partum significantly inhibited the development of sex differences in drug metabolism in adult life.

3 Similar treatment of neonatal female rats with reserpine or chlorpromazine had no effect on the development of hepatic drug metabolism in adulthood.

4 Morphine or phenobarbitone treatment of neonatal rats of either sex had no effect on the development of sex differences in hepatic drug metabolism in adult life.

Introduction

The adult rat is unique amongst laboratory animals in that it exhibits a marked sex difference in the hepatic microsomal metabolism of drugs and xenobiotics (Kato, 1974; El Defrawy El Masry *et al.*, 1974; El Defrawy El Masry & Mannering, 1974; Nerland & Mannering, 1978). In general, liver enzymes from adult male rats metabolize drugs at a faster rate than liver enzymes from adult female rats, resulting in the effects of drugs such as hexobarbitone being more prolonged in adult female rats than in adult male rats (Kato, 1974). Although for the majority of drug substrates sex differences in metabolism are manifest as a difference in the rate of hepatic microsomal metabolism, we have recently shown that the metabolism of 1, 2, 3, 4, 9, 9-hexachloro-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-6, 7-dimethyl-6, 7-epoxy-1, 4-methanonaphthalene (Figure 1), a di-methylated epoxide (DME), exhibits a marked qualitative sex difference (Hassall *et al.*, 1978; Hassall & Adalla, 1979; Finnen & Hassall, 1980a). The hepatic mono-oxygenase system from adult male rats metabolizes DME to produce two metabolites M1 (ring hydroxylated) and M2 (side chain hydroxylated) (Figure 1). The hepatic microsomal mono-oxygenase system from adult female rats however metabolizes DME to produce only metabolite M1 in detectable quantities (Hassall & Adalla, 1979). Sex differences in the metabolism of DME first become apparent at puberty with pre-pubertal rats of both sexes metabolizing DME in an identical manner (Finnen & Hassall, 1980a). The development of sex differences in the metabolism of DME at puberty has been shown to be dependent on neonatal imprinting by testicular androgens (Finnen & Hassall, 1980a; 1983) in a similar

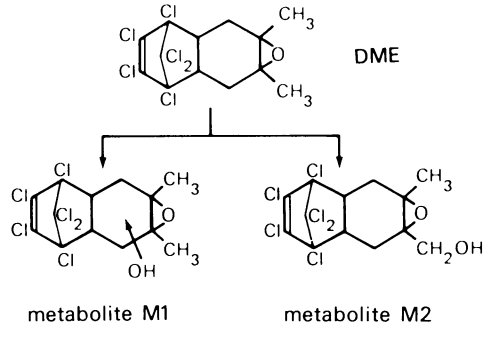

Figure 1 The structure of 1, 2, 3, 4, 9, 9-hexachloro-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-6, 7-dimethyl-6, 7-epoxy-1, 4-methanonaphthalene (DME) and metabolites M1 and M2 based on mass spectrographic evidence (Hassall *et al.*, 1978).

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manner to the imprinting of sex differences in gonadotrophin secretion and sexual behaviour (Plapinger & McEwen, 1978). Castration of male rats before the age of seven days results in the development of a typically feminine pattern of DME metabolism in adulthood whereas castration after this age does not prevent the expression of a typically masculine pattern of metabolism in adult life. The neonatal imprinting of sex differences in gonadotrophin secretion has been shown to be inhibited by various drugs that act principally on the central nervous system (Kikuyama, 1961; Kawashima, 1964; Arai & Gorski, 1968a, b). Arai & Gorski (1968a) have shown that the androgenisation of gonadotrophin secretion in adult female rats caused by the administration of testosterone during the neonatal period, is prevented by the simultaneous administration of reserpine. Furthermore, the inhibitory actions of reserpine on neonatal imprinting of gonadotrophin secretion have also been demonstrated in neonatal male rats (Kawashima, 1964). In addition to reserpine, both chlorpromazine and pentobarbitone have also been found to inhibit the neonatal imprinting of gonadotrophin secretion by neonatal androgens (Arai & Gorski, 1968b). The work presented in the present paper was designed to investigate the effects of centrally acting drugs on the neonatal imprinting of sex differences in the hepatic microsomal metabolism of DME.

Methods

Animals and treatments

Sprague-Dawley rats, CD strain were bred in the departmental animal house and raised with a dam until 21 days of age at which time they were weaned and housed in separate cages. Neonatal male and female rats received one of the following injections on alternate days for two weeks: chlorpromazine hydrochloride (Cpz) 500 μg/injection; reserpine (Res) 300 μg/injection; phenobarbitone (PB) 200 μg/injection; morphine sulphate (MS) 500 μg/injection. All drugs were administered in a final volume of 50 μl by subcutaneous injection in the nape of the neck. Treatments were initiated on the day of birth which was taken to be day 1. Control animals received 50 μl of saline. Rats were left to recover from the injection for 4 to 6 h before being replaced with the dam. The analysis of hepatic enzyme activity was performed when the rats were 90 days of age or older.

Preparation of hepatic microsomes

Rats were killed by a blow to the head and the liver exposed by a cut along the ventral midline. The hepatic vein was located and the liver perfused with ice-cold phosphate buffered KCl solution (1.15% w/v KCl; 0.01 M sodium phosphate pH 7.4). The livers were quickly excised, weighed and chilled on ice. All subsequent procedures were performed at 0–4°C. The weighed livers were finely minced with scissors and homogenized in phosphate buffered KCl solution. The homogenate was centrifuged at 9000 *g* for 20 min and the resulting supernatant decanted and further centrifuged at 100,000 *g* for 1 h. The microsomal pellet was then resuspended and recentrifuged at 100,000 *g* for 1 h. Finally, the resulting pellet was resuspended in phosphate buffered KCl solution such that each ml was equivalent to 500 mg of liver wet weight.

Enzyme assay

Metabolism studies using DME as substrate were performed in a final volume of 1.0 ml containing: 100 μmol sodium phosphate buffer, pH 7.4; NADPH generating mixture comprising NADP 5.0 μmol; glucose-6-phosphate 60 μmol; glucose-6-phosphate dehydrogenase 1.2 units; and liver microsomal preparation equivalent to 5.0 mg liver wet weight. The reaction was started by the addition of DME in the form of 10 μl of an ethanolic solution of 10 $\mu g\,\mu l^{-1}$ and terminated after 3 min incubation in a shaking water bath at 37°C by the addition of 1.0 ml of acetone. Metabolites and residual substrate were partitioned into n-hexane and 5 μl portions of the hexane extract analysed using a Perkin Elmer g.l.c. as previously described (Hassall *et al.*, 1978). Metabolites M1 and M2 were identified by their relative retention times, and quantified using solutions of M1 and M2 of known concentration. Results were expressed as nmol of metabolite produced $min^{-1}\,g^{-1}$ of liver wet weight.

Results

The effects of neonatal administration of phenobarbitone (PB), chlorpromazine (Cpz), reserpine (Res), or morphine sulphate (MS) to male rats on the metabolism of DME in adult life are shown in Table 1. Administration of Cpz on alternate days for the first week of life had no significant effect on the hepatic microsomal metabolism of DME by male rats in adult life. Similarly, treatment with Cpz on alternate days for the second week post-partum was without effect on the production of metabolites of DME in adult life. However, when administered on alternate days for both the first and second week post-partum, Cpz significantly reduced the rate of side chain hydroxylation of DME to metabolite M2, in addition to increasing the rate of ring hydroxylation

to metabolite M1. Similar treatment of neonatal female rats with Cpz had no effect on either the quantitative or qualitative metabolism of DME by hepatic microsomes from female rats in adult life.

Treatment of either neonatal male rats (Table 1), or of neonatal female rats (not shown) with MS or PB for the first two weeks of life had no effect on the differentiation of DME metabolism in adult life, with neither treated male rats nor treated female rats differing significantly from their respective controls in the metabolism of DME. However, Res treatment of neonatal male rats reduced the rate of side chain hydroxylation of DME to metabolite M2, by a similar extent to Cpz (Table 1), while being without effect in female rats. In addition, the effects of Res on DME metabolism, like those of Cpz, were only observed after treatment for the first two weeks post-partum. However Res treatment of neonatal male rats, in contrast to Cpz, did not alter significantly the rate of formation of metabolite M1 by liver enzymes from adult male rats. Res treatment of neonatal female rats had no significant effect on the hepatic metabolism of DME in adult life.

Discussion

The inhibition of the neonatal imprinting of sex differences in gonadotropohin secretion by centrally acting drugs is well established. The tranquilizers reserpine and chlorpromazine (Kikuyama, 1961; 1962; Kawashima, 1964; Arai & Gorski, 1968a) and barbiturates (Sutherland & Gorski, 1972) have been found to attenuate the actions of androgens during the neonatal period. The results presented in the present paper indicate that reserpine and chlorpromazine are also effective in inhibiting the neonatal imprinting of sex differences in hepatic drug metabolism in the rat. However, the data shown in Table 1 suggests that the imprinting of sex differences in drug metabolism is not as sensitive to the actions of Cpz and Res, as the imprinting of gonadotrophin secretion patterns. Firstly, although Cpz and Res both feminized the pattern of metabolism of DME in that the rate of production of metabolite M2 was markedly reduced, they did not reduce M2 production to the non-detectable levels found in the adult female or the adult male castrated at birth (Finnen & Hassall, 1980a). Secondly, unlike the inhibition of the androgenisation of gonadotrophin secretion where one single injection of Cpz or Res has been shown to be effective, the inhibition of the imprinting of drug metabolism required injections of Res or Cpz on alternate days for the first two weeks post-partum (Table 1). This may be a reflection of the differences in the duration of the critical period for neonatal imprinting of these two sexually differentiated parameters. The imprinting of sex differences of gonadotrophin secretion has been shown to be most sensitive to small doses of androgen during the first three days post-partum, whereas imprinting of parameters of sex differences in hepatic drug metabolism have been shown to take place over the first two weeks of life (Chung, 1977; Finnen & Hassall, 1980a; 1983). It is probable therefore that the site of imprinting that determines sex differences in drug metabolism is susceptible to the actions of

Table 1 The effects of neonatal administration of chlorpromazine Cpz), reserpine (Res), phenobarbitone (PB), or morphine sulphate (MS), to male rats, on the metabolism of DME in adult life

	n	*DME* (nmol metabolite $min^{-1} g^{-1}$ liver) *M1*	*M2*
Control male	8	180 ± 10	298 ± 22
Control female	12	257 ± 17*	n.d.
Cpz (1–7)	3	192 ± 14	314 ± 26
Cpz (7–15)	3	172 ± 19	287 ± 29
Cpz (1–15)	6	230 ± 16*	121 ± 12*
Res (1–7)	3	179 ± 24	301 ± 29
Res (7–15)	4	186 ± 17	298 ± 27
Res (1–15)	6	195 ± 14	128 ± 11*
PB (1–15)	6	171 ± 10	309 ± 20
MS (1–15)	4	180 ± 17	311 ± 22

Results are shown as the mean ± s.d.
*Significantly different from control male $P < 0.01$.
n.d. – not detected.
Neonatal male rats were injected subcutaneously with either Cpz, Res, PB or MS, at doses indicated in Methods on days 1, 3, 5, 7 (1–7); 7, 9, 11, 13, 15 (7–15); or 1, 3, 5, 7, 9, 11, 13, 15 (1–15). Analysis of liver enzymes was performed when the rats were 90 days old.

androgens for longer than that for gonadotrophin secretion. The effects of Res and Cpz may therefore be dependent on their continued presence throughout the critical period. PB and MS did not significantly affect the imprinting of masculine characteristics of the hepatic metabolism of DME (Table 1). A similar absence of an inhibitory effect of PB on the androgenisation of gonadotrophin secretion was found by Arai & Gorski (1968a). The absence of an anti-androgenic effect of MS on the neonate is in marked contrast to the effects of MS in the adult male rat, where it greatly diminishes sex differences in hepatic drug metabolism to a similar extent as castration (Sladek *et al.*, 1974).

Three possible mechanisms can be envisaged for the actions of Res and Cpz in the attenuation of imprinting of hepatic drug metabolism. Firstly, a direct antagonism of the actions of testosterone on the hypothalmus is possible. Both these compounds are known to effect levels of monoamines in the brain and monoamines have been implicated in the imprinting process (Raum & Swerdloff, 1981). Secondly, Res and Cpz may have their effects peripherally at the level of the liver. Res and Cpz are both inducers of the hepatic microsomal mono-oxygenase system that metabolizes testosterone and the induction of hepatic mono-oxygenase activity in the neonate has been demonstrated (Guenthner & Mannering, 1977). It is conceivable therefore that Res and Cpz induce the metabolism of testosterone by the liver, and effectively reduce the levels of testosterone available for imprinting in the neonatal male rat. Finally the effects of Res and Cpz could be exerted at the testicular level by reducing the secretion of testosterone either directly or by an effect on pituitary Luteinising hormone (LH) secretion which has been shown to be essential for the production of testosterone in the neonatal male rat (Slob *et al.*, 1980). Gorski and co-workers (Sutherland & Gorski, 1972) have shown that the attenuation of androgenising effects of testosterone on gonadotrophin secretion are the result of actions at the hypothalamic level. Direct infusion of pentobarbitone into the hypothalmus has been shown to attenuate the effects of testosterone administered centrally or peripherally. However, these results were not conclusive as a considerable degree of variation in the effects of centrally administered testosterone was evident. In view of the fact that sex differences in hepatic drug metabolism are thought to be mediated by the pituitary gland via hypothalamic control (Gustafsson *et al.*, 1978; Finnen & Hassall, 1980b), it would seem probable that the effects of Res and Cpz may be due to a direct effect on the hypothalamus, although other explanations are possible. However, the present work further demonstrates the importance of the neonatal period in the determination of sex differences in hepatic drug metabolism in the adult rat, and illustrates the far reaching consequences of the administration of drugs to the neonate of this species.

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