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TESTICULAR NEONATAL IMPRINTING OF SEX DEPENDENT DIFFERENCES IN HEPATIC FOREIGN COMPOUND METABOLISM IN THE RAT

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Abstract—A dimethylated chlorocyclodiene epoxide (DME)* is metabolized by adult male rat liver enzymes to produce two g.l.c. detectable metabolites M1 and M2. Liver enzymes from adult female rats metabolize DME producing only metabolite M1 in detectable quantities. This apparent qualitative sex difference in the metabolism of DME is first manifest between the ages of 35 and 45 days. Liver enzymes from rats of both sexes younger than 35 days metabolize DME in a qualitatively similar manner. The actions of the testes in the development of this apparent qualitative sex difference are exerted between the ages of 7 and 14 days. Castration of male rats at and before the age of 7 days results in the expression of a typically feminine pattern of metabolism of DME by liver enzymes in adult life. Castration of male rats after the age of 14 days does not prevent adult liver enzymes from exhibiting a basically masculine pattern of DME metabolism. At ages between 7 and 14 days castration has a graded effect on the metabolism of DME displayed by liver enzymes in adult life. These results suggest that testicular androgens do not play a direct role at puberty and in adulthood in the expression of the ability of liver enzymes to produce metabolite M2, but that the apparent qualitative sex dependent difference in DME metabolism is determined primarily by neonatal imprinting by testicular androgens.

The oxidative metabolism of many steroids and xenobiotics by rat liver preparations is sex dependent [1–4]. Quantitative differences in the metabolism of substrates such as aminopyrine, ethylmorphine and hexobarbital are well documented [5–7], with the adult male rat metabolizing at a faster rate than the adult female rat. Qualitative sex differences in the metabolism of steroids by rat liver microsomes are common [4, 8–11]. For example, adult male rat liver microsomes hydroxylate testosterone in the 6β, 16α and 7α positions but those from adult female rats only hydroxylate testosterone in the 6β and 7α positions [11]. Equivalent qualitative sex differences in the metabolism of foreign compounds are rare. An apparent qualitative sex difference in the metabolism of DME (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), a dimethylated epoxide, has recently been demonstrated with adult male and female rat liver preparations [12]. The adult male makes two g.l.c. detectable metabolites, M1 and M2 (Fig. 1), whereas the adult female makes only metabolite M1 in detectable quantities. Typical chromatograms are shown for adult rats (60 days of age) in Fig. 2.

The physiological basis for observed sex differences in drug metabolism has in the past been attributed to an anabolic action of circulating testicular androgens on the liver [1,6,13]. Sex differences first became apparent at puberty, pre-pubertal animals exhibiting no sex differences [5]. Castration of adult male rats reduces rates of metabolism of substrates exhibiting a sex difference to values seen in the normal adult female [14]. Furthermore, treatment of castrated adult male rats [14–16] and normal adult female rats [14] with testosterone, increases the rates of metabolism of substrates exhibiting a sex difference. Data has accumulated, however, indicating that the role of the testes in the control of sex differences in drug metabolism is not confined to pubertal and to post-pubertal periods, but that neonatal imprinting by testicular androgens is an important aspect of testicular control of sex dependent differences in drug metabolism. Sex differences in the apparent K_m of hepatic ethylmorphine *N*-demethylase [17] and the half life of cytochrome P-450 [11], have been shown to be determined by

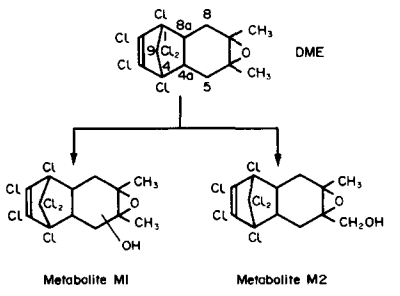

Fig. 1. Formula of 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-dimethyl-6,7-epoxy-1,4-methanonaphthalene (DME), and the tentative structures of metabolites M1 and M2 are based on mass spectrographic evidence (see reference 19).

* Abbreviations used: DME, 1,2,3,4,9,9-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-dimethyl-6,7-epoxy-1,4-methanonapthalene; M1, M2, two metabolites of DME.

testicular androgen during early post-natal life. Castration of male rats when adult has been shown to be ineffective in abolishing these neonatally imprinted sex differences [11, 17]. In this communication the role of the testes in the development of the apparent qualitative sex difference in the metabolism of DME and quantitative sex differences in the metabolism of aminopyrine and ethylmorphine is presented.

MATERIALS AND METHODS

Animals and surgical procedures. Male and female Sprague–Dawley rats, CD strain, were used. Animals were maintained on a pellet and water diet *ad lib.*, and were kept in an atmosphere of constant temperature and humidity, under a 12 hr light cycle (7.00 a.m./7.00 p.m.). Rats were castrated under light ether anaesthesia at the ages indicated. Since some inter-litter variation in the effect of age of castration on the metabolism of DME was found to occur, littermates were used for animals within the same age group.

Incubation procedures. Animals were killed by cervical dislocation at 60–80 days of age except where otherwise indicated. Hepatic 9000*g* × 20 min supernatant fractions were prepared using standard techniques as follows. The livers were perfused *in situ* with ice cold 1.15% (w/v) KCl in 0.01M sodium phosphate buffer (pH 7.4), excised, blotted on tissue paper to remove excess blood and KCl, and weighed. All procedures thereafter were performed in the cold (0–4°C). The livers were homogenized in 2 vol. ice cold buffered KCl, using a rotary homogenizer (Silverson Ltd., Waterside, Bucks., U.K.). The homogenates were diluted with ice cold buffered KCl, so that each milliliter contained 250 mg of liver wet weight, and then centrifuged at 9000 *g* for 20 min. The resulting 9000 *g* × 20 min supernatant fraction was used for all drug metabolism studies.

Where DME was used as substrate, incubations were performed as reported elsewhere [12] with the following modifications. Reaction mixtures contained in a final volume of 3.0 cm^3: 25 μmoles $MgCl_2$; 1.5 μmoles NADP; 20 μmoles glucose-6-phosphate; 1.6 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 1.0 cm^3 of 9000 *g* × 20 min supernatant, equivalent to 0.10 g of liver wet weight; 100 μmoles sodium phosphate buffer, pH 7.4. DME was added in the form of 10 μl of an ethanolic solution containing 3.0 μg/μl. Incubation mixtures containing all ingredients except substrate were preincubated at 37° for 1 min. The reaction was initiated by addition of substrate and terminated after 10 min. by addition of 2.0 cm^3 of acetone.

Aniline hydroxylase activity was measured by the method of Kato and Gillette [16]. Ethylmorphine *N*-demethylase and aminopyrine *N*-demethylase activities were determined by estimation of formaldehyde formed using the method of Nash [18]. Incubation mixtures for the determination of ethylmorphine and aminopyrine *N*-demethylases contained the following components in a final volume of 6.0 cm^3: 45 μmoles semi-carbazide; 25 μmoles $MgCl_2$; 100 μmoles sodium phosphate buffer, pH 7.4; 2.0 μmoles NADP; 20 μmoles glucose-6-phosphate; 1.6 units of glucose-6-phosphate dehydrogenase; liver supernatant equivalent to 250 mg liver wet weight. Substrates were employed at saturating concentrations, namely 5 mM aminopyrine, 1.25 mM aniline, and 10 mM ethylmorphine. Reactions were performed in a metabolic shaker at 37° for periods which had previously been shown to ensure minimal deviation from linear reaction rates.

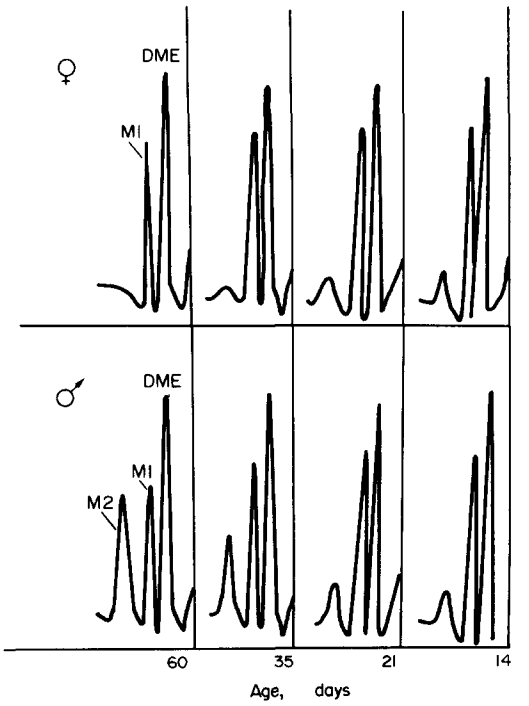

Fig. 2. Gas chromatograms showing the effects of age and sex on the metabolism of DME by rat liver enzymes. The chromatograms are typical of those obtained in eight or more independent experiments. From the injection line (right hand side) the peaks represent residual DME, metabolite M1 and metabolite M2, with relative retention times of 100, 175 and 280 respectively.

Extraction and identification of metabolites of DME. Incubation mixtures were extracted with 2 × 5 cm^3 portions of redistilled hexane and final volumes adjusted to 10 cm^3. Five-microliter portions of the extract were analysed using a Perkin–Elmer F33 g.l.c. fitted with a ^{63}Ni source electron capture detector. All glass columns, 1.75 m in length, containing SE52 were used with Chromosorb W as inert support. Oven and detector temperatures of 195° and 225°, respectively, were used. A more detailed account of the chemical analysis appears elsewhere [19]. Metabolites M1 and M2 (Fig. 1) were identified by their respective relative retention times. Peak areas for metabolites M1 and M2 were determined by triangulation and expressed as metabolite peak area ratio M2/M1. In those cases where metabolite M2 was not detectable the metabolite ratio M2/M1 was taken to be zero. Limits of detection for DME and metabolites M1 and M2 (Fig. 2) were 10, 80 and 120 pg respectively. Typical amounts of M1 formed were in the order of 6–10 μg per incubation.

RESULTS

Influence of age and sex on the metabolism of DME. Hassall and Addala [12] reported that liver preparations from pre-pubertal rats of both sexes apparently produced no metabolite M2. Using g.l.c. columns of greater length it has now been shown that pre-pubertal animals of both sexes make small amounts of metabolite M2 (Fig. 2). The metabolite peak area ratio M2/M1 remains constant for both sexes up to the age of approximately 28 days (Fig. 3). After this age, for the female the relatively small amounts of metabolite M2 produced diminish even further to levels non-detectable using the present assay system. After the age of 28 days the male makes metabolite M2 in increasing amounts up to the age of 45 days when a metabolite ratio of 1.5 is attained. This ratio remains constant for the male throughout adulthood.

Effects of castration at various ages on the metabolism of DME, aniline, aminopyrine and ethylmorphine. Table 1 shows the effects of castration at various ages on the metabolism of DME by adult rat liver preparations. Castration of male rats at and before the age of seven days results in the appearance in adult life of a typically feminine pattern of metabolism of DME, in that only metabolite M1 is produced in detectable quantities. Castration at the ages of 10 and 12 days results in the adult male producing detectable quantities of metabolite M2 but with metabolite peak area ratios significantly ($P < 0.001$) lower than those seen in the adult male rat castrated when mature. Rats castrated at 15 and 25 days, and those castrated when mature, have similar metabolite peak area ratios. It should be noted, however, that results are shown for littermates within each single age group. A small degree of inter-litter variation in the effects of age of castration on the metabolism of DME has been observed, in that for some litters, rats castrated at 7 days old produced detectable amounts of metabolite M2. This is probably a reflection of differing degrees of development between litters on parturition.

Table 1. Effects of castration at various ages on the metabolism of DME by male rat liver 9000 $g \times 20$ min supernatant fraction in adult life*

Age of castration (days)	Metabolite peak area ratio M2/M1	±S.E.
1	0‖	—
5	0‖	—
7	0‖	—
10	0.17§	±0.03
12	0.52§	±0.13
15	1.02	±0.08
25†	1.05	—
60	1.01	±0.03
Control female	0‖	—
Adult female TP‡	0‖	—
Control male	1.52	±0.09

* Male rats were castrated at the ages indicated and killed at 10–12 weeks of age. Values represent the mean ± S.E. of six to eight independent experiments except † which represents two independent experiments only.

‡ Adult females were injected with 2.0 mg testosterone propionate in corn oil daily for 7 days and killed 24 hr thereafter.

§ Statistically significant difference from males castrated at 60 days of age ($P < 0.001$).

‖ 0 metabolite peak area ratio denotes no detectable amount of metabolite M2 formed.

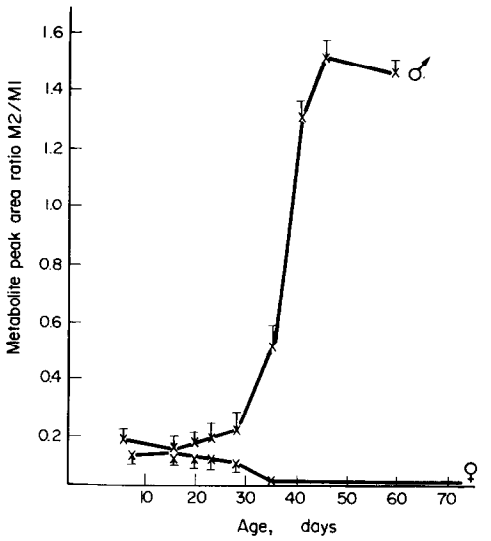

Fig. 3. Effects of age on the metabolism of DME by male and female rat liver 9000 $g \times 20$ min supernatant fraction. Values represent the mean ± S.E. of results from eight independent experiments except that the 60 day results were obtained from twenty independent experiments.

Table 2 shows the effects of age of castration on the hepatic microsomal metabolism of aniline, aminopyrine and ethylmorphine in adult life. Aniline metabolism was not significantly affected by castration at any age, a result consistent with there being no sex difference in the metabolism of aniline [1]. With aminopyrine and ethylmorphine as substrates castration at all ages, as expected, reduced rates of metabolism markedly below values seen in the normal intact male. The effects of castration on the *N*-demethylation of aminopyrine were independent of age of castration, no significant differences in rates of metabolism being evident as a result of castration at different ages. For ethylmorphine *N*-demethylation however, rats castrated at the age of one day old had a significantly ($P < 0.05$) lower rate of metabolism than rats castrated at 5 days and older. Rates of hepatic ethylmorphine *N*-demethylation in all groups of castrated males were significantly higher than those of control females, whereas for aminopyrine *N*-demethylation, rates of metabolism for castrated males and control females were of comparable magnitude.

DISCUSSION

Chung [17, 22] has demonstrated a role for the neonatal testes in the imprinting of sex differences in apparent K_m and in responsiveness to androgens of hepatic ethylmorphine *N*-demethylase, at 2–4 and 12–14 days of age respectively. Sex differences in the half life of cytochrome P-450 have also been shown to be dependent entirely on neonatal imprinting by

Table 2. Effects of castration on the metabolism of aniline, aminopyrine and ethylmorphine by male rat liver 9000 g × 20 min supernatant fraction in adult life*

Age of castration (days)	Aniline (nmoles of PAP† formed/min/g liver)	Aminopyrine (nmoles of HCHO formed/min/g liver)	Ethylmorphine (nmoles of HCHO formed/min/g liver)
1	3.8 ± 0.6	58 ± 3	59 ± 2‡§
5	4.4 ± 0.4	57 ± 3	76 ± 3
7	4.4 ± 0.6	59 ± 3	74 ± 1
10	3.9 ± 0.3	61 ± 3	79 ± 3
12	3.9 ± 0.3	60 ± 3	80 ± 2
15	4.2 ± 0.3	59 ± 2	80 ± 1
60	4.0 ± 0.8	62 ± 4	81 ± 1
Control male	4.6 ± 0.4	148 ± 2	185 ± 6
Control female	3.9 ± 0.2	55 ± 1	42 ± 1

* Male rats were castrated at the ages indicated and killed at 10–12 weeks of age. Values represent the mean ± S.E. of results from six to eight independent experiments.
† Para amino phenol.
‡ Significantly different from castrates 5–60 days ($P < 0.05$).
§ Significantly different from female control ($P < 0.01$).

testicular androgens [11]. Castration of adult male rats, or conversely, treatment of adult female rats with testosterone, has been shown to be ineffective in abolishing these neonatally imprinted sex differences [11, 17]. Results presented in this communication suggest that a similar neonatal imprinting is occurring with respect to the apparent qualitative sex difference in the metabolism of DME (Table 1). The ontogenesis of a masculine pattern of metabolism of DME (i.e. the production of both metabolites M1 and M2) has been shown to be independent of the presence of the testes at puberty. This observation implies that testicular androgens do not play a direct primary role at puberty, and in adult life, in the expression of the ability of liver enzymes to produce metabolite M2. Furthermore, treatment of adult female rats with testosterone propionate does not result in a masculine pattern of hepatic metabolism of DME (Table 1).

The maintenance of a masculine pattern of metabolism of DME also appears to be independent of the presence of the testes in adulthood since castration of the adult male rat does not prevent the formation of metabolite M2 (Table 1), although it does reduce the metabolite peak area ratio M2/M1. In adulthood the testes appear to have a 'fine control' role rather than an on/off role in the regulation of the hepatic microsomal metabolism of DME. The primary actions of the testes in the development of a masculine pattern of metabolism of DME appear to be exerted between the ages of 7 and 14 days. Castration at and before the age of 7 days entirely prevents the formation of metabolite M2, whereas castration after the age of 7 days does not. At ages between 7 and 14 days, castration has a graded effect on the metabolite peak area ratio M2/M1 (Table 1) suggesting that imprinting between these ages is not an 'all or none' effect but that it is, within limits, dependent on the period of exposure to neonatal androgens. Rates of aminopyrine and ethylmorphine N-demethylation in rats that had been castrated at 10, 15 or 60 days were not significantly different from each other (Table 2). This suggests that the gradation of metabolite peak area ratio observed for rats castrated between the ages of 7 and 14 days is not a result of residual testosterone remaining after castration. Since the testes of the male rat are known to be active during the first seven days of life [20], and androgenic imprinting of other sex dependent characteristics is known to occur during the first week of life [21, 22], imprinting with respect to metabolite M2 production is probably determined by the receptivity of the site of imprinting to testicular androgens during the critical period between 7 and 14 days of age.

The studies using aniline, aminopyrine and ethylmorphine as substrates support the observations of Chung [17]. Castration at any age had no significant effect on the metabolism of aniline (Table 2). No significant differences in the rates of aminopyrine N-demethylation were observed between groups of animals castrated at different ages. For ethylmorphine N-demethylation, however, rats castrated at one day old had significantly lower rates of metabolism than those of animals castrated at ages older than one day. It is of interest in this respect that, whereas castration of males reduced the activity of hepatic aminopyrine N-demethylase to levels seen in the normal female (Table 2), ethylmorphine N-demethylase activity in the castrated male was considerably higher than that seen in the female ($P < 0.001$), indicating that the testes are not solely responsible for observed sex differences in hepatic ethylmorphine metabolism.

It has been suggested that the qualitative sex difference in the metabolism of DME is indicative of the existence of multiple forms of cytochrome P_{450} [12]. Present work with DME suggests that some of these forms of P_{450} are under hormonal control, and that the role of the testes in this control is exerted predominantly before puberty rather than at puberty and in adult life. Neonatal imprinting with respect to sex differences in cyclic gonadotrophin secretion, hepatic steroid metabolism and sexual behaviour are well known [8, 9, 21]. Evidence presented here, considered together with other work [11, 17, 22], confirms that neonatal imprinting is instrumental in determining sex differences in drug metabolism.

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