Br. J. Pharmac. (1984), **81**, 049–054

Androgenic repression of hexobarbitone metabolism and action in Crl:CD-1 (ICR)BR mice

Bernard H. Shapiro & Susan M. Szczotka

Laboratories of Biochemistry, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

1 Mice of the Crl:CD-1 (ICR)BR strain exhibit a sexual dimorphism in hexobarbitone metabolism and action. Compared to females, males have a lower V_{max} and a higher K_m for hepatic microsomal hexobarbitone hydroxylase. In agreement with the enzyme studies, hexobarbitone-induced sleeping times were greater for males than for females.

2 Results from experiments measuring hexobarbitone metabolism and action in castrate, testosterone and gonadotropin-treated mice indicate that the sexual differences in drug metabolism and action found in Crl:CD-1 (ICR)BR mice are due to the normally repressive effects of testicular androgens on the activities of the hepatic mono-oxygenases. These findings are in dramatic contrast to studies with rats where it has been shown that androgens induce mono-oxygenases. Furthermore, in the case of the mouse, changes in the activity of hexobarbitone hydroxylase in response to alterations in androgen levels require weeks, while in the rat, androgenic-induced changes are apparent within a matter of days.

Introduction

It is well established that intact rats exhibit sex differences in the metabolism and action of many xenobiotics. Studies have shown that testicular hormones are responsible for the increased activities of hepatic mono-oxygenases found in male rats (Conney, 1967; Gillette, 1971; Kato, 1974). These sex differences are not present in sexually immature rats and have an age-dependent pattern of development, are abolished by orchidectomy, reversed by antagonistic hormones (i.e., oestrogens) and re-established by androgen replacement therapy following castration. Compared to the rat, much less is known about the sexual dimorphism in drug metabolism in the mouse. It appears that sexual differences in drug metabolism are strain-dependent in the mouse (Vesell, 1968). Depending upon the strain, hepatic mono-oxygenase activities may be greater in the male, or in the female, or no sex differences may exist. However, even in those strains that do exhibit a sexual dimorphism it is not clear as to the role of the gonads in maintaining the sexual differences in drug metabolism. In the most prevalent type of sexual dimorphism in the mouse the activities of the drug metabolizing enzymes are greater in the female than in the male. Using these strains it has been reported that the activities of hepatic mono-oxygenases are decreased by ovariectomy (Brown & Greene, 1980) or not affected (Noordhoek, 1972; King & Shapiro, 1981), increased by orchidectomy (Noordhoek, 1972; King & Shapiro, 1981) or not affected (Brown, Greene, Bullock & Bardin, 1978; Brown & Greene, 1980) and decreased by testosterone administration (Noordhoek, 1972; Brown *et al.*, 1978; King & Shapiro, 1981) or not affected (Brown, Bardin & Greene, 1978). It is possible that many of these contradictory findings can be explained by the little noted fact that unlike in the rat, the hepatic mono-oxygenases in some strains of mice may respond very slowly to changes in androgen levels. The activities of hepatic hexobarbitone hydroxylase may not be affected until 3 to 4 weeks following orchidectomy (Noordhoek, 1972; King & Shapiro, 1981) or until 14 days of testosterone treatment (King & Shapiro, 1981). Because the Crl:CD-1 (ICR)BR mouse, originating from the popular Swiss strain, is one of the most commonly used strains in biological research (Hill, 1983), we have investigated the role of the gonads in maintaining the sexual dimorphism in hexobarbitone metabolism and action found in these mice.

Methods

Animal treatment

Adult male and female Crl:CD-1 (ICR)BR mice (Charles River Breeding Laboratories, Wilmington, Massachusetts, U.S.A.) referred to as Crl:CD-1 mice, were housed on hardwood bedding in plastic cages. The animals were given water and commercial mouse diet ad libitum and were kept in air conditioned quarters, 20–23°C, with a photoperiod of 12 h light: 12 h dark.

Bilateral and sham-castrations were performed under Chloropent anaesthesia (chloral hydrate, 162 mg kg^{-1} and pentobarbitone, 34 mg kg^{-1}) as previously described (King & Shapiro, 1981). Animals were allowed to rest 4 to 5 weeks before beginning further treatment. Testosterone propionate (TP) was injected (s.c.) at 50 mg kg^{-1} in corn oil. Human chorionic gonadotropin (HCG) was injected (s.c.) at 250 iu kg^{-1} (1500 iu mg^{-1}) in 0.1 M potassium phosphate buffer containing 1% mannitol (w/v). The hormones were administered daily in the late afternoon for 14 consecutive days and the mice were killed the following morning. Control animals received an equal amount of the appropriate diluent, 2 ml kg^{-1}, for 14 days.

Microsome preparation

Thirty to 60 s after decapitation and exsanguination, livers were perfused with 20 ml of ice-cold 0.9% (w/v) NaCl solution. All subsequent steps were carried out at 0 to 4°C. Each liver was homogenized with 7 ml of ice-cold 0.1 M potassium phosphate buffer, pH 7.4, in a Teflon-glass tissue homogenizer with the aid of a motor driven pestle. The homogenate was centrifuged for 20 min at 10,000 *g* and the supernatant fluid was centrifuged for 60 min at 100,000 *g*. The resulting supernatant fluid was thoroughly drained from the microsomal pellet to remove cytosolic dehydrogenase present in murine liver that could interfere with the hexobarbitone hydroxylase assay by metabolizing the prime metabolite 3-hydroxyhexobarbitone to 3-ketohexobarbitone (Gerber, Lynn, Holcomb, Weller & Bush, 1971). In our hands, this simple precaution prevents the formation of detectable levels of 3-ketohexobarbitone (unpublished observations). The microsomal pellet was then suspended in 2.8 ml of 0.1 M potassium phosphate buffer and stored at −70°C until assayed for hexobarbitone hydroxylase the next morning.

Substrate purification

Within one day before assaying the microsomes, the radioactive hexobarbitone was purified by silica gel thin layer chromatography (t.l.c.) according to the procedure of Dr Barry Dvorchik (personal communications). [^{14}C]-hexobarbitone was applied to a prewashed (methanol/acetone, 1:1, v/v) t.l.c. plate and developed in chloroform/acetone, 9:1 (v/v). The purified [^{14}C]-hexobarbitone was extracted twice with 2 ml of methanol and concentrated under nitrogen gas to 50,000 d.p.m. per 20 µl methanol.

Hexobarbitone hydroxylase

Hepatic microsomal hexobarbitone hydroxylase was assayed by a modification of the procedure of Kupfer & Rosenfeld (1973) and Kupfer (1978). The radioactive hexobarbitone (50,000 d.p.m.) was dried at the bottom of each incubation vessel. A 1.0 ml incubation mixture contained microsomes (1 to 2 mg of protein), potassium phosphate buffer (pH 7.4, 0.1 M), $MgCl_2$ (10 mM), glucose-6-phosphate (12 mM), NADP (0.4 mM), glucose-6-phosphate dehydrogenase (1 I.E.U.) and inert hexobarbitone. The microsomes were added last to initiate the reaction. Linear kinetic data for hexobarbitone hydroxylase were obtained with 6 different hexobarbitone concentrations (0.05 to 0.5 mM). The microsomes were incubated in a shaking water bath (37°C) in air for 10 min. The reaction was terminated by the addition of 0.2 ml of 2.5 M perchloric acid. Blanks containing hexobarbitone, the NADPH generating system and perchloric acid were also incubated. Next, 0.8 ml of chilled 1 M citrate-15% sodium chloride buffer, pH 5.5 and 3.5 ml of 1-chlorobutane were added to the incubation vessels with quick swirling. Vessel contents were immediately transferred to test tubes with teflon screw caps. After vigorous shaking for 15 s, the test tubes were centrifuged for 5 min at 2000 *g*. The hexobarbitone containing organic (upper) phase was aspirated and discarded, and the aqueous phase containing the hexobarbitone metabolite was extracted twice more with 1-chlorobutane. Aliquots of the aqueous phase were mixed with ACS II and monitored for ^{14}C disintegrations in a liquid scintillation counter. Sufficient counts were accumulated to achieve a standard deviation of $< 0.5\%$. Michaelis constants and maximal velocities were determined from linear regression models of the data using the method of Hofstee (1959). The correlation coefficients for all Hofstee plots were positive, exceeded 0.95, and were found to be statistically significant ($P < 0.01$).

Protein determinations

Microsomal protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Table 1 Hexobarbitone-induced sleeping time (min) of intact and castrated male and female Crl:CD-1 mice

	Female	*Male*
Intact	60.1 ± 7.3	76.1 ± 9.4*
Sham-castrate	60.4 ± 6.1	78.5 ± 4.1*
Castrate	62.4 ± 3.7	63.3 ± 7.6

Hexobarbitone (125 mg kg^{-1})-induced sleeping times were measured in 16 intact female and 16 intact male mice at 65 days of age. At 80 days of age half of the animals of each sex were bilaterally castrated and the remaining mice were sham-castrated. Sleeping times were measured again at 118 days of age.
The results are presented as the mean ± s.d.
*$P < 0.001$; different from intact and sham-castrate females.

Statistical analyses

Groups were compared for statistically significant differences by two-way analysis of variance and Student's *t* test.

Materials

Hexobarbitone, glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase and testosterone propionate were purchased from Sigma Chemical Company (St Louis, Missouri, U.S.A.). ACS II was from Amersham Corporation (Arlington Heights, Illinois, U.S.A.) and 1-chlorobutane (HPLC grade) was from Fisher Scientific Company (Fairlawn, New Jersey, U.S.A.). Chloropent was purchased from Fort Dodge Laboratories, Incorporated (Fort Dodge, Iowa, U.S.A.). Radioactive hexobarbitone (cyclohexenyl-3,5-dimethylbarbituric acid, 5-[2-^{14}C]), 8.58 mCi $mmol^{-1}$, was obtained from New England Nuclear (Boston, Massachusetts, U.S.A.). Human chorionic gonadotropin was purchased from A.J. Buck & Son, Incorporated (Cockeysville, Maryland, U.S.A.). All other reagents were of analytical grade.

Results

Intact Crl:CD-1 mice exhibited a sexual dimorphism in hexobarbitone-induced sleeping times (Table 1). In response to the same dose of hexobarbitone, males slept about 25% longer than females. Bilateral gonadectomy abolished the sexual differences in hexobarbitone-induced sleeping times. That is, sleeping times of orchidectomized males were reduced to that of females, which were unchanged following ovariectomy.

Kinetic parameters of hepatic hexobarbitone hydroxylase reflected the sexual dimorphism found in sleeping times. Compared to intact females, the K_m for the mono-oxygenase was higher and the V_{max} lower in hepatic microsomes from intact males (Table 2). While ovariectomy had no effect on the enzyme kinetics, orchidectomy resulted in a decline in the K_m and an elevation in the V_{max} to levels found in intact females. Administration of testosterone propionate to castrate male and female mice did not result in a reappearance of sexually dimorphic enzyme levels. Instead, hexobarbitone hydroxylase in castrate males and females injected with the androgen had the elevated K_m and reduced V_{max} found in intact males.

Administration of chorionic gonadotropin to castrated male mice caused a significant increase in the K_m and a decrease in the V_{max} for hexobarbitone hydroxylase to levels found in intact males (Table 3). Gonadotropin administration to intact males re-

Table 2 Effect of castration and testosterone propionate (TP) on the activity of hexobarbitone hydroxylase in female and male Crl:CD-1 mice

	Female		*Male*	
	K_m	V_{max}	K_m	V_{max}
Sham-castrate + diluent	1.86 ± 0.17	3.15 ± 0.15	2.14 ± 0.14*††	2.32 ± 0.15**††
Castrate + diluent	1.92 ± 0.13	3.20 ± 0.26	1.79 ± 0.19	3.10 ± 0.36
Castrate + TP	2.29 ± 0.19**††	2.59 ± 0.12**†	2.14 ± 0.25†	2.56 ± 0.24**†

Female and male mice were either bilaterally castrated or sham-castrated at 66 days of age. Thirty days later the animals were injected daily with TP (50 mg kg^{-1}) or the corn oil diluent (2 ml kg^{-1}) for 14 consecutive days and killed the following morning.
K_m is expressed as 10^{-4} M and V_{max} is expressed as nmol min^{-1} (mg protein)$^{-1}$
The results are presented as the mean ± s.d. of 6 mice per group.
*$P < 0.01$, **$P < 0.001$; different from diluent-treated, sham-castrated females.
†$P < 0.01$, ††$P < 0.001$; different from diluent-treated, castrated males.

Table 3 Effect of human chorionic gonadotropin (HCG) on the activity of hexobarbitone hydroxylase in sham-castrated and castrated male Crl:CD-1 mice

	Treatment			
	Diluent		*HCG*	
	K_m	V_{max}	K_m	V_{max}
Sham-castrate	2.68 ± 0.27	2.18 ± 0.32	3.01 ± 0.19*	1.36 ± 0.10***†††
Castrate	2.23 ± 0.12**†††	2.73 ± 0.26**††	2.77 ± 0.23	2.21 ± 0.30

Male mice were either bilaterally castrated or sham-castrated at 105 days of age. Thirty-five days later the animals were injected daily with HCG (250 iu kg^{-1}) or the buffer diluent (1.25 ml kg^{-1}) for 14 consecutive days and killed the following morning.
K_m is expressed at 10^{-4} M and V_{max} is expressed as nmol min^{-1} (mg protein)$^{-1}$.
The results are presented as the mean ± s.d. of 6 mice per group.
*$P<0.05$, **$P<0.01$; ***$P<0.001$; different from diluent-treated, sham-castrates.
††$P<0.01$, †††$P<0.001$; different from HCG-treated, castrates.

sulted in a dramatic 40% decline in enzyme activity.

The responsiveness of hepatic hexobarbitone hydroxylase to orchidectomy is reported in Table 4. There were no statistically significant differences in the activity of the mono-oxygenase 17 days following castration. However, after 5 weeks of castration the K_m was lower and the V_{max} higher for hepatic hexobarbitone hydroxylase.

Discussion

Unlike the rat where there is a 200 to 400% difference in the activities of drug metabolizing enzymes between the sexes (Kato, 1974; Sonawane, Yaffe & Shapiro, 1979), we have found, as have others (Noordhoek, 1972; Brown *et al.*, 1978), that there is only a 20 to 40% sex difference in mice. In agreement with those studies measuring sexual differences in hepatic ethylmorphine-N-demethylase activity in Crl:CD-1 mice (Brown *et al.*, 1978; Brown & Greene, 1980), we have found that the females of this strain have a greater level of hexobarbitone hydroxylase than do the males. The V_{max} for the enzyme was 36% greater in the females than in the males, while the K_m for hexobarbitone hydroxylase was significantly greater in the males. Furthermore, we have found that the sex difference in *in vitro* drug metabolism reflects a similar magnitude of change in the biological measure of hexobarbitone-induced sleeping time. Our results, however, are in contrast with those of Brown *et al.* (1978) and Brown & Greene (1980) who found that ovariectomy reduced the activity of hepatic ethylmorphine-N-demethylase while testosterone treatment or orchidectomy had no effect on the activity of the enzyme in Crl:CD-1 mice. We have found that ovariectomy had no effect on hexobarbitone metabolism or action in Crl:CD-1 mice. Orchidectomy, however, resulted in an increase in the V_{max} and a decrease in the K_m for hexobarbitone hydroxylase with a concommitant decrease in hexobarbitone-induced sleeping times to female-like levels, while testosterone administration depressed the activity of the enzyme to pre-castration levels. Studies using the CPB-SE (Noordhoek, 1972) and Tfm (King & Shapiro, 1981) strains of mice have also shown that orchidectomy results in an increase in hepatic hexobarbitone hydroxylase and that testosterone administration decreases the activity of the enzyme in castrated male and female mice. Although it is not clear as to why our findings are in disagreement with previous studies using Crl:CD-1 mice, it

Table 4 The response of hepatic hexobarbitone hydroxylase in male Crl:CD-1 mice at different times following castration

	Age (days)	K_m	V_{max}
Sham-castrate	114	2.36 ± 0.18	2.28 ± 0.19
Castrate	96	2.22 ± 0.16	2.55 ± 0.28
Castrate	114	2.01 ± 0.12*	2.90 ± 0.25**

Male mice were either bilaterally castrated or sham-castrated at 79 days of age and killed 17 or 35 days later.
K_m is expressed as 10^{-4} M and V_{max} is expressed as nmol min^{-1} (mg protein)$^{-1}$.
The results are presented as the mean ± s.d. of 6 mice per group.
*$P<0.01$, **$P<0.001$; different from sham-castrated mice.

seems unlikely that hepatic ethylmorphine-N-demethylase and hexobarbitone hydroxylase would respond differently to castration and androgen treatment as this has not been the case in previous studies (Kato, Takanaka, Takahashi & Onodo, 1969; Noordhoek, 1972; Kato, 1974). However, it might be significant that we measured drug metabolism six weeks after orchidectomy and after two weeks of testosterone treatment, while the other studies were performed following three weeks of castration and six days of androgen treatment. There is some evidence to suggest that unlike in the rat, the hepatic mono-oxygenases in the mouse may respond very slowly to changes in androgen levels (Noordhoek, 1972; King & Shapiro, 1981). In fact, we found that the increase in hepatic microsomal hexobarbitone hydroxylase is twice as great when measured five weeks after orchidectomy as compared to values obtained two and a half weeks after castration. Thus, our finding of an increase (34%) in the V_{max} for hepatic hexobarbitone hydroxylase six weeks after orchidectomy might not have been apparent had the enzyme been measured only two or three weeks after castration.

Although our results show that exogenous administration of testosterone can depress hepatic hexobarbitone hydroxylase activity and thus mimic the effects of the intact testes, the use of pharmacological doses of androgens by us and others (Noordhoek, 1972; Kato, 1974; Brown *et al.*, 1978) prevents our concluding that testicular androgens normally repress the levels of hepatic mono-oxygenases in Crl:CD-1 mice. In order to determine whether androgens produced by the intact testes can reduce the activities of hepatic hexobarbitone hydroxylase, we injected mice with HCG, a gonadotropin shown to stimulate testicular secretion of testosterone with little or no effect on oestrogen production (de Jong, Hey & van der Molen, 1973). Our finding that administration of HCG to intact male mice can significantly reduce the V_{max} and increase the K_m for hepatic hexobarbitone hydroxylase suggests that testicular secretions of androgens can normally suppress the activities of hepatic mono-oxygenases in Crl:CD-1 mice. We also found that HCG reduced hexobarbitone hydroxylase activity in castrate males, although the depressive effects of the gonadotropin were twice as great when administered to intact mice. This extratesticular effect of HCG may be due to gonadotropic stimulation of adrenal androgen secretion (Blichert-Taft, Vejlsted, Kehlet & Albrechtsen, 1975), or possibly, gonadotropins can independently alter the levels of hepatic mono-oxygenases (Gustafsson & Stenberg, 1975). Nevertheless, our findings do suggest that the gonadotropin can reduce the activity of hepatic hexobarbitone hydroxylase by stimulating testicular secretion of androgens.

Thus, the results of our experiments indicate that in contrast to the rat, the sexual dimorphism in drug metabolism and action found in Crl:CD-1 mice is due to the normally repressive effects of testicular androgens on the activities of the hepatic mono-oxygenases.

This study was supported by grants from the United States Public Health Service (GM-26222), the Biomedical Support Program (SO7 RR5464) of the N.I.H. and a U.S.P.H.S. Career Development Award (HD-00260) to B.H.S.

References

BLICHERT-TAFT, M., VEJLSTED, H., KEHLET, H. & ALBRECHTSEN, R. (1975). Virilizing adrenocortical adenoma responsive to gonadotropin. *Acta endocr.*, **78,** 77–85.

BROWN, T.R., BARDIN, C.W. & GREENE, F.E. (1978). Mouse liver N-demethylase activity. Sex differences and androgen responsiveness. *Pharmacology*, **16,** 159–169.

BROWN, T.R. & GREENE, F.E. (1980). Effects of endogenous sex hormones on mouse liver ethylmorphine-N-demethylase. *Arch. int. Pharmacodyn. Thér.*, **248,** 13–25.

BROWN, T.R., GREENE, F.E., BULLOCK, L.P. & BARDIN, C.W. (1978). Effect of the Tfm locus on the hepatic ethylmorphine-N-demethylase system in mice. *Endocrinology*, **103,** 1374–1382.

CONNEY, A.H. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmac. Rev.*, **19,** 317–366.

GERBER, N., LYNN, R., HOLCOMB, R., WELLER, W.L. & BUSH, M.T. (1971). The metabolism of hexobarbital in mice and methodology for isolation and quantitation of its metabolites *in vivo* and *in vitro*. *J. Pharmac. exp. Ther.*, **177,** 234–245.

GILLETTE, J.R. (1971). Factors affecting drug metabolism. *Ann. New York Acad. Sci.*, **179,** 43–66.

GUSTAFSSON, J.-Å & STENBERG, Å. (1975). Partial masculinization of rat liver enzyme activities following treatment with FSH. *Endocrinology*, **96,** 501–504.

HOFSTEE, B.H.J. (1959). Non-inverted versus inverted plots in enzyme kinetics. *Nature (London)*, **184,** 1296–1298.

HILL, B.F. (1983). The CD-1 mouse, history and utilization. In *Charles River Digest* 22 (1), ed. Hill, B.F., Wilminton, Massachusetts: The Charles River Breeding Laboratories, Inc.

DE JONG, F.H., HEY, A.H. & VAN DER MOLEN, H.J. (1973). Effect of gonadotrophins on the secretion of oestradiol-17β and testosterone by the rat testis. *J. Endocrinology*, **57,** 277–284.

KATO, R. (1974). Sex-related differences in drug metabolism. *Drug Metab. Rev.*, **3,** 1–32.

KATO, R., TAKANAKA, A., TAKAHASHI, A. & ONODA, K.

(1969). Species differences in the alteration of drug-metabolizing activities of liver microsomes by thyroxine treatment. *Jap. J. Pharmac.*, **19,** 5–18.

KING, D.K. & SHAPIRO, B.H. (1981). Sexual dimorphism and the effects of the X-linked *Tfm* locus on hexobarbitone metabolism and action in mice. *Br. J. Pharmac.*, **74,** 97–104.

KUPFER, D. (1978). Assay of hexobarbital hydroxylase. *Drug Metab. Dis.*, **6,** 610.

KUPFER, D. & ROSENFELD, J. (1973). A sensitive radioactive assay for hexobarbital hydroxylase in hepatic microsomes. *Drug Metab. Dis.*, **1,** 760–765.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193,** 265–275.

NOORDHOEK, J. (1972). The effect of castration and testosterone on some components of the microsomal drug metabolizing enzyme system in mice. *FEBS Lett.*, **24,** 255–259.

SONAWANE, B.R., YAFFE, S.J. & SHAPIRO, B.H. (1979). Hepatic drug metabolism in pseudohermaphroditic rats. *Drug Metab. Dis.*, **7,** 444–445.

VESELL, E.S. (1968). Factors altering the responsiveness of mice to hexobarbital. *Pharmacology*, **1,** 81–97.

(Received April 26, 1983.
Revised July 25, 1983.)