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Sexually dimorphic response of rat hepatic monooxygenases to low-dose phenobarbital

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It has been clearly established that the activities of most hepatic monooxygenases are greater in male rats than in female rats. Many studies have shown that this sexual dimorphism is due to the inductive effects of testicular androgens [1–3]. In contrast, there are far fewer studies comparing the inductive effects in male and female rats of the much more commonly used inducers, i.e. phenobarbital, methylcholanthrene, benzopyrene, etc. In this regard, while female rats appear to be more responsive to the inductive effect of phenobarbital, in that the same dose of the barbiturate will induce a greater percent increase in the activities of the hepatic monooxygenases in females when compared to males, a sexual difference remains, as induced levels of enzymes are still greater in the males [4, 5]. Unfortunately, studies comparing the responsiveness of hepatic drug-metabolizing enzymes in male and female rats have been confined to the use of only a single, maximally effective dose of the inducing agent. Thus, any alteration in the responsiveness of hepatic monooxygenases to submaximal, or even "subtle" levels of inducers, which are more likely encountered in the environment, is unknown. Although there have been dose–response studies using various inducing agents, they usually have been conducted on a single sex, i.e. male rats [6, 7].

In the present study, we have compared the inductive effects of low doses of phenobarbital on the kinetic parameters of cytochrome P-450-dependent hepatic microsomal hexobarbital hydroxylase and aminopyrine *N*-demethylase in male and female rats. Phenobarbital was chosen as the inducing agent because it is probably used more frequently than any other compound to investigate the mechanism(s) of monooxygenase induction.

Materials and methods

Sprague–Dawley [Crl:CD(SD)BR] rats born and raised in our own animal facilities were housed on hardwood bedding in plastic cages. Animals were given water and commercial rat diet *ad lib.* and were kept in air-conditioned quarters, 20–23°, with a photoperiod of 12 hr light/12 hr dark. At approximately 4 months of age, male and female rats were injected, daily, by the intraperitoneal route, with 1, 3 or 20 mg/kg of sodium phenobarbital (J. T. Baker Chemical Co., Phillipsburg, NJ) or an equivalent amount (2 ml/kg) of the 0.9% NaCl diluent, pH 9.0, for 6 consecutive days and killed on the following day.

Hepatic microsomes were prepared from the 100,000 *g* pellets as previously described [8]. Hepatic microsomal hexobarbital hydroxylase was assayed by our modification [8] of the radioenzyme procedure of Kupfer and Rosenfeld [9]. Basically, the assay measures the rate of microsomal conversion of radioactive hexobarbital, 5-[2-^{14}C]-cyclohexenyl-3,5-dimethylbarbituric acid (14.4 mCi/mmole; NEN Research Products, Boston, MA), to 3-hydroxyhexobarbital. Linear kinetic data for the enzyme were obtained from eight different hexobarbital concentrations (0.033 to 0.33 mM). Hepatic microsomal aminopyrine *N*-demethylase was determined by our modification [10] of the radioassay of Poland and Nebert [11] which measures the production of [^{14}C]formaldehyde from the radioactive aminopyrine substrate [N-*methyl*-^{14}C]antipyrine (112.2 mCi/mmole; NEN Research Products). Kinetic data for aminopyrine *N*-demethylase were obtained with fourteen different aminopyrine concentrations (0.05 to 2 mM). Microsomal protein content was determined by a modification [12] of the method of Bradford [13].

Michaelis constants (K_m) and maximal velocities (V_{max}) were determined from linear regression models of the data using the method of Hofstee [14]. The correlation coefficients for all Hofstee plots were positive, exceeded 0.95, and were found to be statistically significant ($P < 0.01$).

Experimental groups were compared for statistically significant differences by analysis of variance and Student's *t*-test.

Results and discussion

In agreement with previous studies [2–4], we have found a highly significant sexual difference in the kinetics of hepatic microsomal hexobarbital hydroxylase and aminopyrine *N*-demethylase in rats. Basically, the Michaelis constants were lower and the maximal velocities were higher for both hepatic monooxygenases in the males (Tables 1 and 2). Also, in agreement with more recent reports, particularly those using the sensitive radioenzyme assays [10, 11, 15], we have found that aminopyrine *N*-demethylase exhibited biexponential (non-linear) kinetics.

In this study, we proposed to determine if the sex of the animal was a determining factor affecting the ability of phenobarbital to induce hepatic monooxygenases at submaximally effective doses. Following a treatment schedule similar to ours, it has been reported that the maximally effective dose of phenobarbital to induce rat hepatic microsomal monooxygenases is at least 75 mg/kg [7, 16]. In fact, this is probably the most commonly used dose in induction studies. Thus, when we administered phenobarbital at 1, 3 and 20 mg/kg, we were actually administering about 1, 4 and 27% respectively, of the maximally effective dose of the barbiturate.

Our results indicate that the livers of the adult male rats were considerably more sensitive to the inductive effects of low doses of phenobarbital than were the livers of the female rats. As low a dose as 1 mg/kg of the barbiturate produced a significant elevation in the maximal velocities of hepatic hexobarbital hydroxylase, aminopyrine *N*-demethylase, the K_m of hexobarbital hydroxylase, and a decline in the K_{m_1} of aminopyrine *N*-demethylase for the male rats (Tables 1 and 2). In general, the maximal velocities of the enzymes in the males exhibited a dose–response relationship. The 3 mg/kg dose of phenobarbital

Table 1. Effects of phenobarbital treatment on liver weight and hepatic hexobarbital hydroxylase kinetics in male and female rats

Phenobarbital treatment (mg/kg/day)	Liver wt (g/100 g body wt)	Hexobarbital hydroxylase K_m (mM)	Hexobarbital hydroxylase V_{max} (nmoles/min/mg protein)
		Males	
0	3.21 ± 0.26	0.101 ± 0.008	3.96 ± 0.39
1	3.31 ± 0.29	0.137 ± 0.011*	5.92 ± 0.40*
3	3.33 ± 0.28	0.152 ± 0.025	6.91 ± 0.84†
20	3.93 ± 0.16*	0.159 ± 0.036	10.89 ± 1.97*
		Females	
0	3.20 ± 0.29	0.154 ± 0.025‡	1.94 ± 0.27‡
1	3.23 ± 0.44	0.137 ± 0.018	1.99 ± 0.19‡
3	3.21 ± 0.29	0.155 ± 0.032	2.32 ± 0.47‡
20	3.62 ± 0.55	0.104 ± 0.012*‡	5.96 ± 0.71*‡

Adult male and female rats were injected, daily, with either phenobarbital or diluent for 6 consecutive days and killed on day 7. The results are the mean ± S.D. of eight rats per group.

* $P < 0.001$ and † $P < 0.01$, when compared to the effects of the immediately lower dose of phenobarbital in animals of the same sex.

‡ $P < 0.001$, when females are compared to males in the same treatment group.

Table 2. Effects of phenobarbital treatment on hepatic aminopyrine *N*-demethylase kinetics in male and female rats

Phenobarbital treatment (mg/kg/day)	Aminopyrine *N*-demethylase K_{m_2} (mM)	Aminopyrine *N*-demethylase K_{m_1} (mM)	Aminopyrine *N*-demethylase V_{max_2} (nmoles/min/mg protein)	Aminopyrine *N*-demethylase V_{max_1} (nmoles/min/mg protein)
		Males		
0	0.251 ± 0.028	0.939 ± 0.229	4.93 ± 0.82	9.14 ± 2.13
1	0.238 ± 0.032	0.711 ± 0.066*	6.58 ± 1.11†	11.68 ± 1.35†
3	0.292 ± 0.037†	0.636 ± 0.107	9.11 ± 1.42†	14.21 ± 2.20†
20	0.308 ± 0.051	0.805 ± 0.143*	9.91 ± 1.21	19.88 ± 2.94‡
		Females		
0	0.298 ± 0.036§	0.781 ± 0.201	2.05 ± 0.29‖	5.32 ± 1.21‖
1	0.351 ± 0.050*‖	0.739 ± 0.128	2.28 ± 0.31‖	4.93 ± 0.86‖
3	0.396 ± 0.102§	0.709 ± 0.132	3.17 ± 0.96*‖	5.61 ± 1.10‖
20	0.432 ± 0.053‖	0.701 ± 0.126	9.63 ± 1.32‡	17.46 ± 2.85‡

Adult male and female rats were injected, daily, with either phenobarbital or diluent for 6 consecutive days and killed on day 7. The results are the mean ± S.D. of eight rats per group.

* $P < 0.02$, † $P < 0.01$ and ‡ $P < 0.001$, when compared to the effect of the immediately lower dose of phenobarbital in animals of the same sex.

§ $P < 0.01$ and ‖ $P < 0.001$, when females are compared to males in the same treatment group.

produced a greater increase in the activities of the monooxygenases than the 1 mg/kg dose of the barbiturate, and the 20 mg/kg dose increased the activities of the enzymes above that produced by the 3 mg/kg dose. In contrast, the livers of the female rats were basically unresponsive to the inductive effects of the 1 mg/kg and 3 mg/kg doses of phenobarbital. It was only the 20 mg/kg dose that produced significant increases in the maximal velocities of the hepatic enzymes in the female rats.

The effects of the barbiturate on the Michaelis constants of hepatic aminopyrine *N*-demethylase in both sexes were not definitive as there was no clear relationship between the administered dose of phenobarbital and the resulting constants. In general, however, the phenobarbital treatment appeared to reduce the numerical difference between the K_{m_2} and the K_{m_1} so that the two constants tended to approach more intermediate values.

In agreement with the enzyme results, while the 20 mg/kg dose of phenobarbital produced a significant increase in the liver weights of the males, it had no such affect in the females (Table 1).

Our finding that the hepatic monooxygenase system is more sensitive to the inductive effects of low doses of phenobarbital in the male rat is supported by previously published findings. Daily administration of 3 mg/kg of phenobarbital for 6 days is an effective dose in increasing total hepatic cytochrome P-450 in male rats [7]. In contrast, daily injections of either 1 mg/kg or 5 mg/kg of phenobarbital for 30 days have no effect on hepatic microsomal metabolism of hexobarbital or strychnine in adult female rats [17].

Why the hepatic monooxygenase system of the male rat is more sensitive than that of the female to the inductive effects of low doses of phenobarbital is uncertain. It should be noted, however, that the activities of the hepatic drug-metabolizing enzymes of the male rat are more responsive than those of the female to the effects of adrenalectomy, alloxan diabetes, starvation and treatment with morphine, cortisol or thyroxine [2]. Regardless of the reasons for the enhanced sensitivity of the hepatic monooxygenase system in male rats, our results suggest that drug-metabolizing enzymes in the males may be more susceptible to "low"

level exposure of possibly hundreds of environmental inducers.

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

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Changes in hepatic drug metabolism in alloxan-diabetic male rabbits

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In rats, it has been clearly demonstrated that chemically-induced diabetes mellitus produces changes in hepatic microsomal drug metabolism. The activities of sex-dependent enzymes (aminopyrine demethylase, benzo[*a*]pyrene hydroxylase) decrease while those of sex-independent enzymes (aniline hydroxylase) increase following the induction of diabetes in male rats [1–5]. In female rats, by contrast, diabetes causes a more generalized increase in enzyme activities. The effects of diabetes on hepatic monooxygenases can be prevented or reversed by insulin administration to diabetic animals. Some of the changes in drug-metabolizing activities caused by diabetes may be attributable to alterations in the cytochrome P-450 composition of hepatic microsomes. Past and Cook [6] have found far greater amounts of a cytochrome P-450 isozyme, which catalyzes aniline hydroxylation, in microsomal preparations from diabetic than from control rats. Recent studies [7] indicate that the spin state of hepatic cytochromes P-450 may also be modified in diabetic animals. Thus, changes in the ratio of cytochrome P-450 isozymes, as well as in the functional properties of cytochromes P-450, may account for the changes in monooxygenase activities caused by diabetes.

In contrast to the large number of investigations done with rats, there have been relatively few reports on the relationship between diabetes and hepatic drug metabolism in other species. Thus, it is not clear whether the observations made in rats are generally applicable or species-specific. Diabetes seems to have different effects on hepatic monooxygneases in guinea pigs than in rats. In spontaneously diabetic male guinea pigs, there is a decrease in aniline hydroxylase activity and no change in the rate of aminopyrine demethylation [8]. Diabetes has no effect on drug metabolism in female guinea pigs [8]. In untreated human diabetics, the *in vivo* half-lives of acetophenetidin and antipyrine are prolonged, suggesting changes in hepatic drug metabolism [9, 10]. The changes are reversed by insulin treatment. In rabbits, the urinary excretion of unaltered acetophenetidin is greater and metabolite excretion lower in diabetics than in controls following intraperitoneal administration of the drug [11]. Similarly, metabolism of acetophenetidin *in vitro* is lower in microsomal preparations from the diabetic rabbits than those from the controls but the metabolism of other substrates was not evaluated. Because of the relative paucity of data concerning the impact of diabetes mellitus on hepatic drug metabolism in species other than rats, we have pursued the observations of Dajani and Kayyali [11] and studied the effects of diabetes on various hepatic microsomal monooxygenases in rabbits. The results presented in this communication further indicate that the qualitative changes in drug-metabolizing enzymes caused by diabetes are species-specific.

Methods

Adult (1.5 to 2.5 kg) male New Zealand White rabbits obtained from Green Meadows Rabbitry (Murraysville, PA) were used for all experiments. Rabbits were maintained under standard conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°), and they received food and water *ad lib*. Animals were made diabetic following an 18–24 hr fast, by an i.v. infusion of alloxan (100 mg/kg) in 0.02 M citrate saline via the marginal ear vein. Age-matched controls received an infusion of vehicle alone. The induction of diabetes was confirmed by analysis of urine samples with Keto-Diastix (Ames). Rabbits were killed 1 or 2 months after the induction of diabetes (between 8:30 and 9:30 a.m.) by a blow to the head followed by exsanguination. Blood samples were taken, the serum was separated, and serum glucose concentrations were determined using the ABTS method of Bergmeyer and Bernt [12]. Livers were rapidly removed at the time of sacrifice and placed in ice-cold 1.15% KCl containing 0.05 M Tris–HCl (pH 7.4). Tissues were homogenized and microsomes were