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SEXUAL DIMORPHISM IN AVIAN HEPATIC MONOOXYGENASES

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Abstract—Adult white Leghorn chickens exhibited a sexual dimorphism in hepatic microsomal monooxygenases determined from the concentrations of total cytochromes P450 and b_5, and the metabolism of drug (hexobarbital, coumarin and ethoxyresorufin) and steroid (androstenedione and testosterone) substrates that were 2- to 4-fold greater in roosters than in hens. Caponizing at 6 weeks of age reduced the activities of the monooxygenases to levels comparable to those found in intact hens. In spite of the fact that testosterone replacement maximally stimulated comb growth in the capons and elevated (i.e. masculinized) hepatic monooxygenase activities in the hens to male-like levels, androgen replacement was ineffective in increasing the subnormal enzyme levels in the capons. While the failure of testosterone administration to restore monooxygenase levels in the capons may be explained by the immaturity of the birds at orchiectomy, the present results demonstrate, that like some mammals, birds may display gender differences in hepatic monooxygenases that are regulated by the testes.

Ever since its initial discovery in rats in the 1930s [1], gender differences in drug metabolism have been studied extensively in this species. It is now well established that male rats metabolize many drugs at a rate 3–5 times faster than females, and this enhanced metabolism correlates with the shortened pharmacological action of many xenobiotics [2]. While these early studies revealed that testicular androgens were the endogenous agents responsible for inducing the increased activities of the cytochrome P450-dependent hepatic monooxygenases, later findings indicated that it is pituitary growth hormone, regulated by androgens at the hypothalamic-pituitary level, that controls the sex-dependent expression of some half-dozen or more cytochrome P450 isoforms [3, 4]. As a result of both the magnitude and consistency of this dimorphism, studies investigating the developmental and regulatory aspects of gender differences in drug metabolism have been virtually limited to the rat. Curiously, the few reports of sexual differences in drug metabolism in other mammalian species have indicated a dimorphism opposite that found in the rat. Regardless of strain, it is the female mouse that metabolizes drugs more quickly than the male, and the same androgens that indirectly induce hepatic monooxygenases in the rat repress these enzymes in the mouse [5, 6]. Furthermore, androgenic repression of murine hepatic monooxygenases is also mediated by pituitary-secreted growth hormone [7], whereas androgens have no direct effect, *per se*, on the hepatic activities of cytochromes P450 [8]. Unlike the rat where there can be at least a 600% difference in the activities of drug-metabolizing enzymes between the sexes [2, 9, 10], in mice, depending upon strain, only a 30–100% sex difference exists [5, 6]. In addition, this dimorphism in *in vitro* murine drug metabolism is reflected in a similar magnitude of change in *in vivo* drug action [5, 7]. Recent findings in goats [11] have indicated that, like the mouse, the rate of drug metabolism is greater in the female, and the magnitude of this sex difference is also similar to that found in mice.

If reports concerning sexual differences in drug metabolism in non-rat mammals are sparse, then they appear to be non-existent for birds. Mammalian and avian evolution has been highly divergent with a common reptilian-like amphibian ancestor appearing some 320 million years ago. In fact, mammals predate birds by almost 50 million years [12]. Yet, like mammals, the avian liver, which is the principal source of monooxygenase activity, contains putative families of cytochrome P450 isoforms responsible for the metabolism of a wide range of both exogenous (e.g. drugs, pollutants, and pesticides) and endogenous (e.g. steroids and fatty acids) compounds [13, 14]. Thus, using the chicken, we wanted to determine if dimorphisms in hepatic microsomal monooxygenases occurring in some mammals are also evident in birds.

METHODS

Animals were housed in the University of Pennsylvania Laboratory Animal Resources facility, under the supervision of certified Laboratory Animal Medicine veterinarians, and were treated according to a research protocol approved by the University's Institutional Animal Care and Use Committee. Recently hatched white Leghorn chickens obtained from a commercial breeder were housed under a 12-hr light:12-hr dark cycle and had free access to fresh feed and water. At 6 weeks of age, cockerels were

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castrated according to established procedures [15]. At 13 months of age the animals were divided into experimental groups. Caponized birds exhibiting comb growth or radioimmunoassayable [16] plasma testosterone levels greater than those found in known capons were discarded. Capons and hens were daily injected s.c., for 14 continuous days, with either 0.1, 0.5 or 5.0 mg of testosterone propionate per kg body weight. In addition, some of the hens and capons as well as all of the roosters were similarly treated with an equivalent amount of the corn oil vehicle. The birds were decapitated on the morning following the last injection. Throughout the study, the chickens were maintained specific pathogen- and medication-free.

Hepatic microsomes were prepared by our previously described method [17]. Hepatic microsomal hexobarbital hydroxylase was assayed by our modification [18] of the radioenzyme procedure of Kupfer and Rosenfeld [19]. Cytochrome P450 was determined from the reduced carbon monoxide difference spectrum according to the method of Omura and Sato [20]. Cytochrome b_5 was measured as described previously [20]. The activities of 7-ethoxyresorufin *O*-deethylase [21], coumarin 7-hydroxylase and 7-ethoxycoumarin *O*-deethylase [22] were measured spectrofluorimetrically. Lastly, microsomal fractions were assayed for testosterone and androstenedione hydroxylases [23], using 4.6 mm × 15 cm reverse phase C18 columns and Diode Array Detector-HPLC (Perkin Elmer, Norwalk, CT). The hydroxylated steroids were separated by a concave gradient solvent system (2 mL/min) of methanol:water:acetonitrile, using gradient-4 from 38:58:4 to 55:35:10 (for hydroxytestosterones) or 36:60:4 to 55:35:10 (for hydroxyandrostenediones) on Perkin Elmer columns. The products were further verified with a solvent system (1.5 mL/min) of water:tetrahydrofuran (85:15) on Zorbax (MacMod, Chaddsford, PA) columns. [4-^{14}C]Testosterone and [4-^{14}C]-androstenedione were also used to assay these enzymes, and the products were resolved by TLC and quantified by previously reported methods [23].

Data were subjected to analysis of variance, and differences among pairs of means were determined using "t" statistics and the Bonferroni procedure for multiple comparisons [24].

RESULTS

The effectiveness of testosterone propionate administration was evaluated by measuring comb growth in the treated birds. Testosterone at 0.1 mg/kg, the lowest dose used, was found to be maximally effective in all the chickens. A comb weight of 2.4 ± 0.5 g (mean ± SD) in the corn oil-treated capons was increased by a similar 15- to 16-fold after testosterone injections of either 0.1, 0.5 or 5.0 mg/kg. In agreement with the capon findings, there were no dose effects of testosterone on comb growth in the hens. In fact, the mean comb weight of all androgen-treated hens (39.8 ± 7.6 g) was not different from that found in the treated capons. However, since the comb weight in the control hens were 17.4 ± 5.5 g (chicken ovaries secrete substantial amounts of testosterone that maintain some comb growth [25]), their weights were only doubled by androgen treatment.

The effectiveness of testosterone administration was also observed with changes in egg laying activity. The vehicle-treated hens laid 8.2 ± 0.8 eggs during the 14-day treatment period. Whereas the 0.1 mg/kg dose of testosterone propionate had no effect on egg production (8.0 ± 1.0 eggs), both the 0.5 and 5.0 mg/kg doses completely halted egg laying.

Whether hepatic microsomal monooxygenases were determined from concentrations of total cytochromes P450 and b_5, or by measuring the metabolism of drug substrates (i.e. hexobarbital, ethoxyresorufin and coumarin) or endogenous substrates (i.e. androstenedione and testosterone), the enzyme concentrations were consistently greater in males as compared with females (Figs. 1 and 2). In general, we found that the monooxygenase activities were either twice (cytochromes P450 and b_5, hexobarbital hydroxylase, coumarin 7-hydroxylase and testosterone 16α-hydroxylase) or 4 times (androstenedione 6β-, 16α- and 2α-hydroxylases and testosterone 6β-hydroxylase) greater in male liver than in female. (Exceptions were 7-ethoxycoumarin *O*-deethylase which was 3 times greater in males, and testosterone 2α-hydroxylase which exhibited no statistically significant sexual dimorphism.) Caponizing tended to reduce the activities of the monooxygenases to levels equal to or approaching that found in the hens. (The absence of any statistical effect of castration on coumarin 7-hydroxylase and testosterone 16α-hydroxylase activities may be explained by the small sexual differences in the levels of the enzymes and/or the very large standard deviations of the means.) In general, testosterone at all doses was ineffective in increasing the subnormal monooxygenase activities of the capons. (In the case of cytochrome P450, androgen treatment resulted in enzyme concentrations that were not statistically different from that of either intacts or castrates.) In contrast, testosterone administration, particularly at the highest 5.0 mg/kg dose, increased the normally low activities of hexobarbital hydroxylase, coumarin 7-hydroxylase, androstenedione 6β-, 16α- and 2α-hydroxylases and testosterone 6β- and 16α-hydroxylases in the control hens to levels that were statistically the same as those found in roosters. Androgen injections to the hens were only slightly, if at all, effective in increasing the levels of cytochromes P450 and b_5, and ethoxyresorufin and 7-ethoxycoumarin *O*-deethylases.

DISCUSSION

To our knowledge, the present study is the first to report a sexual dimorphism in avian hepatic microsomal monooxygenases. With the exception of testosterone 2α-hydroxylase, whose concentration was only 10% that of androstenedione 2α-hydroxylase, the activities of the other eleven measured monooxygenases were consistently greater in roosters than in hens. Moreover, the fact that the enzyme activities in the capons were reduced to female-like levels suggests that the testes are

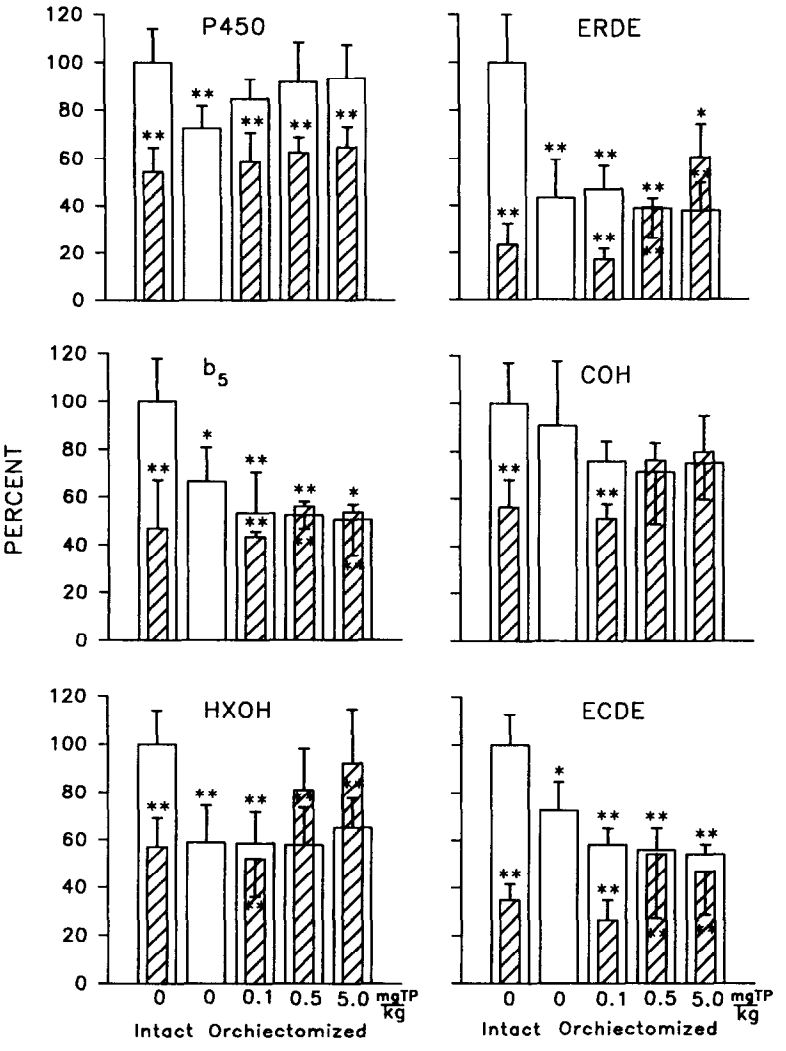

Fig. 1. Sexual dimorphisms in hepatic microsomal drug-metabolizing enzymes of the chicken. Open bars represent intact (roosters) and orchiectomized (capons) males and striped bars are for intact females (hens). Orchiectomies were performed at 6 weeks of age. At 13 months of age, hens and capons were injected (s.c.), daily, with either 0 mg (corn oil vehicle, alone), 0.1 mg, 0.5 mg or 5.0 mg testosterone propionate/kg body weight for 14 consecutive days and were killed on day 15. Roosters were injected only with the vehicle, but otherwise received the same treatment. Enzyme values (mean ± SD, $N \geq 4$) for each treatment group are presented as a percent of the enzyme value of the rooster (= 100%). Key: (*) $P < 0.05$ and (**) $P < 0.01$ when compared with the rooster. The rooster (i.e. 100%) microsomal enzyme values were 0.266 ± 0.037 and 0.336 ± 0.060 nmol/mg protein for cytochromes P450 and b_5, respectively; 3.08 ± 0.43, 1.99 ± 0.34 and 8.78 ± 1.10 nmol/min/mg protein for hexobarbital hydroxylase (HXOH), coumarin 7-hydroxylase (COH) and 7-ethoxycoumarin *O*-deethylase (ECDE), respectively; and 111 ± 24 pmol/min/mg protein for 7-ethoxyresorufin *O*-deethylase (ERDE).

responsible for maintaining the 2- to 4-fold sex differences.

It is not clear, however, why the testosterone injections were able to increase (i.e. masculinize) the monooxygenase activities in the intact hens, but were ineffective in the capons. Both published reports [15] and our own findings that all three doses of testosterone propionate resulted in the same maximal stimulation of comb growth suggest that the capons received greater than physiological androgen replacement. However, it is possible that the age of the cockerels at the time of castration could have contributed to the ineffectiveness of the androgen treatment. Due to the highly diffuse morphology of the chicken gonads, ovariectomies are virtually impossible at any age and orchiectomies tend to be limited to the first 2–8 weeks post-hatching when testicular boundaries are more definitive [26]. Unfortunately, orchiectomies at these early ages, before the completion of hypothalamic-pituitary maturation, may result in an insensitivity of this axis to the feedback effects of testosterone administration

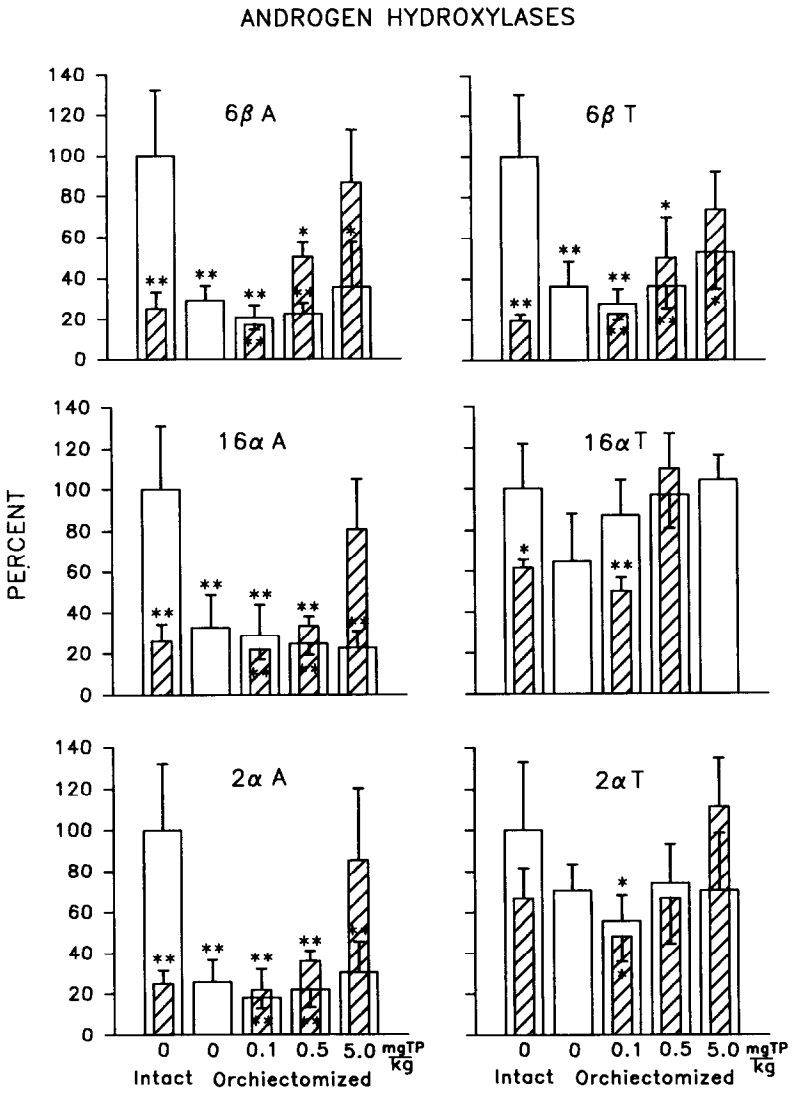

Fig. 2. Sexual dimorphisms in hepatic microsomal steroid hydroxylases of the chicken. Open bars represent intact (roosters) and orchiectomized (capons) males and striped bars are for intact females (hens). Orchiectomies were performed at 6 weeks of age. At 13 months of age, hens and capons were injected (s.c.), daily, with either 0 mg (corn oil vehicle, alone), 0.1 mg, 0.5 mg or 5.0 mg testosterone propionate/kg body weight for 14 consecutive days and were killed on day 15. Roosters were injected with only the vehicle, but otherwise received the same treatment. Enzyme values (mean ± SD; $N \geq 4$) for each treatment group are presented as a percent of the enzyme value of the rooster (= 100%). Key: (*) $P < 0.05$ and (**) $P < 0.01$ when compared with the rooster. The rooster (i.e. 100%) microsomal enzyme values were 580 ± 174, 146 ± 45 and 245 ± 78 pmol/min/mg protein for androstenedione (A) 6β-, 16α- and 2α-hydroxylases, respectively, and 159 ± 48, 124 ± 27 and 27 ± 9 pmol/min/mg protein for testosterone (T) 6β-, 16α- and 2α-hydroxylases, respectively.

[27, 28]. Thus, it is possible that as a result of their early castration, androgen replacement could no longer restore the subnormal monooxygenase levels in the capon. In this regard, it can only be speculated as to whether adult castration would have been as effective as early castration in reducing the activities of the hepatic monooxygenases of the rooster.

Another explanation of the differential effects of testosterone propionate on the hepatic monooxygenases of capons and hens involves the possibility that testosterone is not the testicular factor normally responsible for maintaining the elevated enzyme levels found in the rooster. It is conceivable that the ovaries in the intact hens were capable of metabolizing the administered testosterone to the "active" hormone. Without testes, the capons had no such ability. The avian testis is known to produce various forms of progestins, androgens and estrogens [29], any of which might be the actual enzyme inducer.

Attempts to compare the present findings in the bird to known systems in mammals is limited by our meager knowledge of avian drug metabolism. In contrast to the rat where more than a half-dozen or so constituent isoforms of cytochrome P450 have been characterized extensively at a molecular and regulatory level [3, 4, 10, 17, 23], not a single constituent form of cytochrome P450 has been identified in the bird [30]. Thus, while it is known that increased activities of 7-ethoxyresorufin *O*-deethylase, testosterone 6β-hydroxylase and testosterone 16α- and 2α-hydroxylases are specific indicators of enhanced expression of rat cytochromes P450 1A1, 3A and 2C11, respectively [3, 10, 23], no such conclusion can be made in the bird. Moreover, studies of phenobarbital-, methylcholanthrene-, and ethanol-inducible forms of avian cytochromes P450 have indicated that they are not mammalian orthologues, and are thus undetectable (i.e. immunounreactive) with antibodies raised against inducible forms of mammalian cytochromes P450 [13, 31–33]. Nevertheless, some broad comparisons between these two vertebrate classes can be made. In general, the sexual dimorphisms of the chicken hepatic microsomal monooxygenases are most comparable to those found in the rat. Aside from the obvious similarity that like the male rat, it is the male chicken that exhibits the greater enzyme activities, the magnitudes of the sex differences, if not identical, are often similar. In our hands, rat androstenedione and testosterone 6β-, 16α- and 2α-hydroxylases are about 4- to 7-times greater in the male [10, 17], which is similar to the present findings in the chicken. In contrast, however, the 40% gender difference in hexobarbital hydroxylase found in the chicken is comparable to that reported in mice [5, 6, 18], but quite different from the 500–700% sex difference seen in rats [2, 9, 10]. Moreover, the virtual absence of coumarin 7-hydroxylase from rat liver contrasts with the similar and substantial levels found in mice, rabbits, guinea pigs and humans [34] and with the chicken (present study).

While the sexual differences in the chicken hepatic monooxygenases suggest that the regulatory mechanisms and/or cytochrome P450 compositions are sexually dimorphic, the pharmacologic effects of these differences are not clear. However, since we found that the relative hepatic weights of the hen are 2½ times greater than those of the rooster (29.3 ± 5.3 and 12.1 ± 2.9 g/kg body weight, mean ± SD, respectively), the total drug-metabolizing capacities of the two sexes, although clearly dimorphic, are not nearly so different as the monooxygenase concentrations would indicate.

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