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SEX DIFFERENCES IN PITUITARY-GONADAL REGULATION OF HEPATIC 5α-REDUCTASE AND 16α-HYDROXYLASE

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SUMMARY

In an effort to more fully understand the mechanisms which regulate 5α-reductase and 16α-hydroxylase, these studies have examined the effects of prepubertal hypophysectomy (HYPOX) on day 21, castration (CASTR) on day 42, or HYPOX (day 21) + CASTR (day 42) on adult (day 65) hepatic enzyme activity and enzyme modulation (responsiveness) by postpubertal hormone administration. In intact adult rats, hepatic 5α-reductase is significantly higher in females compared to males whereas 16α-hydroxylase is significantly higher in male livers. Castration results in a feminization of both 5α-reductase (increase) and 16α-hydroxylase (decrease) in the male rat, but has no effect in the female rat. HYPOX or HYPOX-CASTR virtually abolishes 5α-reductase in both sexes; 16α-hydroxylase is also significantly decreased in male rats following HYPOX or HYPOX-CASTR. Conversely, HYPOX or HYPOX-CASTR of the female rat results in an elevation of 16α-hydroxylase. Female enzyme activities are unresponsive to the designated hormone treatment regardless of the surgical (endocrine) status of the animal. In contrast, estradiol-17β (E_2) treatment feminizes both 16α-hydroxylase (decrease) and 5α-reductase (increase) in the intact male rat, while testosterone propionate (TP) treatment masculinizes (increased 16α-hydroxylase and decreased 5α-reductase activity) both enzymes in the castrate male rat. Enzyme levels are unaltered by hormone treatment in the absence of the pituitary. These studies indicate that hepatic 16α-hydroxylase and 5α-reductase are modulated by E_2 and TP in the male rat, whereas equivalent doses do not change enzyme activities in female rats. These findings also suggest sex-dependent differences in sensitivity and/or regulatory mechanisms of these enzymes.

INTRODUCTION

Androgens are required for the morphologic differentiation of the reproductive system during the embryonic phase of development [1]. In adulthood, androgens reversibly stimulate the growth and development of certain androgen dependent tissues [2]. Androgens are also recognized to be important regulators of major metabolic processes in the adult rat, including the regulation of certain enzyme activities [3].

Recently, sexual differences in rat hepatic steroid metabolism have been reported in the literature [4–5]. Male rats have a more active steroid hydroxylating system than female rats [5], while females have a much higher 5α-reductase activity than do males [4]. In the absence of neonatal testosterone exposure (*i.e.* female rats or neonatally castrated male rats), adult rats develop a female-type of metabolism; a masculine-type of adult metabolism appears to require neonatal androgen exposure [6–9]. Sexual differences in metabolism are not evident until puberty (around 30 days of age) [10–11], and are irreversible in adult rats although the hormone exposure responsible for these sexual differences had occurred neonatally [6–9]. Sexual differentiation of hepatic metabolism is not limited to the steroid metabolizing systems, but is also observed in other enzyme systems [12–13]. Such a predetermined but delayed response of adult enzyme activities to neonatal hormone exposure is referred to as programming, or imprinting, and is quite different from the more rapid and reversible response to hormone which is seen in the postpubertal animal [14]. Furthermore, sexual differences in the modulation of adult enzyme activity by hormone treatment have been demonstrated, and are also thought to be the result of neonatal programming [14–15]. It has been suggested that the sexual differentiation of the brain [16–20] may be responsible for the sexual differentiation of hepatic steroid metabolism through the hypothalamo-hypophyseal-gonadal axis, via the release of hormone(s) from the anterior pituitary [21–23].

The present studies were performed to further define the role of the hypothalamo-hypophyseal-gonadal axis in the modulation of adult hepatic 5α-reductase and 16α-hydroxylase by hormone stimulation and in the sexual differentiation of these enzymes. These enzymes were chosen because they exhibit opposite sexual development; hepatic 5α-reductase is higher in female rats, while hepatic 16α-hydroxylase is higher in male rats. Numerous studies have previously examined the effects of hypophysectomy on hepatic enzyme activity [21–22, 24–27], but none of these studies have included 16α-hydroxylase, which is catalyzed by the cytochrome P-450 mono-

oxygenase system [28]. Both enzymes are microsomal, but the enzyme activities are regulated differently since 5α-reductase is not associated with the P-450 system [28]. Testosterone propionate or 17β-estradiol were administered to intact, castrated, hypophysectomized, or hypophysectomized-castrated male or female rats for one week prior to measurement of enzyme activity to assess the direct role of androgens and estrogens in the modulation of adult enzyme activity.

METHODS

Chemicals

The compounds NADPH and EGTA were purchased from Sigma Chemical Co. (St. Louis, Mo.). The radioactive substrates [4-^{14}C]-testosterone and [4-^{14}C]-androst-4-ene-3,17-dione were obtained from New England Nuclear (Boston, MA). Purity of radiolabels was checked in each experiment by thin-layer chromatography. Corresponding unlabeled steroid hormones, in addition to 5α-dihydrotestosterone and androst-4-ene-16α-ol-3,17-dione were purchased from Steraloids, Inc. (Wilton, NH). Thin layer chromatography plates (precoated Silica Gel without fluorescent indicator) were obtained from EM Laboratories, Inc. (Elmsford, NY). Buffer reagents were obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ).

Animals and tissue preparation

Rats were subjected to various surgical procedures at the Charles Rivers Breeding Laboratories, Inc. (Wilmington, Ma.) before being shipped to NIEHS. Hypophysectomy (HYPOX) was performed at 21 days of age, castration (CASTR) was performed at 42 days of age, or both procedures were performed (HYPOX + CASTR) at the appropriate ages. Rats were treated subcutaneously with vehicle (propylene glycol), 17β-estradiol (E_2–100 μg/kg), or testosterone propionate (TP-100 μg/kg) from days 58–64. Rats were sacrificed 24 h after the last injection and livers were rapidly excised and placed on ice. A 20% liver homogenate was prepared in ice cold TMK buffer (10 mM Tris, 14 mM $MgCl_2$ and 0.6 M KCl, pH 7.2) using a Potter–Elvehjem homogenizer equipped with a loosely fitting Teflon pestle. Homogenates were centrifuged at 20,000 *g* for 10 min; the supernatant was decanted and spun at 105,000 *g* for 60 min. The microsomal pellet was resuspended by hand in Tris buffer (150 mM, pH 7.4) with six strokes of the homogenizer such that 1 ml of suspension contained microsomes from 25 mg fresh liver (approximately 0.8 mg microsomal protein).

Enzyme assays

Aliquots of hepatic microsomal suspension containing approximately 50 μg protein were placed into incubation tubes containing the incubation medium. Incubation mixtures consisted of 0.5 mM NADPH, 0.5 mM EGTA, 3.42 μM radio-active substrate (testosterone-4-^{14}C or androst-4-ene-3,17-dione-4-^{14}C), and potassium phosphate buffer (0.1 M, pH 7.4) to make a total volume of 0.2 ml. In addition, unlabeled substrate was added to achieve a total substrate concentration of 40 μM for male liver samples and 107 μM for female liver samples. This concentration of substrate was saturable for both 5α-reductase and 16α-hydroxylase. Samples were incubated in a Dubnoff metabolic shaker for 20 min at 37°C. These conditions provide a linear production of product with respect to time and enzyme or substrate concentrations. Reactions were stopped by adding 1 ml chloroform–methanol (2:1, V/V). Samples were vortexed, and radio-steroids were extracted and dried under nitrogen. Samples were reconstituted with 40 μl chloroform and spotted on thin layer chromatography plates with the appropriate unlabeled metabolites (*i.e.* testosterone and 5α-dihydrotestosterone for the 5α-reductase assay or androst-4-ene-3,17-dione and androst-4-ene-16α-ol-3,17-dione for the 16α-hydroxylase assay). Plates spotted with 5α-reductase metabolites were developed in a solvent system (100 ml) of chloroform–ether (7:3, v/v); 16α-hydroxylase metabolites were developed in 100 ml of chloroform–ethyl acetate–ether (5:3:2, by vol.). Upon removal from the tanks, plates were sprayed with a solution of *p*-anisaldehyde–conc. sulfuric acid–glacial acetic acid (0.5:1:100, by vol.) and heated at 100°C for 30 min to allow visualization of the separated metabolites. Enzymatically-produced radioactive metabolites (*i.e.* [4-^{14}C]-5α-dihydrotestosterone or [4-^{14}C]-androst-4-ene-16α-ol-3,17-dione) were scraped from the plates, placed in scintillation vials and counted on a Beckman LS-9000 liquid scintillation counter. 5α-Reductase activity was determined by the amount of [^{14}C]-5α-dihydrotestosterone (5α-DHT) formed; 16α-hydroxylase activity was calculated by the amount of [^{14}C]-androst-4-ene-16α-ol-3,17-dione-4 formed. All results were expressed as specific enzyme activities (pmol/min/mg protein).

RESULTS

The surgical procedures used produced marked changes in liver and body weights (Table 1). In intact rats, male livers are heavier than female livers. Castration equalizes the liver weights of the two sexes. Hypophysectomy, either alone or in combination with castration, causes arrest in growth of liver weights and body weights of both sexes. Because of the concommitant decrease in body weight following hypophysectomy (not shown), the liver to body weight ratio remains constant for all animals (Table 1).

Hepatic microsomal 5α-reductase activity is at least 10-fold higher in the intact female rat than in the intact male rat (Fig. 1A). Castration (CASTR) produces a significant increase (feminization) of male hepatic 5α-reductase activity, whereas ovariectomy

Table 1. The effects of surgical procedures on liver and body weights of adult rats*

Surgical procedure	Liver weights (gm) Male	Female	Liver/body weight × 10^{-2} Male	Female
Intact	11.6 ± 1.2	8.1 ± 0.9	4.1 ± 0.4	3.8 ± 0.4
Castration	10.9 ± 0.9	10.0 ± 1.3	3.8 ± 0.2	4.4 ± 0.4
Hypophysectomy	2.9 ± 0.3	2.9 ± 0.3	4.0 ± 0.3	4.2 ± 0.3
Hypox-castrate	2.7 ± 0.3	2.6 ± 0.2	3.9 ± 0.2	3.8 ± 0.3

* All weights were recorded at 65 days of age. Castrations were performed on day 42, hypophysectomies were performed on day 21, and combination surgery was performed on these designated days. Values are expressed as mean ± standard deviation, and are derived from at least 6 animals.

does not significantly alter female hepatic 5α-reductase activity. 5α-Reductase activity is approximately 5-fold higher in CASTR females than in CASTR males. Hypophysectomy (HYPOX) or HYPOX plus CASTR results in dramatic decreases of 5α-reductase activity in both males and females. In fact, these surgical procedures (i.e. HYPOX or HYPOX-CASTR) essentially equalize male and female 5α-reductase activities at a negligible level of activity.

In contrast with 5α-reductase, hepatic microsomal 16α-hydroxylase activity is 12-fold higher in intact males than in intact females (Fig. 1B). As seen with 5α-reductase, CASTR produces a feminization (decrease) in 16α-hydroxylase activity in the male, with no alteration in female 16α-hydroxylase activity following gonadectomy. In contrast to 5α-reductase, 16α-hydroxylase activities in CASTR males and CASTR females are equivalent. HYPOX or HYPOX-

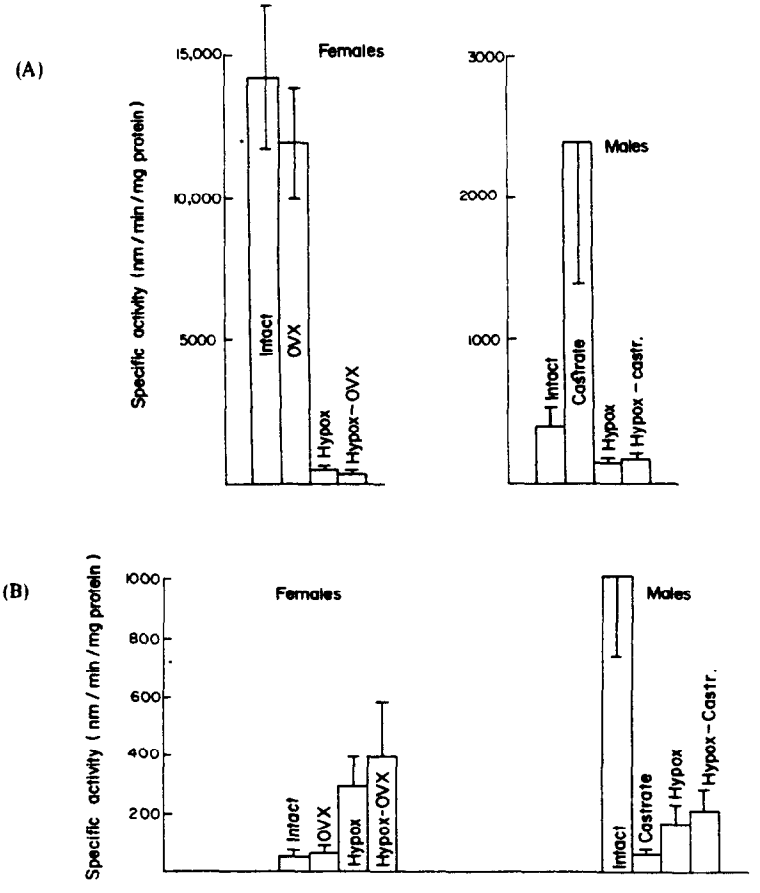

Fig. 1. The effect of surgical manipulations on hepatic 5α-reductase (Fig. 1A) and 16α-hydroxylase (Fig. 1B) activities. Rats were hypophysectomized (HYPOX) on day 21, castrated (CASTR) on day 42, or both. Enzyme activity was measured on day 65. Each bar represents the mean ± standard deviation of at least 6 animals.

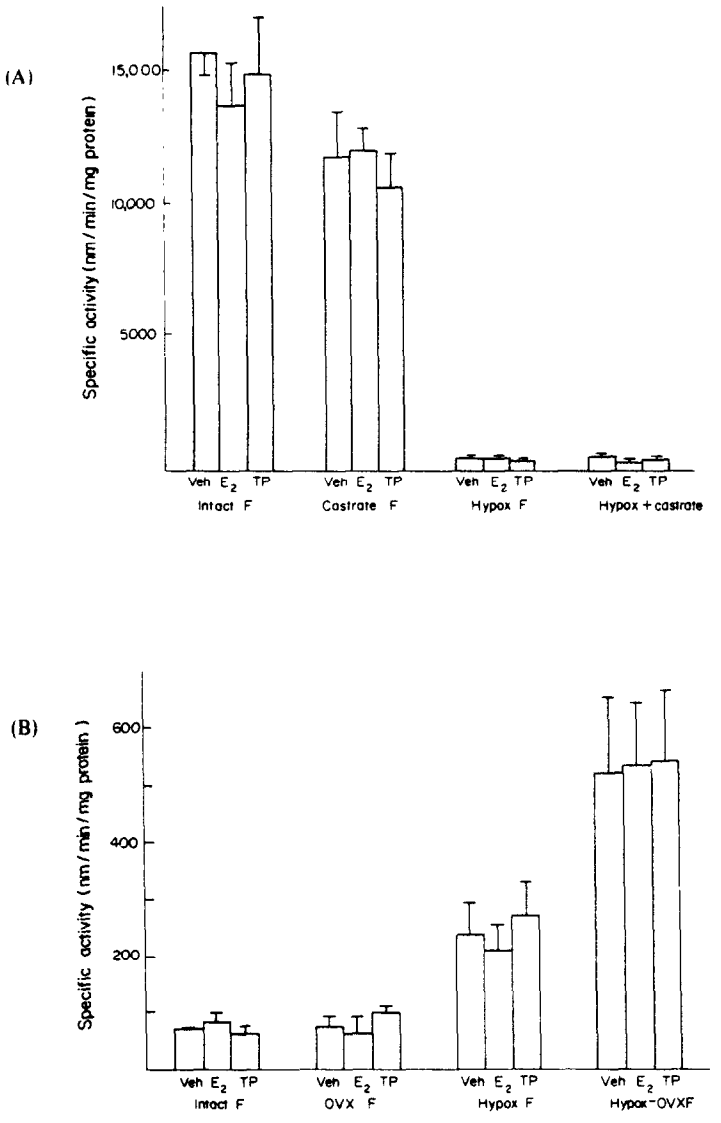

Fig. 2. The effect of 17β-estradiol (E_2) or testosterone propionate (TP) treatment on hepatic 5α-reductase (Fig. 2A) or 16α-hydroxylase (Fig. 2B) activity of surgically modified female rats. Hypox was performed on day 21, castration on day 42; hormones were administered s.c. from days 58–64 at a dose of 100 μg/kg. Enzyme activity was measured on day 65. Each bar represents the mean ± standard deviation of at least 6 animals.

CASTR produces significant increases in female 16α-hydroxylase (masculinization) and decreases in male 16α-hydroxylase (feminization) such that hypophysectomized males and females have equivalent levels of 16α-hydroxylase activity.

The degree of responsiveness of these enzymes to 17β-estradiol (E_2) or testosterone propionate (TP) treatment was examined following each of these surgical manipulations. Neither E_2 nor TP treatment has any effect on either the 5α-reductase (Fig. 2A) or 16α-hydroxylase (Fig. 2B) activity in female rats, regardless of surgery. In contrast, male rats do appear to be responsive to hormone treatment. 5α-reductase is significantly increased (feminized) by E_2 treatment of the intact male and is significantly decreased (masculinized) by TP treatment of the CASTR male (Fig. 3A). Similarly, 16α-hydroxylase is feminized (decreased) by E_2 treatment of the intact male, and was masculinized (increased) by TP treatment of the CASTR male rat (Fig. 3B). Enzyme responsiveness to hormone treatment of both 5α-reductase and 16α-hydroxylase was pituitary dependent since no hormonally-induced changes in enzyme active were evident in hypophysectomized males.

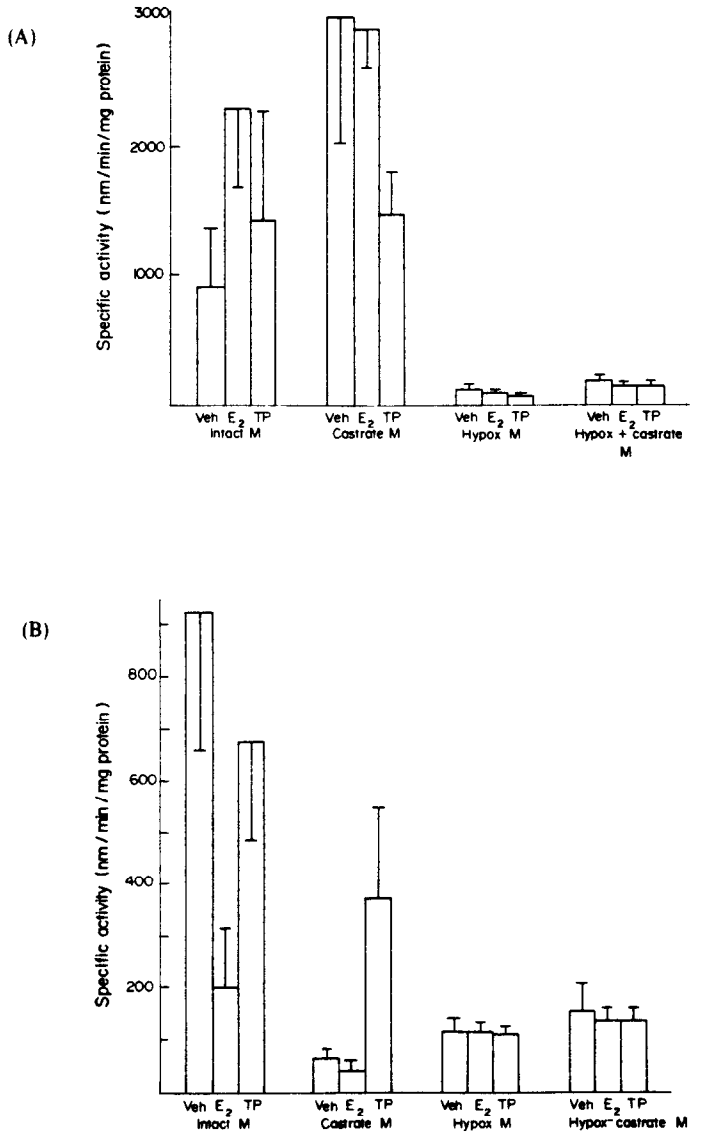

Fig. 3. The effect of E_2 or TP treatment on hepatic 5α-reductase (Fig. 3A) or 16α-hydroxylase (Fig. 3B) activity of surgically modified male rats. Hypox was performed on day 21, castration on day 42; hormones were administered s.c. from days 58–64 at a dose of 100 μg/kg. Enzyme activity was measured on day 65. Each bar represents the mean ± standard deviation of at least 6 animals.

DISCUSSION

Recent reports in the literature have demonstrated that neonatal testosterone administration can alter hepatic steroid metabolizing enzymes in the adult rat [6–9]. In addition to predetermining the resultant absolute enzyme activities of the adult, this "programming" process also appears to alter the ability of certain enzymes to respond to postpubertal steroid hormone administration. Our studies were performed to further define the sex differences in the regulation of adult hepatic 16α-hydroxylase and 5α-reductase following surgical manipulation and/or treatment with steroid hormones. These enzymes were chosen because they exhibit sexual differences in the adult rat; *i.e.* hepatic 5α-reductase activity is higher in females, while 16α-hydroxylase activity is higher in males.

The testes appear to be more important in maintaining normal male hepatic enzyme activities than are the ovaries in maintaining female hepatic enzyme activity, particularly with regard to 16α-hydroxylase. Castration of the male results in a slight but significant feminization (increase) of 5α-reduction and a

complete feminization (decrease) of 16α-hydroxylation to normal female levels. Similarly, Yates[4] and Einarsson *et al.*[6] observed a feminization of male 5α-reductase following castration; but unlike our studies 16α-hydroxylase levels in males were not significantly changed by castration [6]. This difference in results may be attributed to varying protocols; the present studies measure enzyme activities about 3 weeks after castration, while Einarsson *et al.*[6] waited for 13 weeks. In contrast with the male system, gonadectomy of the female does not alter either 5α-reductase or 16α-hydroxylase activity, suggesting that male hepatic enzymes are more sensitive to gonadal steroid regulation than are females. Likewise, these female enzymes were unaltered by replacement therapy with either E_2 or TP. The lack of response of female hepatic steroid-metabolizing enzymes to the removal of estrogen (*i.e.* castration) agrees with previous reports [6], although the lack of response to hormone administration observed in our studies contradicts this report. Stimulation of 16α-hydroxylation and decreased 5α-reduction was seen following long-term TP administration to female rats (1 mg TP per day from 7 to 20 weeks of age) [6]. Previous studies have not examined the effects of E_2 on 16α-hydroxylase activities in both intact and surgically altered animals [6, 26].

The insensitivity of female hepatic enzymes which was observed in the present studies is compatible with the hypothesis [14, 15] that adult enzyme responsiveness is acquired through a neonatal androgen programming process of the developing brain. Accordingly, since female rats are not exposed neonatally to testicular androgens, female hepatic enzyme activities might not be hormone responsive. Male rats, which are neonatally programmed by testicular androgens, are responsive to hormone treatment in these studies. Both enzymes are masculinized in castrate male rats by treatment with TP, and are feminized in intact male rats by treatment with E_2.

Hepatic 5α-reduction and 16α-hydroxylation appear to be under pituitary control in both male and female rats. Prepubertal hypophysectomy appears to prevent sex differentiation of 5α-reductase and 16α-hydroxylase activities suggesting that the pituitary provides a positive modulation of enzyme activity in these cases. These results and others suggest that any programming of hepatic 5α-reductase by the hypothalamo hypophyseal-gonadal axis was complete by puberty [26, 29]. However, 16α-hydroxylase and 5α-reductase appear to be under different regulatory control since we have shown that gonadectomy equalizes male and female 16α-hydroxylase activities whereas a pronounced sex difference on 5α-reductase persists in gonadectomized rats.

Contrary to the positive modulation of the pituitary on male or female hepatic 5α-reduction and on male 16α-hydroxylation, the pituitary appears to be a negative modulator of 16α-hydroxylase activity in female rats, since prepubertal hypophysectomy of the female results in increased 16α-hydroxylase activity. Whether the negative influence of the pituitary is direct or indirect remains unclear. The pituitary has also been observed to elicit a suppressive effect on hepatic histidase [27].

Our studies characterize in a systematic manner the sex differences in the hormonal modulation of these two hepatic enzymes, which have different patterns of sexual differentiation. The male hepatic enzyme responsiveness to hormone treatment is abolished by prepubertal hypophysectomy, as well as by postpubertal hypophysectomy [12, 14, 24–25, 29–30], indicating that an intact pituitary is required for the expression of enzyme responsiveness to be activated. In addition, absolute levels of adult hepatic 16α-hydroxylase and 5α-reductase in the male and female become more similar following prepubertal hypophysectomy, suggesting a role of the pituitary in the maintainence of normal hepatic enzyme activities. The enzyme control mechanisms of 16α-hydroxylase and 5α-reductase activity are probably more complex than can be explained by a pituitary "feminizing factor" and these enzymes are under different types of regulatory control.

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