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Impact of Tamoxifen on Peripubertal Androgen Imprinting of Rat Hepatic Cytochrome P450 2C11, Cytochrome P450 3A2, and Steroid 5α-Reductase

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ABSTRACT. Expression of sex-dependent rat hepatic cytochromes P450 and steroid 5α-reductase is regulated mainly by the sex-specific pattern of growth hormone (GH) secretion and is subject to androgen imprinting. Since tamoxifen suppresses GH pulse amplitude and nadir levels, we investigated the effect of tamoxifen on peripubertal testosterone imprinting of hepatic CYP2C11, CYP3A2, CYP2A1, and steroid 5α-reductase. Prepubertal tamoxifen administration (5 mg once daily s.c. on days 28 and 29 of age) to non-ovariectomized female Sprague–Dawley rats did not affect hepatic microsomal CYP2C11-dependent testosterone 2α-hydroxylase, CYP3A-mediated testosterone 6β-hydroxylase, CYP2A1-dependent testosterone 7α-hydroxylase, or steroid 5α-reductase activity in adult rats. Testosterone treatment (5 μmol/kg, s.c., once daily) of intact female rats during either puberty (days 35–49 of age) or adult life (days 69–77 of age) had no effect on these enzyme activities in adult (78-day-old) female rats, but the same treatment given during both of these periods induced the male-specific testosterone 2α- and 6β-hydroxylase activities and suppressed the female-predominant testosterone 7α-hydroxylase and steroid 5α-reductase activities, indicating that peripubertal testosterone administration imprints the adult androgen responsiveness but not the basal levels of these enzyme activities in non-ovariectomized female rats. However, peripubertal androgen imprinting of the basal levels of testosterone 2α-hydroxylase and steroid 5α-reductase activities was observed in female rats administered tamoxifen prepubertally. Tamoxifen pretreatment also enhanced testosterone imprinting of the adult androgen responsiveness of testosterone 2α- and 6β-hydroxylase and steroid 5α-reductase activities. The enhanced testosterone hydroxylase activities were, however, not associated with an increase in microsomal NADPH-cytochrome P450 reductase activity, but were accompanied by elevated hepatic CYP2C11 and CYP3A2 protein levels. Overall, the present study indicates that prepubertal tamoxifen administration does not interfere with the normal sex differentiation of the gender-dependent hepatic cytochromes P450 and steroid 5α-reductase, but this drug modulates peripubertal androgen imprinting of CYP2C11, CYP3A2, and steroid 5α-reductase in adult female rats. BIOCHEM PHARMACOL 51;3:357–368, 1996.

KEY WORDS. cytochrome P450; steroid 5α-reductase; tamoxifen; testosterone; androgen imprinting; growth hormone

Various proteins in rat liver are expressed in a sex-dependent manner. These include the female-predominant steroid 5α-reductase [1] and certain cytochrome P450 (CYP) enzymes [2], such as the male-specific CYP2A2, CYP2C11, CYP2C13, CYP3A2, and CYP4A2, the female-specific CYP2C12, and the female-predominant CYP2A1 and CYP2C7 [3]. The precise hormonal mechanism for the sex-dependent expression of these enzymes is not understood completely, but the sexually dimorphic pattern of GH‡ secretion appears to play a major regulatory role [3, 4], although GH-independent mechanisms may be relevant in some instances [5, 6]. Whereas GH secretion is intermittent and pulsatile in the male rat, it is more continuous in the female rat [7, 8]. The "male" pattern of GH secretion induces CYP2C11 [9–12], whereas the "female" pattern stimulates CYP2A1 [13], CYP2C7 [14, 15], CYP2C12 [10, 11, 16], and steroid 5α-reductase [12, 17]. In addition to GH, other hormones also influence the expression of the sex-dependent hepatic cytochromes P450 and steroid 5α-reductase. For example, estrogen induces CYP2C12 [18, 19] and steroid 5α-reductase [17, 18] in female rats, whereas androgens

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‡ *Abbreviations:* GH, growth hormone; IgG, immunoglobulin G; and 4-MA, 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one.

are required for the expression of CYP2A2 [20], CYP2C11 [9, 18, 21–23], CYP2C13 [24], CYP3A2 [25], and CYP4A2 [26] in male rats. However, the gonadal hormones are not believed to directly influence the hepatic expression of these enzymes [24, 27]. Rather, they appear to act on the hypothalamus [17], which controls the sex-specific pattern of pituitary GH secretion in the rat [8].

The basal levels and adult androgen responsiveness of specific hepatic cytochromes P450 and steroid 5α-reductase in the rat can be irreversibly programmed or "imprinted" by prior exposure to androgen. Imprinting of these enzymes can occur not only following exposure to androgen during the neonatal period [e.g. Refs. 28 and 29], but also during puberty [30–32]. We have established recently that peripubertal testosterone imprinting of hepatic CYP2C11 and steroid 5α-reductase requires relatively low but prolonged exposure to the androgen and that this occurs by a pretranslational mechanism, leading to long-lasting changes in microsomal drug activation. §The neuroendocrine mechanism(s) responsible for androgen imprinting of these sex-dependent enzymes is not known.

Tamoxifen is a non-steroidal, partial antagonist of the estrogen-receptor, and it is used clinically in the management of breast cancer [33]. Recent animal and clinical studies have demonstrated that this cancer chemotherapeutic drug modifies the secretory dynamics of GH [34, 35]. Tamoxifen administration to male or female rats, with a dosage regimen associated with anti-tumor activity [36], leads to a major suppression of GH pulse amplitude and nadir levels that persists for at least 7 weeks after cessation of drug administration [34].

The sexually dimorphic pattern of pituitary GH secretion is important not only in maintaining the expression of sex-dependent rat hepatic cytochromes P450 and steroid 5α-reductase, but it may also play a role in peripubertal androgen imprinting. Since tamoxifen leads to a prolonged suppression of GH pulse amplitude and nadir levels [34], the present study was undertaken to investigate the effect of tamoxifen on peripubertal testosterone imprinting of CYP2C11, CYP3A2, CYP2A1, and steroid 5α-reducatase in the non-ovariectomized, female rat model. Our results show that prepubertal tamoxifen administration does not interfere with the normal sex differentiation of these hepatic enzymes, but this drug modulates peripubertal androgen imprinting of CYP2C11, CYP3A2, and steroid 5α-reductase.

MATERIALS AND METHODS

Chemicals

Testosterone enanthate, tamoxifen citrate, cytochrome c and NADPH were purchased from the Sigma Chemical Co. (St. Louis, MO), and [4-^{14}C]testosterone (58 mCi/mmol) was obtained from Amersham Canada Ltd. (Oakville, Ontario, Canada). 4-MA was a gift from Merck Sharp and Dohme Research Laboratories (Rahway, NJ).

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Animals

Male and female Sprague–Dawley rats were purchased from the Charles River Co. (Montreal, Quebec, Canada) and were allowed to acclimatize in our animal care facility for at least 7 days prior to initiation of treatment. The temperature of the room was maintained at 22°, and fluorescent lighting in the room was controlled by an automatic timer (8:00 a.m. on, 10:00 p.m. off). The rats were housed on Lobund® corn cob bedding (Paxton Processing Ltd., Paxton, IL, U.S.A.) and were provided with Rodent Laboratory Chow No. 5001 (Ralston Purina Canada Inc., Longueuil, Quebec, Canada) and tap water *ad lib.* up to the time of being killed.

Treatment of Animals

Female rats were injected s.c. with tamoxifen citrate (5 mg free base), testosterone enanthate (5 μmol/kg once daily) or an equal volume of corn oil (vehicle) as indicated in each table or figure legend. The dosage regimen of tamoxifen employed in the present study is known to suppress GH pulse amplitude and nadir levels for up to 7 weeks after drug administration in adult rats [34], and the injection protocol for testosterone is effective in imprinting hepatic CYP2C11 protein and activity levels in adult female rats [32]. Untreated adult male rats were included as controls. All rats were killed on day 78 of age. Livers were quickly excised, washed in ice-cold 1.15% KCl/50 mM Tris (pH 7.5) and used immediately to prepare microsomes by the method of Lu and Levin [37]. The final microsomal pellet was suspended in 0.25 M sucrose, and aliquots of the suspension were stored at −80° until used. Blood was collected and allowed to clot at 4°. Serum was prepared by centrifugation and then stored at −20° until used.

Testosterone Hydroxylase Assay

Microsomal testosterone hydroxylase activities were determined by an HPLC method [38] as described previously [39]. Reactions were carried out at 37° for 5 min in 1-mL incubation mixtures containing 100 mM potassium phosphate (pH 7.4), 3 mM $MgCl_2$, 2.5 μM 4-MA (to inhibit steroid 5α-reductase), 0.25 mM testosterone and 0.5 mg microsomal protein. Microsomal testosterone oxidation was initiated by the addition of NADPH (1 mM final concentration) and stopped 5 min later with 6 mL dichloromethane. 16-Keto-testosterone (3 nmol, internal standard) was then added to the reaction mixture. Hydroxylated testosterone metabolites were extracted with dichloromethane and separated by reverse-phase HPLC as detailed elsewhere [39]. Metabolite quantitation was carried out by the peak height method, using the MAXIMA 820 Chromatography Workstation® (Version 3.3) software program (Millipore Ltd., Mississauga, Canada).

Steroid 5α-Reductase Assay

Microsomal steroid 5α-reductase activity was determined by the reduction of [4-^{14}C]testosterone to 5α-[4-^{14}C]dihydrotestosterone by a TLC method [40]. Reactions were carried out at

37° in 200-μL incubation mixtures containing 100 mM HEPES (pH 7.4) 0.1 mM EDTA, 50 μM ^{14}C-labeled testosterone and 30 μg microsomal protein. Microsomal testosterone metabolism was initiated by the addition of NADPH (1 mM final concentration) and stopped 10 min later with 1 mL ethyl acetate. The reaction mixture was then extracted twice with ethyl acetate. Subsequently, the reaction products were chromatographed on silica gel TLC plates developed with dichloromethane:acetone (4:1, v/v) followed by chloroform: ethyl acetate:absolute ethanol (4:1:0.7, by vol.). Metabolites were localized by autoradiography and quantitated by liquid scintillation counting.

Other Microsomal Assays

Total hepatic microsomal cytochrome P450 content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum using a molar extinction coefficient of 91 cm^{-1} mM^{-1} [41]. Microsomal protein concentration was determined using the Bio-Rad Protein Assay Kit with absorbance measured at 595 nm. Microsomal NADPH–cytochrome P450 reductase activity, expressed as nanomoles cytochrome *c* reduced at 30° per minute per milligram of microsomal protein, was determined spectrophotometrically [42] at 550 nm in 1-mL incubation mixtures containing potassium phosphate (300 mM, pH 7.7), EDTA (0.1 mM), liver microsomes (20 μg), cytochrome *c* (0.5 mg) and NADPH (2 mg).

Anti-cytochrome P450 Antibodies

Monospecific rabbit anti-rat CYP2C11 IgG was purified and immunoabsorbed as described previously [23]. Monoclonal mouse anti-rat CYP3A2 IgG and polyclonal sheep anti-rat CYP2A1 IgG were obtained from Dr. Paul E. Thomas (Rutgers University, Piscataway, NJ, U.S.A.). The anti-CYP3A2 antibody preparation has been shown not to cross-react with rat CYP3A1 [43].

SDS–PAGE and Immunoblot

SDS–PAGE was performed according to the method of Laemmli [44] in a separating gel containing 7.5% acrylamide and a stacking gel containing 3% acrylamide. The amount of microsomal cytochrome P450 loaded in each lane is indicated in the legends to Figs. 5 and 6. Proteins on the SDS electrophoretic gel were electroeluted and transferred onto nitrocellulose membrane, according to a published protocol [45]. Each blot was then probed with anti-CYP2C11 IgG (50 μg/mL), anti-CYP3A2 IgG (0.5 μg/mL), or anti-CYP2A1 IgG (5.5 μg/mL). The secondary antibody used to locate bound anti-CYP2C11 IgG and anti-CYP3A2 IgG is indicated in the legend to Figs. 5 and 6, respectively. Affinity-purified, peroxidases-labeled, rabbit anti-sheep IgG (γ) (Life Technologies, Burlington, Ontario, Canada) at a dilution of 1:250 was used to locate bound anti-CYP2A1 IgG. Immunoreactive CYP2C11 protein was detected by a colorimetric method using the Amplified Alkaline Phosphatase Immuno-Blot Assay Kit® (Bio-Rad Laboratories, Hercules, CA, U.S.A.), whereas immunoreactive CYP3A2 and CYP2A1 proteins were detected by chemiluminescence using the ECL™ Western Blotting Kit (Amersham Life Science, Oakville, Ontario, Canada). CYP2C11, CYP3A2, and CYP2A1 proteins on the immunoblots were quantitated by densitometry (VISAGE 110 Bio Image Analyzer, Bio Image, Ann Arbor, MI, U.S.A.). In the case of CYP2C11, the quantitation was carried out in the presence of purified rat CYP2C11 standards [23].

Serum Hormone Assays

Serum testosterone and 17β-estradiol concentrations were measured by solid-phase ^{125}I radioimmunoassay with the ImmuChem™ Direct Testosterone kit and the ImmuChem™ Direct Estradiol-17β kit, respectively (ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.).

Statistics

The significance of the difference between the means of treatment groups was evaluated by one-way ANOVA and where applicable, was followed by the Newman–Keuls multiple range test. The level of significance was set *a priori* at $P < 0.05$

RESULTS

Peripubertal Androgen Imprinting of Testosterone Hydroxylase and Steroid 5α-Reductase Activities

Hepatic microsomal testosterone 2α-, 6β-, and 7α-hydroxylase activities are selective for CYP2C11 [32, 46], CYP3A [46], and CYP2A1 [13, 47, 48], respectively. Therefore, these activities were employed as catalytic markers for these specific cytochrome P450 enzymes. As shown in Table 1, neither peripubertal nor adult testosterone treatment of non-ovariectomized female rats altered hepatic microsomal testosterone 2α-, 6β-, or 7α-hydroxylase or steroid 5α-reductase activity. By contrast, the combination of peripubertal and adult testosterone administration increased testosterone 2α- and 6β-hydroxylase activities, whereas the same treatment decreased testosterone 7α-hydroxylase and steroid 5α-reductase activities. However, the magnitude of these activities was still significantly different from that found in untreated adult male rats, indicating that complete masculinization was not achieved. This pattern of response of microsomal testosterone 2α-, 6β-, and 7α-hydroxylase activities to androgen is in agreement with our previous finding [32]. Overall, these results establish that peripubertal testosterone administration imprints the adult androgen responsiveness, but not the basal levels, of testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities in non-ovariectomized, adult female rats.

Effect of Prepubertal Tamoxifen Administration on Testosterone Hydroxylase and Steroid 5α-Reductase Activities in Adult Female Rats

Treatment of adult female rats with tamoxifen leads to prolonged suppression of GH pulse amplitude and nadir levels

TABLE 1. Effects of peripubertal and adult testosterone treatment on hepatic microsomal testosterone hydroxylase and steroid 5α-reductase activities in adult female rats

Sex	Treatment		Testosterone hydroxylase*			Steroid 5α-reductase†
	Days 35–49	Days 69–77	2α	6β	7α	
F	Corn oil	Corn oil	0.03 ± 0.01	0.48 ± 0.08	1.14 ± 0.13	17.6 ± 0.5
F	TE	Corn oil	0.06 ± 0.03	0.46 ± 0.07	0.87 ± 0.07	17.1 ± 0.9
F	Corn oil	TE	0.02 ± 0.01	0.39 ± 0.09	0.93 ± 0.06	17.8 ± 0.7
F	TE	TE	0.47 ± 0.06‡	0.84 ± 0.16‡	0.65 ± 0.13‡	7.7 ± 1.3‡
M	UT	UT	0.91 ± 0.14	1.35 ± 0.18	0.21 ± 0.04	1.5 ± 0.2

Non-ovariectomized female (F) rats were injected s.c. with testosterone enanthate (TE: 5 μmol/kg once daily) or corn oil (vehicle) during puberty (days 35–49) and/or adult life (days 69–77). All rats, including untreated (UT) males (M), were killed on day 78 of age. Results are means ± SEM for 6 individual rats per treatment group.

* Activity is expressed as nmol/min/nmol total cytochrome P450.

† Activity is expressed as mol/min/mg microsomal protein.

‡ Significantly different from the corn oil-treated, female control group and significantly different from the untreated male group ($P < 0.05$).

[34]. To determine whether prepubertal tamoxifen administration affects sex-dependent hepatic enzyme activities in adult life, non-ovariectomized female rats were administered tamoxifen (5 mg free base once daily) on days 28 and 29 of age and then were killed on day 78 of age. However, as shown in Table 2, tamoxifen pretreatment did not affect hepatic microsomal testosterone 2α-, 6β-, and 7α-hydroxylase or steroid 5α-reductase activity, suggesting that prepubertal tamoxifen administration did not interfere with the normal sex differentiation of these enzymes in the adult female rat.

Effect of Prepubertal Tamoxifen Administration on Testosterone Hydroxylase and Steroid 5α-Reductase Activities in Female Rats Treated with Androgen during Adulthood

To determine whether prepubertal tamoxifen administration alters the responsiveness of adult rats to androgens, non-ovariectomized female rats were administered tamoxifen (5 mg free base once daily) on days 28 and 29 of age, then were given testosterone on days 69–77, and were killed on day 78. As shown in Fig. 1, tamoxifen did not alter significantly hepatic microsomal testosterone 2α-, 6β-, or 7α-hydroxylase activity and only modestly decreased (25%) steroid 5α-reductase activity in female rats administered testosterone during adulthood. These findings indicate that prepubertal tamoxifen administration has little or no effect on sex-dependent cytochrome P450 and steroid 5α-reductase activities in intact female rats treated with testosterone during adult life.

TABLE 2. Effect of prepubertal tamoxifen administration on hepatic microsomal testosterone hydroxylase and steroid 5α-reductase activities in adult female rats

Enzyme activity	Treatment	
	Corn oil	Tamoxifen
Testosterone 2α-hydroxylase*	0.03 ± 0.01	0.02 ± 0.01
Testosterone 6β-hydroxylase*	0.48 ± 0.08	0.55 ± 0.07
Testosterone 7α-hydroxylase*	1.14 ± 0.13	1.20 ± 0.15
Steroid 5α-reductase†	17.6 ± 0.50	17.7 ± 0.76

Non-ovariectomized female rats were injected s.c. with tamoxifen citrate (5 mg free base once daily) or corn oil (vehicle) on days 28 and 29 of age (prepuberty) and then killed on day 78 of age (adulthood). Shown are mean ± SEM activity values for 6 individual rats per treatment group. No significant difference was found between the groups for each of the activities indicated.

* Activity is expressed as nmol/min/nmol total cytochrome P450.

† Activity is expressed as nmol/min/mg microsomal protein.

Effect of Tamoxifen in Testosterone Hydroxylase and Steroid 5α-Reductase Activities in Adult Female Rats Treated with Androgen during Puberty

To determine the effect of prepubertal tamoxifen administration on peripubertal androgen imprinting of testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities, non-ovariectomized female rats were administered tamoxifen (5 mg free base once daily on days 28 and 29 of age) prior to testosterone administration during puberty (days 35–49) and then killed on day 78 of age. Whereas peripubertal testosterone administration alone did not affect hepatic microsomal testosterone 2α-, 6β-, or 7α-hydroxylase or steroid 5α-reductase activity by 4-fold (Fig. 2A), decreased steroid 5α-reductase activity by 31% (Fig. 2D) and had no statistically significant effect on testosterone 6β- (Fig. 2B) or 7α-hydroxylase activity (Fig. 2C) in adult female rats administered testosterone during puberty. These findings indicate the tamoxifen pretreatment can overcome the inhibitory effect of the ovary on androgen imprinting of basal levels of testosterone 2α-hydroxylase and steroid 5α-reductase activities but apparently not that of testosterone 6β- or 7α-hydroxylase activity.

Effect of Tamoxifen on Testosterone Hydroxylase and Steroid 5α-Reductase Activities in Adult Female Rats Treated with Androgen during Puberty and Adult Life

To examine the effect of tamoxifen on peripubertal androgen imprinting of adult androgen responsiveness of testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities, non-ovariectomized female rats were administered tamoxifen (days 28 and 29 of age) prior to testosterone treatment during puberty (days 35–49 of age) and adult life (days 69–77). As shown in Fig. 3, peripubertal testosterone administration imprinted adult androgen responsiveness of testoster-

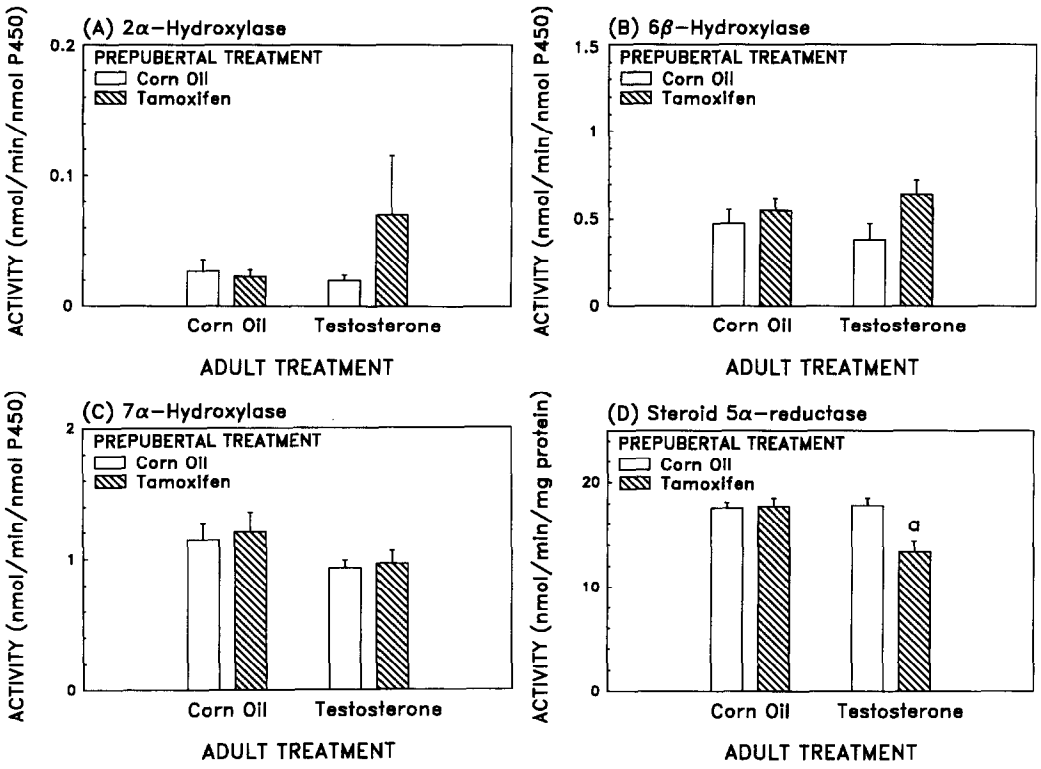

FIG. 1. Effect of prepubertal tamoxifen administration on hepatic testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities in adult female rats administered testosterone during adulthood. Non-ovariectomized female rats were injected s.c. with tamoxifen citrate (5 mg once daily) or corn oil (vehicle) on days 28 and 29 (prepuberty) followed by testosterone enanthate (5 μmol/kg once daily) or corn oil (vehicle) on days 69–77 (adulthood). All rats were killed on day 78 of age. Results are means ± SEM for 6 individual rats per treatment groups. Key: (a) Significantly different from each of the other three groups ($P < 0.05$). Panel A: testosterone 2α-hydroxylase; panel B: testosterone 6β-hydroxylase; panel C: testosterone 7α-hydroxylase; and panel D: steroid 5α-reductase.

one 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities, although the activity levels were still significantly different from those found in untreated adult male rats. Tamoxifen pretreatment of female rats administered testosterone during puberty and adult life, however, resulted in testosterone 2α- (Fig. 3A), 6β-hydroxylase (Fig. 3B), and steroid 5α-reductase (Fig. 3D) activity levels similar to those found in untreated adult male rats. In contrast, the same treatment did not influence adult androgen responsiveness of testosterone 7α-hydroxylase activity (Fig. 3C). These results establish that tamoxifen differentially impacts on peripubertal androgen imprinting of adult androgen responsiveness of sex-dependent hepatic cytochromes P450 and steroid 5α-reductase.

Tamoxifen and NADPH–Cytochrome P450 Reductase Activity

Hepatic NADPH–cytochrome P450 reductase is under pituitary control [49], and it is a rate-limiting component in many microsomal cytochrome P450-catalyzed oxidation reactions [50, 51]. To determine whether NADPH–cytochrome P450 reductase plays a role in the enhanced peripubertal androgen imprinting of microsomal testosterone 2α- and 6β-hydroxylase activities by tamoxifen, hepatic microsomes were assayed for NADPH–cytochrome P450 reductase activity. However, as shown in Fig. 4, tamoxifen did not alter this enzyme activity, indicating that the observed effects of tamoxifen on androgen imprinting of these testosterone hydroxylase activities were not due to increased levels of hepatic microsomal NADPH–cytochrome P450 reductase.

Effect of Tamoxifen on Peripubertal Androgen Imprinting of Hepatic CYP2C11, CYP3A2 and CYP2A1 Protein Levels

The above finding that tamoxifen did not affect microsomal NADPH-cytochrome P450 reductase activity suggests that the elevated testosterone 2α-hydroxylase (Fig. 3A and testosterone 6β-hydroxylase (Figs. 3B) activities in microsomes from female rats administered tamoxifen and testosterone are due to increased CYP2C11 and CYP3A2 protein levels, respectively. Therefore, western blot analysis was performed with antibodies known to be specific for CYP2C11 [23] or CYP3A2 [43]. As shown in Figs 5 and 6, neither CYP2C11 nor CYP3A2 protein was detected in hepatic microsomes from corn oil or tamoxifen-treated adult female rats. Whereas testosterone administration during both puberty and adult life induced CYP2C11

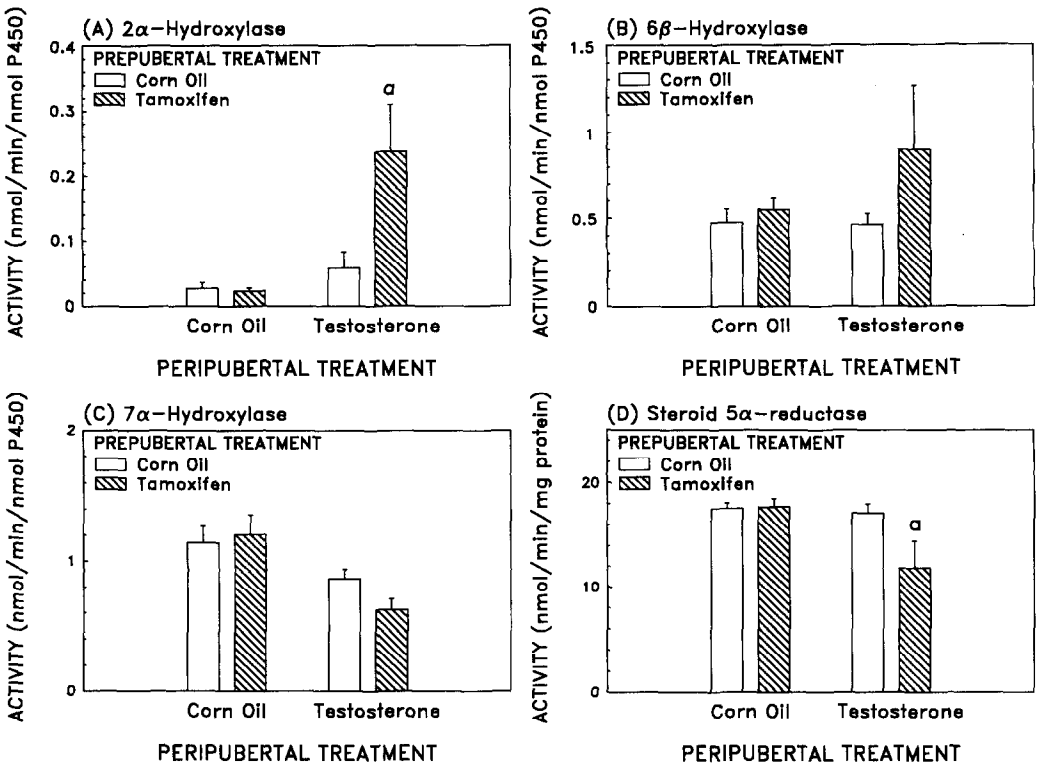

FIG. 2. Effect of prepubertal tamoxifen administration on hepatic testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities in adult female rats administered testosterone during puberty. Non-ovariectomized female rats were injected s.c. with tamoxifen citrate (5 mg once daily) or corn oil (vehicle) on days 28 and 29 (prepuberty) followed by testosterone enanthate (5 μmol/kg once daily) or corn oil (vehicle) on days 35–49 (puberty). All rats were killed on day 78 of age. Results are means ± SEM for 6 individual rats per treatment group. Key: (a) significantly different from each of the other three groups ($P < 0.05$). Panel A: testosterone 2α-hydroxylase; panel B: testosterone 6β-hydroxylase; panel C: testosterone 7α-hydroxylase; and panel D: steroid 5α-reductase.

and CYP3A2, the levels of these proteins were elevated further by prior treatment with tamoxifen (Figs. 5 and 6 and Table 3). It should be noted that the monoclonal anti-CYP3A2 IgG preparation did not cross-react with CYP3A1 because this antibody failed to detect any proteins in hepatic microsomes isolated from dexamethasone-treated adult female rats (Fig. 6, lane 2), where CYP3A1 is present at high levels [43]. In contrast to CYP2C11 and CYP3A2, hepatic CYP2A1 protein content was not significantly different between the tamoxifen-pretreated (0.44 ± 0.07, mean ± SEM densitometric unit) and the corn oil-pretreated (0.44 ± 0.06, mean ± SEM densitometric unit) female rats administered testosterone during puberty and adult life, consistent with the lack of an effect on CYP2A1-dependent testosterone 7α-hydroxylase activity in the same microsomal samples (Fig. 3C).

Serum Steroid Hormone Levels

Androgens and estrogens appear to influence indirectly the hepatic expression of the male-specific CYP2C11 and CYP3A2 and the female-predominant CYP2A1 and steroid 5α-reductase [17, 18, 21]. Therefore, to determine whether the observed changes in testosterone 2α- and 6β-hydroxylase and steroid 5α-reductase activities by tamoxifen were accompanied by altered gonadal hormone levels, we measured serum testosterone and 17β-estradiol concentrations. Consistent with our previous finding [32], only trace levels of testosterone were detected in adult female rats administered androgens during puberty (Table 4). Tamoxifen pretreatment did not affect the serum testosterone levels achieved *in vivo* following exogenous androgen administration. Similarly, exposure to tamoxifen prepubertally did not alter serum 17β-estradiol concentration in adult female rats treated with testosterone during puberty and/or adult life. These results suggest that the enhanced peripubertal androgen imprinting of specific cytochromes P450 and steroid 5α-reductase by tamoxifen is not due to permanently altered circulating levels of gonadal hormones in the adult rats.

DISCUSSION

The sex-dependent rat hepatic cytochromes P450 and steroid 5α-reductase are subject to neonatal androgen imprinting [3]. However, androgen imprinting of these enzymes is not restricted to the neonatal period because testosterone administration during puberty can also lead to imprinting of the basal

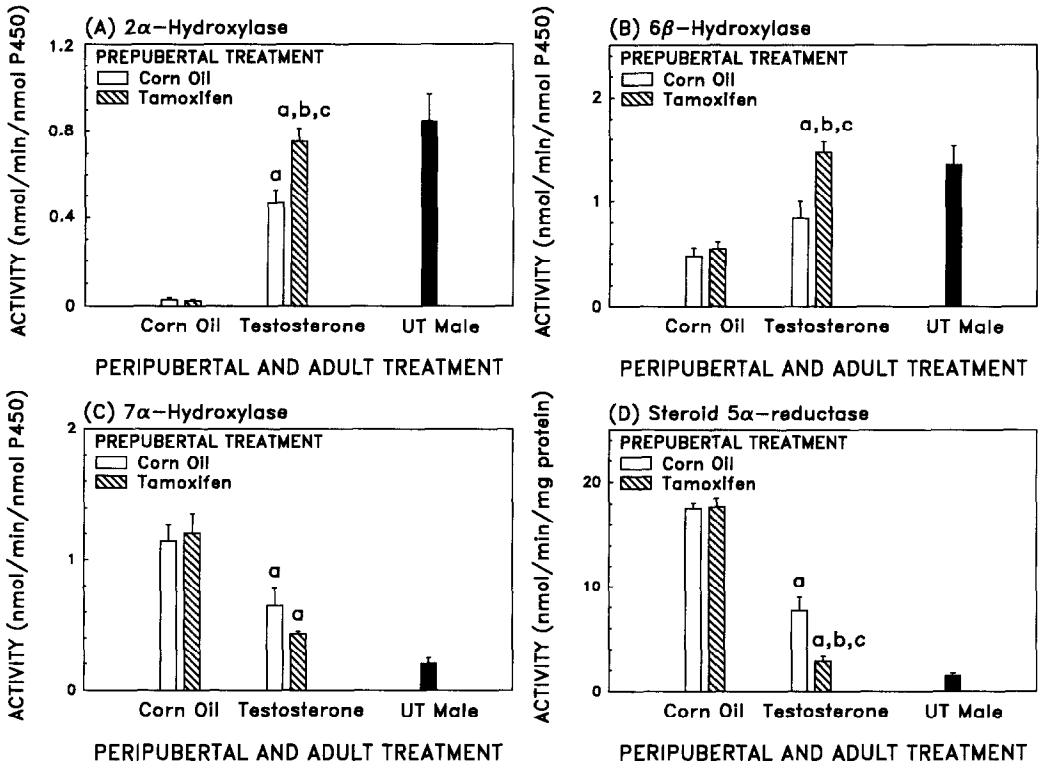

FIG. 3. Effect of prepubertal tamoxifen administration on hepatic testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities in adult female rats administered testosterone during puberty and adulthood. Non-ovariectomized female rats were injected s.c. with tamoxifen citrate (5 mg once daily) or corn oil (vehicle) on days 28 and 29 (prepuberty) followed by testosterone enanthate (5 μmol/kg once daily) or corn oil (vehicle) on days 35–49 (puberty) and days 69–77 (adulthood). Untreated (UT) male rats were included as a control. All rats were killed on day 78 of age. Results are means ± SEM for 6–8 individual rats per treatment group. Key: (a) Significantly different from the corn oil control group ($P < 0.05$); (b) significantly different from the testosterone group pretreated with corn oil ($P < 0.05$); and (c) not significantly different from the untreated male group. Panel A: testosterone 2α-hydroxylases; panel B: testosterone 6β-hydroxylase; panel C: testosterone 7α-hydroxylase; and panel D: steroid 5α-reductase.

levels and adult androgen responsiveness of specific hepatic microsomal cytochrome P450 and steroid 5α-reductase activities in ovariectomized, adult female rats [30–32]. By comparison, in non-ovariectomized female rats, peripubertal testosterone treatment results in imprinting of adult androgen responsiveness, but not the basal levels, of hepatic CYP2C11 protein and testosterone 2α-, 6β-, and 7α-hydroxylase activities [32]. The present study confirmed this observation in intact female rats and further showed that adult androgen responsiveness of hepatic steroid 5α-reductase can be imprinted by peripubertal testosterone administration. Our results also indicate that prepubertal tamoxifen administration does not affect the normal sex differentiation of hepatic microsomal CYP2C11, CYP3A2, CYP2A1, or steroid 5α-reductase. Moreover, tamoxifen does not impair, but rather enhances, peripubertal androgen imprinting of CYP2C11, CYP3A2, and steroid 5α-reductase in adult female rats.

Neither peripubertal nor adult testosterone administration alone altered the basal levels of hepatic microsomal testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities in non-ovariectomized, adult female rats (Table 1). By contrast, the same treatment results in a partial masculin-

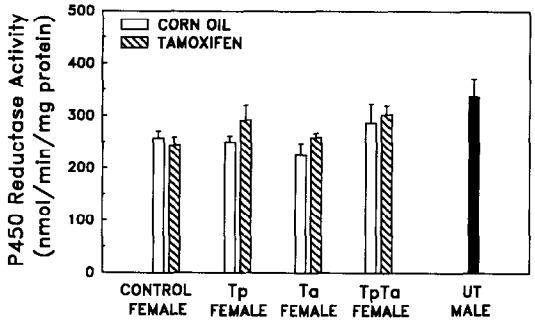

FIG. 4. NADPH–cytochrome P450 reductase activity in hepatic microsomes isolated from tamoxifen-pretreated female rats administered testosterone during puberty and adulthood. NADPH–cytochrome P450 reductase activity was measured in the same microsomal samples used for the determination of cytochrome P450 and steroid 5α-reductase activities (cf. Figs. 1–3). Results are means ± SEM for 6 individual rats per treatment group. No significant difference was found between any of the groups. Abbreviations: Tp, testosterone treatment during puberty; Ta, testosterone treatment during adulthood; TpTa, testosterone treatment during both puberty and adulthood; and UT, untreated.

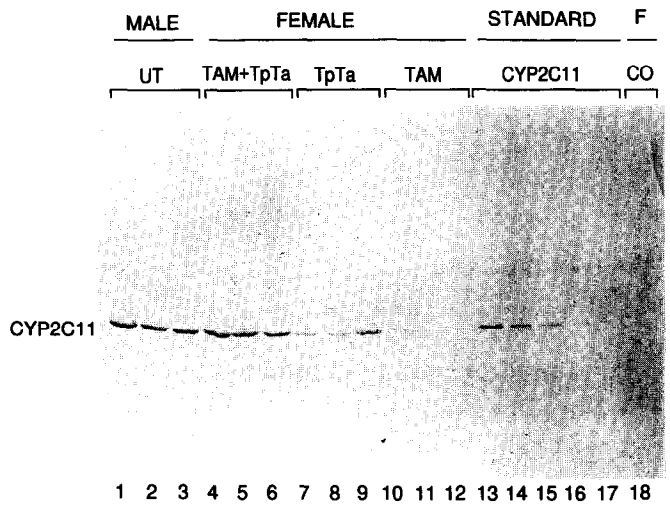

FIG. 5. Western blot of hepatic CYP2C11 protein levels. Hepatic microsomes isolated from adult male and female rats (10 pmol of total cytochrome P450 per lane, one individual microsomal sample per lane) and purified CYP2C11 standard (0.031 to 0.5 pmol CYP2C11 per lane) were subjected to SDS–PAGE [44]. Proteins from the SDS gel were electroeluted and transferred onto nitrocellulose membrane [45]. The immunoblot was probed with monospecific, rabbit anti-rat CYP2C11 IgG [23] at a final concentration of 50 μg/mL followed by alkaline phosphatase-conjugated goat $F(ab')_2$ anti-rabbit IgG (gamma and light chain specific, 1:3000 dilution) purchased from TAGO, Inc. (Burlingame, CA, U.S.A.). Immunoreactive CYP2C11 protein was detected as described under Materials and Methods. Lanes 1–3: untreated male; lanes 4–6: tamoxifen-pretreated female administered testosterone during puberty and adult life; lanes 7–9: female treated with testosterone during puberty and adult life; lanes 10–12: tamoxifen-treated female; lane 13: 0.5 pmol CYP2C11; lane 14: 0.25 pmol CYP2C11; lane 15: 0.125 pmol CYP2C11; lane 16: 0.063 pmol CYP2C11; lane 17: 0.031 pmol CYP2C11; and lane 18: corn oil-treated female.

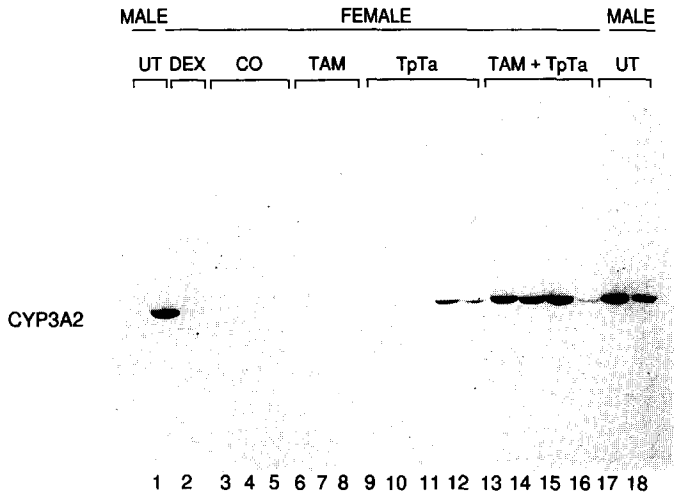

FIG. 6. Western blot of hepatic CYP3A2 protein levels. Hepatic microsomes isolated from adult male and female rats (20 pmol of total cytochrome P450 per lane and one individual microsomal sample per lane) were subjected to SDS–PAGE [44]. Proteins from that SDS gel were electroeluted and transferred onto nitrocellulose membrane [45]. The immunoblot was probed with monoclonal, mouse anti-rat CYP3A2 IgG [43] at a final concentration of 0.5 μg/mL followed by affinity-isolated horseradish peroxidase-conjugated, goat $F(ab')_2$ anti-mouse IgG (gamma and light chain specific, 1:3000 dilution) purchased from TAGO Inc. Immunoreactive CYP3A2 protein was detected by chemiluminescence as indicated under Materials and Methods. Lane 1: untreated male; lane 2: dexamethasone (DEX)-treated female; lanes 3–5, corn oil-treated female; lanes 6–8, tamoxifen-treated female; lanes 9–12, female treated with testosterone during puberty and adult life; lanes 13–16, tamoxifen-pretreated female administered testosterone during puberty and adult life; lanes 17 and 18, untreated male. No band was found in lane 2 (hepatic microsomes from DEX-treated female rats), verifying the lack of cross-reactivity of CYP3A1 by the anti-CYP3A2 antibody preparation [43].

ization of these activities in ovariectomized, adult female rats [31, 32]. These findings indicate that the intact ovary inhibits peripubertal testosterone imprinting of the sex-dependent cytochromes P450 and steroid 5α-reductase. Indeed, estrogen is known to suppress CYP2C11 [9, 18, 23] and CYP3A2 [18] expression in male rats, whereas it positively regulates CYP2A1 [13] and steroid 5α-reductase [18] levels in female rats. While other studies have shown that testosterone treatment has no effect [23] or completely masculinized [18, 24] CYP2C11 protein or activity levels, it should be noted that the androgen treatment protocol was not identical among the various studies. The higher magnitude of CYP2C11 expression reported by Dannan *et al.* [18] and McClellan-Green *et al.* [24] may be related to a longer treatment period (continuous androgen exposure for 5 weeks via a silastic capsule implanted s.c.) and a higher dosage. In fact, we observed recently a time-dependent increase in CYP2C11 activity in ovariectomized, adult female rats treated with testosterone during puberty and/or adult life.*

* Chang TKH and Bellward GD, Manuscript submitted for publication.

Prepubertal tamoxifen administration did not affect hepatic microsomal testosterone 2α-, 6β-, or 7α-hydroxylase or steroid 5α-reductase activity in non-ovariectomized, adult female rats. Whereas stimulation of testosterone 2α-hydroxylase activity requires intermittent GH pulses, testosterone 7α-hydroxylase and steroid 5α-reductase activities can be maintained in female rats as long as GH is secreted in a continuous manner, regardless of the GH pulse amplitude or nadir levels [4, 12]. In a recent study, treatment of adult female rats with two consecutive daily doses of tamoxifen was found to progressively decrease plasma GH levels to that by 7 weeks after discontinuation of tamoxifen treatment, not only was the GH pulse amplitude substantially reduced, but the nadir levels were decreased by > 80% to ~1 ng/mL [34]. The present study indicates that prepubertal tamoxifen administration does not interfere with the normal sex differentiation of testosterone 2α-, 6β-, and 7α-hydroxylase and steroid 5α-reductase activities in female rats. However, postpubertal tamoxifen treatment may affect hepatic microsomal cytochrome P450 expression because a moderate increase in testosterone 6β-hydroxylase and a small, but statistically significant, decrease in testosterone 7α-hydroxylase activity have been reported in adult female

TABLE 3. Hepatic CYP2C11 and CYP3A2 protein levels in tamoxifen-treated female rats administered testosterone during puberty and adult life

Treatment			Cytochrome P450 protein	
Days 28–29	Days 35–49	Days 69–77	CYP2C11*	CYP3A2†
(A) Female rats				
Corn oil	Corn oil	Corn oil	None detected	None detected
TAM	Corn oil	Corn oil	None detected	None detected
Corn oil	TE	TE	40 ± 5‡	0.15 ± 0.09‡
TAM	TE	TE	66 ± 8‡§	1.99 ± 0.62‡§
(B) Untreated male rats			102 ± 9§	3.66 ± 0.56§

Non-ovariectomized female rats were injected s.c. with tamoxifen citrate (TAM: 5 mg free base once daily), testosterone enanthate (TE: 5 μmol/kg once daily) or corn oil (vehicle) on the days shown. All rats, including untreated males, were killed on day 78 of age. Data are means ± SEM for 6–8 individual rats per treatment group.

* Protein level is expressed as pmol CYP2C11/nmol total microsomal cytochrome P450.

† Protein level is expressed in densitometric units.

‡ Significantly different from untreated adult male rats ($P < 0.05$).

§ Significantly different from corn oil-pretreated female rats administered testosterone on days 35–49 and days 69–77 ($P < 0.05$).

rats treated with tamoxifen at 8 weeks of age and killed 5 weeks later.† Normal sex differentiation of gender-dependent hepatic cytochromes P450 and steroid 5α-reductase has been found in dwarf rats [4, 52], which have low circulating GH levels as a consequence of a genetic defect in GH synthesis [53], and in rats neonatally administered a moderate dosage of monosodium glutamate (2 mg/g body weight) [54, 55], which selectively destroys neurons in the arcuate nucleus so that levels of GH-releasing factor are reduced [56]. Although GH pulse amplitude is diminished in these two models of GH suppression, the sexually dimorphic pattern of GH secretion still exists [4, 54]. Similarly, sex differences in GH secretory profile are observed in adult rats treated with tamoxifen (5 mg once daily for two consecutive days) [34].

Tamoxifen pretreatment of intact female rats enhanced peripubertal androgen imprinting of the basal levels of hepatic microsomal testosterone 2α-hydroxylase and steroid 5α-reductase activities to levels comparable to those found in ovariectomized rats treated with testosterone peripubertally [32]. Thus, it appears that the lack of androgen imprinting of the basal levels of these two enzyme activities in female rats with intact ovaries can be overcome by tamoxifen pretreatment. However, the overall pattern of response to androgen treatment found in tamoxifen-pretreated female rats is not identical to that in ovariectomized rats. Whereas treatment of ovariectomized rats with testosterone peripubertally increases testosterone 6β-hydroxylase activity and decreases testosterone 7α-hydroxylase activity [32], the same treatment has no effect on these enzyme activities in tamoxifen-pretreated female rats (Fig. 2, B and C). Furthermore, adult testosterone administration increases testosterone 2α- and 6β-hydroxylase activities and decreases testosterone 7α-hydroxylase and steroid 5α-reductase activities in ovariectomized rats [31, 32], but the same androgen treatment only modestly decreases steroid 5α-reductase and does not affect testosterone 2α-, 6β-, and 7α-hydroxylase activities in tamoxifen-pretreated rats (Fig. 1).

Prepubertal tamoxifen administration enhanced peripubertal androgen imprinting of the adult androgen responsiveness of hepatic microsomal testosterone 2α- and 6β-hydroxylase in intact female rats. Since hepatic NADPH–cytochrome P450 reductase is under pituitary control [49], induction of this microsomal enzyme is a potential biochemical mechanism whereby tamoxifen enhances androgen imprinting of testosterone 2α- and 6β-hydroxylase activities. However, prepubertal tamoxifen administration did not affect NADPH–cytochrome P450 reductase activity. Rather, western blot analysis indicated that tamoxifen pretreatment elevated hepatic CYP2C11 and CYP3A2 protein levels in adult female rats administered testosterone during puberty and adult life. Conceivably, the elevated CYP2C11 protein level might be a consequence of increased CYP2C11 gene expression because transcript initiation is an important control point in the expression of rat hepatic CYP2C11 [57, 58]. In addition to CYP2C11 and CYP3A2 activity and protein levels, prepubertal tamoxifen administration also modulated peripubertal androgen imprinting of the adult androgen responsiveness of steroid 5α-reductase but not the CYP2A1-dependent testosterone 7α-hydroxylase activity. Differential response of these two female-predominant hepatic enzyme activities has been reported following perturbation of the hypothalamic-pituitary axis. Whereas hypophysectomy abolishes steroid 5α-reductase activity (and also CYP2C12 protein level), the same surgical operation has little or no effect on CYP2A1 protein content [13]. Therefore, the lack of an effect of tamoxifen on peripubertal androgen imprinting of testosterone 7α-hydroxylase activity provides additional evidence that CYP2A1 is not regulated by the same underlying hormonal mechanism as steroid 5α-reductase in rat liver.

As mentioned previously, the sex-specific pattern of pituitary GH secretion is a major regulator of hepatic expression of sex-dependent cytochromes P450 and steroid 5α-reductase [3].

† Holsmer SL and Bandiera SM, Effect of tamoxifen on hepatic cytochrome P450 expression in adult female rats. *Proceedings of the 10th International Symposium on Microsomes and Drug Oxidations, Toronto, Canada, 18–21 July 1994*, p. 412.

TABLE 4. Serum testosterone and 17β-estradiol levels in adult female rats treated with tamoxifen and testosterone

Treatment			Serum testosterone (ng/mL)	Serum 17β-estradiol (pg/mL)
Days 28–29	Days 35–49	Days 69–77		
(A) Female rats				
Corn oil	Corn oil	Corn oil	<0.2	85 ± 12
TAM	Corn oil	Corn oil	<0.2	70 ± 8
Corn oil	TE	Corn oil	0.3 ± 0.1	72 ± 8
TAM	TE	Corn oil	0.7 ± 0.2	56 ± 12
Corn oil	Corn oil	TE	1.7 ± 0.3	67 ± 10
TAM	Corn oil	TE	1.9 ± 0.5	69 ± 13
Corn oil	TE	TE	4.9 ± 0.3	69 ± 12
TAM	TE	TE	3.5 ± 0.4	52 ± 11
(B) Untreated male rats			3.5 ± 0.7	85 ± 12

Non-ovariectomized female rats were injected s.c. with tamoxifen citrate (TAM: 5 mg free base once daily), testosterone enanthate (TE: 5 μmol/kg once daily), or corn oil (vehicle) on the days shown. All rats, including untreated males, were killed on day 78 of age. Results are means ± SEM for 6 individual rats per treatment group. No significant differences in serum testosterone or 17β-estradiol concentration were found between the tamoxifen group and the corresponding corn oil-treated control group.

However, sex differences in the levels of these enzymes are more pronounced in adult rats than in immature rats [59], probably because sex differences in the plasma GH secretory pattern in rats are not apparent until 30 days of age [60], just prior to the onset of puberty. Whereas androgen is responsible for the higher pulse amplitude and the lower basal level of GH found in male rats [61], estrogen causes the higher basal levels and the shorter interpulse interval in female rats [62]. Recent efforts to identify the gender-specific signalling elements in GH secretory dynamics have led to the conclusion that a signal recognized as "masculine" by the hepatocytes is a minimum of "off-time" during the interpulse interval in which GH levels are undetectable [12]. The hormonal mechanism by which tamoxifen modulates peripubertal androgen imprinting of CYP2C11, CYP3A2, and steroid 5α-reductase is not known. However, a recent study reported that tamoxifen suppresses GH pulse amplitude and nadir levels in adult male and female rats by increasing the release of the GH-inhibiting somatostatin [34]. Whether GH suppression by tamoxifen is a consequence of its antagonistic action on estrogen receptors is not known, but estrogen receptors are present in the arcuate-ventromedial and preoptic areas of the hypothalamus [63] where control of GH secretion occurs. Our hypothesis is that in tamoxifen-treated female rats administered testosterone during puberty and adulthood, the plasma GH secretory profile consisted of undetectable GH levels during the interpulse period caused by tamoxifen and intermittent GH pulses generated by the androgen, a profile that is characteristic of male rats. However, a detailed analysis of GH secretory dynamics will be required to test this possibility

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