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Imprinting of Hepatic Microsomal Cytochrome P-450 Enzyme Activities and Cytochrome P-450IIC11 by Peripubertal Administration of Testosterone in Female Rats

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SUMMARY

The influence of peripubertal exposure to physiological doses of testosterone on the adult androgen responsiveness of hepatic microsomal cytochrome P-450 was investigated. Male and female Sprague-Dawley rats were sham-operated or gonadectomized before puberty, at 25 days of age. They were injected subcutaneously with testosterone enanthate (5 μmol/kg/day) during the pubertal time period, on days 35–49. Responsiveness to this same dose of testosterone was tested by administering the compound during adulthood, on days 81–89. The females provided a model that had not been exposed to neonatal androgen imprinting, in contrast to the males. Testosterone 2α-hydroxylase activity and cytochrome P-450IIC11, which are normally expressed only in adult males, were expressed in the gonadectomized females administered testosterone during puberty with no further exposure to the hormone for the next 40 days. The levels found were similar to those in the gonadectomized male group. When the combined pubertal and adult testosterone regimen was used, a synergistic effect was produced; the 2α-hydroxylase activity reached control male levels in both gonadectomized and sham-operated females and, in addition, cytochrome P-450IIC11 attained control male levels in the gonadectomized females. Testosterone 6β-hydroxylase and erythromycin *N*-demethylase activities were used as indicators of the cytochrome P-450IIIA subfamily. These activities were significantly increased only in the females treated with testosterone during both the pubertal and adult periods, reaching control male levels of 6β-hydroxylation. A similar effect, but in the opposite direction, was found with testosterone 7α-hydroxylase, an enzyme activity indicative of cytochrome P-450IIA1. A decrease in this enzyme was produced in the females administered testosterone during both time periods, resulting in levels equivalent to those found in control males. In general, a highly significant interaction was found between the pubertal and adult treatment periods for the females, indicating a chronic effect of the pubertal exposure. The experiments with castrated males did not result in synergistic interactions, although there was some evidence of an additive effect. The results of this study support the hypothesis that the peripubertal period is a time during which testosterone imprinting of both increased basal levels and adult androgen responsiveness of some hepatic cytochrome P-450 enzymes can occur in the female rat.

Cytochromes P-450 are an important family of enzymes involved in the biotransformation of both endogenous compounds, such as steroids, and xenobiotics. Multiple forms of cytochrome P-450 have been identified in mammalian liver (1), and much current research is directed at elucidating the factors and mechanisms involved in the regulation of the different cytochrome P-450 enzymes. Sex-specific and developmental variations in expression of enzyme activities have been observed for some of these enzymes, and their regulation often involves hormones (2–16). Early studies of the effects of castration and testosterone treatment on rat liver cytochrome P-450-dependent steroid metabolism have indicated the partial dependence of some enzyme activities on reversible stimulation by androgens in the adult male animal (17, 18). This adult androgen responsiveness, as well as the basal level of activity, has been considered a phenomenon that is irreversibly "imprinted" or programmed. It has been demonstrated clearly that such imprinting occurs during the neonatal period (19–21). However, Pak *et al.* (22) reported that the adult androgen responsiveness of hepatic microsomal benzo[*a*]pyrene hydroxylation could be imprinted by administering testosterone to female gonadectomized rats at a later date, at the time of puberty. Therefore, we hypothesized that exposure to physiological doses of testosterone during puberty would permanently affect the responsiveness of hepatic cytochrome P-450 to testosterone administered later in the life of the adult animal.

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ABBREVIATIONS: HPLC, high performance liquid chromatography; 4-MA, 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one.

The model we used to test this hypothesis was the Sprague-Dawley rat, sham-operated or gonadectomized prepubertally at 25 days of age, which is past the critical time for neonatal imprinting to occur in the male. Corn oil or physiological doses of testosterone enanthate were administered either pubertally, during adulthood, or during both time periods. Subsequently, selective hepatic microsomal cytochrome P-450 enzyme activities and enzymes were measured, in order to determine the pattern of changes that occurred. Several cytochrome P-450 enzymes have demonstrated catalytic activity for benzo[*a*]pyrene hydroxylase (6, 23). Analysis of the study by Pak *et al.* (22) suggested that the quantitatively most important candidates in uninduced animals were cytochrome P-450IIC11 and the cytochrome P-450IIIA subfamily. Therefore, assays directed towards these enzymes were used in the present experiments.

Materials and Methods

Chemicals. HPLC-grade organic solvents were obtained from British Drug House (Toronto, Ontario, Canada). The following steroids were purchased from Steraloids Inc. (Wilton, NH): 7α-, 6β-, 16α-, and 16β-hydroxytestosterone. Prof. D. N. Kirk of the Steroid Reference Collection, MRC (London, UK), provided samples of 2α- and 2β-hydroxytestosterone. The 15α-hydroxytestosterone was a gift from the Upjohn Company (Kalamazoo, MI), provided by L. T. Bell and verified as authentic by S. A. Mizsak. The 15β-hydroxytestosterone and 18-hydroxytestosterone were gifts from Searle Canada Inc. (Oakville, Ontario). The steroid 5α-reductase inhibitor 4-MA was a gift from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). Testosterone enanthate, androstenedione, 16-ketotestosterone, and erythromycin base were obtained from Sigma Chemical Co. (St. Louis, MO), NADPH from Boehringer-Mannheim (Dorval, Quebec, Canada), and a Bradford protein assay kit from Bio-Rad (Mississauga, Ontario, Canada).

Animals and treatments. Male and female Sprague-Dawley rats were purchased from Charles River Co. (Montreal, Quebec, Canada). The animals were housed in polycarbonate cages, with corncob bedding free of antibacterial agents (Paxton Processing Co., Paxton, IL), under conditions of controlled temperature (23°) and lighting (lights on from 8:00 a.m. to 10:00 p.m.). *Ad libitum* access to food (Purina rodent laboratory chow 5001; Ralston Purina of Canada, Ltd., Woodstock, Ontario, Canada) and water was provided. Gonadectomies and sham operations were performed by the breeder, under ether anesthesia, when the animals were 25 days of age. Animals were given subcutaneous injections of either testosterone enanthate (5 μmol/kg/day) or an equal volume of corn oil, in both the peripubertal (days 35–49 of age) and the adult (days 81–89) periods (Fig. 1). This dose of testosterone had been previously shown to be the minimum necessary to maintain prostate weight in castrated male rats (22).

Microsome preparation. When the animals reached 90 days of age, they were sacrificed by decapitation. Livers were quickly removed, placed in ice-cold 0.05 M Tris, 1.15% KCl, pH 7.5 (Tris/KCl), and minced. All subsequent steps were performed at 4°. Each liver was added to 20.0 ml of Tris/KCl buffer and homogenized using a Potter-Elvehjem glass mortar and a motor-driven pestle. The homogenate was centrifuged at 10,000 × *g* for 20 min. The supernatant was centrifuged at 100,000 × *g* for 60 min. The resulting microsomal pellet was resuspended in 10 mM EDTA, 1.15% KCl, pH 7.4, and centrifuged again at 100,000 × *g* for 60 min. The pellet was resuspended in 4.0 ml of 0.25 M sucrose, resulting in a final microsomal protein concentration of 20–40 mg/ml. Aliquots were stored at −80° for subsequent assays.

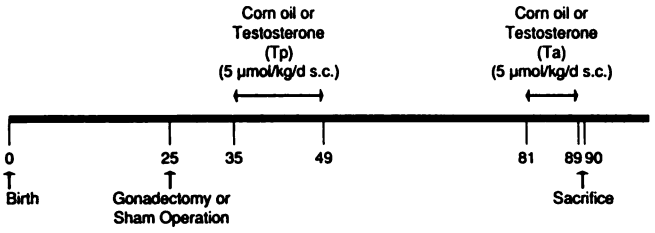

Fig. 1. Summary of treatment regimen for male and female rats.

Erythromycin *N*-demethylase assay. Erythromycin *N*-demethylase is a catalytic activity that has been linked to the cytochrome P-450IIIA subfamily by immunoinhibition studies (16, 23–25). Therefore, erythromycin demethylase activity was determined according to the method of Arlotto *et al.* (16), with spectrophotometric measurement of formaldehyde by the method of Nash (26). The assay was performed under conditions where product formation was proportional to both protein concentration and incubation time. The incubation mixture contained 0.4 mM erythromycin base, 3 mM magnesium chloride, 5 mM semicarbazide HCl, 1 mM NADPH, microsomes diluted in 0.25 M sucrose to give a final protein concentration of 0.8 mg/ml (females) or 0.35 mg/ml (males), and 0.2 M NaK phosphate buffer, pH 7.4, in a total volume of 1.5 ml. The reaction was initiated by addition of NADPH. After a 20-min incubation at 37°, the reaction was stopped by the addition of 0.6 ml of ice-cold 17% perchloric acid. The tubes were centrifuged at 1000 × *g* for 15 min at 4°. Double Nash reagent (ammonium acetate, 7.5 g; acetylacetone, 0.1 ml; distilled water to 25 ml) was freshly prepared, and 0.5 ml was added to 1.5 ml of each supernatant. After incubation at 60° for 15 min, absorbance was measured at 412 nm with a Hewlett Packard 8452A diode array spectrophotometer.

Assay of testosterone hydroxylation. Microsomal samples containing 0.1–1.0 nmol of cytochrome P-450 were incubated at 37°, in a 1.0-ml final volume, with 1 mM NADPH, 3 mM magnesium chloride, 50 mM sucrose, 50 mM potassium phosphate buffer, pH 7.4. In the female samples, 2.5 μM 4-MA was used to inhibit 5α-reductase. After the addition of 250 μM testosterone in 20 μl of methanol, the reaction was incubated for 5 min and then terminated by the addition of 6 ml of dichloromethane, followed by vigorous mixing for 30 sec. The internal standard, 3 nmol of 11β-hydroxytestosterone or 16-ketotestosterone in 100 μl of dichloromethane, was added to each sample. After centrifugation for 1 min at 800 × *g*, 4 ml of the organic phase were transferred to a 16- × 125-mm culture tube and evaporated under a stream of nitrogen at 35°. The residue was dissolved in 200 μl of methanol, and a 10-μl aliquot was analyzed by HPLC (Waters Division, Millipore, Milford, MA). The HPLC method was essentially that of Wood *et al.* (27), except that a 40-μm Pelliguard C_{18} guard column (Supelco Canada Ltd., Oakville, Ontario, Canada) was used. The minimum level of detection of metabolites was 50 pmol/min/nmol of cytochrome P-450. Details of individual HPLC components and the computer system used are as previously described.[2]

Other Assays. Rat cytochrome P-450IIC11 was purified to electrophoretic homogeneity by a modification of the method of Ryan *et al.* (6), as outlined in a recent study from our laboratory.[2] Polyclonal antibody against cytochrome P-450IIC11 was purified from rabbit serum and used for immunoinhibition of catalytic activity and immunoquantitation of cytochrome P-450IIC11 by competitive enzyme-linked immunosorbent assay, as described (10).[2]

The effects of polyclonal rabbit anti-rat cytochrome P-450IIC11 IgG on testosterone 2α- and 16α-hydroxylase activities in rat hepatic microsomes were determined as previously described.[2] Microsomes (0.3 nmol of total cytochrome P-450) were preincubated with either the anti-cytochrome P-450IIC11 IgG (2.5 mg of IgG/nmol of total cytochrome P-450) or an equivalent amount of control rabbit IgG. Based on initial studies in our laboratory, this quantity of anti-cytochrome

[2] Chang, T., M. Levine, S. M. Bandiera, and G. D. Bellward. Selective inhibition of rat hepatic microsomal cytochrome P-450. I. Effect of the *in vivo* administration of cimetidine. *J. Pharmacol. Exp. Ther.* **260**:1441–1449 (1992).

P-450IIC11 IgG is known to inhibit testosterone 2α- and 16α-hydroxylase activities maximally. Control rabbit IgG did not have an inhibitory effect on either of these enzyme activities.

Hepatic microsomal protein was measured by the method of Bradford (28), using bovine serum albumin as the standard. Total cytochrome P-450 was measured according to the method of Omura and Sato (29). Total unconjugated plasma 17β-estradiol and testosterone were measured at 90 days of age, using ^{125}I radioimmunoassay kits (ICN Biomedical Inc., Carson, CA), essentially as previously described (30, 31).

Statistical procedures. Statistical analyses were performed using either one-way or two-way analysis of variance, followed by the Student-Newman-Keuls multiple-range test. Differences were considered significant at $p < 0.05$.

Results

Testosterone 2α- and 16α-hydroxylase activities. In hepatic microsomes from uninduced rats, hydroxylation of testosterone at the 2α- and 16α-positions is carried out predominantly by cytochrome P-450IIC11, a male-specific enzyme (8, 32). Our anti-cytochrome P-450IIC11 antibody inhibited rat hepatic microsomal testosterone 2α-hydroxylation by 90–100% and 16α-hydroxylation by 80–90%, in control gonadectomized male and female rats (Fig. 2). Because the pattern of results with these two enzyme activities was similar, in general only the data for the 2α-hydroxylation are described.

Essentially minimum detectable levels of testosterone 2α-hydroxylation were found in the sham-operated control females, even when testosterone was administered, demonstrating a lack of androgen responsiveness for this enzyme activity in intact females (Fig. 3). However, in the gonadectomized females, hepatic 2α-hydroxylase activity was present after either pubertal or adult testosterone treatment, at levels equivalent to that found in the gonadectomized males. This did not occur in the sham-operated females with functioning ovaries (Fig. 4A). Of greater importance is the finding that the combination pubertal and adult testosterone regimen, in either the gonadectomized females (Fig. 3) or the sham-operated group (Fig. 4A), resulted in 2α-hydroxylation at the control adult male level of activity. The interaction between the two treatment regimens in these female groups was highly significant ($p < 0.0001$), indicating a synergistic effect of exposure to testosterone in the two time periods. The level of testosterone 16α-hydroxylase activity was found to be similarly increased ($p < 0.0002$) (Fig. 4B). In male rats, gonadectomy at 25 days of age reduced the levels of testosterone 2α-hydroxylase, indicating a dependence on circulating androgen (Fig. 3). The gonadectomized males remained sensitive to the administration of testosterone during the adult period (Fig. 3). However, there was no interaction between the two treatment periods.

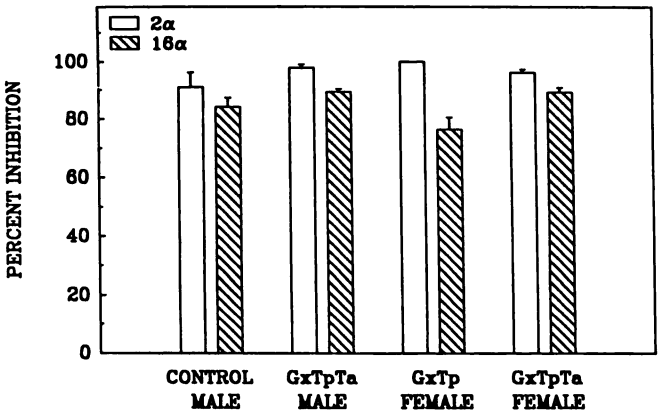

Fig. 2. Specificity of testosterone 2α- and 16α-hydroxylase activity as a measurement of cytochrome P-450IIC11. Values are mean ± standard error (n = 7–9). Anti-cytochrome P-450IIC11 antibody inhibition (percentage of inhibition) of these two enzyme activities was measured *in vitro* in the various experimental groups. *Gx*, gonadectomized; *Tp*, testosterone enanthate (5 μmol/kg/day, subcutaneously), during days 35–50 of life; *Ta*, testosterone enanthate (5 μmol/kg/day, subcutaneously), during days 81–90 of life; *TpTa*, both treatment regimens administered.

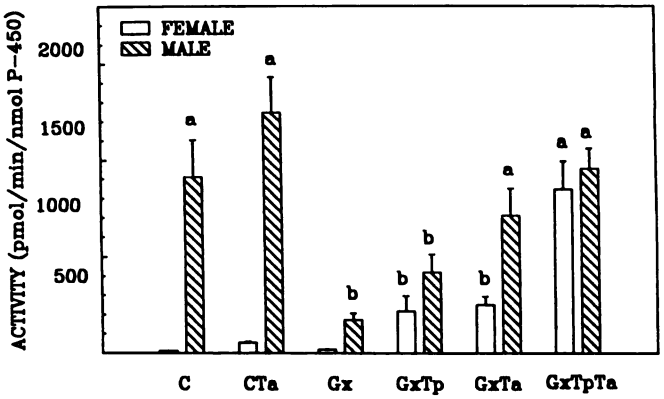

Fig. 3. Rat hepatic microsomal testosterone 2α-hydroxylation activity in the various treatment groups. *C*, corn oil control; *ND*, below the level of detection; *GX*, *Tp*, and *Ta*, as in the legend to Fig. 2. *a*, Groups not significantly different from control male values; *b*, groups not significantly different from each other. Values are mean ± standard error (n = 7–9).

Immunoquantitation of cytochrome P-450IIC11. Antibodies monospecific for cytochrome P-450IIC11 were used in a competitive enzyme-linked immunosorbent assay, to quantitate levels of this protein in liver microsomes from the various treatment groups (Fig. 5). No cytochrome P-450IIC11 was detected in control females, control females treated with testosterone during adulthood, or ovariectomized females. However, cytochrome P-450IIC11 constituted 1.5 ± 0.4% of the total cytochrome in the gonadectomized females treated with testosterone during the pubertal period, 2.9 ± 0.5% in those treated with testosterone as adults, and 13.9 ± 1.1% after both treatments. This latter value did not differ from the control male levels. Again, there was a highly significant interaction between the pubertal and adult treatment regimens in the females ($p < 0.001$), indicating a synergistic effect of exposure to testosterone during the two periods.

In the males, 14.7 ± 2.6% of the total cytochrome P-450 was the P-450IIC11 form (Fig. 5). This was significantly decreased, to 3.0 ± 0.7%, by castration. Additive increases occurred when pubertal or adult treatment with testosterone was administered, reaching a maximum level, with the combination, that was similar to control male values. In contrast to the results with the females, no significant interaction was found between the two treatment periods in the male rats.

In summary, the immunoquantitation results for cytochrome P-450IIC11 protein levels paralleled those found for testosterone 2α- and 16α-hydroxylase activities.

Testosterone 6β-hydroxylation and erythromycin *N*-demethylase activity. Testosterone 6β-hydroxylation activity is indicative of the cytochrome P-450IIIA subfamily (9, 14), as is erythromycin *N*-demethylase activity (16, 23, 24, 33). Control female levels for testosterone 6β-hydroxylation and erythromycin demethylase activities were lower than control

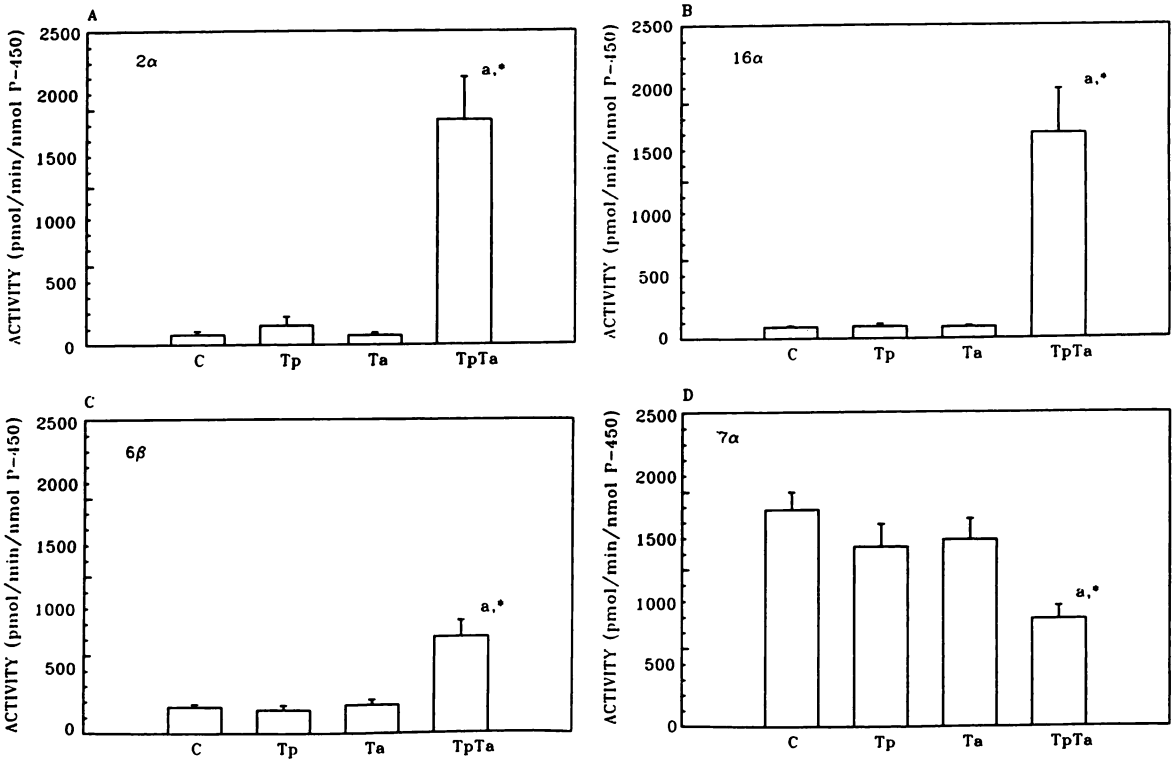

Fig. 4. Hepatic microsomal enzyme activities in sham-operated female rats. Values are mean ± standard error (n = 7 or 8). *C*, corn oil control group; *Ta*, testosterone treatment during days 81–90 of life; *Tp*, testosterone therapy during days 35–50 of life; *TpTa*, treatment during both time periods. *a*, Significantly different from all others in the group; *, significant interaction between the two treatment periods. A, Testosterone 2α-hydroxylation; B, testosterone 16α-hydroxylation; C, testosterone 6β-hydroxylation; D, testosterone 7α-hydroxylation.

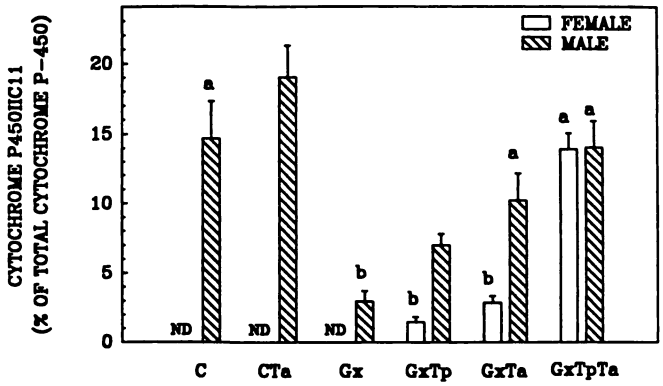

Fig. 5. Hepatic microsomal cytochrome P-450IIC11 levels in the various treatment groups (percentage of total cytochrome P-450). Values are mean ± standard error (n = 7–9). *a*, Groups not significantly different from control male values; *b*, groups not significantly different from gonadectomized male values. Other abbreviations are as in Figs. 2 and 3.

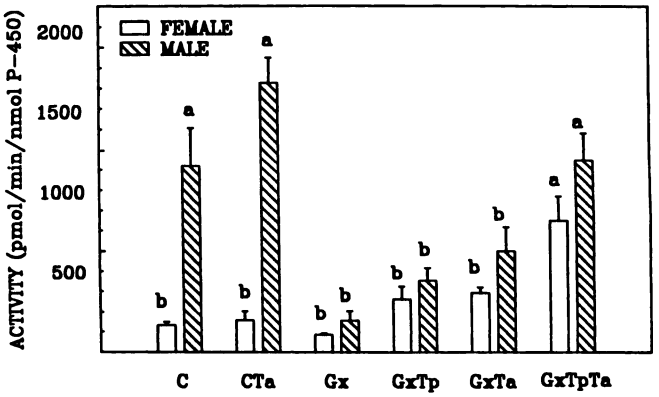

Fig. 6. Hepatic microsomal testosterone 6β-hydroxylation activity in the various groups. Values are mean ± standard error (n = 7–9). *a*, Groups not significantly different from control male values; *b*, groups not significantly different from the gonadectomized males treated with testosterone as adults (*GxTa*). Other abbreviations are as in Figs 2 and 3.

male levels, and an adult androgen response was not observed in intact females (Figs. 4C, 6, and 7). In contrast to the results with testosterone 2α-hydroxylation and cytochrome P-450IIC11, treatment of gonadectomized females with testosterone during the pubertal period alone did not significantly alter the level of testosterone 6β-hydroxylation (Fig. 6) or erythromycin *N*-demethylase activity (Fig. 7), when measured 40 days later in adulthood. However, females treated with testosterone during both the pubertal and adult periods had significant increases in hepatic testosterone 6β-hydroxylation (Figs. 4C and 6) and erythromycin demethylase activities (Fig. 7). A highly significant interaction between the two treatment periods was found in sham-operated females with respect to testosterone 6β-hydroxylation ($p < 0.001$) (Fig. 4C). The magnitude of the enzyme activities attained in females treated during both pubertal and adult time periods was the same as that in the gonadectomized males treated in both periods.

The relatively high control male levels of 6β-hydroxylation and erythromycin *N*-demethylase were reduced by gonadectomy on day 25 of age, consistent with their expected responsiveness to circulating androgen. In contrast to the females, gonadectomized males treated with testosterone during both puberty and adulthood had additive, not synergistic, increases

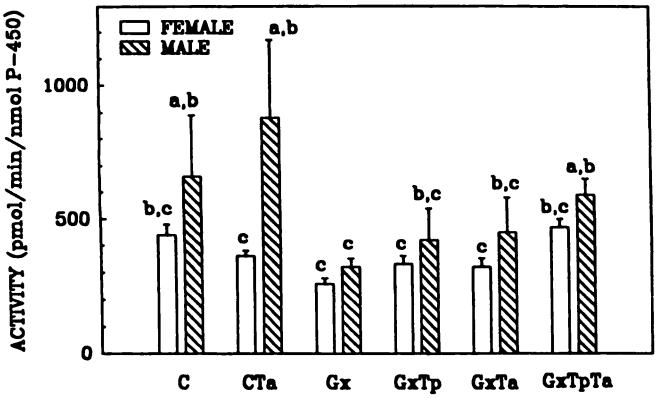

Fig. 7. Hepatic microsomal erythromycin *N*-demethylase activity in the treatment groups. Values are mean ± standard error (n = 7–9). *a*, Groups not significantly different from control males; *b*, groups not significantly different from gonadectomized males treated with testosterone during both the pubertal and adult time periods (GxTpTa); *c*, groups not significantly different from each other. Other abbreviations are as in Figs. 2 and 3.

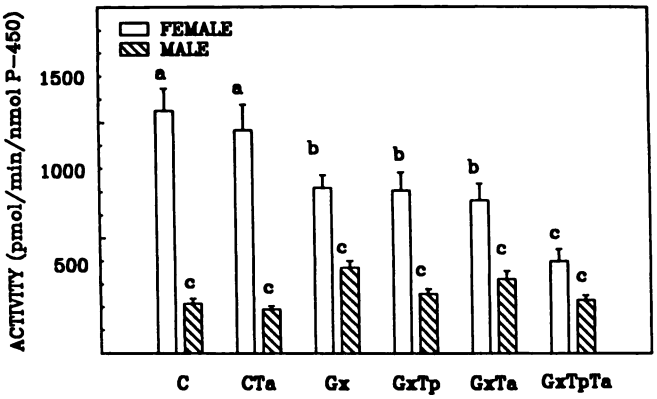

Fig. 8. Hepatic microsomal testosterone 7α-hydroxylation activity. Values are mean ± standard error (n = 7–9). *a–c*, Groups labeled with the same letter are not significantly different from one another. Other abbreviations are as in Figs. 2 and 3.

in these two enzyme activities. The levels reached in gonadectomized males treated with testosterone only during adulthood were lower than those of control males and gonadectomized males treated in both pubertal and adult time periods.

Testosterone 7α-hydroxylase activity. The hydroxylation of testosterone at the 7α-position is catalyzed in rat liver almost exclusively by cytochrome P-450IIA1 (27, 34). In contrast to the other cytochromes in this study, cytochrome P-450IIA1 is present in greater amounts in adult female rat liver than in the male and, therefore, provides an interesting internal control. Gonadectomy decreased this activity in the female, but not to male levels (Fig. 8). In the gonadectomized or sham-operated females, neither pubertal nor adult testosterone affected the 7α-hydroxylation activity (Figs. 4D and 8). However, the combination regimen decreased this enzyme activity, resulting in a significant interactive effect in intact females (Fig. 4D). In the ovariectomized females, the combined treatment resulted in 7α-hydroxylation at the same level as that found in the males. There were no significant differences between any of the male treatment groups (Fig. 8).

Plasma sex hormone levels. The mean plasma 17β-estradiol levels for female and control male rats at the conclusion of the study are given in Table 1. Plasma estradiol levels in normal adult female rats range from 20 to 330 pg/ml (30), depending on the stage of estrus. The control, sham-operated, female levels of 17β-estradiol in this study were at the lower end of the normal range. Estradiol levels in the ovariectomized groups were significantly lower than in the intact females, confirming the expected effects of the surgery. The lower estradiol levels in the ovariectomized females were comparable to those found in the control male group.

In the normal adult male rat, levels of plasma testosterone range from <1 to approximately 7 ng/ml, with large fluctuations (31, 35–37). The testosterone levels measured in the present study for control males were at the lower end of the normal range, whereas the female levels were below the level of detection (Table 1). In all groups injected with testosterone in adulthood, 24 hr before blood sampling, the plasma testosterone levels were also within the normal physiological range for males. No testosterone remained in the plasma of animals treated only during the pubertal period.

Discussion

Cytochrome P-450IIC11 and testosterone 2α-hydroxylase activity. Gustafsson and co-workers (19–21) have previously described neonatal androgen imprinting of both soluble and microsomal hepatic enzymes. The critical time period for such imprinting was found to be the first few days of life. It is clear from the results of the present experiments that an imprinting effect can also occur during another developmental period, because exposure of gonadectomized female rats to physiological doses of testosterone during puberty resulted in a masculinized pattern of expression of various cytochrome P-450 enzymes. Low but significant levels of cytochrome P-450IIC11 continued to be expressed 1 month after the gonadectomized females had been administered testosterone peripubertally. In addition, a synergistic effect was seen when testosterone was administered during both the pubertal and adult time periods, resulting in the expression of cytochrome P-450IIC11 protein levels equal to that of control adult males. The significant interaction between the two treatment periods indicated that pubertal exposure sensitized the expression of this enzyme to a subsequent testosterone exposure. It is noteworthy that this sensitization also occurred in intact females.

Of interest, previous workers have shown that supraphysiological doses of androgen induce steroid 16α-hydroxylase activity in intact adult female rats (8). Thus, pharmacological doses of testosterone may overcome the need for "priming" by physiological doses in the peripubertal period.

In contrast to the females, the results of our experiments with the castrated male rats, where neonatal androgen imprinting had already occurred, showed that the level of cytochrome P-450IIC11 after adult testosterone treatment was not different from that resulting from administration of both pubertal and adult androgen. It is of interest, however, that, after pubertal testosterone, an elevated level of the cytochrome was found 1 month after cessation of treatment. The levels of cytochrome P-450IIC11 in the various castrated male treatment groups indicated additive effects.

The data from the testosterone 2α-hydroxylation assay exhibited a pattern identical to that of the cytochrome P-450IIC11 protein levels. As well, there was a similar pattern of results for the 16α-hydroxylation activity (data not shown).

Testosterone 6β-hydroxylase and erythromycin *N*-de-

TABLE 1

Plasma 17β-estradiol and testosterone levels at 90 days of age

Each value is the mean ± standard error of seven to nine animals per group.

Treatment group		Plasma 17β-estradiol		Plasma testosterone	
Pubertal	Adult	Females	Males	Females	Males
		pg/ml		*ng/ml*	
Corn oil (sham-operated)	Corn oil	25.4 ± 1.7	14.3 ± 1.1[a]	0.03 ± 0.004	0.98 ± 0.18[b]
Corn oil (sham-operated)	Testosterone	21.8 ± 2.6	ND[c]	2.02 ± 0.28[b]	5.56 ± 0.29[b]
Corn oil (gonadectomized)	Corn oil	18.8 ± 1.3[a]	ND	0.02 ± 0.002	0.03 ± 0.001
Corn oil (gonadectomized)	Testosterone	17.8 ± 1.0[a]	ND	2.63 ± 0.28[b]	3.29 ± 0.31[b]
Testosterone (gonadectomized)	Corn oil	16.4 ± 1.4[a]	ND	0.07 ± 0.009	0.08 ± 0.008
Testosterone (gonadectomized)	Testosterone	15.1 ± 1.3[a]	ND	4.04 ± 0.23[b]	5.62 ± 0.53[b]
Corn oil (sham-operated)	Corn oil	48.1 ± 4.5	ND	0.26 ± 0.07	ND
Corn oil (sham-operated)	Testosterone	47.9 ± 3.6	ND	2.91 ± 0.41[b]	ND
Testosterone (sham-operated)	Corn oil	42.6 ± 3.2	ND	0.45 ± 0.05	ND
Testosterone (sham-operated)	Testosterone	48.3 ± 3.1	ND	6.54 ± 0.73[b]	ND

[a] Significantly different from sham-operated, corn-oil-treated females (estradiol).
[b] Significantly different from sham-operated, corn-oil-treated females (testosterone).
[c] ND, not done.

methylase activities. As was expected from previous work (16), hepatic microsomal erythromycin *N*-demethylase activity was higher in the adult male than the adult female rat. Control female rats demonstrated a lack of adult androgen responsiveness. However, a male level of adult androgen responsiveness was found for 6β-hydroxylation of testosterone in the gonadectomized females treated with testosterone during the pubertal period. These results are comparable to those observed for testosterone 2α-hydroxylation and cytochrome P-450IIC11. A significant interaction between the two treatment periods was observed in sham-operated females with respect to 6β-hydroxylation, indicating a lack of inhibition by the ovaries of peripubertal imprinting of adult androgen responsiveness.

It should be noted that the adult androgen responsiveness of erythromycin *N*-demethylase and testosterone 6β-hydroxylase in gonadectomized males was lower than that in intact males or gonadectomized males exposed to testosterone in puberty. This indicates that the adult androgen responsiveness was not maximally imprinted in the males during the neonatal period but could be increased by testosterone exposure after 25 days of age.

In order to identify which members of the cytochrome P-450IIIA subfamily are being affected by testosterone treatment, specific antibodies should be used (23, 33). It is probable that cytochrome P-450IIIA2 is the enzyme involved, because it is a male-specific form (9).

Testosterone 7α-hydroxylation. A useful enzyme to monitor effects that occur in the direction opposite to those previously studied is testosterone 7α-hydroxylation activity. This corresponds to levels of cytochrome P-450IIA1 (34), which are higher in the adult female rat liver than in the male. Gonadectomy of the female rats decreased the hepatic microsomal 7α-hydroxylase activity, although not to male levels. Testosterone administration did not affect the level of testosterone 7α-hydroxylase activity of either intact or gonadectomized females in either time period, nor that of the males. There was a significant decrease in the 7α-hydroxylase activity of both intact and gonadectomized females after the combined testosterone treatment. The significant interaction occurring between these two treatment periods in the sham-operated females, in the direction opposite to that of the other enzymes, adds further strength to our hypothesis.

Conclusions. Taken together, these results support the findings of Pak *et al.* (22) and our hypothesis that the peripubertal period is a time during which imprinting of various cytochrome P-450 enzymes by testosterone can occur. Current concepts of hormonal influences on a number of cytochrome P-450 enzymes are based on the seminal work by Gustafsson and co-workers (18–21, 38). These investigators concluded that the mechanism for neonatal androgen imprinting is mediated by sex-dependent growth hormone secretion patterns. Future experiments to determine whether the diurnal growth hormone secretory pattern is "masculinized" in the females treated with testosterone during both peripubertal and adult time periods are planned.

It was interesting to find that the intact sham-operated females also developed a masculinized response to the combined testosterone treatment during puberty and adulthood, considering the physiological dose of testosterone used. In contrast, previous studies have reported an inhibition by estrogen or intact ovaries of neonatal testosterone imprinting of adult male patterns of liver steroid metabolism (39) and growth hormone secretion (40). As well, Pak *et al.* (22) demonstrated an inhibition of peripubertal testosterone imprinting of adult androgen responsiveness in benzo[*a*]pyrene hydroxylase activity in intact female rats, compared with gonadectomized animals. However, our results are similar to those of Dannan *et al.* (41), where intact (i.e., nonovariectomized) female rats administered testosterone on days 1 and 3 of life, followed by a testosterone implant at age 5 weeks, demonstrated adult male levels of 16α- and 6β-testosterone hydroxylases and their corresponding cytochromes P-450.

Bullock *et al.* (42) reported normal sex differences in dwarf rats and pointed out that our current understanding of the regulation of sex-dependent cytochrome P-450 enzymes is not yet complete. Very low levels of growth hormone appear to maintain normal sex differentiation of cytochromes P-450IIC11 and P-450IIIA2, and their various enzyme activities, as long as the normal ultradian pattern occurs (43). However, in situations where there is no detectable level of circulating growth hormone, sex differences are abolished (42–44). With the cytochrome P-450IIC11 isoenzyme, the sexually undifferentiated level in both hypophysectomized males and females is approximately half that found in normal males (7). Morgan and coworkers (45) have concluded that there is evidence for a growth

hormone-independent regulation of cytochrome P-450IIC11, using streptozotocin-diabetic rats. From the data in the present study, it is not possible to conclude which underlying mechanism(s) is/are responsible for the results.

In summary, the present study provides evidence for imprinting of adult androgen responsiveness of certain sex-dependent cytochrome P-450 enzymes in female rats administered physiological doses of testosterone during the peripubertal time period. Specifically, testosterone 2α-, 16α-, 6β-, and 7α-hydroxylation and erythromycin *N*-demethylase reactions were masculinized.

In addition, cytochrome P-450IIC11 was partially expressed in prepubertally gonadectomized adult female rats given testosterone during puberty and was fully expressed at adult male levels in female rats treated with testosterone during both peripubertal and adult periods. These results indicate that puberty is a time during which androgen imprinting of adult androgen responsiveness and basal levels of hepatic cytochrome P-450 enzymes can occur in the female rat.

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