EFFECT OF ANDROGEN ADMINISTRATION DURING PUBERTY ON HEPATIC CYP2C11, CYP3A, AND CYP2A1 EXPRESSION IN ADULT FEMALE RATS

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ABSTRACT:

This biochemical and pharmacokinetic investigation was undertaken to evaluate the effects of androgen administration during puberty on sex-dependent cytochrome P450 (CYP or P450) enzyme expression in adult female rats. Hepatic testosterone 2α-hydroxylase activity and CYP2C11 and CYP3A protein levels were elevated in prepubertally ovariectomized rats injected subcutaneously with testosterone enanthate at 35–49 days of age and killed 41 days after discontinuation of treatment. In contrast, testosterone 6β- and 7α-hydroxylase activities and CYP2A1 protein content were not affected. The increase in CYP2C11 and CYP3A was likely not due to circulating testosterone because plasma testosterone was not affected. The increase in CYP2C11 and CYP3A was found to be reversible and was likely due to the slower elimination of the more lipophilic testosterone enanthate ester when compared with unesterified testosterone (elimination half-life was 2.0 ± 0.3 hr, mean ± SE). Once- or twice-daily dosing (5 or 2.5 μmol/kg/injection, respectively) during days 35–49 of age did not increase the mean testosterone 2α-hydroxylase activity and CYP2C11 protein content were elevated in three of the eight rats injected twice daily. Neither dosing regimen increased CYP3A or decreased CYP2A1 expression. In summary, the results indicate that treatment with testosterone enanthate during puberty resulted in a prolonged but reversible increase in hepatic expression of CYP2C11 and CYP3A.

Cytochrome P450 (CYP or P450) enzymes comprise a family of related hemeproteins that catalyze the oxidation of steroids, drugs, and other lipophilic compounds. Some of these enzymes are sexually and developmentally regulated. For example, in the rat, CYP2C11 is male-specific (Kamataki et al., 1983; Waxman, 1984; Morgan et al., 1985), and CYP2C12 is female-specific (Kamataki et al., 1983; MacGeoch et al., 1984; Ryan et al., 1984). By comparison, other hepatic CYP forms, such as CYP2A1 (Thomas et al., 1981; Arlotto and Parkinson, 1989; Waxman et al., 1989), CYP2C7 (Bandiera et al., 1986), and CYP3A9 (Mahnke et al., 1997), are more abundant in adult female than in adult male rats, whereas CYP3A2 is male-specific in untreated, adult rats (Cooper et al., 1993; Wright et al., 1997). The precise hormonal mechanism for the sex- and age-dependent expression of these enzymes is not understood completely, but the sexually dimorphic pattern of GH secretion appears to play a major regulatory role (Legraverend et al., 1992; Waxman, 1992). In addition to GH, gonadal hormones are also essential for hepatic expression of sexually differentiated P450 enzymes and are thought to influence hepatic P450 expression primarily by acting on the hypothalamic-pituitary axis, thereby altering GH secretory patterns (Waxman, 1988). However, gonadal hormones may also affect hepatic P450 expression independently of GH (Thummel and Schenkman, 1990).

Neonatal androgen exposure is thought to irreversibly program or “imprint” basal levels and adult androgen responsiveness of several rat hepatic P450 enzymes, such as CYP2C11 (Gustafsson et al., 1983; Morgan et al., 1985; Waxman et al., 1985). However, neonatal androgen exposure alone is not sufficient for full expression of CYP2C11 in adulthood (Waxman et al., 1985; Bandiera and Dworschak, 1992). Imprinting of P450 expression has also been suggested to occur at a developmental stage other than the neonatal period, based on the observation that treatment of prepubertally ovariectomized female rats with testosterone enanthate during puberty increases hepatic microsomal benzo[a]pyrene hydroxylase activity in adult life (Pak et al., 1984). Studies from our laboratory indicated that peripubertal administration of this androgen increases levels of CYP2C11 protein (Cadario et al., 1992) and mRNA (Chang and Bellward, 1996) in prepubertally ovariectomized adult female rats. However, it remains to be determined if androgen exposure during puberty affects the hepatic levels of other age- and sex-dependent P450 enzymes in adult animals.

We recently reported that continuous androgen release via an sc implant containing unesterified testosterone, in contrast to once-daily sc injections of testosterone enanthate during puberty, produced only a transient and reversible increase in CYP2C11-mediated enzyme activity, so that by adulthood, the effect was no longer observed (Chang and Bellward, 1996). A possible explanation for this observation is that the increased adult CYP2C11 expression by daily sc testosterone enanthate injections during puberty reflects prolonged enzyme induction due to the slower elimination of the more lipophilic enantiate ester of testosterone when compared with unesterified tes-
testosterone. Alternatively, a periodic rather than a continuous pattern of plasma androgen levels during puberty is required for increased expression of CYP2C11 in adulthood. This second possibility is consistent with the trimodal rhythmicity in the endogenous release of testosterone in adult male rats (Mock et al., 1978).

The present study was undertaken to compare and contrast the influence of peripubertal administration of testosterone enanthate on adult expression of hepatic CYP2C11, CYP3A, and CYP2A1 proteins in prepubertally ovariectomized female rats, to characterize the time course of the testosterone enanthate–mediated modulation of P450 enzymes, and to compare the effect of once- and twice-daily sc injections of unesterified testosterone during puberty on adult expression of these enzymes. The results obtained show a differential effect of peripubertal administration of testosterone enanthate on adult expression of hepatic CYP2C11, CYP3A, and CYP2A1. They also indicate that the peripubertal testosterone enanthate–mediated effects observed in adult rats are due to reversible enzyme induction rather than a permanent effect and suggest that the magnitude of CYP2C11 expression may be influenced by the frequency of androgen administration.

Materials and Methods

Chemicals. Testosterone and testosterone enanthate (testosterone 17β-heptanoate) (fig. 1) were purchased from Sigma Chemical Co. (St. Louis, MO). Authentic monohydroxytestosterone metabolite standards were bought from Steraloids, Inc. (Wilton, NH). NADPH was purchased from Boehringer Mannheim Canada, Ltd. (Laval, Quebec, Canada). All other chemicals were obtained from sources indicated previously (Wong and Bandiera, 1996).

Animals. Adult male and female Sprague–Dawley rats were purchased from 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. Female rats were ovariectomized at 25 days of age by the breeder. All the rats were housed on corn-cob bedding under controlled temperature (23°C) and lighting (7 a.m. on and 7 p.m. off), provided with Rodent Laboratory Diet™, No. 5001 (PMI Feeds, Inc., Richmond, IN) and tap water ad libitum up to the time of sacrifice, and cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Prepubertally (day 25 of age) ovariectomized female rats were injected subcutaneously once daily with testosterone enanthate (5 μmol/kg) or corn oil (1 ml/kg, vehicle control) at days 35–49 of age and sacrificed 41 days later (day 90 of age). Results are expressed as mean ± SE for seven individual rats per treatment group. Hepatic microsomal testosterone 2α-, 6β-, and 7α-hydroxylase activities (mean ± SE) in untreated adult male rats were 0.88 ± 0.12, 1.19 ± 0.13, and 0.17 ± 0.01 nmol/min/mmol total microsomal CYP, respectively. *, Significantly different from the control group (p < 0.005).

The immunoblot was incubated with monospecific anti-CYP2C11 lgG (panel A) or anti-CYP3A polyclonal IgG (panel B) at a concentration of 50 μg/ml. Hepatic microsomes from individual rats treated at days 35–49 of age and killed at day 90 of age were applied to the gel at an amount of 20 pmol of total microsomal P450 per lane for samples from female rats and 10 pmol for the sample from the male rat. In panel A, the lane designations are as follows: lanes 1–3, prepubertally ovariectomized rats injected subcutaneously with corn oil (CO); lanes 4–6, testosterone enanthate (TE; 5 μmol/kg once daily); lanes 7–11, purified CYP2C11 in amounts of 0.06, 0.2, 0.5, 0.6, and 1.2 pmol per lane, respectively; lanes 12–14, unesterified testosterone (T) at a dosage of 5 μmol/kg once daily (1X); lanes 15–17, unesterified testosterone at a dosage of 2.5 μmol/kg twice daily (2X); and lane 18, untreated (UT) adult male rat. In panel B, the lane designations are as follows: lanes 1–4, prepubertally ovariectomized rats injected subcutaneously with corn oil (CO); lanes 5–8, testosterone enanthate (TE; 5 μmol/kg once daily); lanes 9–12 contained purified CYP3A1 in amounts of 0.0625, 0.125, 0.2, and 0.25 pmol per lane, respectively; lanes 13–15, unesterified testosterone (T) at a dosage of 5 μmol/kg once daily (1X); and lanes 16–18, unesterified testosterone (T) at a dosage of 2.5 μmol/kg twice daily (2X).

FIG. 1. Chemical structures of testosterone and testosterone enanthate.

FIG. 2. Testosterone hydroxylase activities in hepatic microsomes isolated from prepubertally ovariectomized adult female rats treated with testosterone enanthate during puberty.

The immunoblot was incubated with monospecific anti-CYP2C11 lgG (panel A) or anti-CYP3A polyclonal IgG (panel B) at a concentration of 50 μg/ml. Hepatic microsomes from individual rats treated at days 35–49 of age and killed at day 90 of age were applied to the gel at an amount of 20 pmol of total microsomal P450 per lane for samples from female rats and 10 pmol for the sample from the male rat. In panel A, the lane designations are as follows: lanes 1–3, prepubertally ovariectomized rats injected subcutaneously with corn oil (CO); lanes 4–6, testosterone enanthate (TE; 5 μmol/kg once daily); lanes 7–11, purified CYP2C11 in amounts of 0.06, 0.2, 0.5, 0.6, and 1.2 pmol per lane, respectively; lanes 12–14, unesterified testosterone (T) at a dosage of 5 μmol/kg once daily (1X); lanes 15–17, unesterified testosterone at a dosage of 2.5 μmol/kg twice daily (2X); and lane 18, untreated (UT) adult male rat. In panel B, the lane designations are as follows: lanes 1–4, prepubertally ovariectomized rats injected subcutaneously with corn oil (CO); lanes 5–8, testosterone enanthate (TE; 5 μmol/kg once daily); lanes 9–12 contained purified CYP3A1 in amounts of 0.0625, 0.125, 0.2, and 0.25 pmol per lane, respectively; lanes 13–15, unesterified testosterone (T) at a dosage of 5 μmol/kg once daily (1X); and lanes 16–18, unesterified testosterone (T) at a dosage of 2.5 μmol/kg twice daily (2X).

FIG. 3. Immunoblots of CYP2C11 and CYP3A proteins in hepatic microsomes from prepubertally ovariectomized adult female rats treated with androgen during puberty.

The immunoblot was incubated with monospecific anti-CYP2C11 lgG (panel A) or anti-CYP3A polyclonal IgG (panel B) at a concentration of 50 μg/ml. Hepatic microsomes from individual rats treated at days 35–49 of age and killed at day 90 of age were applied to the gel at an amount of 20 pmol of total microsomal P450 per lane for samples from female rats and 10 pmol for the sample from the male rat. In panel A, the lane designations are as follows: lanes 1–3, prepubertally ovariectomized rats injected subcutaneously with corn oil (CO); lanes 4–6, testosterone enanthate (TE; 5 μmol/kg once daily); lanes 7–11, purified CYP2C11 in amounts of 0.06, 0.2, 0.5, 0.6, and 1.2 pmol per lane, respectively; lanes 12–14, unesterified testosterone (T) at a dosage of 5 μmol/kg once daily (1X); lanes 15–17, unesterified testosterone at a dosage of 2.5 μmol/kg twice daily (2X); and lane 18, untreated (UT) adult male rat. In panel B, the lane designations are as follows: lanes 1–4, prepubertally ovariectomized rats injected subcutaneously with corn oil (CO); lanes 5–8, testosterone enanthate (TE; 5 μmol/kg once daily); lanes 9–12 contained purified CYP3A1 in amounts of 0.0625, 0.125, 0.2, and 0.25 pmol per lane, respectively; lanes 13–15, unesterified testosterone (T) at a dosage of 5 μmol/kg once daily (1X); and lanes 16–18, unesterified testosterone (T) at a dosage of 2.5 μmol/kg twice daily (2X).
Prepubertally (day 25 of age) ovariectomized female rats were injected with testosterone enanthate as described in the legend to fig. 1. Data are shown as mean ± SE for seven or eight individual rats per treatment group. Protein level is expressed as nmol/nmol total microsomal CYP for CYP2C11 and CYP2A1, and as relative optical density unit for CYP3A, CYP2C11, CYP3A1, and CYP2A1 protein levels (mean ± SE) in hepatic microsomes isolated from untreated adult male rats (n = 8) were 0.13 ± 0.01 nmol/nmol total microsomal P450, 0.078 ± 0.008 relative optical density units, and 0.014 ± 0.001 nmol/nmol total microsomal P450, respectively. * Significantly different from the control group (p < 0.05).

15,000g for 10 min at 4°C. The plasma supernatant was carefully removed and stored in cryovials at –20°C until analysis.

Preparation of Microsomes. Rats were killed by decapitation. Livers were excised quickly, washed in ice-cold 50 mM Tris (pH 7.5) buffer containing 1.15% KCl, and used immediately to prepare microsomes by differential ultracentrifugation (Lu and Levin, 1972). The final microsomal pellet was stored in cryovials at 275°C until use.

Total P450 and Microsomal Protein Assays. Total P450 content was determined from the sodium dithionite–reduced carbon monoxide difference spectrum, using a molar extinction coefficient of 91 cm−1 mM−1 (Omura and Sato, 1964). Microsomal protein concentration was determined by the method of Lowry et al. (1951).

Testosterone Hydroxylase Assay. Microsomal testosterone 2α-, 6β-, and 7α-hydroxylase activities were measured by high-performance liquid chromatography as described previously (Wong and Bandiera, 1996) but with the following modifications. Reactions were carried out at 37°C for 10 min.

Testosterone and its metabolites were resolved at 40°C on a Supelco LC-18 (3 μm particle size, 15 x 4.6 mm i.d.) reversed phase column. The column was eluted at a flow rate of 1.4 ml/min with solvent A (methanol:water: acetonitrile, 35:64:1) and solvent B (methanol:water:acetonitrile, 80:18:2) using 100% solvent A for the first 10 min, followed by linear gradients to 40% solvent B from 10 to 30 min, to 55% solvent B from 30 to 35 min, and to 100% solvent from 35 to 36 min. Solvent B was held at 100% from 36 to 40 min, followed by a return to 100% solvent A from 40 to 41 min, and re-equilibration with solvent A from 41 to 45 min. Testosterone metabolites were monitored at 254 nm and quantified as described previously (Wong and Bandiera, 1996). Assay validation experiments indicated that the limits of quantitation (LOQ) of the testosterone 2α-, 6β-, and 7α-hydroxylase assays were 0.033, 0.067, and 0.017 nmol product formed/min/nmol total microsomal P450, respectively. The intraday and interday coefficients of variation of the testosterone hydroxylase assay were less than 15% at the metabolite concentrations tested. Hepatic microsomal testosterone 2α-hydroxylase activity was used as an enzyme-selective catalytic marker for CYP2C11 (Waxman, 1984), testosterone 6β-hydroxylase activity for CYP3A (Halvorson et al., 1990), and testosterone 7α-hydroxylase activity for CYP2A1 (Levin et al., 1987).

Purified P450 Standards. Purified rat CYP2C11, CYP3A1, and CYP2A1 proteins were included as positive controls in the immunoblot assays. CYP2C11 was purified from hepatic microsomes prepared from untreated, adult male Long-Evans rats as described previously (Bandiera and Dworschak, 1992). CYP3A1 was purified from dexamethasone-treated adult female Long-Evans rats according to the method outlined previously (Cooper et al., 1993). CYP2A1 was purified from Long-Evans rats and was provided by Dr. A. Parkinson (University of Kansas Medical Center, Kansas City, KS).

Preparation of Antibodies. Monospecific rabbit anti-rat CYP2C11 polyclonal IgG and rabbit anti-rat CYP3A polyclonal IgG were prepared as described previously (Bandiera and Dworschak, 1992; Panesar et al., 1996). The anti-CYP3A IgG was back-absorbed as reported (Panesar et al., 1996) and reacted with CYP3A1 predominantly, although it also recognized CYP3A2 (Wong and Bandiera, 1998), which is unresolved from CYP3A1 on SDS-PAGE. This antibody may also recognize other CYP3A proteins that are not resolved by SDS-PAGE. Mouse anti-rat CYP3A1 monoclonal IgG (P108), mouse anti-rat CYP3A2 monoclonal IgG (L171), and sheep anti-rat CYP2A1 polyclonal IgG were provided by Dr. P. E. Thomas (Rutgers University, Piscataway, NJ). The anti-CYP2A1 IgG reacts primarily with CYP2A1 but also recognizes CYP2A2. However, these two proteins can be resolved by SDS-PAGE.

SDS-PAGE and Immunoblot Assay. SDS-PAGE and electrophoretic transfer were performed as described previously (Wong and Bandiera, 1996). The primary antibodies included monospecific anti-CYP2C11 IgG (50 μg/ml), polyclonal anti-CYP3A IgG (50 μg/ml), monoclonal anti-CYP3A1 IgG (1 μg/ml), monoclonal anti-CYP3A2 IgG (1 μg/ml), or polyclonal anti-CYP2A1 IgG (10 μg/ml) at the concentrations listed. Assay conditions for the reaction between alkaline phosphatase and the substrate were optimized to ensure that color development did not proceed beyond the linear response range of the phosphatase reaction. Staining intensities of the bands were measured with a pdi 420 oe scanning densitometer connected to an IBM-type personal computer using Quantity OneVersion 3.0 software (pdi Inc., Huntington Station, NY). Calibration curves were constructed with purified rat CYP2C11, CYP3A1, and CYP2A1. A single concentration of the appropriate purified P450 was included on each blot as an internal standard. The LOQ of CYP2C11, CYP3A1, and CYP2A1 were 0.01 nmol/nmol total microsomal P450, 0.0015 relative optical density units, and 0.006 nmol/nmol total microsomal CYP, respectively. The intraday and interday coefficients of variation of the immunoblot assays were generally less than 10% at purified P450 concentrations greater than the LOQ.

Enhanced chemiluminescence detection (Amersham Life Sciences Inc., Oakville, Ontario, Canada) of immunoblots probed with monoclonal antibodies to CYP3A1 and CYP3A2 was also used. Blots were incubated with primary antibody as described above but the secondary antibody was horseradish peroxidase–conjugated goat anti-mouse IgG.

Plasma Testosterone Assay. Total unconjugated plasma testosterone concentration was measured by solid-phase 125I radioimmunoassay with the Im-

![Fig. 4. CYP2C11, CYP3A1, and CYP2A1 protein levels in hepatic microsomes isolated from prepubertally ovariectomized adult female rats treated with testosterone enanthate during puberty.](image)

![Fig. 5. Plasma testosterone profile in female rats administered testosterone enanthate during puberty.](image)
Results

Effect of Testosterone Enanthate Administration During Puberty on Adult Expression of Hepatic CYP2C11, CYP3A, and CYP2A1. To compare and contrast the effect of testosterone enanthate treatment during puberty on adult expression of hepatic CYP2C11, CYP3A, and CYP2A1, prepubertally ovariectomized rats were injected sc with testosterone enanthate (5 µmol/kg) once daily at days 35–49 of age and sacrificed 41 days later, at day 90 of age. In agreement with our previous finding, hepatic microsomal testosterone 2α-hydroxylase activity was increased in female rats treated with testosterone enanthate, whereas testosterone 7α-hydroxylase activity was unchanged (fig. 2). By comparison, testosterone 6β-hydroxylase activity was increased, but the effect was not statistically significant because of a large intersample variability. To measure hepatic levels of CYP2C11, CYP3A, and CYP2A1, immunoblot analyses were performed with monospecific anti-CYP2C11 polyclonal IgG, anti-CYP3A polyclonal IgG, and anti-CYP2A1 polyclonal IgG, respectively. As illustrated on a representative immunoblot (fig. 3A), the monospecific anti-CYP2C11 IgG detected a single protein band in microsomes isolated from testosterone enanthate–treated female rats (lanes 1–3), but no band was apparent in samples from corn oil–treated female rats (lanes 4–6). Polyclonal anti-CYP3A IgG detected an immunoreactive CYP3A protein in liver microsomes from corn oil–treated (fig. 3B, lanes 1–4) and testosterone enanthate–treated (fig. 3B, lanes 5–8) adult female rats. The electrophoretic mobility of this immunoreactive CYP3A protein is indistinguishable from that of purified CYP3A1 (fig. 3B, lanes 9–12), but it is apparent that there was considerable variation in CYP3A expression among the individual female rats tested. Densitometric analysis of the immunoblots indicated that the CYP2C11 protein content (mean ± SE) was 0.033 ± 0.001 nmol/nmol total microsomal P450 in female rats treated with testosterone enanthate, whereas it was undetectable in the corn oil–treated control group (fig. 4). Similarly, the hepatic level of CYP3A3 was greater in the testosterone enanthate–treated group (0.057 ± 0.006 relative optical density units) than in the control group (0.037 ± 0.004 relative optical density units). In contrast to the observed increase in CYP2C11 and CYP3A protein levels, peripuberal administration of testosterone enanthate did not affect the adult expression of CYP2A1 (fig. 4).

Plasma Elimination Half-Life of Testosterone Enanthate. The increase in hepatic CYP2C11 and CYP3A expression (fig. 4) in 90-day-old female rats administered testosterone enanthate during days 35–49 of age was not accompanied by detectable levels of plasma testosterone (data not shown). To substantiate this observation, serial blood sampling was performed in a separate group of prepubertally ovariectomized rats after discontinuation of testosterone enanthate administration at days 35–49 of age. The mean plasma testosterone concentration increased to a maximum level approximately 6 hr after the last dose of testosterone enanthate, then declined gradually (fig. 5) and was below the limit of detection by 10 days after the last injection. The calculated elimination half-life was 51 ± 6 hr (mean ± SE, N = 9). On the basis of pharmacokinetic considerations, the increased expression of CYP2C11 and CYP3A observed in ovariectomized female rats at 41 days after the last dose of testosterone enanthate is not likely to be due to the presence of circulating testosterone.

Statistics. The significance of the difference between the means of two treatment groups was assessed by the Mann-Whitney rank sum test. In cases where three treatment groups were compared, the Kruskal-Wallis one-way analysis of variance was used and was followed by Dunn’s multiple comparison test, if applicable. The level of significance was set a priori at p < 0.05.

muChem Direct Testosterone kit (ICN Biomedicals, Inc., Costa Mesa, CA). The limit of quantitation was 0.2 ng/ml. A preliminary experiment indicated that the antiseraum provided by the manufacturer did not crossreact with testosterone enanthate at concentrations of 0.3 and 3 ng/ml, and only 1.3% crossreactivity was obtained at 15 ng/ml.

Calculation of Elimination Half-Life. The elimination half-life was calculated as 0.693/K, where K is the elimination rate constant and is derived from the slope of the terminal elimination phase of the log plasma testosterone concentration vs. time curve, according to the following relationship: slope = −K/2.303.


determination of CYP3A content in microsomes from female rats was not possible because CYP3A1 was the only purified CYP3A enzyme available for use as a calibration standard for quantitation. Thus, CYP3A levels are expressed as the optical density (OD x mm) of the stained band relative to the optical density of an internal standard.
Time Course of Testosterone Enanthate–Mediated Effects on Hepatic CYP2C11 and CYP3A. The relatively slow elimination of testosterone enanthate suggests that this esterified androgen may produce a prolonged alteration in P450 levels. To investigate if the testosterone enanthate–mediated effects are permanent, a time-course experiment was conducted whereby prepubertally ovariectomized rats were injected subcutaneously once daily with the androgen at days 35–49 of age and killed at 41, 80, and 120 days after the last dose. As shown in fig. 6A and fig. 6B, microsomal testosterone 2α-hydroxylase activity and CYP2C11 protein level declined progressively so that they were near or below the limit of quantitation at 80 and 120 days after discontinuation of treatment. The observed increase in the level of immunoreactive CYP3A protein at 41 days after the last injection was also not permanent. By 80 days after discontinuation of androgen administration, the level of CYP3A was similar to that of control female rats (fig. 6C).

Plasma Elimination Half-Life of Unesterified Testosterone. In contrast to once daily sc injections of testosterone enanthate during puberty, continuous androgen release via a sc implant containing unesterified testosterone produced only a transient and reversible increase in CYP2C11-mediated enzyme activity (Chang and Bellward, 1996). A possible explanation for these data is that a periodic increase in CYP2C11-mediated enzyme activity (Chang and Bellward, 1996). A possible explanation for these data is that a periodic increase in CYP2C11-mediated enzyme activity (Chang and Bellward, 1996).

Effect of Once-daily and Twice-daily sc injections of unesterified testosterone during puberty on CYP2C11 enzyme activity and protein content in hepatic microsomes isolated from prepubertally ovariectomized adult rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Testosterone 2α-Hydroxylase Activity*</th>
<th>CYP2C11 Protein Content/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil (vehicle control)</td>
<td>7</td>
<td>&lt;0.03d</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Unesterified testosterone (5 µmol/kg once daily)</td>
<td>7</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Unesterified testosterone (2.5 µmol/kg twice daily)</td>
<td>8</td>
<td>0.04 ± 0.03</td>
<td>0.010 ± 0.005</td>
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*Prepubertally (day 25 of age) ovariectomized rats were injected subcutaneously with unesterified testosterone (5 µmol/kg once daily or 2.5 µmol/kg twice daily) or corn oil (1 ml/kg, vehicle control) at days 35–49 of age (puberty) and sacrificed at day 90 of age (adult). Results are expressed as mean ± SE for the number (N) of rats per treatment group.

Activity expressed as nmol/min/nmol total microsomal P450. Protein content expressed as nmol/nmol total microsomal P450. Below the limit of quantitation of 0.01 nmol/nmol total microsomal P450.

Effect of Peripubertal Administration of Androgen on Hepatic Expression of CYP3A1 and CYP3A2. The rat CYP3A subfamily comprises several forms, including CYP3A1 and CYP3A2 (Nelson et al., 1996). Therefore, to determine whether hepatic expression of CYP3A1 or CYP3A2 is modulated in ovariectomized adult female rats by peripubertal administration of androgen, immunoblots were probed with monoclonal antibodies against rat CYP3A1 or rat CYP3A2. As shown in fig. 8A, CYP3A1 was not expressed in liver microsomes from ovariectomized female rats that were treated with corn oil (lane 2), testosterone enanthate (lanes 3 and 4), or unesterified testosterone (lanes 5–8), or in liver microsomes from an intact untreated male rat (lane 9). In contrast, the monoclonal antibody to CYP3A2 detected a protein in microsomes from an untreated adult male rat (fig. 8B, lane 9) and in one of two randomly chosen individual microsomal samples isolated from prepubertally ovariectomized rats treated with testosterone enanthate (fig. 8B, lane 4). Interestingly, this immunoreactive CYP3A protein was not detected in the other six animals in this treatment group or in any other female rat tested. The results indicate that the protein band detected by the polyclonal antibody in liver microsomes from corn oil–treated and androgen-treated female rats does not correspond to either CYP3A1 or CYP3A2, with the exception of a single rat, which apparently expresses CYP3A2.

Discussion

Previous studies have indicated that treatment of prepubertally ovariectomized rats with testosterone enanthate during puberty results in elevated hepatic expression of CYP2C11 protein in adult life (Cadario et al., 1992; Chang and Bellward, 1996). The present study confirmed this finding and further showed that the same androgen treatment increased CYP3A protein expression in prepubertally ovariectomized 90-day-old female rats. The rat CYP3A subfamily consists
with the monoclonal antibody to CYP3A2 also had the highest CYP2C11 protein content in the treatment group, suggesting that the animal was unusually responsive to the androgen treatment. However, the mean hepatic CYP3A level was still significantly greater (p = 0.02) in the testosterone enanthe-treated group than in the corresponding control group when the data were reanalyzed without including results from this one sample. Therefore, another CYP3A protein(s), such as CYP3A9, CYP3A18, CYP3A23, or a currently unidentified CYP3A form(s), must have contributed to the observed effect in the rest of the samples in the group. It is not known presently whether androgen influences the hepatic expression of CYP3A9, CYP3A18, or CYP3A23.

CYP2A1 is another sex-dependent, developmentally regulated P450 enzyme. The level of hepatic CYP2A1 expression is similar in immature male and female rats (Thomas et al., 1981; Arlotto and Parkinson, 1989). However, in postpubertal rats, CYP2A1 content is severalfold greater in females than in males (Waxman et al., 1989). The decreased expression of CYP2A1 in postpubertal males has been attributed to the pubertal increase in endogenous androgen, which stimulates a pulsatile pattern of GH secretion that is associated with suppression of CYP2A1 (Waxman et al., 1989). In the present study, peripubertal administration of testosterone enanthe did not alter hepatic CYP2A1 expression in prepubertали ovarioctomized adult female rats. This finding is consistent with the previous observation that neonatal androgen exposure has little or no effect on CYP2A1 levels in adult life (Waxman et al., 1989). Testosterone can suppress CYP2A1 expression if the androgen is administered repeatedly to gonadectomized rats so that the circulating testosterone levels at the end of the study are comparable to those of untreated adult male rats (Dannan et al., 1986; Waxman et al., 1989). In the present study, plasma testosterone was no longer detectable in 90-day-old female rats treated with testosterone enanthe at days 35–49 of age.

A previous study postulated that treatment of ovarioctomized female rats with testosterone enanthe during puberty implants hepatic P450 expression in adult life (Pak et al., 1984). However, subsequent studies have produced conflicting data. In one study, sc implantation of testosterone propionate at days 35–71 of age increased P450-mediated ethylmorphine N-demethylase activity in birth-castrated male rats, but the effect was fully reversible by 2 weeks after removal of the implant (Virgo, 1991). We reported that testosterone enanthe administration during days 35–49 of age elevated CYP2C11-mediated activity, and CYP2C11 protein and mRNA levels in 90-day-old, prepubertally ovarioctomized female rats (Cadario et al., 1992; Chang and Bellward, 1996), but the effects were observed only with daily sc injections of testosterone enanthe and not with continuous androgen release via a sc implant containing unesterified testosterone (Chang and Bellward, 1996). In other studies, testosterone propionate administration by daily sc injections at days 35–49 of age did not result in detectable CYP2C11 protein content in neonatally ovarioctomized, 70-day-old female rats (Bandiera and Dworschak, 1992), whereas hepatic expression of CYP2C11, CYP2C13, and CYP3A was completely masculinized in neonatally ovarioctomized 70-day-old female rats that had been implanted with a continuous-release capsule containing testosterone propionate at 5 weeks of age (Dannan et al., 1986; McClellan-Green et al. 1989). The higher P450 levels reported by Dannan et al. (1986) and McClellan-Green et al. (1989) may reflect a longer treatment period because androgen was released continuously from the implanted capsules, which were left in place until the rats were killed. Collectively, these data suggest that peripubertal administration of androgen does not lead to a permanent alteration in P450 expression in adult life. Consistent with this proposal, the present study shows that the observed increase in CYP2C11 and CYP3A...
expression at 41 days after discontinuation of testosterone enanthate administration was no longer apparent by 80 and 120 days after treatment. The differences noted in the previous studies may also reflect pharmacokinetic differences among the various testosterone preparations used. Our pharmacokinetic analysis indicates that the elimination half-life of testosterone enanthate is considerably greater than that of unesterified testosterone. The step that limits the appearance of testosterone in the bloodstream is the rate of absorption of the androgen ester from the site of injection and this is governed by the oil/water partition coefficient (Minto et al., 1997), which is a measure of the lipophilicity of the compound. Once the ester appears in the extracellular fluid, it is rapidly hydrolyzed by esterases to the biologically active androgen. After injection of a testosterone ester, both esterified and hydrolyzed free testosterone appear in the circulation, although only the unesterified form is measured by the radioimmunoassay. The plasma concentration of unesterified testosterone has been shown to correlate well with salivary testosterone and provides a good measure of the steroid available to target tissues (Wang et al., 1985). The lipophilicity and duration of action of the various testosterone preparations is influenced by the length of the side-chain ester. Thus testosterone enanthate, which has a linear seven-carbon aliphatic side-chain (Fig. 1), has a longer duration of action than the short propionate (three-carbon) ester of testosterone, and both have a longer duration of action than unesterified testosterone (Gerrity et al., 1982).

Testosterone is not secreted in adult male rats at a constant level. Rather, a tridomal rhythmicity of plasma testosterone concentration is observed over an 24-hr period (Mock et al., 1978). Whether the pattern of testosterone secretion has any physiological significance is not known. In the present study, altering the frequency of administration of unesterified testosterone from once daily (at a dosage of 5 μmol/kg) to twice daily (at a dosage of 2.5 μmol/kg) resulted in elevated CYP2C11 activity and protein levels in three of the eight rats in the treatment group. The reason for this interindividual variability is not known, but the effects were still apparent 41 days after discontinuation of androgen administration and they were not accompanied by detectable levels of plasma testosterone. Further studies will be required to determine whether there is an optimal frequency of testosterone administration associated with CYP2C11 expression and whether the effect is long-lasting.

In summary, peripubertal administration of testosterone enanthate resulted in elevated expression of hepatic CYP2C11 and CYP3A but not CYP2A1 in prepubertally ovarectomized, 90-day-old rats. However, the increase in CYP2C11 and CYP3A levels was fully reversible, indicating that the observed effects in the 90-day-old rats was not imprinting but reflected prolonged enzyme induction as a result of the slower elimination of the more lipophilic ester androgen. The conflicting data from previous studies (Pak et al., 1984; Dannan et al., 1986; McLellan-Green et al., 1989; Virgo, 1991; Bandiera and Dworschak, 1992; Cadario et al., 1992; Chang and Bellward, 1996) investigating the effect of peripubertal administration of androgen on P450 activity and expression in adult life may be explained on the basis of pharmacokinetic differences between the different testosterone preparations and treatment protocols used in those studies. Finally, our data suggest that the frequency of androgen injections may influence the magnitude of hepatic CYP2C11 expression.

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