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INVERSE RELATIONSHIP BETWEEN OVARIAN AROMATASE CYTOCHROME *P*450 AND 5α-REDUCTASE ENZYME ACTIVITIES AND mRNA LEVELS DURING THE ESTROUS CYCLE IN THE RAT

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Summary—In the present study, we examined the changes in enzyme activity and mRNA levels of aromatase cytochrome *P*450 ($P450_{AROM}$) and 5α-reductase in ovarian tissue from adult cyclic rats. For each stage of the estrous cycle, the enzymatic activities were quantified by means of the ^3H_2O-release assay in the case of $P450_{AROM}$ and thin-layer chromatography in the case of 5α-reductase. Levels of mRNA encoding $P450_{AROM}$ and 5α-reductase in the ovary were determined by Northern blot analysis utilizing ^{32}P-labeled rat cDNAs as probes. Serum LH levels were determined by RIA. Three $P450_{AROM}$ mRNA species were detected (at 1.7, 2.2 and 2.7 kb) in ovarian tissue from cyclic rats. All three $P450_{AROM}$ transcripts were expressed in a co-ordinated fashion throughout the cycle. The $P450_{AROM}$ levels were highest during diestrus and proestrus, decreased during estrus while at metestrus the levels were nearly nondetectable. Conversely, one 5α-reductase mRNA species at 2.5 kb was detected in ovarian tissue from cyclic animals. Ovarian 5α-reductase mRNA levels were lowest during diestrus and proestrus, increased at estrus and were most abundant during metestrus; a pattern opposite to that of $P450_{AROM}$.

The pattern of change in $P450_{AROM}$ and 5α-reductase activities paralleled that of the respective mRNA profiles but lagged behind the mRNA profiles by about 24 h, or one stage of the estrous cycle. Aromatase activity was 1.5 pmol/h/mg protein during diestrus, increased over 3-fold at proestrus (≈5.5 pmol/h/mg protein), decreased at estrus and declined to the lowest values at metestrus (≈1.0 pmol/h/mg protein). In contrast, the 5α-reductase activity pattern was essentially the mirror image of the $P450_{AROM}$ activity pattern during the estrous cycle. 5α-Reductase levels were lowest during proestrus (≈5 pmol/h/mg protein) and estrus (≈8 pmol/h/mg protein), increased over 3-fold during metestrus, while the highest activity levels occurred during diestrus (≈36 pmol/h/mg protein).

The normalization of the $P450_{AROM}$ and 5α-reductase mRNA levels and their respective enzyme activities revealed a correspondence between mRNA abundance and subsequent increases (24 h later) in enzyme activity levels during the estrous cycle. These findings suggest that: (a) a temporal relationship exists between the profiles of the enzymatic activities that follows the changes in the levels of their respective mRNAs and (b) an inverse pattern exists between $P450_{AROM}$ and 5α-reductase in terms of both enzymatic activity and mRNA expression during the estrous cycle in rat.

INTRODUCTION

It is well established that androgens are converted to estrogens in the ovary [1, 2]. During the rat estrous cycle serum estradiol levels [3–5] parallel aromatase cytochrome *P*450 ($P450_{AROM}$) enzyme activity levels in a cyclic pattern [5, 6]. While the reduction of androgens by the 5α-reductase enzyme has not been investigated as extensively as the $P450_{AROM}$ pathway of androgen metabolism, 5α-reductase enzyme activity has been demonstrated in both immature [7, 8] and adult ovarian tissue [9]. The aromatase enzyme has been identified in granulosa cells [10], while the 5α-reductase enzyme has been localized, mainly, in thecal and interstitial cells [11] of the ovary.

Recent evidence indicates that there is an inverse relationship between the stimulatory effects of estrogens and inhibitory effects of dihydrotestosterone on the events of follicular development. Estrogens appear to enhance the

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responsiveness of ovarian follicles to gonadotropin stimulation [12], increase the proliferation of granulosa cells [13], augment ovarian weight [14] and exert a direct anti-atretic effect [15]. On the other hand, dihydrotestosterone has been shown to inhibit the FSH induction of LH receptors in granulosa cells [16, 17], reduce the ovulation rate by decreasing the number of granulosa cells [18] and decrease estrogen biosynthesis in these cells [18, 19].

Androgen metabolism throughout the different phases of the rat estrous cycle may be regulated by the expression of the $P450_{AROM}$ and 5α-reductase mRNA species encoding these steroidogenic enzymes. Previous studies, in rat and other species, indicate that regulation of mRNA encoding steroidogenic enzymes is an important component of the regulation of steroidogenesis [20–22]. To elucidate a possible relationship between aromatase and 5α-reductase, we characterized the changes in enzyme activity and mRNA levels of both $P450_{AROM}$ and 5α-reductase in ovarian tissue during the estrous cycle in the rat. These findings indicate that an inverse pattern exists between $P450_{AROM}$ and 5α-reductase in terms both of enzymatic activity and mRNA expression, and that the profiles of both enzyme activities during the estrous cycle follow the changes in the levels of their respective mRNAs.

MATERIALS AND METHODS

Animals

Adult cyclic (70–90 day old) Sprague–Dawley rats were purchased from Holtzman (Madison, WI), and were housed in a controlled environment (lights on 0600–1800 h; temperature, 23–25°C) with free access to tap water and rat laboratory chow (Teklad Rat Diet, Madison, WI). The cyclic stage of each animal was determined by daily vaginal cytology for 10 days before tissue was collected. The animals were classified according to the different stages of the estrous cycle (i.e. diestrus, proestrus, estrus and metestrus) immediately before euthanasia (by ether). The animals in this study displayed 4-day estrous cycles. From one group of animals, $P450_{AROM}$ and 5α-reductase enzyme activities were determined (1100–1600 h), while from another group of animals ovarian and liver tissues were collected and RNA was immediately prepared for Northern analysis (1000–1400 h). Systemic blood samples were obtained from the inferior vena cava from each animal, and serum samples were stored at −20°C until LH levels were determined by radioimmunoassay.

Steroids and reagents

[$1\beta,2\beta$-^{3}H]testosterone (T; 42 Ci/mmol) and deoxycytidine 5′-[α-^{32}P]triphosphate (dCTP; 3000 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [$1\alpha,1\beta$-^{3}H]T was prepared as described previously [23]; and purified by celite–ethylene glycol column chromatography before use. The final specific activity was 20.1 Ci/mmol. Nonradioactive steroids were purchased from Steroids, Inc. (Pauling, NY) or Sigma Chemical Co. (St Louis, MO). All other materials and chemicals for the enzyme assays and RNA preparation and analysis have been listed previously [22–27].

RNA isolation and enriched selection of poly A$^+$ RNA

Total RNA was prepared from ovarian tissue (pooled from; $n = 8$ to 10 animals/stage; according to the different stages of the estrous cycle) and liver samples (pooled from animals during all stages of the estrous cycle) by homogenization in guanidinium thiocyanate followed by centrifugation through a 5.7 M cesium chloride (CsCl) gradient as described [22, 25, 26].

Poly A$^+$-enriched RNA was prepared by centrifugation of total RNA over oligo (dT) cellulose columns (one pass) according to the manufacturer's instructions (Pharmacia, Pistcataway, NJ); however, in this procedure ribosomal RNA was not completely eliminated.

RNA blot analysis and preparation of probes

Poly A$^+$-enriched RNA was applied to a 1.1% agarose gel, electophoresed and transferred to a nylon membrane as described previously [22]. The membrane was dried and prehybridization and hybridization was performed using a solution containing 50% formamide (w/v), 50 mM Na_2HPO_4, 10% dextran sulfate (w/v), 5 × Denhardt's solution and 100 μg/ml sonicated/denatured salmon sperm DNA at 42°C, as outlined previously [22]. The probe for $P450_{AROM}$ was isolated from a cDNA library prepared from a rat Leydig tumor cell (R2C) line [27]. A probe specific for the 5′ terminus was prepared by isolating a DNA segment with EcoR1 and BamH1 as described by Lephart *et al.* [27], [^{32}P]dCTP, specific

oligonucleotide primers and Taq polymerase [22]. This probe detects all three $P450$ mRNA species. The probe for 5α-reductase was prepared, as described previously [25], using a rat 5α-reductase cDNA, [^{32}P]dCTP, by the method of random hexamers [26]. The prehybridization and hybridization conditions and procedures were performed as detailed elsewhere [26]. In the case of $P450_{AROM}$, after hybridization (18–24 h), the blot was washed in 2 × SSC (sodium chloride–sodium citrate, w/v) and 0.5% (w/v) sodium dodecyl sulfate (SDS) to a final stringency of 0.1% (w/v) SSC, twice for 25 min at 45°C. For 5α-reductase the blot was washed twice for 30 min in 0.1% SSC and 0.1% SDS at 58°C [25]. The blot was exposed to Kodak XAR-2 film at −70°C with two intensifying screens for 4 h in the case of $P450_{AROM}$ and 24 h in the case of 5α-reductase. After exposure, the blot was washed in 0.1% SDS and 0.1% SSC at 100°C for 45 min (stripped), exposed overnight, before rehybridization was performed.

For the 18S rRNA probe, an oligonucleotide (30 base pairs; 5′→3′; CTTCCTCTAGATA-GTCAAGTTCGACCGTCT) complementary to 18S ribosomal RNA was end-labeled using [^{32}P]ATP and T4 polynucleotide kinase [28]. Prehybridization (25 h) was performed at 57°C in a buffer containing 5 × Denhardt's, 1% SDS, 6 × SSC (final concentration, v/v), 5 mM sodium pyrophosphate and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed under identical conditions in the same buffer with the addition of the ^{32}P-labeled oligonucleotide probe for 18 h. The blot was washed with 2 × SSC (w/v) and 1.0% SDS, 30 min at room temperature, then two times in 2 × SSC and 1.0% SDS for 35 min each at 50°C. the blot was exposed to Kodak XAR-2 film −70°C with two intensifying screens for 2 h.

Enzyme activity assays

Aromatase and 5α-reductase activities in ovarian tissue were measured simultaneously using [1α,1β-^{3}H]T as the substrate. In these experiments we used ovarian tissue slices since this may maintain thecal/granulosa cell interrelationships and better reflect the activities present *in vivo*. In aromatase assays only the tritium atom in the β-position is incorporated into water during the aromatization reaction (i.e. isolated and quantified), whereas, in 5α-reductase assays, the tritium atom in the α-position is retained in the steroid structure (i.e. extracted into the organic phase, resolved by TLC and quantified by scintillation counting). The enzyme activities were determined in individual ovarian tissues samples which were classified according to the different stages of the estrous cycle [n = 8 to 10 independent determinations (animals)/stage]. The protein content of each tissue slice was assessed by the method of Lowry *et al.*, as described previously [24–26]. The aromatase and 5α-reductase activities were expressed as pmol/h of incubation/mg protein.

Aromatase. Aromatase activity was measured in ovarian tissue samples by the tritiated water release assay, as described in detail elsewhere [24, 29]. However, in brief, ovarian tissue was trimmed of adherent fat and cut into small slices. The tissue slices were incubated for 1 h at 37°C in 0.2 ml of Dulbecco's Modified Eagle Medium (DMEM), pH 7.4, containing 4.5 μM of [1α,1β-^{3}H]T under an atmosphere of 95% O_2 and 5% CO_2. The incubations were terminated by the addition of 5 vol (1 ml) of chloroform–methanol (2:1, v/v) and steroids were extracted by vortexing for 1 min. From each tube the aqueous phase was purified and an aliquot of the ^3H_2O formed during the incubation was quantified by scintillation counting. As previously demonstrated in our laboratory, maximal rates of aromatase activity in ovarian tissue are obtained at a substrate concentration of 0.5 μM [6] and the identity of the [^{3}H]estrogen formed has been confirmed by recrystallization of the derivatized estradiol to constant specific activity [6].

5α-Reductase. 5α-Reductase activity was determined in ovarian tissue slices by incubation with [1α,1β-^{3}H]T as substrate, followed by TLC as described elsewhere [24–26]. In brief, the incubation of the reaction mixture (as described above) was stopped and steroids extracted with 1 ml chloroform–methanol (2:1, v/v). From each tube, a portion of the organic phase (100–200 μl) was evaporated to dryness, redissolved in 30 μl (twice) of chloroform containing 10 μg each of five nonradioactive steroids [5α-androstane-3β,17-dione (5α-dione), androstenedione, 5α-dihydrotestosterone (5α-DHT), T, and 5α-androstane-3α,17β-diol (3α-diol)], applied to plastic sheets (20 × 20 cm) precoated with silica gel, and developed with one ascent of the solvent system (dichloromethane–ethyl acetate–methanol, 85:15:3; by vol). The five reference standards were visualized by spraying with water and marked. Within each lane, the entire TLC strip was cut out from the origin to

the solvent front, each area corresponding to the five reference steroids was isolated and assayed for tritium by scintillation counting. In this method the total radioactivity per lane was determined which automatically corrects for procedural losses. The radioactivity recovered in 5α-dione, 5α-DHT, 3α-diol areas was expressed as a fraction of the total radioactivity recovered within each lane. To calculate the enzymatic rate, the percentage for each steroid was multipled by the substrate concentration added at the beginning of the incubation, as previously detailed [24–26].

In preliminary experiments using cyclic (metestrus and diestrus) rats, maximal rates of 5α-reductase activity (5α-DHT only) were obtained with a substrate concentration of 3.0 μM (data not shown). The calculated apparent K_m was 2.4 μM and a maximal velocity of 14 pmol/h/mg protein was obtained. Therefore, standard incubation conditions were employed in which tissue slices were incubated for 1 h at 37°C in 0.2 ml DMEM containing 4.5 μM substrate.

In vitro inhibition of 5α-reductase activity was demonstrated by preincubating ovarian tissue slices for 30 min in 0.1 ml of DMEM containing 2.0 μM of the 5α-reductase inhibitor, MK-906, at 37°C before the addition of 9.0 μM [^{3}H]T in 0.1 ml DMEM. The reaction mixture was incubated for 1 h as described [6] and the reactions were stopped by the addition of 1 ml chloroform–methanol (2:1, v/v) for the extraction of labeled steroids. Subsequently TLC was performed to isolate the 5α-reduced metabolites and quantification and calculation of 5α-reductase activity rates were determined. Using samples from proestrous and estrous animals, 5α-reductase activity (Control: 5α-DHT = 0.8 pmol/h/mg protein and 3α-diol = 6.0 pmol/h/mg protein) in ovarian tissue was significantly decreased by ≈90% when incubated in the presence of the 5α-reductase inhibitor, MK 906 (data not shown).

Normalization of activity measurements and mRNA levels

The autoradiograms of northerns of RNA from ovarian tissue were analyzed by densitometry using a Helena scanning densitometer (Beaumont, TX). The levels of $P450_{AROM}$ mRNA were determined by scanning the RNA blot autoradiogram in which the largest mRNA species (at 2.7 kb) was quantified since this appears to be the functional $P450_{AROM}$ mRNA in rat ovarian tissue [27]. The levels of 5α-reductase mRNA were also quantified and the relative levels of RNA loaded into each lane were determined by scanning the 18S rRNA bands.

To compare activity measurements to mRNA levels the highest levels obtained in each assay or RNA blot was assigned a value of 100%. All other values were expressed as a percentage of this maximal value. The total 5α-reductase enzymatic activity for each phase of the estrous cycle was obtained by combining the rates of the three 5α-reduced metabolites (i.e. 5α-dione, 5α-DHT and 3α-diol).

Radioimmunoassay

Serum was obtained from blood samples by centrifugation and stored at −20°C until assayed. Random samples (4 to 5) were chosen from each stage of the estrous cycle across the two groups of animals [from which enzyme activity (individual samples) or mRNA levels (from pooled tissue) had been evaluated (total $n = 8$ to 10 animals/stage of the estrous cycle)]. Luteinizing hormone (LH) levels were determined by double-antibody radioimmunoassay (RIA) as described previously [24, 26]. Serum samples from these animals were analyzed in a single assay. The intra-assay coefficient of variation was 9%.

RESULTS

mRNA levels

$P450_{AROM}$. The changes in $P450_{AROM}$ mRNA levels in ovarian tissue as classified by the different stages of the estrous cycle was shown in Fig. 1. During the 4-day cycle, all three $P450_{AROM}$ mRNA species (i.e. at 1.7, 2.2 and 2.7 kb) were detected when the 5′ terminus of the rat $P450_{AROM}$ cDNA was utilized as probe. The three $P450_{AROM}$ mRNA species were most abundant during diestrus and proestrus. However, the most abundant band throughout the estrous cycle appeared to be the largest 2.7 kb species. During estrus the abundance of all three mRNA species decreased, however, during metestrus the levels were markedly reduced to nearly nondetectable levels. (Longer exposures of this blot revealed faint bands for the three $P450_{AROM}$ mRNA species, during metestrus).

5α-Reductase. Conversely, only one 5α-reductase mRNA species (at 2.5 kb) was detected in ovarian tissue, throughout the estrous cycle (Fig. 2). Moreover, 5α-reductase mRNA

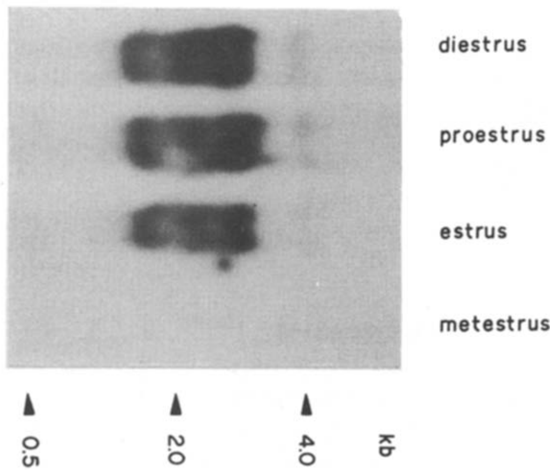

Fig. 1. RNA blot analysis of $P450_{AROM}$ mRNA in pooled ovarian tissue collected during various stages of the rat estrous cycle. Total cellular RNA was extracted from ovaries of normal adult cyclic rats according to the estrous cycle classifications ($n = 8$ to 10 animals/stage of cycle). Poly A^+-enriched RNA (40 μg) per lane was fractionated by gel electrophoresis, transferred to Zetaprobe membrane, and hybridized to a ^{32}P-labeled rat 5′ $P450_{AROM}$ cDNA. A ^{32}P-labeled λ HindIII DNA ladder served as standard, for size estimation (kilobase, kb).

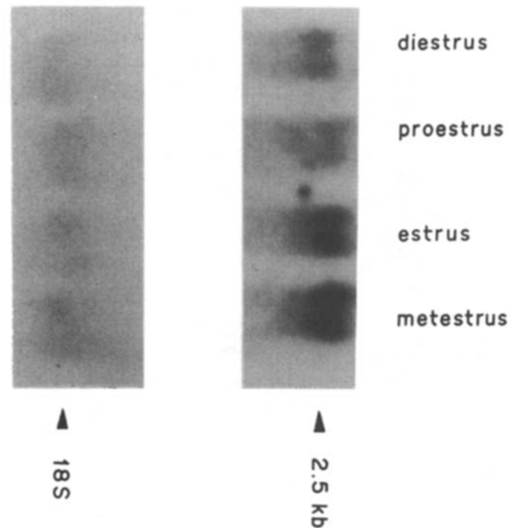

Fig. 2. RNA blot analysis of 5α-reductase mRNA in pooled ovarian tissue collected during various stages of the rat estrous cycle. *Right panel*: following the determination of $P450_{AROM}$ mRNA levels, the blot was washed in 0.1% SDS and 0.1% SSC at 100°C for 45 min (stripped), exposed overnight, before rehybridization was performed utilizing a ^{32}P-labeled probe complementary to the coding region of the rat 5α-reductase cDNA. *Left panel*: following autoradiographic exposure for 5α-reductase mRNA, the blot was stripped and hybridized to an 18S rRNA probe to quantify the relative amounts of RNA loaded in each lane.

levels displayed a pattern opposite to that of the $P450_{AROM}$ mRNA profile (see Fig. 1). The most abundant levels of ovarian 5α-reductase mRNA occurred during metestrus, the levels decreased during diestrus and proestrus, but increased during estrus. To serve as a positive control for the 5α-reductase probe, in another blot containing adult rat liver and ovarian RNA, an intense band was observed in the liver sample (at 2.5 kb) that was processed and hybridized in parallel with the blot above (data not shown), as previously demonstrated by our laboratory [24, 25].

To confirm that equal amounts of RNA were loaded into each well, the blot was hybridized with an 18S rRNA probe. The intensity of the 18S bands was relatively constant across all lanes (Fig. 2).

Enzyme activities

$P450_{AROM}$. As shown in Fig. 3, the pattern of $P450_{AROM}$ activity paralleled that of the

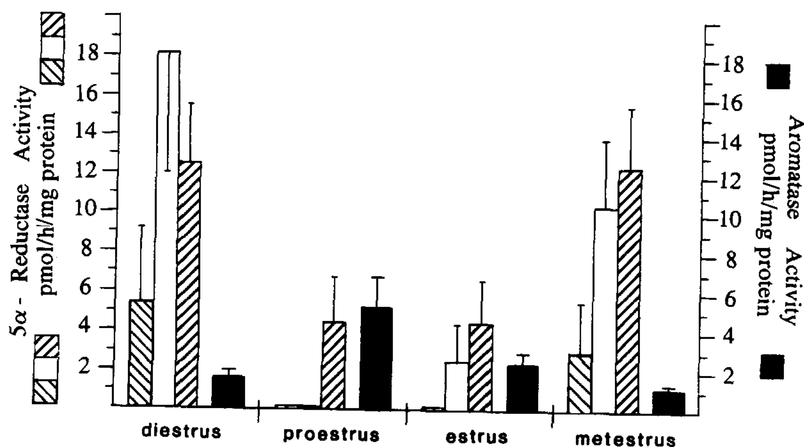

Fig. 3. Histogram of the changes in $P450_{AROM}$ and 5α-reductase enzyme activities during the estrous cycle of the rat. Ovarian tissue slices were incubated in 0.2 ml DMEM, pH 7.4, containing 4.5 μM [^{3}H]T for 1 h at 37°C. Each point represents the mean ± SEM of data obtained from 8 to 10 animals/stage of the estrous cycle. ■ = aromatase activity. For 5α-reductase activity, ▧ = 5α-dione. □ = 5α-DHT and ▨ = 3α-diol levels.

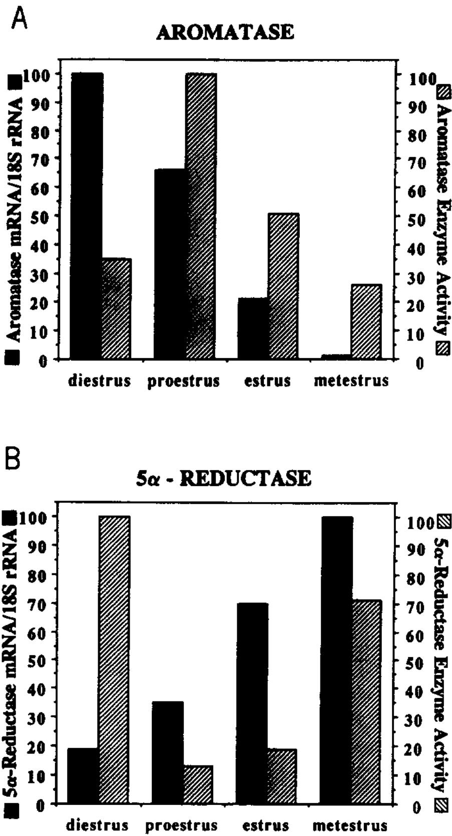

Fig. 4. (A) Aromatase and (B) 5α-reductase: histograms comparing normalized mRNA levels and enzyme activities expressed as percentages during the estrous cycle. The abundance of the mRNAs were determined by scanning densitometry. The relative levels of poly A^+-enriched RNA loaded into each lane were determined by scanning the 18S rRNA bands. After the mRNA levels were normalized against the 18S rRNA levels, the highest level was assigned a value of 100% (i.e. diestrus for aromatase and metestrus for 5α-reductase). All other normalized values were expressed as a percentage of this maximal value. To express relative enzyme activities (i.e. pmol/h/mg protein), the highest level of aromatase (during proestrus) and 5α-reductase (during diestrus) was assigned a value of 100%, and all other values were expressed as percentages of this maximal value.

$P450_{AROM}$ mRNA profile (see Fig. 1), however, the activity levels appeared to lag behind the mRNA profile (Fig. 1) by about 24 h, or one stage of the estrous cycle. Aromatase activity was recorded at 1.5 pmol/h/mg protein during diestrus, displayed over a 3-fold increase during proestrus (≈5.5 pmol/h/mg protein), then decreased by one-half during estrus and further declined to the lowest recorded values at ≈1.0 pmol/h/mg protein, during metestrus.

5α-Reductase. In comparison (and in a coordinate manner with the respective mRNA profiles), the 5α-reductase activity pattern was opposite to the $P450_{AROM}$ activity pattern during the estrous cycle. As shown in Fig. 3, 5α-reductase activity was lowest during proestrus; 5α-dione and 5α-DHT were undetectable, while 3α-diol levels were low (≈5 pmol/h/mg protein). During estrus, 5α-dione and 3α-diol levels remained at proestrus values. Whereas, 5α-DHT levels that were nondetectable during proestrus, increased to ≈3 pmol/h/mg protein during the estrous stage of the cycle. However, all three 5α-reduced metabolites increased markedly (over 3-fold) during metestrus. While the highest activity levels were recorded during diestrus (5α-dione ≈5.5 pmol/h/mg protein; 5α-DHT ≈18.2 pmol/h/mg protein and 3α-diol ≈12.5 pmol/h/mg protein).

Comparison of $P450_{AROM}$ and 5α-reductase enzyme activities to $P450_{AROM}$ and 5α-reductase mRNA levels

In the ovary, the expression of $P450_{AROM}$ and 5α-reductase mRNA levels appeared (≈24 h) before the corresponding changes in the respective activities during the estrous cycle. To correlate the relationship between mRNA levels and enzyme activity the content of the mRNA levels for each enzyme was quantified by densitometric scanning. After the mRNA levels and enzyme activities were normalized they were expressed as percentages of the maximal values obtained in each experiment for a given phase of the estrous cycle. A comparison of the normalized mRNA abundance and enzyme activities is shown in Fig. 4. The abundance of $P450_{AROM}$ mRNA was highest during diestrus [100%; see Fig. 4(A)]. At proestrus $P450_{AROM}$ mRNA levels were two-thirds of proestrus values, decreased during estrus (21%) and were nearly nondetectable during metestrus (1%). The normalized $P450_{AROM}$ enzyme activity pattern was similar to the $P450_{AROM}$ mRNA profile, however the changes in activity lagged behind the mRNA profile during the estrous cycle [Fig. 4(A)]. The activity was highest (100%) during proestrus, decreased by ≈one-half at estrus and declined further to low levels during metestrus (≈26%) and diestrus (≈34%).

Conversely, the changes in the normalized values of 5α-reductase enzyme activity and mRNA levels are shown in Fig. 4(B). The 5α-reductase mRNA levels were lowest during diestrus (≈18%), increased in a stair-step fashion during proestrus (≈34%) and estrus (≈68%), whereas, the highest mRNA abundance were recorded during metestrus (100%). This 5α-reductase mRNA pattern is the inverse

to that of the $P450_{AROM}$ mRNA profile. The normalized 5α-reductase enzyme pattern trailed the mRNA profile by ≈24 h [Fig. 4(B)]. The activity was lowest during proestrus (≈13%) increased during estrus (≈19%) and metestrus (≈71%), with the highest activity levels recorded at diestrus (100%).

Serum LH levels

To provide additional confirmation of the classification of the cyclic rats into the different phases of the estrous cycle, serum LH levels were determined by RIA. During estrus, metestrus and diestrus, LH levels were low ($\approx 1.0 \pm 0.2$ ng/ml), however, LH levels significantly increased by more than 8-fold (8.7 ± 2.8 ng/ml) during proestrus ($n = 8$ to 10 animals/stage of the estrous cycle), in agreement with previous reports [3, 4, 30].

DISCUSSION

Previous reports indicate that the end-products of androgen metabolism, via the enzymes $P450_{AROM}$ and 5α-reductase, apparently have opposing effects on follicular development. Estrogens stimulate and enhance [12–15] while DHT inhibits [16–19] follicular growth. This prompted us to examine the changes in ovarian $P450_{AROM}$ and 5α-reductase mRNA levels and record the activities of both enzymes using quantitative assays by an indirect ($P450_{AROM}$ by the ^3H_2O-release method) or direct measurement (5α-reductase by TLC) during the rat estrous cycle. While considerable attention has been devoted to the $P450_{AROM}$ pathway of androgen metabolism in the ovary [1, 2], little is known about the changes in the 5α-reductase enzyme during the estrous cycle [9].

Although in previous reports the detection of 5α-reductase activity has been demonstrated in both immature [7, 8] and adult ovarian tissue [9], the relationship between the pattern of 5α-reductase (and $P450_{AROM}$) mRNA levels and activity has not been presented heretofore. Northern blot analysis of ovarian RNA (from the different stages of the cycle) hybridized to a rat 5α-reductase cDNA revealed the presence of a single band at 2.5 kb similar in size to the 5α-reductase mRNA species previously detected in rat liver [31], prostate [31], adrenal [26] and brain [25]. The 5α-reductase mRNA levels were lowest during diestrus and proestrus but increased at estrus and peaked during metestrus. The changes in 5α-reductase mRNA levels preceded the changes in enzymatic activity.

For $P450_{AROM}$, Northern analysis revealed three species of mRNA (at 1.7, 2.2 and 2.7 kb) which hybridized to a 5′ segment of a rat cDNA as described previously [27]. The pattern of the peak (i.e. at diestrus and proestrus) and decline (i.e. at estrus and metestrus) of ovarian $P450_{AROM}$ RNA levels displayed a similar pattern to 5α-reductase RNA expression in terms of the relationship to that of the enzyme activity profile during the estrous cycle. In both instances the changes in mRNA levels preceded the changes in enzymatic activity by about 24 h, or one stage of the estrous cycle. The reason for the apparent lag interval between peak mRNA levels and enzyme levels is unknown. Presumably, as previously demonstrated in other studies [20–22], the regulation of steroidogenesis is at the level of mRNA expression of these species encoding the steroidogenic enzyme(s). Apparently this may be the case for both the 5α-reductase and $P450_{AROM}$ enzymes.

Conversely, the increase and decrease of the normalized $P450_{AROM}$ mRNA levels within the estrous cycle was opposite to that of 5α-reductase. When $P450_{AROM}$ mRNA levels were high during diestrus and proestrus, the normalized 5α-reductase mRNA levels were low. On the other hand, 5α-reductase mRNA levels were high while $P450_{AROM}$ mRNA levels were declining or low during estrus and metestrus, respectively. The inverse relationship between the expression of $P450_{AROM}$ and 5α-reductase mRNA during the estrous cycle may be due, in part, to the compartmentalization of these enzymes in granulosa [10] and thecal [11] cells, respectively. This raises the possibility of thecal–granulosa cell interaction and that growth factors [32] may be involved in the regulation of both $P450_{AROM}$ and 5α-reductase in the rat ovary during the estrous cycle. Although more data is available concerning the effects of growth factors on ovarian cell growth/proliferation and inhibition of growth [32], a number of growth factors have shown to modulate ovarian steroidogenesis. For example, epidermal growth factor (EGF) has been shown to inhibit FSH stimulated estrogen biosynthesis [33] and effect $P450_{AROM}$ mRNA levels [34]. Conversely, insulin-like growth factor-I (IGF-I) has been shown to increase $P450_{AROM}$ mRNA levels [34] and enzymatic activity [35], while transforming growth factors (TGF-α and -β) also appear to have the ability

to alter the rates of several steps in ovarian steroidogenesis [36, 37].

While a considerable amount of information is available concerning *in vitro* regulation of $P450_{AROM}$ enzymatic activity [1, 2, 33] much less is known about the modulation of 5α-reductase activity. Previous studies suggest that LH may stimulate 5α-reductase activity in the rat ovary [7, 38]. In the present study LH was measured and the highest levels were recorded during the proestrous stage of the cycle. The peak in serum LH preceded the increase in 5α-reductase mRNA levels, suggesting that a temporal relationship may exist between serum LH levels and increased abundance of 5α-reductase mRNA observed during estrus and metestrus. However, whether serum LH levels directly regulate ovarian 5α-reductase mRNA levels will require further study.

It is noteworthy that the $P450_{AROM}$ enzyme displays an inverse pattern of mRNA levels and enzymatic activities to that of 5α-reductase during the estrous cycle since the steroid products of each (i.e. estradiol and 5α-DHT, respectively) have opposing effects on regulating the aspects of ovarian function [12–19]. The changes in the pattern of $P450_{AROM}$ mRNA levels which precede the enzyme activities and hence estrogen biosynthesis are in agreement with the known changes in the serum estradiol profile in cyclic rats [3, 4, 30]. On the other hand, it has been shown that serum testosterone, the major substrate for the 5α-reductase enzyme, displays a similar pattern to estradiol throughout the estrous cycle [39, 40]. In the present study, however, the 5α-reductase activities were lowest during proestrus when high substrate (testosterone) levels have been observed [39, 40]. Conversely, 5α-reductase enzyme activities were high during metestrus when testosterone values are low [39, 40], suggesting that the conversion of low levels of testosterone to 5α-DHT may be maximized during this stage of the estrous cycle, when estradiol production is low [3, 4, 30]. Therefore, based upon the change in the mRNA levels and activity profiles in the present study and the known changes in steroid hormone levels during the rat estrous cycle, it is reasonable to suggest that the $P450_{AROM}$ and 5α-reductase enzymes may be directly involved in regulating steroid hormone secretion patterns and hence follicular development.

In summary, during the estrous cycle: (1) three mRNA species encoding rat $P450_{AROM}$ were detected (at 1.7, 2.2 and 2.7 kb); (2) only a single 5α-reductase band was observed at 2.5 kb (similar in size to 5α-reductase mRNA species previously detected in rat liver, prostate [31], brain [25] and adrenal [26]; (3) an inverse pattern was seen between $P450_{AROM}$ and 5α-reductase for both enzymatic activity and mRNA expression and; (4) the profiles of both enzyme activities follow the changes in the levels of their respective mRNAs.

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REFERENCES

1. Engel L. L.: The biosynthesis of estrogens. In *Handbook of Physiology-Endocrinology II (Part I)* (Edited by R. O. Greep). Waverly Press, Baltimore, MD (1973) p. 467.
2. Erickson G. F., Magoffin D. A., Dyer C. A. and Hofeditz C.: The ovarian androgen producing cells: A review of structure/function relationships. *Endocrine Rev.* **6** (1985) 371–399.
3. Butcher R. L., Collins W. E. and Fugo N. W.: Plasma concentrations of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day estrous cycle of the rat. *Endocrinology* **94** (1974) 1704–1708.
4. Nequin L. G., Alvarez J. and Schwartz N. B.: Measurement of serum steroid gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat. *Biol. Reprod.* **20** (1979) 659–670.
5. Brandt M. E., Puett D. and Zimniski S. J.: Divergence between ovarian aromatase activity, estrogen, and androgen levels in the cycling rat. *Endocrinology* **126** (1990) 72–79.
6. Lephart E. D., Mathews D., Noble J. F. and Ojeda S. R.: The vaginal epithelium of immature rats metabolizes androgens through an aromatase-like reaction: Changes during the time of puberty. *Biol. Reprod.* **40** (1989) 259–267.
7. Terakawa N., Kondo K., Aono T., Kurachi K. and Matsumoto K.: Hormonal regulation of 4-ene-5α-reductase activity in prepubertal rat ovaries. *J. Steroid Biochem.* **9** (1978) 307–311.
8. Eckstein B. and Lerner N.: Changes in ovarian 5α-steroid reductase and 20α-hydroxysteroid dehydrogenase activity produced by induction of first ovulation with gonadotropin. *Biochim. Biophys. Acta* **489** (1977) 143–149.
9. Meijs-Roelofs H. M. A., Kramer P., van Cappellen W. A., Gribling-Hegge L. and Woutersen P. J. A.: Periovulatory changes in serum concentration and ovarian content of 5α-androstane-3α, 17β-diol in the adult rat. *Biol. Reprod.* **35** (1986) 890–896.
10. Yoshinaga-Hirabayashi T., Ishimura K., Fujita H., Kitawaki J. and Osawa Y.: Immunocytochemical localization of aromatase in immature rat ovaries treated with PMSG and hCG, and in pregnant rat ovaries. *Histochemistry* **93** (1990) 223–228.
11. Aono, T., Kitamura Y., Fukuda S. and Matsumoto K.: Localization of 4-ene-5α-reductase, 17β-ol-dehydrogenase and aromatase in immature rat ovary. *J. Steroid Biochem.* **14** (1981) 1369–1377.

12. Richards J. S.: Hormonal control of ovarian follicular development: a 1978 perspective. *Recent Prog. Horm. Res.* **35** (1979) 343–373.
13. Rao M. C., Midgley, A. R. Jr and Richards J. S.: Hormonal regulation of ovarian cellular proliferation. *Cell* **14** (1978) 71–78.
14. Louvet J.-P., Harman S. M. and Ross G. T.: Effects of human chorionic gonadotropin, human interstitial cell stimulating hormone, and human follicle-stimulating hormone on ovarian weights in estrogen-primed hypophysectomized immature female rats. *Endocrinology* **96** (1975) 1179–1186.
15. Harman S. M., Louvet J.-P. and Ross G. T.: Interaction of estrogen and gonadotropins on follicular atresia. *Endocrinology* **96** (1975) 1145–1152.
16. Farookhi R.: Effects of androgen on induction of gonadotropin receptors and gonadotropin-stimulated adenosine 3′,5′-monophosphate production in rat ovarian granulosa cells. *Endocrinology* **106** (1980) 1216–1223.
17. Jia C., Kessel B., Welsh T. H. and Hsueh A. J.: Androgen inhibition of follicle-stimulating hormone stimulated luteinizing hormone receptor formation in cultured rat granulosa cells. *Endocrinology* **117** (1985) 13–22.
18. Conway B. A., Mahesh V. B. and Mills T. M.: Effect of dihydrotesterone on the growth and function of ovarian follicles in intact immature female rats primed with PMSG. *J. Reprod. Fert.* **90** (1990) 267–277.
19. Hillier S. G., Agnes M. J. van den Boogaard, Reichert L. E. and van Hall E. V.: Alterations in granulosa cell aromatase activity accompanying preovulatory follicular development in the rat ovary with evidence that 5α-reduced C_{19} steroids inhibit the aromatase reaction *in vitro*. *J. Endocr.* **84** (1980) 409–419.
20. Rodgers R. J., Waterman M. R. and Simpson E. R.: Levels of messenger ribonucleic acid encoding cholesterol side-chain cleavage cytochrome *P*-450, 17α-hydroxylase *P*-450, adrenodoxin, and low density lipoprotein receptor in bovine facilities and corpora lutea throughout the ovarian cycle. *Molec. Endocr.* **1** (1987) 274–279.
21. Doody K. J., Lorence M. C., Mason J. I. and Simpson E. R.: Expression of mRNA species encoding steroidogenic enzymes in human follicles and corpora lutea throughout the menstrual cycle. *J. Clin. Endocr. Metab.* **70** (1990) 1041–1045.
22. Doody K. J., Lephart E. D., Stirling D., Lorence M. C., Magness R. R., McPhaul M. J. and Simpson E. R.: Expression of mRNA species encoding steroidogenic enzymes in the rat ovary. *J. Molec. Endocr.* **6** (1991) 153–162.
23. Leshin M., Griffin J. E. and Wilson J. D.: Hereditary male pseudohermaphroditism associated with an unstable form of 5α-reductase. *J. Clin. Invest.* **62** (1978) 685–691.
24. Lephart E. D. and Ojeda S. R.: Hypothalamic aromatase activity in male and female rats during juvenile peripubertal development. *Neuroendocrinology* **51** (1990) 385–393.
25. Lephart E. D., Andersson S. and Simpson E. R.: Expression of neural 5α-reductase messenger ribonucleic acid: Comparison to 5α-reductase activity during prenatal development in the rat. *Endocrinology* **127** (1990) 1121–1128.
26. Lephart E. D., Simpson E. R. and Trzeciak W. H.: Rat adrenal 5α-reductase mRNA content and enzyme activity are sex hormone dependent. *J. Molec. Endocr.* **6** (1991) 163–170.
27. Lephart E. D., Peterson K. G., Noble J. F., George F. W. and McPhaul M. J.: The structure of cDNA clones encoding the aromatase *P*-450 isolated from a rat Leydig cell tumor line demonstrates differential processing of aromatase mRNA in rat ovary and a neoplastic cell line. *Molec. Cell. Endocr.* **70** (1990) 31–40.
28. Sambrook J., Fritsch E. F. and Maniatis T.: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, Vol. 1 (1989) p. 5.70.
29. Lephart E. D. and Simpson E. R.: Techniques for the assay of aromatase cytochrome *P*450. In *Methods in Enzymology* (Edited by M. R. Waterman and E. F. Johnson). Academic Press, Orlando, FL, Vol. 206 (1991) pp. 477–483.
30. Feder H. H.: Estrous cyclicity in mammals. In *Neuroendocrinology of Reproduction* (Edited by N. T. Adler). Plenum Press, New York, NY (1981) pp. 279–305.
31. Andersson S., Bishop R. W. and Russell D. W.: Expression cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation. *J. Biol. Chem.* **264** (1989) 16,249–16,255.
32. Skinner M. K.: Transforming growth factor production and action in the ovarian follicle: Theca cell-granulosa cell interactions. In *Growth Factors and The Ovary.* Serono Symposia, U.S.A. (Edited by A. N. Hirshfield). Plenum Press, NY (1989) pp. 141–150.
33. Hsueh A. J. W., Adashi E. Y., Jones P. B. C. and Welsh T. H.: Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocrine Rev.* **5** (1984) 76–126.
34. Steinkampf M. P., Mendelson C. R. and Simpson E. R.: Effects of epidermal growth factor and insulin-like growth factor I on the levels of mRNA encoding aromatase cytochrome *P*-450 of human ovarian granulosa cells. *Molec. Cell. Endocr.* **59** (1988) 93–99.
35. Bendell J. J. and Dorrington J. H.: Epidermal growth factor influences growth and differentiation of rat granulosa cells. *Endocrinology* **127** (1990) 533–540.
36. Caubo R., DeVinna R. S. and Tonetta S. A.: Regulation of steroidogenesis in cultured porcine theca cells by growth factors. *Endocrinology* **125** (1989) 321–326.
37. Adashi E. Y., Resnick C. E., Hernandez E. R., May J. V., Purchio A. F. and Twardzik D. R.: Ovarian transforming growth factor-β (TGF-β): cellular site(s), and mechanism(s) of action. *Molec. Cell. Endocr.* **61** (1989) 247–256.
38. Fukuda S., Terakawa N., Sato B., Imori T. and Matsumoto K.: Hormonal regulation of activities of 17β-ol-dehydrogenases, aromatase and 4-ene-5α-reductase in immature rat ovaries. *J. Steroid Biochem.* **11** (1979) 1421–1427.
39. Dupon C. and Kim M. H.: Peripheral plasma levels of testosterone, androstenedione, and oestradiol during the rat oestrous cycle. *J. Endocr.* **59** (1973) 653–654.
40. Mathews D., Andrews W. W., Parker R. P. Jr and Ojeda S. R.: A role of aromatizable androgens in female rat puberty. *Biol. Reprod.* **36** (1987) 836–843.