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Expression of 17β-Hydroxysteroid Dehydrogenase Type 1 and Type 2, P450 Aromatase, and 20α-Hydroxysteroid Dehydrogenase Enzymes in Immature, Mature, and Pregnant Rats*

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ABSTRACT

In the present study, we evaluated the expression and regulation of 17β-hydroxysteroid dehydrogenase (17HSD) type 1 and type 2, cytochrome P450 aromatase (P450arom), and 20α-hydroxysteroid dehydrogenase (20HSD) in mature and pregnant rats. Immunohistochemical analysis of rat 17HSD type 1 showed that the enzyme is exclusively expressed in the granulosa cells of developing, healthy, primary, secondary, and tertiary follicles at all stages of the estrous cycle and pregnancy, and is not detected in the corpora lutea. The data showed that the amount of the enzyme expressed in the follicle increases as follicular maturation progresses and is highest in tertiary and Graafian follicles. However, Northern blot analysis of total RNA from whole ovaries showed a rather constitutive expression of the 17HSD type 1 enzyme. It is evident that compared with P450arom, 17HSD type 1 is more widely expressed in the follicles during the various maturational stages of folliculogenesis. Hence, the data indicate distinct localization, expression, and regulation patterns for 17HSD type 1 and P450arom during the rat estrous cycle and pregnancy. Furthermore, compared with the two estradiol biosynthetic enzymes, a different expression pattern was detected for 20HSD messenger RNA. During the estrous cycle the enzyme was detected in the ovaries throughout the cycle, and in the ovaries of pregnant animals the enzyme showed an expression pattern the opposite of that observed for P450arom. Rat 17HSD type 2, not detected in the ovaries, was constitutively expressed in both female and male liver and small intestine in 21-day-old fetuses up to 6-week-old mature animals. Similarly, in these tissues the enzyme was constitutively expressed in normal cycling and pregnant animals, but it showed increasing expression in the placenta as pregnancy advanced. The relatively constitutive expression of the enzyme at all physiological stages of the animals suggests a general role for the enzyme in the inactivation of circulating sex steroids. (*Endocrinology* **138:** 2886–2892, 1997)

TWO OF THE key enzymes catalyzing the last steps of estradiol (E_2) biosynthesis in the ovarian follicles are P450 aromatase (P450arom) and 17β-hydroxysteroid dehydrogenase (17HSD). P450arom catalyzes the formation of the phenolic A ring of the C_{17} and C_{19} oxosteroids. The expression and regulation of P450arom in rat granulosa cells have been widely studied and have been found to be associated with follicular development and luteinization (1–5 and references therein). 17HSDs catalyze interconversion between the low activity neutral and phenolic 17-oxosteroids, such as androstenedione and estrone, into highly active 17β-hydroxysteroids, such as testosterone and E_2, respectively. To date, three rat 17HSD enzymes with varying biochemical properties have been reported (6–9). The current data indicate that distinct tissue specificities of the enzymes are responsible for the predominant reductive or oxidative activities found in various steroidogenic and peripheral tissues (7–9). In both humans and rodents, 17HSD type 1 has been localized to the ovarian granulosa cells, and it has predominantly reductive activity (6, 10–12). In contrast, 17HSD type 2 is highly expressed in the liver, small intestine, and placenta, and it predominantly oxidizes the highly active 17-hydroxysteroids into less active 17-ketosteroids (7, 13, 14).

In addition to its 17HSD activity, human 17HSD type 2 also possesses 20α-hydroxysteroid dehydrogenase (20HSD) activity (13), interconverting 20α-hydroxyprogesterone (20αOHP) and progesterone (P). In addition to P, a high concentration of 20αOHP is secreted from the rat ovary (15). 20αOH-P is mainly formed via the activity of 20HSD, which is widely distributed in several steroid (adrenal, ovarian, placental, and uterine) and nonsteroid-producing cells (16–19). As P is a necessary hormone during mammalian pregnancy, enzymes with 20HSD activity could play a pivotal role in the establishment and maintenance of pregnancy. The present study was carried out to study the expression and regulation of 17HSD type 1 and type 2, P450arom, and 20HSD enzymes in immature, mature, and pregnant rats, especially during the estrous cycle and pregnancy.

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Materials and Methods

Chemicals and reagents

[α-^{32}P]Deoxy-CTP (3000 Ci/mmol) and [α-^{32}S]deoxy-ATP (800 Ci/mmol) were purchased from Amersham International (Aylesbury, UK). Guanidine isothiocyanate was obtained from Fluka (Buchs, Switzerland). Cesium chloride and oligo(deoxythymidine)-cellulose were pur-

chased from Boehringer Mannheim (Mannheim, Germany). Agarose was obtained from FCM Bioproducts (Rockland, ME), and RNA mol wt markers (RNA ladder) were purchased from BRL (Gaithersburg, MD). Restriction enzymes were obtained from New England Biolabs (Beverley, MA) or Pharmacia (Piscataway, NJ), and T4 DNA ligase was obtained from Boehringer Mannheim. Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Other reagents not mentioned in the text were purchased from Sigma Chemical Co. (St. Louis, MO) or Merck (Darmstadt, Germany) and were of the highest purity grade available.

Animals and histological techniques

The ovaries, liver, and small intestine were excised from adult female regularly cycling Sprague-Dawley rats at proestrus, estrus, metestrus, and diestrus and from pregnant rats on various days of gestation. The stages of the estrous cycle were determined by light microscopic examination of vaginal fluids of the animals. In addition, placental tissues from the pregnant rats were analyzed. The liver and small intestine were also removed for analysis from 21-day-old fetuses and from immature male and female rats. All tissue specimens, except those used for immunohistochemistry, were immediately frozen in liquid nitrogen and stored at −70 C before processing. For immunohistochemistry, ovarian tissues were excised and immediately placed in 4% paraformaldehyde-PBS for 1 h at 4 C. The tissues were further fixed in 4% paraformaldehyde-5% sucrose-PBS for 1 h at 4 C. Thereafter, the ovaries were incubated overnight in 20% sucrose-PBS at 4 C. The fixed tissues were washed in PBS and frozen in isopentane cooled with liquid nitrogen. For immunohistochemical staining, 7-μm cryosections were cut and mounted onto polylysine-coated slides.

Immunohistochemistry

Immunohistochemical staining for rat 17HSD type 1 was carried out as described previously (6, 10). Nonspecific binding of the antibodies was blocked by incubating the slides in 10% goat serum-PBS for 30 min at room temperature. Thereafter, the slides were further incubated overnight at 4 C with a 1:200 dilution (in 10% goat serum-PBS) of a rabbit antiserum raised against human 17HSD type 1 protein. All subsequent steps were carried out at room temperature. The slides were washed three times with PBS for 5 min each time and incubated for 2 h in a 1:50 dilution of biotinylated goat antirabbit antibody (Dakopatts, Copenhagen, Denmark) in 10% goat serum-PBS. The slides were washed and treated for 1 h in a 1:50 dilution of streptavidin-conjugated fluorescein isothiocyanate (Dakopatts) in 10% goat serum-PBS. The slides were then washed five times with PBS, for 5 min each time, and mounted with a drop of Immu-mount (Shandon, Pittsburgh, CA).

Northern analysis

Total RNA was extracted from liver, ovary, placenta, and small intestine by homogenization in guanidium isothiocyanate buffer, followed by centrifugation through a cesium chloride density gradient. Twenty micrograms of total RNA were subjected to electrophoresis in 1% (wt/vol) agarose-formaldehyde gel, transferred overnight onto a Hybond nylon membrane (Amersham), and fixed by UV irradiation. The membranes were prehybridized for 2 h at 46 C in 5 × SSPE (1 × SSPE = 0.15 M NaCl, 10 mM PBS, and 0.1 mM EDTA, pH 7.4) with formamide (50%), BSA (0.1%), Ficoll (0.1%), polyvinylpyrrolidone (0.1%), SDS (0.5%), and 20 mg salmon sperm DNA/liter. Hybridization was carried out overnight at 46 C with ^{32}P-labeled complementary DNAs (cDNAs) of rat

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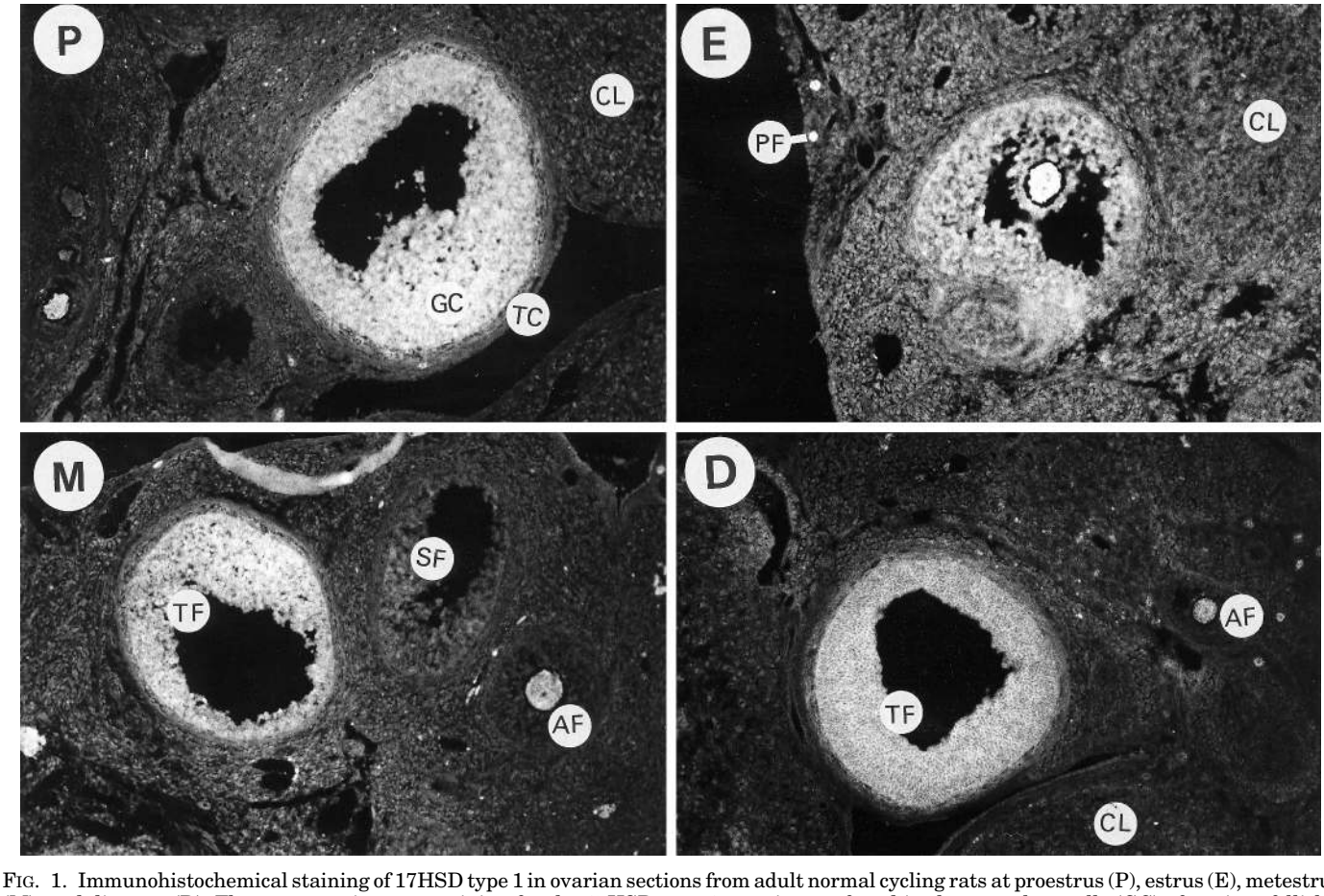

FIG. 1. Immunohistochemical staining of 17HSD type 1 in ovarian sections from adult normal cycling rats at proestrus (P), estrus (E), metestrus (M), and diestrus (D). The strongest immunostaining for the 17HSD type 1 protein was found in the granulosa cells (GC) of tertiary follicles (TF) throughout the estrous cycle. Immunostaining was also detected in growing primary (PF) and secondary follicles (SF), but not in the thecal cells (TC), atretic follicles (AF), or CL.

17HSD type 1 [1.0-kilobase (kb) *Eco*RI-SacI fragment) (6), rat 17HSD type 2 (1.3-kb full-length *Eco*RI fragment) (7), rat P450 aromatase (1.2-kb *Eco*RI fragment; provided by Prof. JoAnne Richards, Department of Cell Biology, Baylor College of Medicine, Houston, TX), and rat 20HSD (981-bp full-length *Apa*I fragment). After hybridization, the membranes were washed twice at 46 C in 2 × SSPE-0.1% SDS and once in 1 × SSPE-0.1% SDS for 15 min each time and exposed to Kodak X-AR films (Eastman Kodak, Rochester, NY) for 6–96 h. In between the different hybridizations, the bound probes were removed by treating the membrane with 0.1% SDS at 95 C. The amounts of 17HSD type 1, P450arom, and 20HSD messenger RNAs (mRNAs) expressed in the pregnant rat ovary were analyzed by laser densitometric scanning of the autoradiographs (Molecular Dynamics, Sunnyvale, CA) after 18 h of exposure and were normalized against the values obtained for γ-actin. The normalized values were used to analyze the relative expression of the mRNAs. The experiments by Northern analysis and immunohistochemistry were repeated several times with identical results, and the data presented in this manuscript are typical examples.

The 20HSD cDNA used was cloned by RT-PCR using polyadenylated RNA from adult rat ovaries. Oligo(deoxythymidine)-primed cDNA synthesis was carried out using a ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). A PCR reaction was then carried out using pfu DNA polymerase (Stratagene) together with primers corresponding to nucleotides −3 to 18 of the open reading frame and 954–978 of the complimentary strand (18).

Results

In our previous studies using hormonally treated immature rats, we have shown that 17HSD type 1 is predominantly expressed in the ovarian granulosa cells, and that the enzyme is under multihormonal regulation. The present results demonstrate that throughout the rat estrous cycle, 17HSD type 1 is expressed in the developing primary, secondary, and tertiary follicles. The enzyme is down-regulated in luteinizing follicles and is not significantly expressed in atretic follicles or corpora lutea (CL; Fig. 1). The amount of the enzyme expressed in the follicle increases as follicular maturation progresses and is highest in tertiary and Graafian follicles. Northern blot analysis of RNA extracted from whole ovaries, however, showed constitutive expression of 17HSD type 1 mRNA at proestrus, estrus, metestrus, and diestrus (Fig. 2). Compared with 17HSD type 1, P450arom was more efficiently regulated during the estrous cycle. The greatest expression of P450arom mRNAs was detected at proestrus; this was lower but still significant at metestrus and diestrus, but was barely detectable at estrus (Fig. 2). Among the analyzed mRNAs, 20HSD mRNA showed the strongest expression throughout the estrous cycle, with a gradual decline from proestrus to diestrus (Fig. 2). In diethylstilbestrol (DES)-treated immature rat ovaries, no significant expression of P450arom or 20HSD mRNAs was detected, indicating that of the enzymes analyzed, only 17HSD type 1 is expressed in the granulosa cells at the early stages of follicular maturation induced by DES.

Conversion of E_2 into estrone is a central metabolic reaction in the inactivation of E_2 in the liver, and of the 17HSD enzymes characterized to date, the type 2 isoform is suggested to have the most important role in this process. Hence, 17HSD type 2 expression was evaluated in rats with differing circulating sex steroid concentrations. The data, however, indicate that in the liver and small intestine the enzyme is constitutively expressed throughout the estrous cycle (Fig. 2). Similarly, no variation in the amount of the enzyme was detected during rat pregnancy or at any age of the animal

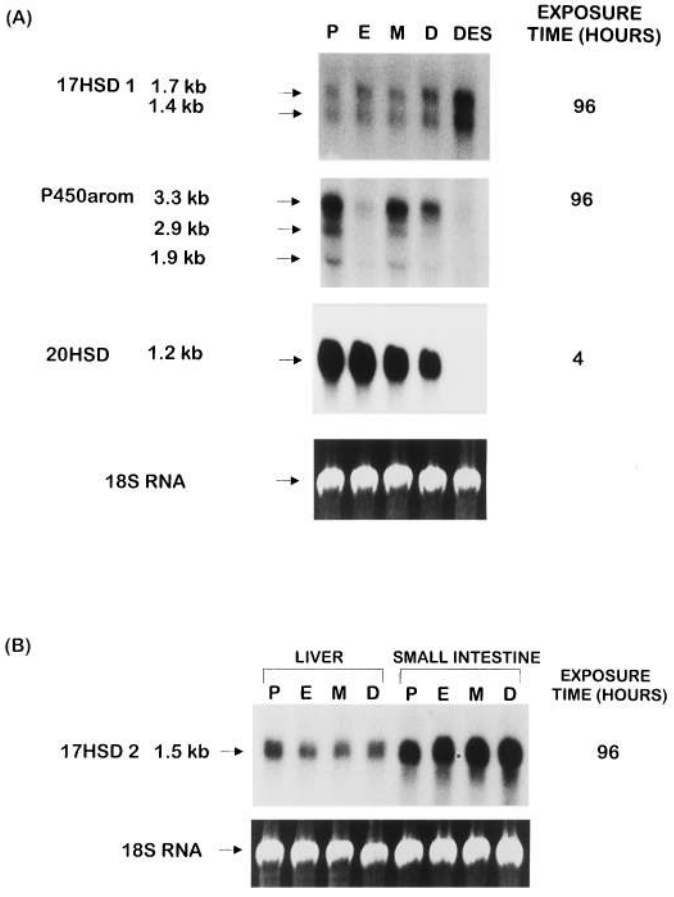

FIG. 2. Northern blot analysis of RNA (40 μg total RNA/lane) extracted from the ovaries (A) and liver and small intestine (B) of adult, normal cycling rats at proestrus (P), estrus (E), metestrus (M), and diestrus (D) and from the ovaries of DES-treated rats. Fragments of rat 17HSD type 1 and type 2, P450arom and 20HSD cDNAs were used as probes. Sample loading was controlled by ethidium bromide staining of 18S ribosomal RNA.

from late fetal life up to 6 weeks of age, regardless of sex (data not shown). This indicates constitutive expression of 17HSD type 2 enzyme in the liver and small intestine throughout the life span of the animals. In contrast, the expression of 17HSD type 2 in the placenta increased from a very low amount on day 8 to a relatively strong expression detected at the end of pregnancy (Fig. 3).

In the pregnant rat E_2 is produced in the ovaries from the androgenic precursors (mainly androstenedione) synthesized in the placenta. Steroidogenic enzymes, 17HSD type 1 and P450arom, were detected only in the ovaries of pregnant rats and were not found in the placenta at any stage during gestation (Fig. 3). Interestingly, immunohistochemical analysis of rat 17HSD type 1 expression throughout pregnancy showed strong expression of the enzyme only in the granulosa cells of developing ovarian follicles, similar to that found during the estrous cycle (Fig. 4). No significant expression of the enzyme was detected in the CL at any stage of gestation. Constitutive expression of the two 17HSD type 1 mRNAs in the ovary, with a slight increase from day 5 to day 15, was confirmed by Northern blot analysis (Fig. 3). Furthermore, our results using Northern analysis agree with

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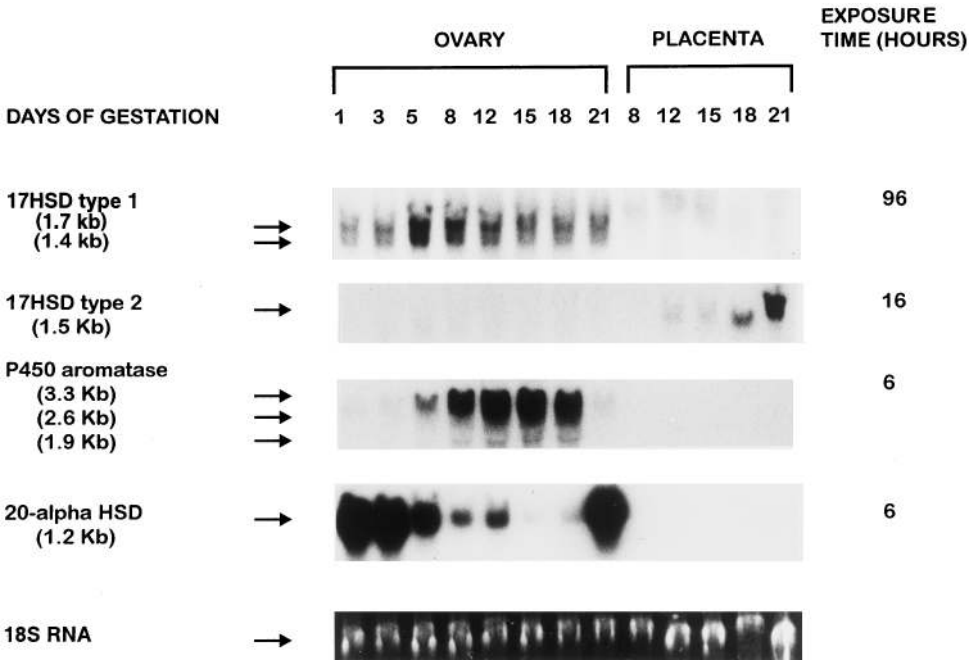

FIG. 3. A, Northern blot analysis of RNA (40 μg total RNA/lane) extracted from the ovaries and placental tissues of pregnant rats on days 1, 3, 5, 8, 12, 15, 18, and 21 and on days 8, 12, 15, 18, and 21 of gestation, respectively. Fragments of rat 17HSD type 1 and type 2, P450arom, and 20HSD were used as probes. Sample loading was controlled by ethidium bromide staining of 18S ribosomal RNA.

previous reports of strong up-regulation of ovarian P450arom expression starting on day 5 and reaching a plateau on day 12, lasting until day 18 of rat pregnancy. Thereafter, the amount of enzyme decreased precipitously to very low levels on day 21. Hence, 17HSD type 1 expression does not follow the pattern of P450arom regulation in pregnant rat ovaries. Furthermore, 17HSD type 1 is present in developing follicles, whereas P450arom is expressed in the CL, and the relative concentration of P450arom mRNA is about 30-fold higher than that of 17HSD type 1 on days 15–18 of pregnancy (Fig. 5).

We next studied the expression of 20HSD mRNA in pregnant rat ovaries. It showed a clearly different pattern compared with that of either 17HSD type 1 or P450arom. In fact, the expression of 20HSD mRNA was strictly opposite that detected for P450arom (Fig. 3). The high amount of mRNA expression detected throughout the estrous cycle continued on days 1 and 2 of pregnancy. Thereafter, a gradual decrease in the mRNA was observed, reaching a nadir on days 15–18. However, on day 21 a dramatic rise in the amount of 20HSD mRNA expression was observed, resulting in expression comparable to that detected on days 1 and 2 of gestation (Fig. 3).

Discussion

The preovulatory period of the estrous cycle in the rat is characterized by the growth of a cohort of ovarian follicles and enhanced biosynthesis and secretion of estradiol. FSH action via cAMP is known to increase rat P450arom and 17HSD type 1 mRNAs and enzyme activities in developing follicles and cultured granulosa cells (3, 10, 11, 20). Circulating E_2 concentrations reach a peak at midproestrus, just before the preovulatory surge of LH and subsequent ovulation (15, 21). Our present results show, however, that the expression of 17HSD type 1 is present in granulosa cells throughout the various maturational stages of the follicles during the normal estrous cycle. The expression of this enzyme becomes visible at the beginning of follicular development, increases as the follicles matures, and decreases sharply during luteinization. P450arom shows more extensive and stage-specific regulation. The amount of the enzyme, which is high at proestrus, shows strong down-regulation at estrus, in line with the decline in ovarian biosynthesis and secretion of E_2 observed during the periovulatory period (3, 15, 21–23). Hence, our results suggest that compared with 17HSD type 1, P450arom is more closely associated with the changes observed in ovarian E_2 biosynthesis and secretion. After ovulation and subsequent formation of the CL, estradiol synthesis begins to rise steadily through metestrus and diestrus in the rat (15). This possibly explains the significant level of P450 aromatase enzyme detected at metestrus and diestrus. The present results agree with our previous findings using hypophysectomized immature rats, in which DES stimulation results in the formation of large numbers of preantral follicles with very high amounts of 17HSD type 1 mRNA expression and 17HSD activity, whereas P450arom is absent (6). During the rat estrous cycle, the dominant progestin secreted by the ovary is 20αOHP (15), a progesterone metabolite formed by the activity of the 20HSD enzyme. Previous reports have also shown that the peripheral plasma concentrations of 20αOHP are variable, highest at proestrus and lowest at diestrus, probably mirroring similar variations in plasma progesterone concentrations (15). These findings agree with our present data that show a high level of expression of 20HSD mRNA during the whole estrous cycle with a gradual decline from proestrus to diestrus.

Prolongation of luteal function in the rat and, hence, maintenance of pregnancy during the first week of gestation are modulated by pituitary PRL and LH (24). During the latter half of pregnancy, however, gestation is maintained by the placental production of rat placental lactogen and andro-

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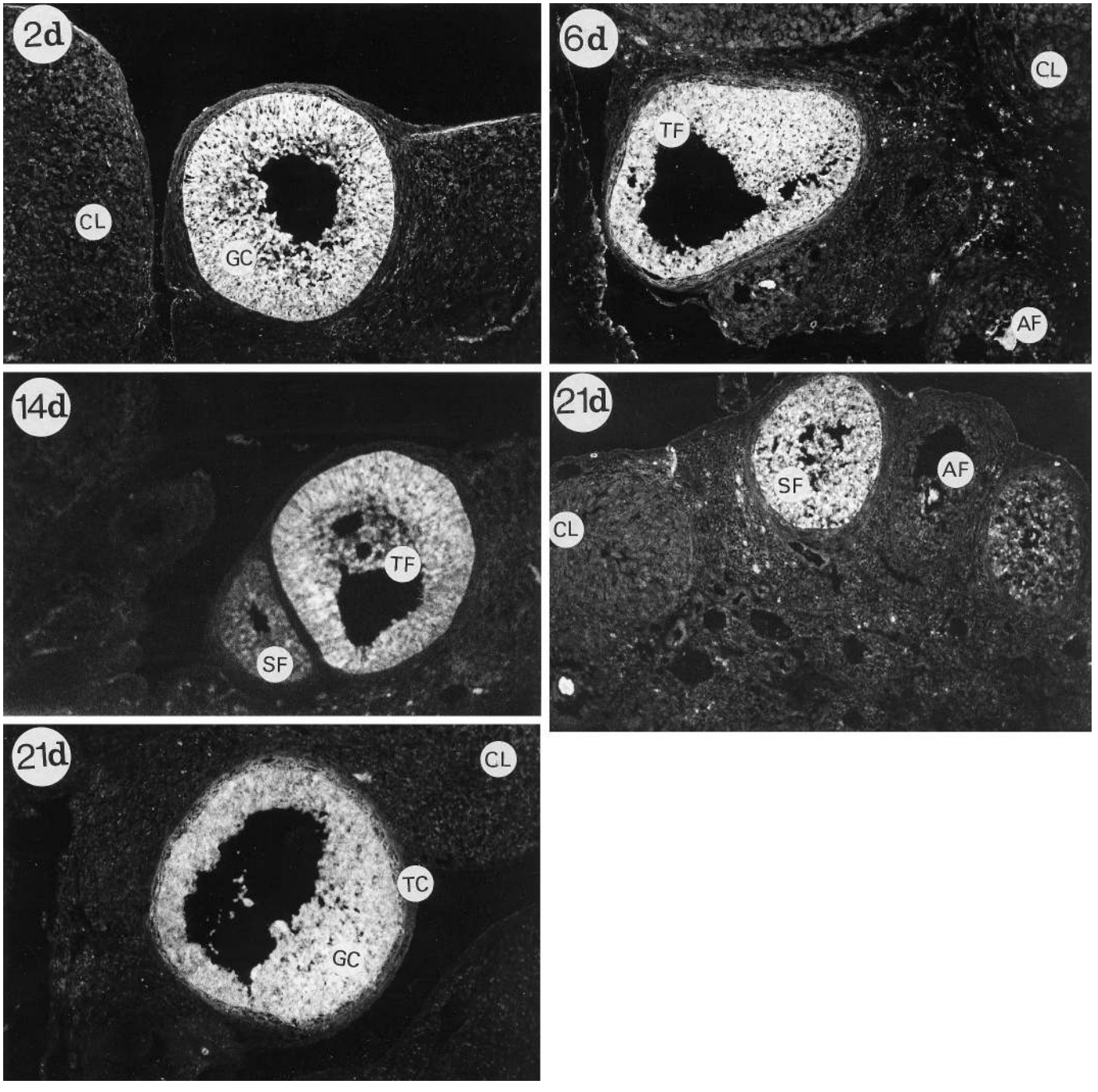

FIG. 4. Immunohistochemical staining of 17HSD type 1 in ovarian sections from pregnant rats on days 2, 6, 14, and 21 of gestation. Throughout pregnancy, the strongest immunostaining of the 17HSD type 1 protein was detected in the granulosa cells (GC) of tertiary follicles (TF). The staining was lower, but detectable, in growing secondary follicles (SF). No staining for the enzyme was found in the thecal cells (TC), atretic follicles (AF), or CL at any stage of gestation.

gens, and by E_2 and P produced by the CL (2, 25, 26). During pregnancy, the size of the CL increases rapidly in parallel to its capacity to secrete E_2 and P to sustain normal pregnancy in the rat. Our data, however, indicate 17HSD type 1 expression only in the granulosa cells of the growing follicles in pregnant rat ovaries, whereas no significant amount of the enzyme was detected in the CL. Hence, the expression pattern of 17HSD type 1 during rat pregnancy appears to be identical to that found during the normal estrous cycle. These results suggest that once 17HSD type 1 expression is turned on in the granulosa cells by FSH and/or paracrine factors (6, 10, 11), it is maintained in the growing follicles and down-regulated in the CL by luteinization and perhaps by all hormonal factors that sustain CL functions during pregnancy. Whether the relatively low expression of 17HSD type 1 is sufficient for E_2 production in pregnant rat ovaries from the placental androgenic precursor, androstenedione, remains to be clarified. Similar to previous reports, our results show

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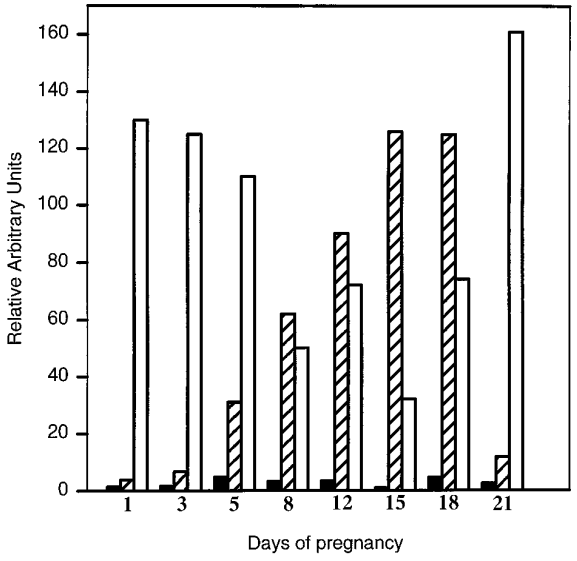

FIG. 5. Comparison of the relative expression of 17HSD type 1 (■), P450arom ( ), and 20HSD (□) mRNAs in pregnant rat ovaries on days 1, 3, 5, 8, 12, 15, 18, and 21 of gestation. Northern hybridization signals were quantified and normalized against γ-actin signals.

strong up-regulation of P450arom on days 5–18 of rat pregnancy. These effects have been attributed to the luteotropic effects of PRL and rat placental lactogen during the first and second weeks of pregnancy in rats, respectively (2, 24). Hence, it is evident that these hormones are not similarly involved in the regulation of 17HSD type 1 in the pregnant rat.

The luteotropic effects of E_2 secreted by the CL are necessary in the latter half of pregnancy to sustain the functions of the CL and thus for continued P production (2, 27). The mechanism of regulation of 20HSD has yet to be clarified, but the enzyme activity is significantly up-regulated in immature rats treated with equine CG/hCG (18). The expression and regulation of 20HSD (which inactivates P to 20αOHP) in the ovary might also determine P secretion and its activities during rat gestation. Our results as well as those of previous reports (16) show a significant drop in the amount of 20HSD mRNA in the rat ovary from day 8 to day 18 of pregnancy. This period is also associated with undetectable 20HSD activity in the mesometrial/visceral yolk sac and spontaneous fetal loss in the rat (16). This might support the hypothesis that 20HSD activity protects the fetuses from the cytotoxic effects of P by inactivating it to 20αOHP. Interestingly, the patterns of expression of P450arom and 20HSD during rat gestation appear to be opposite ones, suggestively maintaining an optimal E_2/P ratio at each stage of gestation.

The significance of the high degree of expression of 17HSD type 2 mRNA noted in the rat placenta, especially toward the end of pregnancy, remains to be clarified further. However, previous results have shown that the type 2 enzyme has a predominant oxidative 17HSD activity (7). This together with the absence of 17HSD type 1 and P450arom enzymes in the rat placenta is in line with the fact that the rat placenta does not contribute significantly to E_2 biosynthesis (28–31). It is, therefore, hypothesized that 17HSD type 2 in the rat placenta could also protect the fetuses by inactivating the high activity 17β-hydroxy forms of estrogens and androgens to their less active 17-keto forms. In addition, the significance, if any, of the putative 20HSD activity of the rat 17HSD type 2 enzyme in relation to progesterone metabolism during rat gestation remains to be clarified. To our surprise, the data suggest that in the liver and small intestine the enzyme is constitutively expressed throughout the life span of the animal, regardless of sex. The expression of rat 17HSD type 2 in the liver and small intestine does not appear to correlate with any of the changes in the circulating sex hormone concentrations in the animals. Thus, the enzyme is suggested to have a general constitutive role in the metabolic inactivation of potent systemic sex hormones to their less active forms.

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