Participation of ovarian 20 α -hydroxysteroid dehydrogenase in luteotrophic and luteolytic processes during rat pseudopregnancy

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Summary. In normal rats, before Day 12 of pseudopregnancy, minimal levels of 20 α -HSD activity were detected in functional CL whereas those in the residue were 3–5 times higher. When ovulation was blocked for more than 2 weeks by placing rats in a continuously lit environment before the induction of pseudopregnancy, only minimal levels of 20 α -HSD activity were detectable in the functional CL and residue before Day 12. In normal pseudopregnant rats, there was a linear increase in 20 α -HSD activity from Day 12 to 15 in the functional CL and residue, but the rate of elevation was much higher in functional CL. This tendency was much more clear-cut in rats in the continuous lighting. In immature rats in which pseudopregnancy was induced by PMSG and hCG treatment, 20 α -HSD activity peaked twice. The first small peak was attributed to the early regression of some of the large number of corpora lutea, and the changes in 20 α -HSD activity in most of the corpora lutea paralleled those in rats in continuous lighting. Bromocriptine abolished the prolactin surges, and in normal pseudopregnant rats an increase in 20 α -HSD activity in functional CL started from 12 h and the rate of the increase was accelerated from 36 h afterwards, while a relatively small increase was observed in the residue at 18 h and later. These results suggest that, in the early–middle phase of pseudopregnancy, 20 α -HSD activity in the functional CL is suppressed under the control of prolactin and at the end of pseudopregnancy 20 α -HSD activity appears mainly in the functional CL without suppressive action of prolactin.

Keywords: 20 α -hydroxysteroid dehydrogenase; progestagen; pseudopregnancy; corpus luteum; rat

Introduction

Ovarian 20 α -hydroxysteroid dehydrogenase (20 α -HSD) (EC 1.1.1.149) mediates the catabolism of progesterone to a biologically inactive steroid, 20 α -hydroxypregn-4-en-3-one (20 α -dihydroprogesterone or 20 α -OHP) (Wilcox & Wiest, 1960; Wiest & Forbes, 1964). During pregnancy or pseudopregnancy in the rat, ovarian secretory rate of progesterone is inversely correlated with that of 20 α -dihydroprogesterone (Hashimoto *et al.*, 1968), and it is generally believed that this relationship between two progestagens is regulated by ovarian 20 α -HSD (Wiest *et al.*, 1968). Pupkin *et al.* (1966) reported the distribution of this enzyme in the rat ovary during normal oestrous cycles and found that relatively older corpora lutea (CL) possessed high enzyme activity. It is therefore presumed that during pseudopregnancy the age or functional state of the CL is well correlated with changes in 20 α -HSD activity, although the details still remain to be clarified.

In the present study, changes in 20 α -HSD activity of functional CL and the remainder of the ovary were investigated with reference to peripheral progestagen concentrations throughout pseudopregnancy. In addition to normal rats, we used other preparations in which only one generation of

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CL was generated in the ovary. The effect of treatment with bromocriptine, a dopamine agonist, was also investigated to clarify the effect of prolactin on 20 α -HSD activity.

Materials and Methods

Animals

Animals used were of the Wistar-Imamichi strain and bred in our laboratory. Food and water were available *ad libitum*. Lighting was controlled automatically, giving 14 h of light (lights on at 05:00 h) and 10 h of darkness, except for Group L rats as mentioned below. The room temperature was kept at $23 \pm 1^\circ\text{C}$.

Group N. Rats, 2–4 months old, that had shown more than 2 normal vaginal cycles were used. Pseudopregnancy was induced by stimulating the uterine cervix with a glass rod for 1 min between 18:00 and 19:00 h on the day of pro-oestrus. The day following cervical stimulation was designated Day 0 of pseudopregnancy. Some of the rats were treated with bromocriptine (Sandoz, Ltd, Basle, Switzerland) s.c. at a dose of 150 $\mu\text{g}/0.1\text{ ml}$ 3% tartaric acid solution at 00:00 h on Day 4 of pseudopregnancy.

Group L. Rats which had shown regular 4-day oestrous cycles for at least 10 days under a light regimen of 14 h light and 10 h darkness were transferred to an isolated room in which continuous lighting was supplied. Ovulation and subsequent pseudopregnancy were induced by cervical stimulation between 18:00 and 19:00 h (Murakami *et al.*, 1978) in a group of these animals which had shown continual vaginal oestrus for 14–16 days. The day following cervical stimulation was designated Day 0 of pseudopregnancy.

Group I. Normal rats, 23–26 days old, were injected with 50 i.u. PMSG (Sankyo Zoki Co. Ltd, Tokyo, Japan) and 25 i.u. hCG (Sankyo Zoki Co. Ltd) 72 h later to induce pseudopregnancy after the occurrence of superovulation (Parlow, 1958; Tan & Robinson, 1977). Each hormone preparation was dissolved in 0.9% (w/v) NaCl (0.2 ml) and injected s.c. between 18:00 and 18:30 h.

Collection of functional luteal tissue

Group N. Ovaries were separated into functional corpora lutea (CL) and the remaining tissue (residue) which consisted of old CL and non-luteal tissue. The ovaries of these animals contained 3–4 generations of CL: 5–10 of the largest CL were selected as functional CL and these were enucleated with tweezers.

Group L. New CL were enucleated, and were easily identifiable from old ones since ovulation had not occurred for at least 2 weeks.

Group I. Usually, > 30 CL and a few follicles were present in each ovary of these rats. The 15 biggest corpora lutea were enucleated and used for the experiments.

Blood collection and ovarian cytosol preparation

Rats were killed by decapitation between 11:00 and 15:00 h except as otherwise indicated. Blood samples collected from trunks were allowed to clot at room temperature for several hours and centrifuged for 15 min at 1500 *g*. The sera thus obtained were stored at -20°C until hormone assays. The ovaries were removed, freed from adhering tissues and weighed. Functional CL and residue were separated as described above within 30 min after killing the animals, then homogenised with a Potter-type Teflon–glass blender in 500 μl 100 mM-Tris-HCl (pH 8.0) containing 1 mM-EDTA and 5 mM-dithiothreitol, and centrifuged at 105 000 *g* for 60 min. Then the cytosol fraction (supernatant) was filtered through a fibre-glass filter (GC 50; Toyo Roshi Co., Tokyo, Japan) to remove any insoluble substances including fat. All of these procedures were performed at 4°C .

Hormone assays

Serum progesterone and 20 α -dihydroprogesterone concentrations were determined by radioimmunoassays using specific antibodies originally raised in our laboratory as described previously (Takahashi *et al.*, 1980; Matsuyama *et al.*, 1987). Progesterone and 20 α -dihydroprogesterone were purchased from Sigma Chemical Co., St Louis, MO, USA. Tritium-labelled progesterone and 20 α -dihydroprogesterone were from New England Nuclear, Boston, MA, USA. A serum sample (0.1 ml) was diluted to 1 ml with distilled water and extracted twice with 2 ml diethyl ether, and the extracts were radioimmunoassayed without chromatography. The progesterone antiserum cross-reacts 0.2% with 20 α -dihydroprogesterone, 3.1% with corticosterone and < 1.0% with other common progestagens, androgens and corticoids. The 20 α -dihydroprogesterone antiserum cross-reacts 0.1% with progesterone and < 1.0% with other common progestagens and corticoids. The minimal detectable levels of progesterone and 20 α -dihydroprogesterone were both 50 pg. The within- and between-assay coefficients of variation for each assay did not exceed 7%.

Measurement of 20 α -hydroxysteroid dehydrogenase activity

The activity of 20 α -hydroxysteroid dehydrogenase was measured spectrophotometrically by the method of Wiest *et al.* (1968) with a few modifications. The 20 α -dihydroprogesterone (20 α -hydroxypregn-4-en-3-one), 30 μ M, was catalysed to progesterone in 100 μ M-Tris-HCl buffer (pH 8.0) containing 300 μ M-NADP, 1 mM-EDTA, 5 mM-dithiothreitol (DTT) and 3% ethanol (used to solubilize the steroids). DTT and NADP were added immediately before use. The assay mixture (500 μ l) was warmed to 37°C in an assay cuvette, and then the enzyme reaction was initiated by introducing 25 μ l cytosol preparation into the cuvette with rapid mixing. The temperature during the assay was maintained at 37 $\pm$ 0.1°C. Initial velocities of NADPH accumulation were recorded spectrophotometrically at 340 nm for 3 min with an Hitachi 100-60 spectrophotometer (a molar absorbance of 6220 at 340 nm was used to calculate NADPH concentrations). Enzyme activity was determined by defining 1 unit of enzyme as the amount capable of reducing 1 μ mol NADP per min at 37°C.

Protein assay

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) originally developed by Bradford (1976).

Statistical analysis

Duncan's new multiple range test was used for statistical evaluation of the results. Differences at a probability of $P < 0.05$ were considered to be statistically significant.

Results

Group N (Figs 1a, 2a)

An almost linear increase in ovarian cytosolic 20 α -HSD activity was found from Day 12 to Day 15 in the functional CL and residue. Although only minimal amounts of 20 α -HSD activity were detected in functional CL from Days 4 to 12, activities detected in the residue were 3–5 times higher.

The 20 α -dihydroprogesterone concentration decreased during the early phase of pseudo-pregnancy. A decrease in progesterone concentration from Day 12 to Day 15 coincided with a rapid rise in 20 α -dihydroprogesterone concentration.

Group L (Figs 1b, 2b)

Only extremely low activities of ovarian cytosolic 20 α -HSD were detected from Days 4 to 12 in the functional CL and residue. An increase in the activity began on Day 12 and the rate of the increase was much higher in functional CL than in residue.

There was a high progesterone concentration from Days 4 to 8 and a rapid decrease from Days 12 to 15. The serum 20 α -dihydroprogesterone concentration rose significantly at Day 15.

Group I (Figs 1c, 2c)

The very low ovarian cytosolic 20 α -HSD activity on Day 0 represents the value before the ovulation induced by hCG treatment. Between Days 0 and 4, a small but significant increase was observed and a large increase occurred between Days 12 and 16.

The peak value of progesterone concentration on Day 4 was 10 times higher than that in Group N rats. The highest 20 α -dihydroprogesterone concentration was 3 times higher than that in Group N rats.

There was a rapid increase in cytosolic progesterone concentration between Days 0 and 4 and high levels were maintained until Day 8. Values of 20 α -dihydroprogesterone remained low until Day 12, when luteolysis was considered to occur (Fig. 3).

Fig. 1. Ovarian cytosolic 20 α -hydroxysteroid dehydrogenase (20 α -HSD) activities in functional CL and other ovarian tissues (residue) in rats in Groups N (a), L (b) and I (c). Each symbol represents the mean $\pm$ s.e. for 4–5 animals. The activity of 20 α -HSD was expressed as mU enzyme activity/mg cytosol protein, with 1 unit of activity defined as the amount capable of reducing 1 μ mol NADP/min at 37°C.

Effects of bromocriptine

20 α -Hydroxysteroid dehydrogenase activity in the functional CL after bromocriptine treatment in Group N rats started to increase from 12 h and the rate of the increase was accelerated from 36 h after the treatment. The activity in the residue was increased 18 h after the treatment, but no significant changes in the activity could be observed from 18 to 60 h after the treatment when a rapid rise was observed in the functional CL (Fig. 4a).

Progesterone concentrations after bromocriptine treatment were lowered when 20 α -HSD activity in the functional CL rose (Fig. 4b).

Fig. 2. Serum progesterone and 20 α -dihydroprogesterone (20 α -OHP) concentrations in rats in Groups N (a), L (b) and I (c). Each symbol represents the mean $\pm$ s.e. for 4–5 animals.

Discussion

The age of old crops of corpora lutea differs between the ovaries of rats in Groups N and L. In Group N rats on Days 0 or 15 of pseudopregnancy for example, the age of the latest crop of corpora lutea carried over from the preceding oestrous cycles is 4 or 19 days, respectively. On the other hand, in Group L rats, the corresponding age is 16 or 31 days. Pupkin *et al.* (1966) observed histochemically that the 20 α -HSD activity during oestrous cycles began to appear at 3.5 days of age in newly generated corpora lutea and remained at least until the end of the following 2 cycles, i.e. for 9.5 days. In Group L rats, 20 α -HSD activity was extremely low in non-luteal tissue (residue) in comparison with the moderate activity observed in the residue of the Group N rat ovary. 20 α -HSD activity present in residual tissue during early pseudopregnancy in normal rats may depend upon the old corpora lutea which are carried over from the previous oestrous cycle, and the elevation of 20 α -HSD activity at the end of pseudopregnancy may occur specifically in the new corpora lutea.

Fig. 3. Ovarian cytosolic progesterone and 20 α -dihydroprogesterone (20 α -OHP) concentrations in Group I rats. Each symbol represents the mean $\pm$ s.e. for 4–5 animals. Broken line shows the lower limit of the 20 α -dihydroprogesterone assay.

This observation reaffirms the earlier report of Wiest *et al.* (1968) and is also supported by the results of bromocriptine treatment (see below).

Correlations between ovarian 20 α -HSD activity and serum 20 α -dihydroprogesterone concentration in pregnant and pseudopregnant rats have been studied (Wiest *et al.*, 1968; Hashimoto & Wiest, 1969). The patterns of ovarian 20 α -HSD activity and serum progestagen concentrations in Group N rats are consistent with these previous investigations. It is generally believed that old corpora lutea retain a high level of 20 α -HSD activity, and are carried over to contribute to the high serum 20 α -dihydroprogesterone concentrations in the early-middle phase of pseudopregnancy (Day 0–Day 12); the new corpora lutea regress and acquire a high 20 α -dihydroprogesterone content at the end of pseudopregnancy (Days 13–15).

In Group L rats, however, relatively high concentrations of serum 20 α -dihydroprogesterone were maintained up to Day 12 of pseudopregnancy, although there was an extremely low ovarian 20 α -HSD activity. Moreover, as shown in the Group I rats, the peripheral serum progestagen concentrations were clearly different from the cytosolic concentrations. 20 α -HSD activity has been found in mature T lymphocytes (Weinstein, 1977) and several other tissues, such as adrenal gland (Matthijssen *et al.*, 1964), liver (Recknagel, 1957) and placenta (Purdy *et al.*, 1964). Lu & Judd (1982) have also reported that extra-ovarian system(s) can convert large amounts of circulating progesterone into 20 α -dihydroprogesterone. Peripheral progestagen concentration is therefore not necessarily correlated with ovarian 20 α -HSD activity.

In the immature rats, the serum 20 α -dihydroprogesterone concentration peaked twice on Days 4 and 12 of pseudopregnancy, and this fluctuation was correlated with 20 α -HSD activity. The significance of these two peaks of 20 α -HSD activity in immature rats remains to be studied further. However, the close relationship between serum 20 α -dihydroprogesterone concentration and ovarian 20 α -HSD activity suggests that a proportion of the corpora lutea actually regress on Day 3–5, similar to the situation during normal oestrous cycles, and that the other corpora lutea remain functional throughout the normal period of pseudopregnancy. The occurrence of 20 α -HSD activity in the luteal tissue is thought to be irreversible (Wiest *et al.*, 1968). If this is the case, the decrease in 20 α -HSD activity on Day 8 cannot be explained by a transient inhibition of activity, but rather that the corpora lutea in the immature rat appear to consist of two types with different functions. One type of corpora lutea might be refractory to prolactin by which 20 α -HSD activity is thought to be suppressed, and the other type might be sensitive to prolactin.

On Day 4 of pseudopregnancy, prolactin surges occur early in the morning (nocturnal surge) and in the evening (diurnal surge) (Smith *et al.*, 1975; Murakami *et al.*, 1979). Prolactin release from the anterior pituitary gland is tonically inhibited by dopamine (MacLeod & Lehmeyer, 1974). In the present study, administration of bromocriptine, a dopamine agonist, at 00:00 h on Day 4 would have begun to inhibit the series of prolactin surges from the nocturnal surge on the same day. A marked increase in 20 α -HSD activity was observed in functional CL about 40 h after the last prolactin surge, but an increase in the residue was not so remarkable. This suggests that the 20 α -HSD activity in the functional CL is effectively suppressed by the surge of prolactin and 20 α -HSD activity appears mainly in the functional CL when prolactin surges are abolished.

The long latency, of about 40 h, between prolactin withdrawal and the increase in 20 α -HSD activity is surprising. Saito *et al.* (1988) reported that macrophages or splenic adherent cells were involved in expression of the luteotrophic action of prolactin and this interposition of myelogenic cells between prolactin and luteal cells is one possible explanation for this long latency.

At the end of pseudopregnancy, it is known that prolactin surges do not cease if serum progesterone concentrations are maintained experimentally at high levels (de Greef & Zeilmaker, 1978; Murakami *et al.*, 1979). Under this condition, or if the animals are treated with prolactin, luteolysis occurs at the scheduled time, i.e. at 2 weeks (Naito & Suzuki, 1978; Takahashi *et al.* 1978). It is therefore generally believed that pseudopregnancy ends not as a result of extinction of prolactin surges but by refractoriness of the corpus luteum to prolactin, the action of which has been inhibiting 20 α -HSD activity for 2 weeks. It is concluded that the refractoriness of the corpus luteum to

prolactin causes an increase in 20 α -HSD activity in newly formed corpora lutea, which in turn causes a decrease in progesterone secretion.

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