The Relationship between Ovarian Progesterone and Proteolytic Enzyme Activity during Ovulation in the Gonadotropin-treated Immature Rat

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To clarify the mechanisms by which progesterone acts as a mediator in the ovulatory process, ovulation rate and proteolytic enzyme activities were investigated in immature 22-day-old rats treated with PMSG/hCG, RU486 (10 mg/kg), synthetic anti-progesterone, and RU486 (10 mg/kg) + progesterone (10 mg/kg). The number of ova was significantly decreased when RU486 (10 mg/kg) was given from 2 h before to 4 h after the hCG injection. In addition, its inhibitory action on ovulation was reversed by exogenous progesterone (10 mg/kg) at 2 or 4 h after the hCG injection. Serum progesterone and estradiol concentrations in the rats treated with RU486 did not show any significant differences compared to controls. The proteolytic enzyme activities were measured by using the synthetic substrates n-N-benzoyl-DL-Arg-β naphthylamide (BANA) and dinitrophenyl peptide (DNP). Activities were significantly increased after hCG injection in the control group during 8--9 h for BANA hydrolase and 7--10 h for DNP peptidase. The preovulatory increase of these activities was totally suppressed by RU486 with hCG. After administration of progesterone (10 mg/kg) following hCG and RU486 injection, the elevation of proteolytic enzyme activities in the preovulatory phase was effectively reversed, and levels became similar to those in the control group.

These results suggest that progesterone plays an indispensable role in the first 4 h of the ovulatory process by regulating proteolytic enzyme activities.

INTRODUCTION

The rupture of ovarian follicles requires the decomposition of collagenous connective tissue in the thecal layers of the follicle wall. The injection of minute amounts of collagenase into rabbit follicles induces rupture [1], and collagenolytic activity has been detected in cultured rabbit follicles [2]. Changes in such activity during ovulation have been measured by methods using synthetic naphthylamide (BANA) [3, 4] and dinitrophenyl peptide (DNP) [5, 6] substrates.

Ovarian steroidogenesis changes markedly after the LH surge. The involvement of ovarian steroidogenesis in follicular rupture has been studied for almost two decades by using several inhibitors of steroid biosynthesis [7-12]. However, it has not yet been established how the preovulatory change in steroidogenesis, especially progesterone, is involved in the ovulatory process.

The aim of the present study was to determine the influence of progesterone on ovarian proteolytic enzyme activity during the process of ovulation in rats. The proteolytic activity was measured after rats were treated with an ovulation-inhibiting dose of 17β-hydroxy-11β-(4-dimethylamino-phenyl)-17α-(propynyl-1)-estradiene-3-one (RU 486), a synthetic steroid that blocks progesterone activity at the receptor level [13]; it was again measured after the ovulation rate was restored with exogenous progesterone. The results show that ovarian proteolytic activity and ovulation are dependent on ovarian progesterone during the ovulatory process.

MATERIALS AND METHODS

Ovulation Induction

Immature Wistar rats (22 days old) received s.c. injections at 0900 h of 10 IU PMSG (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) in 0.1 ml of saline. The ovulatory process was induced 48 h later by an s.c. injection of 5 IU hCG (Mochida Pharmacological Corp., Tokyo, Japan). Approximately 24 h after the hCG injection, the rats were killed by chloroform vapor. The oviducts were separated from the ovaries, placed on a glass slide, and examined under a dissecting microscope for counting the number of ova released from the ovaries.

Agent Injection

To determine the optimum time to inhibit ovulation with RU486 (RU38486, Roussel-Uclaf, Romainville, France), this agent was dissolved in 70% ethanol (10 mg/ml), and a dose of 10 mg/kg was administered s.c. at 2 h before, or at 0, 2, 4, 6, 8, or 10 h after the hCG injection. To assess the ability of progesterone (Proluton, Schering AG, Berlin, Germany) to reverse the effects of RU486, this hormone was injected at 0, 2, 4, 6, 7, or 8 h after hCG.

Steroids Assays

Blood samples were obtained by puncture of the inferior vena cava. Serum was separated from blood and stored at -20°C until assayed for progesterone and estradiol. Both steroids were measured by RIA kits (Sorin Biomedica, Saluggia, Italy). The sensitivities of the assays for progesterone and estradiol were 0.10 ng/ml and 10 pg/ml, respectively.
The progesterone antiserum cross-reacted with other steroids as follows: corticosterone, 2.5%; deoxycorticosterone, 2.5%; 17-hydroxyprogesterone, 2.0%; and cortexolone, testosterone, pregnenolone, 20-dihydroxyprogesterone, cortisol, androstenedione, cholesterol, cortisol, dehydroepiandrosterone, estradiol, estrone, and etiocholanolone, <0.25%. The estradiol antiserum cross-reacted with other steroids as follows: estrone, 0.7%; estriol, 0.55%; 20-dihydroprogesterone, 0.007%; and cortisol, cortisone, corticosterone, cholesterol, progesterone, 17-hydroxyprogesterone, etiocholanolone, pregnenolone, androstenedione, testosterone, dehydroepiandrosterone, and deoxycorticosterone, <0.002%. The intraassay variations were 7.6% for progesterone and 3.4% for estradiol. The interassay variations for progesterone and estradiol were 8.5% and 9.0%, respectively.

**BANA Hydrolase Activity Assay**

BANA hydrolase activity was measured by a modification [3, 4] of the original method [14]. The ovaries were washed in saline (0.9% w/v NaCl) and homogenized in 0.25 M sucrose containing 0.1% Triton-X 100 at a final concentration of 30 mg tissue/ml. The homogenates were centrifuged at 20,000 × g for 10 min, and the supernatant fluids were used as the enzyme source. The reaction mixture, which consisted of 50 μl enzyme solution, 50 μl BANA substrate (40 mg/ml in dimethylsulfoxide), α-N-benzoyl-ε-Arg-β-naphthylamide (Sigma Chemical Co., St. Louis, MO), and 450 μl assay buffer (0.1 M phosphate buffer containing 1 mM EDTA, pH 6.0), was incubated at 37°C for 10 min. The reaction was stopped by the addition of 600 μl of a coupling reagent, which consisted of 300 μl p-chloromercuribenzenzamide (Sigma) in EDTA-PBS and 300 μl 6% Fast Garnet GBC (Sigma). The released β-naphthylamide was extracted with 1.2 ml of butanol and its activity was then determined by measuring its absorbance at 520 nm. The intraassay and interassay variations were 4.6% and 6.5%, respectively.

**DNP Peptidase Activity Assay**

DNP peptidase activity was assayed by a modification [3, 4] of the method of Morales et al. [5]. The assay buffer was 50 mM Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl and 5 mM CaCl2. The ovaries were weighed, placed in assay buffer at a concentration of 30 mg tissue/ml buffer, and homogenized for 30 sec with a glass-glass homogenizer. The homogenates were centrifuged at 20,000 × g for 10 min, and then the supernatant fluids were passed through a 45-μm Millipore filter (Millipore Corp., Bedford, MA). The filtrates were used as the enzyme solution. Synthetic DNP-peptide substrate, dinitrophenol Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg OH (Peptide Institute, Protein Research Foundation, Osaka, Japan) was dissolved in assay buffer to make up 0.45 mM and centrifuged at 10,000 × g for 10 min. The supernatant fluids were used as the substrate solution. The reaction mixture, which consisted of 150 μl substrate, 50 μl enzyme solution, and 100 μl assay buffer, was incubated at 37°C for 3 h. The reaction was terminated with 300 μl 1.0 N HCl. The cleaved peptide was extracted with 1.5 ml ethylacetate and its activity was determined by measuring the absorbance at 365 nm. The activity was expressed as the difference in optical density between a 0 time blank and a reaction-mixture tube that had been incubated for 3 h. The intraassay and interassay variations were 5.1% and 7.2%, respectively. The method of Lowry et al. [15] was used for the protein determination, with BSA as a standard.

**Statistical Analysis**

All data are presented as means ± SEM. The significance of the differences in steroid levels and enzyme activities between untreated and treated groups at any given hour during the ovulatory process was determined by Duncan's Multiple Range tests after a two-factor (two-way) ANOVA of the means of all experimental groups that could be paired across the 12-h period of the study. In the other experimental groups, the significance of differences was determined by Duncan's Multiple Range tests after a completely randomized one-way ANOVA of the means of the groups. The threshold of significance was set at p = 0.05.

**RESULTS**

**Inhibition of Ovulation by RU486**

The ovulation rate in the control group was 36.5 ± 2.0 ova/rat. This was significantly lower when RU486 (10 mg/kg) was administered at 2 h before, or 0, 2, or 4 h after hCG, but not at 4 h before, or 6, 8, or 10 h after, hCG (Fig. 1).
Reversal of RU486 Action by Progesterone

When progesterone (10 mg/kg) was administered at 2 or 4 h after hCG to rats that had been treated with RU486 (10 mg/kg) at 0 h, the ovulation rates were significantly greater than in the RU486 control group that was treated with RU486 only at 0 h (Fig. 2). However, there was no significant recovery in the ovulation rate when progesterone was given at 0, 6, or 8 h after hCG.

Effect of RU486 on Serum Progesterone and Estradiol Levels

In untreated controls, serum progesterone levels rose significantly within 6 h after hCG, reached a peak at 8 h, and then declined slightly by 12 h (Fig. 3). There was no significant difference in this pattern of progesterone secretion when RU486 (10 mg/kg) was administered at the same time as hCG. In untreated controls, serum estradiol levels decreased significantly between 8 and 10 h after hCG (Fig. 3). There were no significant differences in estradiol levels between the two groups within 8 h after hCG.

Effect of RU486 on Ovarian BANA Hydrolase Activity

In controls, BANA hydrolase activity increased significantly at 7 h after hCG and reached a peak at 8–9 h (Fig. 4). The administration of RU486 (10 mg/kg) at the same time as hCG prevented the increase in BANA hydrolase activity, whereas treatment with 10 mg/kg progesterone (at 4 h after hCG) completely reversed this inhibitory effect of
RU486 (Fig. 4). Progesterone treatment at 2 or 6 h (but not at 0, 7, or 8 h) after hCG also led to the recovery of BANA hydrolase activity when the activity was measured at 9 h after hCG (Fig. 5).

**Effect of RU486 on Ovarian DNP Peptidase Activity**

The effect of RU486 (10 mg/kg at 0 h) on DNP peptidase activity was similar to the action of this drug on BANA hydrolase activity, except that at 12 h after hCG the ovarian enzyme activity in the RU486-treated group was significantly higher than in the control group (Fig. 6). As with BANA hydrolase activity, the optimum time to administer progesterone to reverse the inhibitory action of RU486 on DNP peptidase activity (measured at 9 h after hCG) was 4–6 h after hCG (Fig. 7).

**DISCUSSION**

There are conflicting reports concerning the role of ovarian progesterone in the ovulatory process. Bullock and Kappauf [16] found a dissociation between ovulation and steroidogenesis when administering the appropriate dose of aminoglutethimide or cyanoketone to rats. Using the in vitro perfusion system, Yoshimura et al. [17, 18] showed that aminoglutethimide and cyanoketone inhibit ovarian steroidogenesis but not ovulation in rabbits. In contrast, the importance of ovarian progesterone in ovulation has been demonstrated by using several inhibitors of steroidogenesis: aminoglutethimide [7, 8], cyanoketone [7, 8], epistane [9, 10, 12], and compound A [11], or by using an antiserum against progesterone [19]. This conflict may come from variety of experimental designs. Above all, the time when drugs are administered or when steroid levels are checked in serum or in ovarian follicles may be critical. We have indicated that ovarian steroid levels show a different pattern of inhibition in the time-course study when epistane is given at 3 h, 6 h, or 7 h after hCG [20, 21]. Therefore, it is important to know the critical time when ovarian progesterone is required for ovulation.

In the present study, we confirmed the finding of Tsafirri et al. [10] that RU486, a progesterone receptor antagonist, blocks ovulation in rats. However, they did not examine the optimum time for RU486 administration. The ovulation rate was significantly decreased when RU486 was administered to immature female rats treated with PMSG and hCG from 2 h before to 4 h after the hCG injection. In addition, the inhibitory effect of RU486 on ovulation was reversed by exogenous progesterone at 2 or 4 h after hCG injection. These results suggest that during the first 4 hours after hCG injection the presence of progesterone may play an important role in ovulation.

Serum progesterone concentration has a peak at 8 h after hCG, but progesterone carries out its most important role during the first 4 h after hCG. A small amount of progesterone during the first 4 h after hCG seems to be critical for ovulation, and the peak of progesterone production at 8 h after hCG may have roles other than that in the ovulatory process.

Serum progesterone levels in RU486-treated rats did not differ significantly from those in controls. It seems that RU486 has no inhibitory effect on the production of progesterone but antagonizes progesterone receptor in this experimental design.

The involvement of collagenolytic enzymes in ovulation has been thoroughly studied [22–24]. DNP peptidase and BANA hydrolase degrade native collagen at neutral and acid pH, respectively, and there is biochemical [3, 5] and morphological [25] evidence to suggest that such enzymes are involved in the dissociation of the collagen matrix in the follicle wall at the time of ovulation. In the rabbit, these
enzymes start to increase around 7 h after hCG to a significant level at 1–2 h before follicle rupture [4, 6]. In the present study of immature rats, both enzyme activities significantly increased about 9 h after hCG and then decreased up to the time of ovulation. These results correlate with the previous data from rabbits.

Progestrone may activate the enzyme activity that digests the collagen framework to increase the distensibility of a follicular wall [26]. Tsafritri et al. [10] indicated that follicular steroids, including progesterone, influence the activity of ovarian plasminogen activator, one of the proteolytic enzymes. It has been reported that sulpiride-induced hyperprolactinemia inhibits progesterone production and collagenolytic activity in the rabbit [27]. Using the in vitro perfused rat ovary, Brannstrom et al. [24] investigated the effect of two collagenase inhibitors on ovarian progesterone. However, the precise effect of progesterone on collagenolytic enzyme in the ovary during the ovulatory process remains unclear. In the present study, the preovulatory elevation of both enzyme activities, which reached a maximum around 3–4 h before ovulation in the control, were completely inhibited by the administration of RU486. Furthermore, when progesterone was administered at 4 or 6 h after RU486 following hCG, both enzyme activities were completely restored to the level of the control group. These results suggest that the regulatory effect of progesterone on the ovulatory process is initiated about 4 h after the LH surge through its effect on a proteolytic enzyme, possibly collagenase.

In conclusion, progesterone plays an indispensable role in the ovulatory process within a short time after the LH surge through its effect on proteolytic enzyme activities in the follicular wall.

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REFERENCES