Effects of progesterone and anti-progesterone RU486 on ovarian 3 β -hydroxysteroid dehydrogenase activity during ovulation in the gonadotrophin-primed immature rat

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The effects of progesterone and RU486, a synthetic anti-progesterone, on ovarian 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity, a key enzyme of progesterone production, were studied during ovulation in immature 22-day-old rats primed with pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG). Ovarian 3 β -HSD activities had increased significantly 4 h after hCG injection. These increases were inhibited at 4 and 6 h after hCG when 20 mg RU486 kg⁻¹ was administered 2 h before hCG. However, RU486 had no influence on the activity of 3 β -HSD when administered at the same time as hCG injection. A histochemical study revealed that 3 β -HSD activities in the granulosa cell layer, but not in the theca cell layer, were inhibited when RU486 was given 2 h before hCG. Serum progesterone concentrations, but not oestradiol concentrations, were significantly suppressed by RU486 treatment 4 and 6 h after hCG. The effect of progesterone on ovarian 3 β -HSD activity was tested by administering graded doses of progesterone exogenously to rats 2 h before hCG injection. Ovarian 3 β -HSD activity was increased in a dose-dependent manner, and more than 20 mg progesterone kg⁻¹ significantly stimulated the activity. Although 10 mg progesterone kg⁻¹ did not stimulate ovarian 3 β -HSD activities, the RU486-inhibited activities were recovered by the concomitant administration of 10 mg progesterone kg⁻¹ with RU486. These results indicate that ovarian 3 β -HSD activity depends on progesterone concentrations, and suggest an autocrine regulation of progesterone production during ovulation in immature rat ovaries stimulated with PMSG and hCG.

Introduction

Although ovarian steroidogenesis is primarily controlled by pituitary gonadotrophins, the possibility of autocrine and paracrine controls of steroid production in the ovary has been debated. The hypothesis of autocrine regulation of ovarian progesterone secretion is based on experiments in hypophysectomized animals and granulosa cell culture. Removal of the pituitary just after ovulation has no influence on the pattern of progesterone secretion and the function of the corpus luteum (Smith *et al.*, 1975). A synthetic progestin, R5020, stimulates progesterone secretion in cultured granulosa cells (Fanjul *et al.*, 1983). 17 β -Hydroxy-11 β -(4-dimethylamino-phenyl-1)-17 α -(propynyl-1)-estra-4,9-diene-3-one (RU486), a synthetic anti-progesterone without agonist effect (Herrman *et al.*, 1982; Healy *et al.*, 1983), decreases progesterone secretion from cultured human granulosa cell (Dimattina, 1986).

It is known that RU486 can inhibit ovulation. Rao and Mahesh (1986) suggested that RU486 inhibits ovulation through an antagonism of the action of progesterone on the

preovulatory surge of gonadotrophins in rats. We have previously demonstrated that progesterone production changed significantly during the first 4 h of the ovulatory process after hCG injection, that this increase plays an important role on ovulation by regulating proteolytic enzyme activities, and that RU486 inhibits ovulation through an antagonism of the action of progesterone on proteolytic enzyme activities in PMSG/hCG-primed immature rats (Iwamasa *et al.*, 1992). In this study, autocrine regulation of progesterone production during ovulation was examined by investigating biochemically and histochemically ovarian 3 β -hydroxysteroid dehydrogenase (3 β -HSD, EC 1.1.1.51) activity, which is responsible for the conversion of pregnenolone to progesterone as well as other Δ^5 to Δ^4 steroids, in rats treated with RU486 or progesterone *in vivo*.

Materials and Methods

PMSG/hCG-primed rat model

Immature Wistar rats (22 days old) received an s.c. injection at 09:00 h of 10 iu pregnant mares' serum gonadotrophin (PMSG;

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Fig. 1. Effect of RU486 on ovarian 3β -hydroxysteroid dehydrogenase (3β -HSD) activity. Control (●—●). RU486, 20 mg kg^{-1} , was given (○—○) 2 h before hCG injection or (△—△) at the same time as hCG injection. Points with the same letter differ significantly (a: $P < 0.05$; b, c, d: $P < 0.01$). Each point represents the mean $\pm$ SEM from six rats.

Teikoku Hormone Manufactory Co. Ltd, Tokyo) in 0.1 ml of saline (0.9%). The ovulatory process was induced 48 h later by an s.c. injection of 5 iu human chorionic gonadotrophin (hCG; Mochida Pharmacological Corp., Tokyo).

Injection of RU486

RU486 (RU38486; Roussel-Uclaf, Romainville, France) was dissolved in 70% ethanol (10 mg ml^{-1}), and a dose of 20 mg kg^{-1} was administered s.c. 2 h before or at the same time as hCG injection. Progesterone, 10, 20 or 100 mg kg^{-1} (Proluton; Schering AG, Berlin) was given s.c. 2 h before hCG injection. The effects of RU486 were reversed by injecting 10 mg progesterone kg^{-1} concomitantly with 20 mg RU486 kg^{-1} 2 h before hCG.

Measurement of 3β -HSD activity

Rats were killed by chloroform vapour at the designated times. Ovaries were removed and stored at -80°C until determination of 3β -HSD activity. 3β -HSD activity was assayed by the methods of Kuhn and Briley (1970), Okazaki *et al.* (1976), and Kawano *et al.* (1988) with a slight modification. Ovaries were weighed and homogenized in 0.25 mol sucrose l^{-1} at 0°C with a glass-glass homogenizer at a concentration of 20 mg wet weight ml^{-1} . The homogenates were centrifuged at 105 000 g for 60 min. The precipitates were rehomogenized at the same concentration as before, and centrifuged at 800 g for 5 min. The supernatant fluids were used as the enzyme solution.

The assay mixture, which consisted of 40 μmol glycine-NaOH (pH 9.4), 0.9 mg bovine serum albumin, 0.5 μmol oxidized nicotinamide adenine dinucleotide (NAD^{+}), and 50 μl enzyme solution, was preincubated at 37°C for 5 min. The reaction was started by the addition of the substrate solution containing 5 μg pregnenolone (0.1 mg ml^{-1} in ethanol) in a final volume of 800 μl . The activity was determined spectrophotometrically by measuring the absorbance of NADH at 340 nm. One unit of the enzyme activity was defined as the amount of enzyme producing 1 μmol NADH. The intra- and interassay coefficients of variation were 4.3% and 9.6%, respectively. The values were expressed as mU mg^{-1} protein. The method of Lowry *et al.* (1951) was used for the protein determination with bovine serum albumin as the standard.

Histochemical study of 3β -HSD activities

Ovaries in control rats and RU486-treated rats were resected 4 h after hCG injection. Thin frozen sections (14 μm) of ovaries were prepared with a cryostat (Sakura Seiki Co. Ltd, Tokyo), air-dried, and incubated at 37°C for 30 min with the reaction mixture containing 5 mg dehydroepiandrosterone (dissolved in 0.2 ml dimethyl sulfoxide), 15 mg nitro-blue tetrazolium, 20 mg NAD, and 30 ml Tris buffer (0.1 mol l^{-1} , pH 8.0). The activities are represented as brown deposits of formazan, which were formed when 3β -hydroxysteroids were oxidized and then tetrazolium salts were coupled with hydrogen ion (Wattenberg, 1958).

The preovulatory follicles more than 300 μm in diameter were studied 4 h after hCG injection. The number of these follicles was 33.6 ± 2.1 (mean $\pm$ SEM) in a pair of ovaries, and this number was consistent with the number of ovulated ova in our rat models. The follicles were classified into three groups (1+, 2+ and 3+) according to the staining strength of enzyme activities in the granulosa cell layer.

Steroid assays

Blood samples were obtained by puncture of the inferior vena cava. Serum was separated and stored at -20°C until assayed for progesterone and oestradiol. Both steroids were measured by radioimmunoassay kits (Sorin Biomedica, Saluggia, Italy). The sensitivities of the assays for progesterone and oestradiol were 0.10 ng ml^{-1} and 10 pg ml^{-1} , respectively. The progesterone antiserum crossreacted with other steroids as follows: corticosterone, 2.5%; deoxycorticosterone, 2.5%; 17-hydroxyprogesterone, 2.0%; and cortisone, testosterone, pregnenolone, 20-dihydroxyprogesterone, cortisone, androstenedione, cholesterol, cortisol, dehydroepiandrosterone, oestradiol, oestrone, and etiocholanolone, $<0.25\%$. The oestradiol antiserum crossreacted with other steroids as follows: oestrone, 0.7%; oestriol, 0.55%; 20-dihydroprogesterone, 0.007%; and cortisol, cortisone, corticosterone, cholesterol, progesterone, 17-hydroxyprogesterone, aetiocholanolone, pregnenolone, androstenedione, testosterone, dehydroepiandrosterone, and deoxycorticosterone, $<0.002\%$. The intra-assay variations were 7.6% for progesterone and 3.4% for oestradiol. The interassay variations for progesterone and oestradiol were 8.5% and 9.0%, respectively.

Fig. 2. Histochemical demonstration of ovarian 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity. (a) Strong 3 β -HSD activities were observed in the granulosa and theca cell layers 4 h after human chorionic gonadotrophin (hCG) injection. (b) 20 mg RU486 kg⁻¹ given 2 h before hCG injection inhibited the activities in the granulosa cell layer, but not in the theca cell layer, at 4 h after hCG injection. Bars represent 50 μ m.

Statistical analysis

All data are presented as means $\pm$ SEM. The significance of the differences in steroid concentrations and in enzyme activities between untreated groups and treated groups at any given hour during the ovulatory process was determined by Duncan's multiple range test after a two-factor (two-way) ANOVA of means of all experimental groups that could be paired across the 6 h 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. Chi-square analysis (χ^2) was used (Table 1). The threshold of significance was set at $P = 0.05$.

Table 1. Histochemical study of effect of RU486 on 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activity. Preovulatory follicles seen 4 h after hCG injection were classified into three groups (1+, 2+ and 3+) according to the staining strength of 3 β -HSD activities in the granulosa cell layer. 20 mg RU486 kg⁻¹ given 2 h before human chorionic gonadotrophin (hCG) reduced the number of follicles showing high activity in the granulosa cell layer

	Percentage of follicles		
	Group 1 (1+)	Group 2 (2+)	Group 3 (3+)
Control (35)	3 ^a	48	48 ^b
RU486 treated (54)	30 ^a	42	28 ^b

The numbers in parentheses equal numbers of follicles analysed from five rats in control group and eight rats in RU486 group. a, b: $P < 0.05$.

Results

Effect of RU486 on ovarian 3 β -HSD activity

In control rats, ovarian 3 β -HSD activities increased significantly ($P < 0.05$) 4 h after the hCG injection (Fig. 1). When 20 mg RU486 kg⁻¹ was administered 2 h before hCG, the activities were significantly ($P < 0.01$) inhibited 4 and 6 h after hCG injection. However, when RU486 was administered at the same time as hCG injection, the activities showed no significant difference compared with those of control rats (Fig. 1).

Histochemical study of ovarian 3 β -HSD activity

In control rats, strong 3 β -HSD activities were observed in the granulosa and theca cell layers 4 h after hCG injection (Fig. 2a). When 20 mg RU486 kg⁻¹ was administered 2 h before hCG, the number of follicles showing high activity in the granulosa cell layer was significantly ($P < 0.01$) decreased 4 h after hCG injection (Table 1). However, RU486 did not affect the activity in the theca cell layer (Fig. 2b).

Effect of RU486 on serum steroid concentrations

In control rats, serum progesterone concentrations rose significantly ($P < 0.01$) within 4 h after hCG (Fig. 3a). When 20 mg RU486 kg⁻¹ was given 2 h before hCG, progesterone concentrations were significantly suppressed 4 h ($P < 0.01$) and 6 h ($P < 0.05$) after hCG (Fig. 3a). Serum oestradiol concentrations did not change significantly within 6 h after hCG (Fig. 3b). RU486 had no effect on serum oestradiol concentrations.

Fig. 3. Effect of RU486 on steroid concentrations in serum. Control (●—●). 20 mg RU486 kg⁻¹ was given 2 h before hCG injection (○—○). Points with the same letter differ significantly (a, b, c: $P < 0.01$; d: $P < 0.05$). Each point represents the mean $\pm$ SEM from six rats.

Fig. 4. Effect of progesterone on ovarian 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. Control (●—●). 100 mg kg⁻¹ progesterone was given exogenously 2 h before hCG injection (○—○). Points with the same letter differ significantly (a: $P < 0.05$; b, c: $P < 0.01$). Each point represents mean $\pm$ SEM from six rats.

Effect of progesterone on ovarian 3β-HSD activity

When 100 mg progesterone kg⁻¹ was exogenously administered 2 h before hCG, ovarian 3β-HSD activities were

Fig. 5. Dose-dependent stimulation of progesterone on ovarian 3β-hydroxysteroid dehydrogenase (3β-HSD) activity. Graded doses of progesterone were given exogenously 2 h before human chorionic gonadotrophin (hCG) injection, and the activities were measured 4 h after hCG injection. Columns with the same letter differ significantly (a: $P < 0.05$; b: $P < 0.01$). The numbers in parentheses represent numbers of rats.

significantly ($P < 0.01$) higher than control activities 4 h after hCG injection (Fig. 4). When graded doses of progesterone were administered 2 h before hCG injection, the activities were stimulated in a dose-dependent manner 4 h after hCG (Fig. 5).

Fig. 6. Reversal of RU486 action by progesterone. 20 mg RU486 kg⁻¹ in presence and absence of 10 mg progesterone kg⁻¹ was given 2 h before human chorionic gonadotrophin (hCG) injection, and the activities were measured at (a) 4 h or (b) 6 h after hCG injection. Columns with the same letter differ significantly ($P < 0.01$). The numbers in parentheses equal numbers of rats.

Reversal of RU486 action by progesterone

Although 10 mg progesterone kg⁻¹ did not stimulate ovarian 3 β -HSD activities (Fig. 5), the RU486-inhibited activities were recovered at 4 (Fig. 6a) and 6 h (Fig. 6b) after hCG injection by the concomitant administration of 10 mg progesterone kg⁻¹ with 20 mg RU486 kg⁻¹ given 2 h before hCG.

Discussion

Although follicular development and steroidogenesis are primarily controlled by pituitary gonadotrophins, recent studies have demonstrated that autocrine and paracrine controls by steroid and peptide substances in the ovary are also involved (Franchimont *et al.*, 1988). Oestradiol inhibits progesterone secretion in human granulosa cells (Veldhuis *et al.*, 1983) and in bovine granulosa and theca cells (Fortune and Hansel, 1979). Progesterone inhibits the induction of aromatase activity in rat granulosa cells (Fortune and Vincent, 1983). These results from experiments performed *in vitro* suggest that steroids act as local mediators within the follicle to modulate production of other steroids and follicular development. Chakravorty *et al.* (1991) proposed that oestradiol may act as an autocrine or paracrine mediator of follicular development via the synthesis of a specific peptide factor in immature rat ovaries treated with diethylstilboestrol. Fanjul *et al.* (1983) suggested that progestins may exert an autoregulatory positive feedback action to enhance gonadotrophin-stimulated production of progesterone and 20 α -hydroxyprogesterone by cultured rat granulosa cells. In

the present study, the autocrine regulation of progesterone production was examined by investigating the effects of progesterone and anti-progesterone RU486 on ovarian 3 β -HSD activity in rats *in vivo*. We showed that RU486 inhibits ovarian 3 β -HSD activity, and that progesterone stimulates the activity and restores the RU486-inhibited activity. These results indicate that ovarian 3 β -HSD activity depends, at least in part, on progesterone concentrations, and suggests that there is autocrine control of progesterone production in the ovary.

RU486 is a synthetic steroid that binds to progesterone receptors and acts as a progesterone antagonist (Herrman *et al.*, 1982; Healy *et al.*, 1983). Dimattina *et al.* (1986) reported that RU486 may directly affect ovarian progesterone production through the reduction of 3 β -HSD activity by *in vitro* cultured human granulosa cells. Our *in vivo* experiments may support this finding. However, it is questionable whether RU486 directly and simply inhibits ovarian 3 β -HSD activity in our experimental design because we used 20 mg RU486 kg⁻¹ *in vivo*, and up to 1 mmol RU486 l⁻¹ had no influence on 3 β -HSD activity in our *in vitro* assay system. In addition, RU486 administration 2 h before hCG injection inhibited the activity at 4–6 h after hCG, and RU486 administration at the same time as hCG injection had no influence on the activity. Our previous data using epistane, a 3 β -HSD inhibitor, demonstrated that epistane rapidly decreases ovarian progesterone concentrations whenever it is administered *in vivo* (Espey *et al.*, 1990; Espey *et al.*, 1991; Tanaka *et al.*, 1991). It seems likely, therefore, that RU486 may inhibit 3 β -HSD activity through the reduction of enzyme induction in the granulosa cells by acting as a progesterone receptor antagonist. The suggestion that RU486

acts as a progesterone receptor antagonist in ovaries is supported by report of the presence of progesterone receptors and binding of RU486 to progesterone receptors in ovaries of diethylstilboestrol-treated immature rats (Schreiber *et al.*, 1983).

Activity of 20 α -HSD increases after hCG stimulation in rat preovulatory follicles (Tsafiri and Eckstein, 1986) and RU486 stimulates ovarian 20 α -HSD activity in rat corpora lutea (Kawano *et al.*, 1988). It is therefore possible that the decrease in hCG-stimulated progesterone production by RU486 is due to the decrease of 3 β -HSD activity and the increase of 20 α -HSD activity. In this study we focused on 3 β -HSD activity, and our histochemical observation that RU486 inhibited 3 β -HSD activity in the granulosa cell layer, but not in the theca cell layer, supports the suggestion that RU486 affects the process of induction of 3 β -HSD. Fortune (1986) reported from studies using bovine theca and granulosa cell culture that theca 3 β -HSD is less sensitive to inhibition by both oestradiol and epostane.

Ovarian progesterone concentration changes markedly during the first 4 h of the ovulatory process (Espey *et al.*, 1990; Espey *et al.*, 1991). This is the time when ovarian 3 β -HSD activity increases significantly. We have previously demonstrated that a small amount of progesterone during the first 4 h after hCG injection plays a critical role in ovulation through its effect on proteolytic enzyme activities, although progesterone production reaches a peak 8 h after hCG; in addition RU486 was shown to inhibit ovulation by inhibiting this action of progesterone (Iwamasa *et al.*, 1992). The present study indicates that a small amount of progesterone just before hCG injection, together with hCG, seems critical for the expression of 3 β -HSD activity. A small amount of progesterone just before and after the LH surge plays a regulatory role in the ovulatory process by modulating 3 β -HSD activity and proteolytic enzyme activities.

In conclusion, the results suggest that there is autocrine regulation of progesterone production in the rat ovary, and that this autocrine regulation may play an important role in the ovulatory process.

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