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Decreased Luteinizing Hormone-Stimulated Progesterone Secretion by Preovulatory Follicles Isolated from Cyclic Rats Treated with the Progesterone Antagonist RU486

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ABSTRACT

Since administration of the antiprogesterone RU486 to cyclic female rats at metestrus and diestrus results in increased serum levels of LH, estradiol, and testosterone at proestrus, we investigated whether RU486 affects follicular steroidogenesis.

Female rats with a 4-day estrous cycle, induced experimentally by a single injection of bromocriptine on the morning of estrus, were given RU486 (2 mg) twice daily (0900 and 1700 h) on metestrus and diestrus. At proestrus the preovulatory follicles were isolated and incubated for 4 h in the absence and presence of LH. In the absence of LH, accumulation of estradiol, testosterone, and progesterone in the medium was not different for RU486-treated rats and oil-treated controls. In contrast, LH-stimulated estradiol, testosterone, and progesterone secretions were significantly lower in RU486-treated rats compared with controls. Addition of pregnenolone to the incubation medium resulted in a significantly lower increase of progesterone in follicles from RU486-treated rats compared with those from oil-treated controls. This suggests that 3β-hydroxysteroid dehydrogenase (3β-HSD) activity is decreased by administration of RU486 in vivo. Aromatase and 17α-hydroxylase/C_{17-20} lyase activities were not affected: addition of substrate (androstenedione and progesterone respectively) did not affect differently the amount of product formed (estradiol and testosterone) in RU486- and oil-treated rats. However, LH-stimulated pregnenolone secretion was lower in follicles from RU486-treated rats compared with follicles from oil-treated controls, suggesting that either cholesterol side-chain cleavage activity or LH responsiveness is decreased.

At proestrus the preovulatory follicles from RU486- and oil-treated rats were not morphologically different. However, at estrus follicles from RU486-treated rats seemed functionally atretic: these follicles, isolated at estrus after an ovulation-blocking dose of pentobarbital at proestrus, secreted a low amount of estradiol compared with follicles from oil-treated rats.

It is concluded that the low LH-stimulated steroid secretion by preovulatory follicles derived from RU486-treated rats is mainly due to a reduced activity of 3β-HSD. This demonstrates that RU486 has a negative effect on steroidogenic enzymes, either directly or indirectly, by increasing LH.

INTRODUCTION

RU486 binds to the progesterone receptor with high affinity and acts as a progesterone antagonist [1]. Administration of RU486 to cyclic female rats results in increased basal levels of serum LH and decreased basal levels of FSH [2, 3]. Furthermore, ovulation can be blocked by antiprogestagens [2–5]. The decreased ovulation rate can be explained by a diminished preovulatory surge of LH on the afternoon of proestrus [2, 5]. However, administration of an ovulatory dose of hCG does not restore full ovulation [5]. Thus either the quality of the follicles or the ovulation process itself is affected. There is evidence that with high doses of RU486 the number of large follicles undergoing atresia is increased at proestrus [2]. A parameter to test the quality of preovulatory follicles is their ability to secrete estradiol in vitro. It has been shown that a decreased follicular estradiol secretion in vitro in response to LH is an early sign of atresia [6–8].

In the present study, follicles isolated at proestrus from RU486-treated rats were incubated with LH to test the capacity of these follicles to produce estradiol, testosterone, and progesterone in vitro. Since LH-stimulated steroid production was found to be decreased, we investigated at what site of the steroidogenic pathway a deficiency occurs.

MATERIALS AND METHODS

Animals

Locally bred (R×U) F_1 hybrid Wistar rats were used. These animals display almost exclusively 5-day ovarian cycles as assessed from daily vaginal smears. The animals were 3–5 mo old and housed under controlled conditions (20–23°C, lights-on from 0500 to 1900 h).

Treatment

Animals were given daily injections of RU486 or oil from metestrus onwards. RU486 (11β-[4-dimethylaminophenyl]-17β-hydroxy-17α-[1-propinyl]-estra-4,9-diene-3-one; Roussel- Uclaf, Romainville, France) was obtained in micronized crystalline form and suspended in olive oil. The injections were given s.c. twice daily at 0900 and 1700 h at a dose of 2 mg per 0.1 ml. Controls received oil vehicle alone.

From a previous study, it appeared that daily injections with antiprogestagens in 5-day cyclic rats from metestrus onwards advanced the forthcoming ovulation by 1 day [5]. To compare follicles isolated at proestrus in RU486-treated

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rats with those in oil-treated rats, a single injection of bromocriptine (Sandoz, Basel, Switzerland) was given to both groups on the morning of the day of estrus. This treatment decreases progesterone production by the corpora lutea and consequently shortens the estrous cycle length from 5 to 4 days [9–12] but has no effect on ovulation rate [9]. Bromocriptine was dissolved in 70% ethanol and injected s.c. at a dose of 1 mg per 0.25 ml.

Animals treated with RU486 or oil were killed at proestrus or at estrus between 0900 and 1000 h. A small group of animals killed at proestrus was used for ovarian histology; the other animals killed at proestrus were used to isolate the 10–12 largest follicles. These follicles were incubated for 4 h under various conditions to measure steroid secretion in vitro. The animals killed at estrus were divided into two groups: one group was given an i.p. injection of sodium pentobarbital (PB, 35 mg/kg body weight) at proestrus at 1300 h, followed by a single injection of 10 IU hCG (Pregnyl, Organon, Oss, The Netherlands) at 1500 h. In these animals, ova in the oviduct were counted the next morning. The other group of animals received an i.p. injection of PB at proestrus (1300 h); at estrus, the 10–12 largest follicles were isolated to measure steroid secretion in vitro.

Follicle Incubations

Intact follicles isolated at proestrus or estrus were incubated individually in 1 ml Medium 199 (Gibco, Grand Island, NY), pH 7.4, containing 25 mM Hepes (Sigma Chemical Co., St. Louis, MO) and an antibiotic-antimycotic mixture (250 μg amphotericin, 10^5 IU penicillin, and 100 mg streptomycin sulfate per liter; Gibco). The incubations were carried out at 37°C under an atmosphere of 95% O_2 and 5% CO_2 in a shaking water bath. After incubation for 4 h, the follicles were discarded and the medium was stored at −20°C until assayed for steroids.

The responsiveness of the follicles to LH was measured by incubating follicles with various amounts of LH (NIH-LH S19, LH activity 1.01 × NIH-LH S1, FSH activity 0.05 × NIH FSH S1). After incubation the concentrations of estradiol, testosterone, and progesterone in the medium were measured.

Total steroid production was measured by incubating follicles in medium containing an inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (Epostane, Sterling-Winthrop, New York, NY; 10 μM) and an inhibitor of 17α-hydroxylase activity (SU-10603, Ciba-Geigy, Basel, Switzerland; 20 μM). After incubation for 4 h, accumulation of pregnenolone in the medium was measured.

Aromatase activity was measured by incubating follicles with androstenedione (0.1–2.0 μM) and measuring the amount of estradiol formed in the medium. The 17α-hydroxylase/C_{17-20} lyase complex was measured by incubating follicles with progesterone (1–20 μM) and measuring the amounts of testosterone and estradiol in the medium after 4 h of incubation. 3β-HSD activity was measured by incubating follicles with pregnenolone (1–20 μM) in the presence of SU-10603 (20 μM) to inhibit 17α-hydroxylase activity and measuring the amount of progesterone formed.

Ovarian Histology

After fixation in Bouin's fluid, ovaries were sectioned at 10 μm and stained with hematoxylin and eosin. Serial sections were studied to count the number of healthy and atretic follicles. Only the number of follicles larger than 450 μm in diameter was counted since they represented the preovulatory follicles that were isolated for incubation studies. Atresia was classified according to criteria reported by Osman [13]. Briefly, early atresia includes the presence of degenerative changes only in the granulosa wall (cell shrinkage and pyknosis); late atresia also includes changes in the oocyte (resumption of meiosis).

Hormone Assays

Concentrations of estradiol, testosterone, progesterone, and pregnenolone in the medium were measured by RIA without extraction as described earlier [14]. The sensitivity of the estradiol assay was 37 fmol/tube, and the interassay variation was 8.3%. The sensitivity of the testosterone assay was 87 fmol/tube, and the interassay variation was 6.0%. The sensitivities of the progesterone and pregnenolone assays were 80 and 158 fmol, and the interassay variations were 12.5% and 2.5%, respectively. Progesterone concentrations measured in the presence of pregnenolone were corrected for the cross-reactivity of pregnenolone to the progesterone antibody (0.3%).

Statistical Analysis

Results are expressed as means ± SEM. Statistical analysis consisted of Student's *t*-test and two-way analysis of variance (ANOVA). Provided that the overall test was significant, comparison between groups was made by the Least Significance Difference (LSD) test. A difference was considered significant if the probability (p) was < 0.05 (two-tailed).

RESULTS

Ovulation Rate and Numbers of Follicles at Proestrus

Treatment with RU486 at metestrus, diestrus, and proestrus resulted at estrus in a low ovulation rate (3 of 6 animals ovulated with 2.3 ± 0.7 ova per ovulating rat) compared with that of oil-treated rats (6/6 with 11.3 ± 0.2 ova). Injection with PB at proestrus followed by an injection with 10 IU hCG did not induce ovulation in any of the RU486-treated rats (0/5), whereas all oil-treated rats ovulated (6/6 with 10.2 ± 0.8 ova).

Morphometric measurement of preovulatory follicles larger than 450 μm in diameter present at proestrus revealed no significant differences in the number and size of

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TABLE 1. Effect of RU486 on the numbers of healthy and atretic follicles larger than 450 μm in diameter at proestrus.

	Healthy follicles[b]		Atretic follicles[b]	
Treatment[a]	Number	Diameter (μm)	Number	Diameter (μm)
oil	11.0 ± 0.9	566 ± 9	1.0 ± 0.6	521 ± 9
RU486	11.0 ± 0.4	583 ± 6	1.6 ± 0.2	508 ± 12

[a]The rats were given a single injection with bromocriptine (1 mg) at estrus and were treated with oil or RU486 in oil (2 mg at both 0900 and 1700 h) at metestrus and diestrus.
[b]Values are means ± SEM of 5 animals.

healthy and atretic follicles from rats treated with RU486 and from oil-treated controls (Table 1).

Follicle Incubations in the Absence of LH

Follicles isolated at proestrus from oil- and RU486-treated rats and incubated in the absence of LH secreted a high amount of estradiol and a low amount of progesterone. No significant difference appeared between the two treatment groups (groups 1 and 2, Table 2). Follicles isolated at estrus from oil-treated rats in which ovulation was blocked by an injection of PB at proestrus (group 3) also secreted a high amount of estradiol and a low amount of progesterone. However, follicles isolated at estrus from RU486-treated rats given PB at proestrus (group 4) secreted a significantly lower amount of estradiol ($p < 0.01$). Follicles isolated from RU486-treated rats that were not given an injection of PB at proestrus (group 5) secreted a low amount of estradiol, but a high amount of progesterone.

Follicle Incubations in the Presence of LH

Follicles isolated at proestrus from oil-treated rats and incubated in the presence of various amounts of LH secreted a high amount of estradiol. A dose of 10 ng LH resulted in maximal stimulation (Fig. 1a). In contrast, at every dose of LH used, estradiol secretion by follicles from RU486-treated rats was significantly lower than that by follicles from oil-treated rats. At a dose of 200 ng LH, the accumulation of estradiol in the medium was decreased in both treatment groups compared with a dose of 100 ng LH. The accumulation of testosterone in the medium by follicles from RU486-treated rats was significantly lower than that by follicles from oil-treated rats (Fig. 1b). An even larger difference between RU486-treated rats and oil-treated controls was found for the accumulation of progesterone in the medium (Fig. 1c).

Enzyme Activities

To determine whether the low estradiol secretion by follicles isolated from RU486-treated rats was due to a decrease in aromatase activity, follicles isolated from oil- and RU486-treated rats were incubated with various amounts of androstenedione. Addition of androstenedione resulted in a dose-dependent increase of estradiol in the medium (Fig. 2). No significant difference appeared between follicles isolated from oil- and RU486-treated rats.

Incubation in the presence of progesterone (1–20 μM) resulted in testosterone and estradiol concentrations in the medium that were not significantly different between oil- and RU486-treated rats (estradiol $p = 0.53$, testosterone $p = 0.06$; Fig. 3, a and b). This demonstrates that the 17α-hydroxylase/C_{17-20} lyase complex was not affected by the treatment.

Incubation in the presence of pregnenolone at doses of 10 and 20 μM, however, resulted in a significantly lower amount of progesterone in the medium by follicles isolated from RU486-treated rats compared with those from oil-treated rats ($p < 0.05$ at least; Fig. 4). This suggests that 3β-HSD activity is decreased by administration of RU486 in vivo.

To measure total steroid production, follicles were incubated in the presence of SU-10603 (an inhibitor of 17α-hydroxylase) and Epostane (an inhibitor of 3β-HSD). Addition of LH resulted in a dose-related increase in both

TABLE 2. Effect of administration in vivo of RU486 on follicular estradiol and progesterone secretion by preovulatory follicles in vitro.

Group	Treatment[a]	Pentobarbital at proestrus	Estradiol[b] (pmol/follicle/4 h)	Progesterone[b] (pmol/follicle/4 h)
			proestrus	
1	oil		5.92 ± 0.58	0.71 ± 0.16
2	RU486		6.82 ± 0.60	0.90 ± 0.05
			estrus	
3	oil	+	5.56 ± 0.90	0.86 ± 0.11
4	RU486	+	2.44 ± 0.13*	0.79 ± 0.16
5	RU486	−	0.76 ± 0.07**	3.63 ± 0.81**

[a]The rats were given a single injection with bromocriptine (1 mg) at estrus and were treated with oil or RU486 in oil (2 mg at both 0900 and 1700 h) from metestrus through proestrus. Preovulatory follicles were isolated at proestrus (groups 1 and 2) or at estrus (groups 3, 4 and 5) and incubated for 4 h in the absence of LH. In groups 3 and 4, an injection of sodium pentobarbital was given at 1300 h on the day of proestrus to block the expected preovulatory LH surge.
[b]Values are means ± SEM of 20–25 follicles (4–6 follices per rat)
*$p < 0.01$ compared with group 3 (Student's t-test).
**$p < 0.01$ compared with group 4 (Student's t-test).

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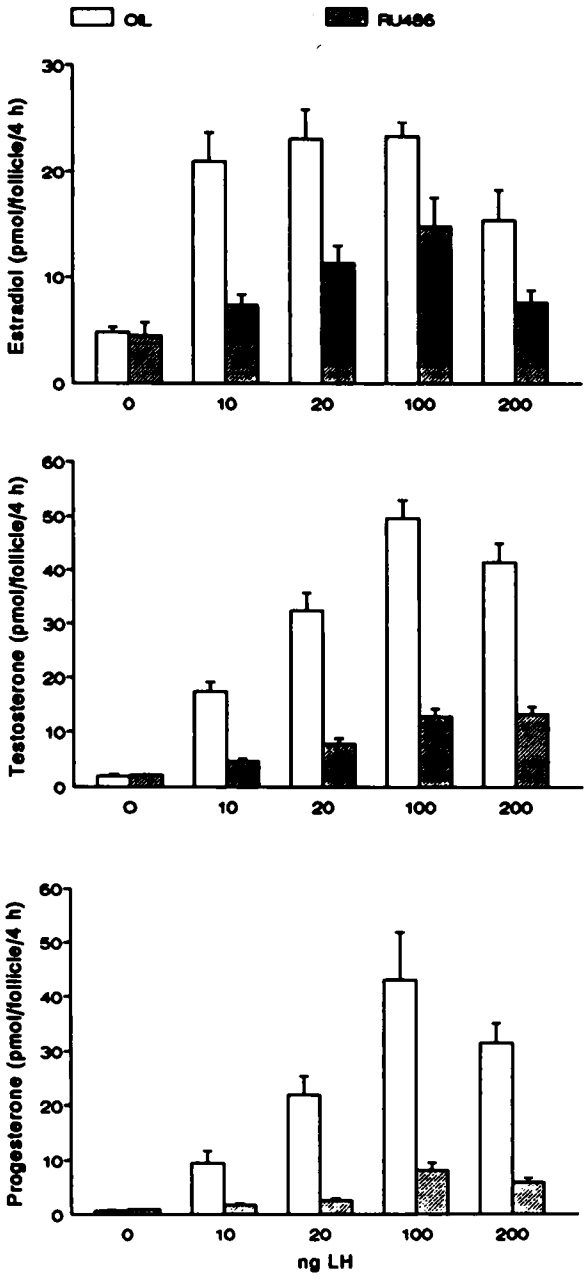

FIG. 1. Effect of LH on follicular (a) estradiol, (b) testosterone, and (c) progesterone production. The follicles were isolated at proestrus and incubated with increasing amounts of LH for 4 h. The rats were given a single injection with bromocriptine (1 mg) at estrus and were treated with oil or RU486 in oil (2 mg twice daily, 0900, and 1700 h) at metestrus and diestrus. Each bar represents the mean ± SEM of 8 follicles (2 follicles per rat).

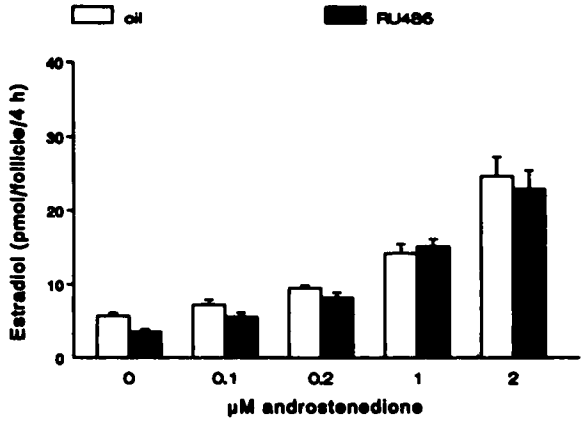

FIG. 2. Effect of androstenendione on follicular estradiol production. The follicles were isolated at proestrus and incubated with increasing amounts of androstenedione for 4 h. The rats were treated as described in Figure 1. Each bar represents the mean ± SEM of 8 follicles (2 follicles per rat).

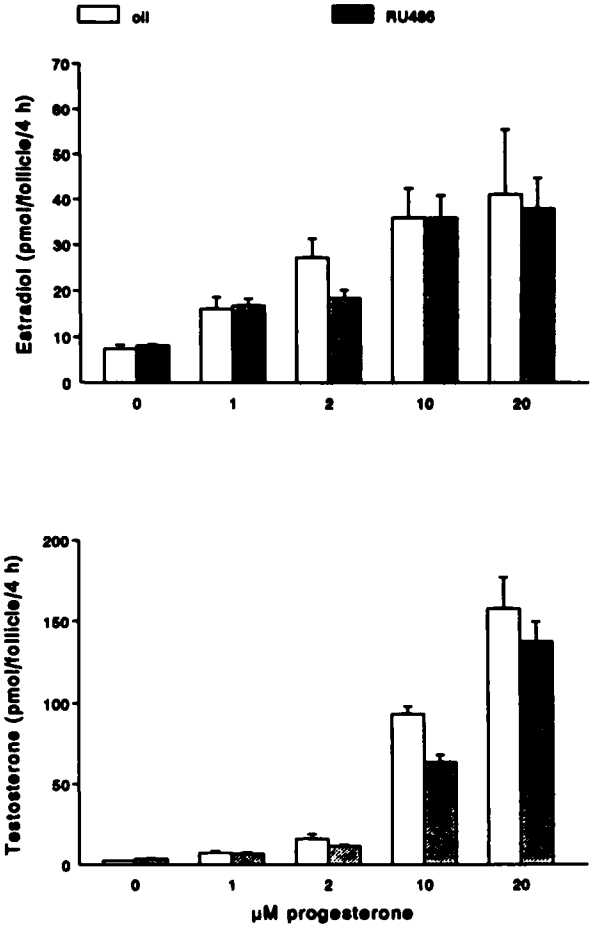

FIG. 3. Effect of progesterone on follicular (a) estradiol and (b) testosterone production. The follicles were isolated at proestrus and incubated with increasing amounts of progesterone for 4 h. The rats were treated as described in Figure 1. Each bar represents the mean ± SEM of 6 follicles (2 follicles per rat).

treatment groups (Fig. 5). At 20, 100, and 200 ng LH, the accumulation of pregnenolone was significantly lower in RU486-treated rats compared with oil-treated rats ($p < 0.05$ at least), demonstrating that total steroid production in response to LH was diminished after administration of RU486 in vivo.

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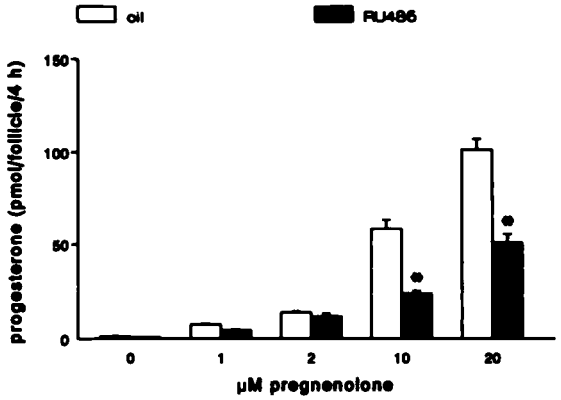

FIG. 4. Effect of pregnenolone on follicular progesterone production. The follicles were isolated at proestrus and incubated with increasing amounts of pregnenolone and an inhibitor of 17α-hydroxylase activity (SU-10603, 20 μM) for 4 h. Rats were treated as described in Figure 1. Each bar represents the mean ± SEM of 6 follicles (2 follicles per rat). *P<0.05 compared with oil-treated rats (two-way ANOVA followed by LSD-test).

DISCUSSION

The decreased ovulation rate after administration of RU486 (4 mg/day) and the failure to ovulate after injection of an ovulatory amount of hCG are in agreement with earlier findings [2, 5]. The question is whether follicular steroidogenesis is affected as well. The present study shows that LH-stimulated accumulation of estradiol, testosterone, and progesterone in the medium was reduced in follicles isolated from RU486-treated rats. In the absence of LH, however, follicular steroid secretion was not different in follicles isolated from RU486-treated rats and from controls. The decreased LH-stimulated estradiol secretion was not due to a decrease in aromatase or 17α-hydroxylase/C_{17-20} lyase activity, for LH-stimulated testosterone and progesterone secretions (Fig. 1, b and c) were also decreased. In accordance, aromatase activity did not fall since incubation with androstenedione showed no difference in estradiol levels between oil- and RU486-treated rats (Fig. 2). Moreover, 17α-hydroxylase/C_{17-20} lyase actitivity did not decrease since incubation with progesterone showed no difference in levels of testosterone and estradiol between oil-treated rats and RU486-treated rats (Fig. 3). In contrast, a significant difference was found between progesterone levels of oil- and RU486-treated rats after incubation with 10 and 20 μM pregnenolone (Fig. 4), thus showing a decrease in 3β-HSD activity. Also, accumulation of pregnenolone after incubation with LH was significantly lower in follicles isolated from RU486-treated rats than in follicles from oil-treated controls (Fig. 5). This suggests that either cholesterol side-chain cleavage activity or LH responsiveness was also diminished. An inhibitory effect of RU486 on steroidogenesis has been reported for cultured human granulosa cells [15] and whole testis [16]. In agreement with our observation, Dimattina et al. [15] found decreased progesterone production due to inhibition of 3β-HSD activity after culturing human granulosa cells with RU486. In contrast, Sanchez et al. [16], using whole ovaries from hypophysectomized rats treated for 7 days with hCG, reported a decrease of 17α-hydroxylase without a decrease of 3β-HSD after administration of RU486 in vivo. These seemingly contrasting results may be related to the difference in follicular population in these two studies. Hypophysectomized rats treated with hCG do not contain large follicles, but only small follicles and interstitial tissue.

It is surprising that, while LH-stimulated estradiol and testosterone secretions by preovulatory follicles in vitro are decreased, serum levels of estradiol and testosterone in RU486-treated rats at proestrus are increased [3]. This suggests that either these steroids are not of follicular origin or that the increased levels of LH compensate for the decreased responsiveness of the follicles. In line with this suggestion, the interstitial compartment of RU486-treated rats contains a high amount of testosterone, while high levels of estradiol are present in the corpora lutea [3].

The decreased ability of the preovulatory follicles to secrete progesterone after LH stimulation might be due to a direct effect of RU486 or be secondary to changed serum levels of gonadotropins. A direct effect of RU486 is conceivable since progesterone receptors are present in the ovary [17] and RU486 binds to these receptors with high affinity [18]. The present study shows that in the absence of progesterone action the activity of the enzymes stimulating progesterone synthesis are reduced. This supports the concept that progesterone stimulates its own production by a stimulation of 3β-HSD activity [19]. This concept is based on the autonomy of corpus luteum progesterone secretion in hypophysectomized rats. A direct effect of RU486 on ovarian steroidogenesis has been demonstrated by Dimattina et al. [15] using human granulosa cells in culture.

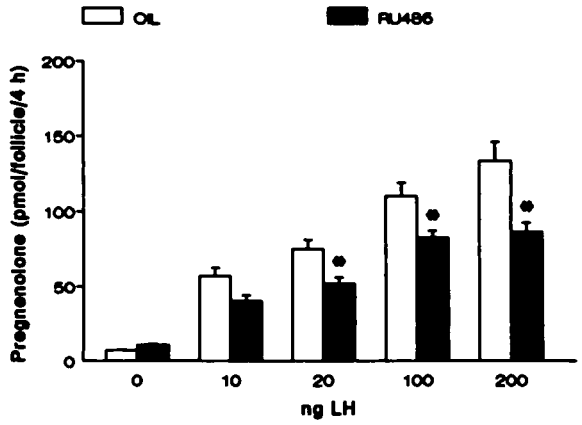

FIG. 5. Effect of LH on follicular pregnenolone production. The follicles were isolated at proestrus and incubated with increasing amounts of LH in the presence of an inhibitor of 17α-hydroxylase activity (SU-10603, 20 μM) and an inhibitor of 3β-hydroxysteroid dehydrogenase activity (Epostane, 10 μM) for 4 h. Rats were treated as described in Figure 1. Each bar represents the mean ± SEM of 6–8 /follicles (2 follicles per rat). *$p <$ 0.05 compared with oil-treated rats (two-way ANOVA followed by LSD-test).

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The decreased LH-stimulated progesterone production by isolated follicles, however, could be secondary to the changed levels of gonadotropins. In rats treated with RU486 from metestrus through proestrus, basal serum levels of LH are increased at diestrus and proestrus, while those of FSH are decreased from the afternoon of metestrus onwards [2, 3]. It has been demonstrated for cultured granulosa cells that both gonadotropins are involved in the regulation of cholesterol side-chain cleavage and 3β-HSD activities [20]. Furthermore, serum levels of testosterone and estradiol are increased [3]. Increased levels of androgens are favorable for follicular atresia [21]. However, at proestrus no morphological signs of atresia were present as shown by the absence of differences in the number and size of healthy and atretic follicles between RU486-treated rats and controls (Table 1). This is consistent with earlier findings in which an increase in atresia was found only after administration of 5 mg RU486 twice daily, but not with 1 mg RU486 twice daily [2]. There is, however, some evidence that follicles from RU486-treated rats become atretic earlier. In rats and hamsters with a 4-day cycle, estradiol secretion by explanted follicles can be prolonged by 24 h when ovulation is blocked by an injection of PB at proestrus before the expected time of the LH surge [7, 8]. The present study shows that in RU486-treated rats such a treatment results in diminished follicular estradiol secretion (Table 2). This indicates that, in contrast to follicles from oil-treated rats, follicles from RU486-treated rats isolated at estrus (after PB treatment at proestrus) are already functionally atretic.

Because of the failure to ovulate, preovulatory follicles in RU486-treated rats are also present at estrus without an injection of PB at proestrus. Incubation of such follicles revealed a low concentration of estradiol, but a high concentration of progesterone (group 5, Table 2). Such follicles at estrus show dispersion of cumulus cells and luteinization as a consequence of the subovulatory release of LH on the afternoon of proestrus [5]. The low concentration of estradiol and the high concentration of progesterone illustrate the shift from estradiol to progesterone production by the LH surge.

In summary, administration of high doses of RU486 for 2 days reduces LH-stimulated steroid secretion by large follicles in vitro. This is mainly due to lower 3β-HSD activity. Whether or not this lower enzyme activity is caused by a direct effect of RU486 on granulosa cells or is mediated by changed levels of gonadotropins will be a subject for further study.

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