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LUTEOLYTIC EFFECT OF THE ANTIPROGESTIN AND ANTIGLUCOCORTICOID AGENT RU486 IN RATS

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Summary—Ovarian cells of pregnant rats were cultured with synthetic progestins (R5020, R2323), dexamethasone and RU486. Progesterone and 20α-hydroxy-pregn-4-en-3-one (20α-dihydroprogesterone) in the medium were measured by specific radioimmunoassay. Both R5020 and R2323 increased concentrations of these intrinsic progestins. RU486 decreased concentrations of progesterone, however, the addition of R5020 or R2323 counteracted this action.

Immature hypophysectomized rats treated with pregnant mare serum gonadotropin (PMS) and human chorionic gonadotropin (hCG) were administered with RU486; the serum levels of progesterone and 20α-dihydroprogesterone tended to decrease.

R5020 and R2323 inhibited the effect of 3β-hydroxysteroid dehydrogenase (3β-HSD), whereas RU486 did not. Inhibition of the cholesterol side chain cleavage enzyme (CSCC) by RU486 was more marked than that by R5020 or R2323.

These results show that RU486 decreases progesterone synthesis in cultured ovarian cells. A part of the mechanism may involve an inhibition of CSCC.

INTRODUCTION

The antiprogesterone and antiglucocorticoid agent RU486 has an abortifacient effect in pregnant rats and decreases levels of serum progesterone [1]. The decrement of progesterone is considered to be a secondary effect of abortion [1]. However, it is possible that RU486 acts directly on the ovary and causes luteolysis, because it binds both the progesterone receptor and the glucocorticoid receptor [2] which have been identified in the ovary [3, 4]. It is reported that RU486 decreases the *in vitro* output of progesterone and the 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in cultured human granulosa cells [5], but little information is available concerning its effect on corpus luteum cells. The following study was carried out to investigate the luteolytic effect of RU486 in luteal cells from pregnant rats *in vitro* and in hypophysectomized immature rats treated with pregnant mare serum gonadotropin (PMS) and human chorionic gonadotropin (hCG) *in vivo*. The *in vitro* effect of RU486 on 3β-HSD and cholesterol side chain cleavage enzyme (CSCC) was also studied in rat luteal cells.

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Abbreviations: RU486: 17β-hydroxy-11β-(4-dimethylaminophenyl)-17-(prop-1-ynyl)-estra-4,9-dien-3-one; R5020: 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; R2323: 13-ethyl-17α-ethinyl-17-hydroxy-gona-4,9,11-trien-3-one (gestrinone); dexamethasone: 9-fluoro-16α-methyl-11β,17,21-trihydroxy-1, 4-pregnadiene-3,20-dione.

EXPERIMENTAL

Experiment 1

Ovarian cell preparation. Pregnant rats of the Wistar–Imamichi strain were sacrificed by decapitation on day 13 of gestation. Ovaries were dissected out and the surrounding tissue was removed. Each ovary was cut into 5–6 pieces and washed with Hanks' calcium- and magnesium-free balanced salt solution (HCMF) containing 1% bovine serum albumin (BSA).

Cells were dispersed by incubating tissue pieces for 60 min at 37°C in HCMF containing 0.4% collagenase, 10 μg/ml DNAse, and 1% BSA. The dispersed ovarian cells were collected by centrifugation (1000 rpm, 5 min) and washed twice in HCMF. Aliquots of the cell suspension were diluted with equal volumes of Trypan blue stain and samples were taken for counting in a hemocytometer.

Cell culture procedure. Ovarian cells (1×10^5 viable) were cultured in a tissue culture plate (Corning, with 24 wells) in 1 ml Dulbecco's modified Eagle's Medium (DMEM). The steroids used in the experiment, R5020, R2323, RU486 (10^{-6} M) and dexamethasone (10^{-7}–10^{-6} M), were dissolved in 10 μl of ethanol and added to the incubation medium (1 ml). After incubation for 2 days, the cells were washed twice with 1 ml portions of HAM F-12 and subsequently reincubated in 1 ml HAM F-12. After a 3-h incubation period, media were collected and stored at −20°C until radioimmuno assay (RIA) was performed for progesterone and 20α-hydroxy-pregn-4-en-3-one (20α-dihydroprogesterone).

Radioimmunoassay of progesterone and 20α-dihydroprogesterone. Steroids in the media were directly measured by RIA. The specific antibodies against progesterone and 20α-dihydroprogesterone were raised in our laboratory; Japanese albino rabbits were immunized by injections of progesterone-11α-hemisuccinate-BSA or 20α-dihydroprogesterone-3-oxime-BSA and produced specific antisera. The cross reactions of the anti-progesterone antibody (diluted at 1:200,000) to 20α-dihydroprogesterone and of the anti-20α-dihydroprogesterone antibody (diluted at 1:5000) to progesterone were 6.73 and 4.40%, respectively [6, 7].

Experiment 2

Animals. Immature female rats (22-days-old) were hypophysectomized and at 0900 h on day 26, 10 IU of PMSG were injected subcutaneously (s.c.). 30 IU of hCG were injected s.c. at 1400 h on day 28. The rats were then separated into 2 groups, and at 1000 h on day 29, vehicle oil (control group) or 10 mg/kg body wt of RU486 (RU group) were injected s.c. The animals were decapitated at 1000 h on day 31 and blood samples were collected.

RIA of serum progesterone and 20α-dihydroprogesterone. The blood sample was centrifuged at 3000 rpm for 20 min to separate serum, which was extracted twice with ether. The residue of the extract was subjected to RIA of progesterone and 20α-dihydroprogesterone.

Experiment 3

Preparation of enzymes. Immature female rats of the Wistar–Imamichi strain were used. 10 IU of PMS were injected s.c. at 0900 h on day 23 and these rats were decapitated at 1700 h 2 days later. Another group of rats were administered with 30 IU of hCG at 1400 h 2 days after the PMS injection, and sacrificed 3 h after the last treatment. Ovaries were dissected out, homogenized in a Teflon glass homogenizer with 0.25 M sucrose/0.05 M Tris–HCl buffer, and then centrifuged at 800 *g* for 20 min. The supernatant thus obtained was used as the enzyme source. Protein in the supernatant was determined by the Bradford's method [8].

3β-HSD activity. Each incubation mixture (1 ml) contained a tissue preparation from PMS-hCG-treated rats (20 μg protein), 0.5 μmol of NAD, and [^{14}C]pregnenolone (about 0.03 μCi, 10 nmol). The mixture was incubated in a Dubnoff-type incubator at 37°C for 20 min with or without added steroid inhibitors ($0.1–100 \times 10^{-6}$ M). The incubation was terminated by chilling in an ice bath. Steroids were extracted with ether (5 ml). A mixture of nonradioactive carrier steroids was added to each extract. Then the residue of each extract was applied to a silica gel thin-layer plate and developed in a solvent system of benzene–acetone (4:1, v/v). Spots of the carrier steroids on thin-layer chromatograms were located under u.v. light (254 nm) or by developing color in iodine vapor. Each spot was scraped off the plate, added to 0.1 ml methanol and 3 ml toluene scintilator, and the radioactivity was measured in a liquid scintillation spectrometer (Aloka LSC 703). The enzyme activity was expressed as the amount of [^{14}C]progesterone produced from pregnenolone. The K_m value of 3β-HSD for pregnenolone, as 10 μM of the substrate was used, was 2.73 ± 1.34 μM [9].

CSCC activity. Each incubation mixture (1 ml) contained tissue preparation (81 μg protein), 0.5 μmol of NADPH, and 26 [^{14}C]cholesterol (about 0.03 μCi, 0.2 nmol). The mixture was incubated in Tris–HCl buffer in a Dubnoff type incubator for 10 min at 37°C with or without steroid inhibitors. The reaction was stopped by an addition of 0.25 ml of 1 N NaOH and unchanged [^{14}C]cholesterol was removed by shaking the incubation mixture with dichloromethane (3 ml). [^{14}C]Isocaproic acid derived from cholesterol in the water phase was counted in the liquid scintillation spectrometer using a Triton–toluene scintillator. The enzyme activity was expressed as the amount of [^{14}C]isocaproic acid formed [10].

Chemicals

HCMF, DMEM and HAM F-12 were purchased from Gibco Life Technologies Inc. (Grand Island, N.Y., U.S.A.). BSA (fraction V), 20α-dihydroprogesterone, progesterone, and dexamethasone were obtained from Sigma Chemical Company (St Louis, Mo., U.S.A.). Collagenase (type I: 137 U/mg) was a product of Worthington Biochemical Co. (Freehold, N.J., U.S.A.). R5020, R2323 and RU486 were provided by Roussel Uclaf (Paris, France). 26[^{14}C]Cholesterol (53.0 mCi/mmol) and 4[^{14}C]pregnenolone (57.2 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Mass, U.S.A.).

Statistical analysis

Data were analyzed using the Student's *t*-test; a *P*-value of less than 0.05 was considered significant.

RESULTS

Experiment 1

R5020 significantly increased both progesterone and 20α-dihydroprogesterone release *in vitro* ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 1a). R2323 increased progesterone and 20α-dihydroprogesterone release, and the increment of progesterone was significant ($P < 0.05$) (Fig. 1b). RU486 decreased progesterone output significantly ($P < 0.05$), but it did not change the level of 20α-dihydroprogesterone. RU486 counteracted the increased release of progesterone induced by R5020 and R2323 ($P < 0.05$ and $P < 0.01$, respectively). RU486 did not change the increased output of 20α-dihydroprogesterone caused by R5020 or R2323. Dexamethasone caused no significant change in the release of progestins (Fig. 1c).

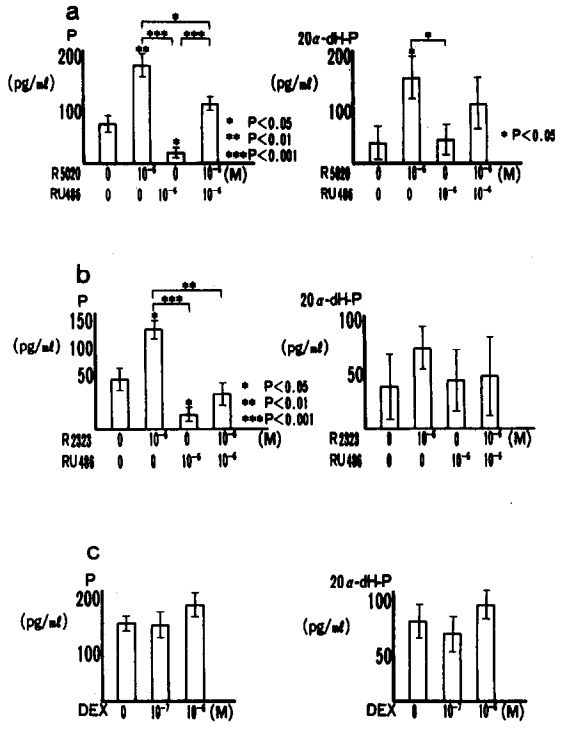

Fig. 1. The effects of RU486, R5020, R2323, and dexamethasone on the release *in vitro* of P and 20α-dH-P from cultured cells of the rat corpus luteum. The vertical bars indicate standard errors. P: progesterone; 20α-dH-P: 20α-dihydroxyprogesterone; DEX: dexamethasone.

Experiment 2

Administration of RU486 to hypophysectomized, gonadotropin-treated rats decreased the levels of serum progesterone and 20α-dihydroprogesterone, but the difference was not significant (Fig. 2).

Experiment 3

3β-HSD. In the control experiment, 7.6% of labelled pregnenolone was converted to progesterone in 20 min, therefore the 3β-HSD activity of the control group was 0.114 μM/μg protein/h. R5020 and R2323 inhibited the 3β-HSD activity significantly at a concentration of 2 or 20 μM, but RU486 showed no effect at concentrations up to 100 μM (Fig. 3a).

CSCC. In the control experiment, 9.5% of labelled cholesterol added was metabolized in 10 min, therefore the CSCC activity of the control group was 0.0148 μM/μg protein/h. R5020 and R2323 inhibited

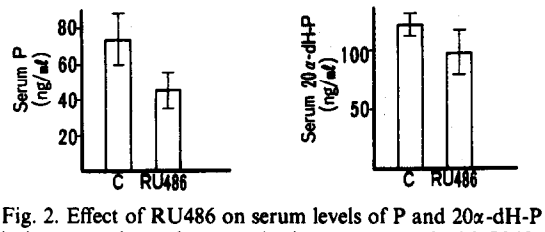

Fig. 2. Effect of RU486 on serum levels of P and 20α-dH-P in immature hypophysectomized rats pretreated with PMS-hCG. The vertical bars indicate standard errors. Differences as compared to controls were not significant.

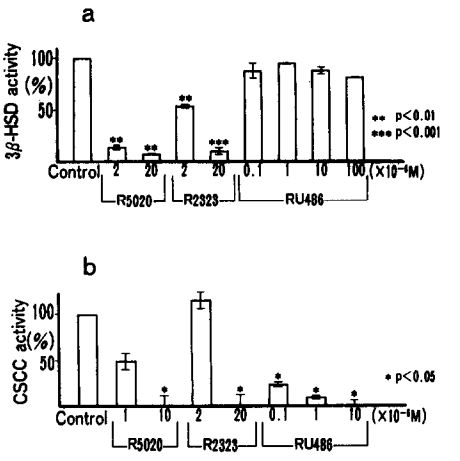

Fig. 3. Effect of R5020, R2323 and RU486 on the enzymic activity of rat ovarian (a) 3β-HSD and (b) CSCC. The vertical bars indicate standard errors. Enzyme activities for controls: 3β-HSD 0.114 μM/μg protein/h; CSCC 0.0148 μM/μg protein/h.

the CSCC activity significantly at concentrations of 10 and 20 μM, respectively. The inhibition of this enzyme by RU486 was more marked than that by R5020 or R2323; CSCC was inhibited significantly ($P < 0.05$) at a concentration of 0.1 μM (Fig. 3b).

DISCUSSION

The present experiments showed that the progestational compounds R5020 and R2323 increased whereas RU486 decreased the release of progesterone *in vitro* from ovarian cells of pregnant rats.

It is reported that 10^{-5}–10^{-6} M of R5020 increases the release of progesterone and 20α-dihydroprogesterone from cultured rat granulosa cells [11]. A similar result was obtained using rat luteal cells in the present experiment. However, according to Schreiber, R5020 decreases the release of progestins from rat granulosa cells [12]. Under the present experimental conditions R5020 and R2323 inhibited steroidogenic enzymes at concentrations over 2 μM. It seems probable that the enhanced release of progesterone from luteal cells by these synthetic steroids predominates their suppressive effect against steroidogenic enzymes in the subcellular fractions. These inconsistent data may be the result of different experimental conditions. Further studies will be needed to clarify this point.

RU486 decreased the release of progesterone from rat luteal cells in the present experiment. It was also indicated that RU486 directly inhibited CSCC, but not 3β-HSD, at the level of enzyme molecules. Consequently, it seems probable that the decreased release of progesterone is, at least in part, due to inhibition of CSCC.

Rat granulosa cells also have glucocorticoid receptors [3] and dexamethasone stimulates progesterone release from granulosa cells under the influence of FSH [13, 14]. The present experiment, however,

showed that dexamethasone had no effect on the release of progesterone from luteal cells. Different experimental conditions may be responsible for the inconsistency, and further study will be needed to clarify this problem.

The possibility that RU486 exerted toxic effects on corpus luteum cells under the conditions used is minimal, because there was no difference in the number of viable cells among the groups at the end of the experiment (data not shown).

To investigate a direct effect of RU486 on the corpus luteum, pregnant rats may not be suitable for experimental animals because placental hormones are involved. Thus, we used PMS-hCG-treated immature rats to obtain the uniformly developed corpus luteum for the source of steroidogenic enzymes.

RU486 exhibited a minor effect *in vivo* on the serum level of progesterone. A sufficient dose of RU486 (10 mg/kg body wt), which induced complete abortion in pregnant rats (day 16) [1], was administered in the present study. The peripheral serum level of progesterone in PMS-hCG-treated hypophysectomized immature rats was over 70 ng/ml (Fig. 2) and this high concentration of the hormone was the result of its larger production rate in the ovary, which could not be sufficiently blocked by RU486.

When luteolysis is induced in pregnant rats by RU486, a decrease of placental tropic hormones following abortion may play an important role. It is reported that the rat placenta secretes rat chorionic gonadotropin [15] and rat placental lactogen [16], both of which are luteotropic. When abortion occurs, secretions of these placental hormones decrease and consequently induce luteolysis.

Hypophysectomy or administration of anti LH serum to rats on days 8–11 of pregnancy causes abortion [17, 18]. However, hypophysectomy on days 11–20, delays parturition [17], and administration of LH blocks this effect [19]. In the early stage of pregnancy, prolactin also plays an important role in the maintenance of pregnancy [20]. RU486 affects the release of pituitary hormones *in vitro* [21] or *in vivo* [22], and there is an additional possibility that RU486 causes abortion via its effects on pituitary hormones.

Progesterone synthesis in the corpus luteum is mainly regulated by LH, but Rothchild proposed progesterone has a luteotropic effect maintaining its own synthesis [23]. The results of our present experiment suggest there may be a short positive feedback loop of progestins to ovarian progesterone synthesis; synthetic progestins stimulated the release of intrinsic progestins and a smaller amount of RU486 inhibited CSCC. These facts suggest that the progesterone secretion from the corpus luteum is regulated by progesterone itself, too.

On the basis of the present experiment, it was demonstrated that RU486 had a direct luteolytic effect on the rat corpus luteum, but a possible indirect effect via ablation of placental and/or pituitary hormones following abortion must not be overlooked in pregnant rats administered with this agent systemically.

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