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Antiprogestagen RU486 Prevents the LH-Dependent Decrease in the Serum Concentrations of Inhibin in the Rat

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SUMMARY

1. In the rat, the LH-dependent ovarian progesterone rise mediates several actions of the primary surge of LH on the ovary. This experiment was aimed at elucidating the effects of the antiprogestagen RU486 on the LH-dependent decrease in both the serum concentrations and the ovarian content of inhibin.

2. All rats in this experiment were treated with an antagonist of LHRH (1 mg/200 μl saline at 0800 h in proestrus) to supress the endogenous release of LH. One group of rats received 32 μg LH/250 μl saline at 1200 h in proestrus. Other group was given 4 mg RU486/200 μl oil at 0800 h in proestrus. The third group was injected with both RU486 and LH. Rats from the control group were injected with 250 μl saline and 200 μl oil. Animals were decapitated at 1700 h in proestrus and trunk blood and ovaries collected to determine the serum concentrations of LH, FSH, progesterone, 17ß-estradiol and inhibin as well as the ovarian content of inhibin.

3. The ovulatory dose of LH in LHRHa-treated rats decreased both the serum concentrations and the ovarian content of inhibin and increased the serum concentrations of FSH. The administration of RU486 blocked the effect of LH on the serum concentrations of inhibin but not that on the ovarian content of inhibin.

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4. Since the antiprogestagen RU486 blocked the effect of LH on the serum concentrations of inhibin, we conclude that ovarian progesterone, besides mediating the effects of the primary LH surge on the ovulatory process and luteinization, participates in the LH-dependent drop in the serum concentrations of inhibin in proestrous afternoon.

INTRODUCTION

In the cyclic rat, the maximal release of gonadotropins (primary surge of LH and FSH) is in the afternoon of proestrus shortly before ovulation. While the concentrations of LH fall in the last hours of proestrus, the secretion of FSH remains high during the first hours of estrus (secondary surge of FSH) and reachs pre-surge values at noon in estrus (Freeman 1981). The control of the secondary surge of FSH, which is implicated in the recruitment of a new set of growing follicles (Greenwald and Terranova 1988), is not completely known. The preovulatory surge of gonadotropins is completely coupled to the LHRH secretion (Sarkar *et al.*, 1976), while the release of FSH during early estrus is independent of LHRH (Hasegawa *et al.*, 1981; Sánchez-Criado *et al.*, 1994). It is well established, however, that the secondary surge of FSH depends on both the preovulatory release of LH (Schwartz and Talley 1978) and the LH-dependent drop in the serum concentrations of inhibin (Shander *et al.*, 1980; Rivier *et al.*, 1989).

The administration of the antiprogesterone and antiglucocorticoid RU486 blunts the primary surge of LH and FSH and blocks the secondary surge of FSH (Sánchez-Criado *et al.*, 1992a; Knox and Schwartz 1992). Moreover, the injection of an ovulatory dose of LH does not restore the secondary surge of FSH in rats treated with RU486 (Knox *et al.*, 1993). These data suggest that another factor, which is blocked by RU486, is necessary, together with the drop in the serum concentrations of inhibin, to evoke the secondary surge of FSH. Recently, we have demonstrated that the secondary surge of FSH during early estrus is evoked by the combined effects of the primary surge of LH and the LH-independent rise of serum corticosterone in proestrus afternoon (Tébar *et al.*, 1995a).

It is known that the LHRH-dependent primary surge of LH induces progesterone receptors, luteinization and secretion of progesterone by the preovulatory follicles, resulting in a rise in the serum concentrations of progesterone (Natraj and Richards 1993; Uchida *et al.*, 1969). In addition, there are evidences that ovarian progesterone mediates the effects of the primary surge of LH on the ovulatory events and luteinization (Iwamasa *et al.*, 1992; Tanaka *et al.*, 1993; Natraj and Richards 1993). Besides, a preliminary observation from our laboratory showed that the administration of RU486 in proestrus tends to prevent the LH-dependent drop in the serum concentrations of inhibin (Sánchez-Criado *et al.*, 1993b).

Overall, these data suggest that progesterone, through a local inhibitory

effect, could mediate the action of the primary surge of LH on the serum concentrations of inhibin. The present work was carried out to investigate the effect of RU486 on the serum concentrations and on the ovarian content of inhibin in the presence or in the absence of the preovulatory surge of LH.

METHODS

Animals

Adult female Wistar rats weighing 180–220 g were used. The rats were housed (5 per cage) under standard light conditions (lights on from 0500 to 1900 h) and temperature (20–23°C) with water and food provided "ad libitum". Vaginal smears were examined daily. Only rats exhibiting at least two consecutive 4-day estrous cycles were used.

Treatments

Antiprogestagen RU486 (Mifepristone, 11 ß-(4-dimethylaminophenyl)-17 ß-hydroxy-17 α-(1-propinyl)-estra-4,9-diene-3-one) was donated by Dr R. Deraedt (Roussel-Uclaf, Romainville, France). This compound has a high affinity for progesterone and glucocorticoid receptors (Philibert *et al.*, 1985; van der Schoot *et al.*, 1989). Rats were injected (s.c.) with 4 mg/200 μl oil at 0800 h in proestrus. Control injections consisted of 200 μl oil.

The LHRH antagonist (LHRHa) used was ORG.30276 (Ac-D-p-Cl-Phe-D-p-Cl-Phe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2.CH_3.COOH) (van den Dungen *et al.*, 1989) Organon international B.V., Oss, The Netherlands. Immediately before use the peptide was dissolved in saline and rats were injected (s.c.) with 1 mg/200 μl saline at 0800 h in proestrus. This dosage causes maximal suppression of endogenous LH secretion (Sánchez-Criado *et al.*, 1993a).

Ovine LH (oLH) NIDDK oLH-26 was injected (i.v.) under ether anesthesia at 1200 h in proestrus at a dosage of 32 μg/250 μl saline. This LH preparation contained less than 0.5% contamination of FSH. Control injections consisted of 250 μl saline.

Experimental Groups

All the rats were treated with the LHRHa (1 mg/200 μl saline) at 0800 h in proestrus. Animals were divided in four groups (n = 7–9) and they were treated in proestrus as follows: in the first group, rats were injected with vehicles (200 μl oil at 0800 h and 250 μl saline at 1200 h); in the second group, rats were given injections of RU486 (4 mg/200 μl oil at 0800 h) and of saline; in the third group, rats were given oil and an ovulatory dose of LH (32 μg/250 μl saline at 1200 h); and, in the fourth group, rats were injected with RU486 and LH. All rats were decapitated at 1700 h in proestrus and trunk blood and ovaries were collected.

Blood was centrifuged for 20 minutes at 2000 × g and the serum was stored frozen until the radioimmunoassays were run. The ovaries were handly homogenated (2 ml medium 199/ovary) and centrifuged for 1 h at 71000 × g and the supernatants were stored frozen until the radioimmunoassay was run.

Radioimmunoassay of Gonadotropins

Serum concentrations of LH and FSH were measured in duplicate in 50 and 25 μl samples, respectively, using double-antibody RIA methods with the RIA kits supplied by NIH (Bethesda, MD) and following the microassay method described previously (Sánchez-Criado *et al.*, 1993a). Rat LH-I-9 and FSH-I-8 were labeled with ^{125}I by the chloramine T method (Greenwood *et al.*, 1963). LH and FSH concentrations were expressed as ng/ml of serum of the reference preparation LH-rat-RP-3 and FSH-rat-RP-2, respectively. All samples were run in the same assay. The intra-assay coefficients of variation were 7% and 8% for LH and FHS, respectively. The sensitivities of the assays were 7.5 and 50 pg/tube for LH and FSH, respectively.

Radioimmunoassay of Steroids

Serum 17ß-estradiol and progesterone concentrations were measured by using a commercially obtained kits (Diagnosis Products Corporation, Los Angeles, CA). All samples were run in the same assay. The sensitivities of the assays were 1 pg/tube and 10 pg/tube for estradiol 17ß and progesterone, respectively. The intra-assay coefficients of variation were 6.5% and 6% for estradiol 17ß and progesterone, respectively.

Radioimmunoassay of Inhibin

Serum and ovarian extracts inhibin-like immunoactivity were measured in duplicate in 10 μl samples following the method described previously (Robertson *et al.*, 1989; Sánchez-Criado *et al.*, 1992b). A bFF preparation with an arbitrary potency of 1 U/μg protein was used as the standard. The International Research Standard of inhibin (86/690) has a relative specific activity of 60 ± 10 U/μg (mean ± S.E.M., $n = 5$) when expressed in units of this bFF standard. All samples were run in the same assay. The intra-assay coefficient of variation was 24.4%.

Data Evaluation and Statistical Analysis

Results are given as the mean ± SEM. Data were evaluated for statistically significant differences using one-way analysis of variance (ANOVA) followed by Tukey's Q test. A difference was considered to be statistically significant if $P < 0.05$ in relation of LHRHa-treated rats injected with vehicles.

RESULTS

Serum Concentrations of LH and FSH (Fig. 1)

The injection of an ovulatory dose of LH to LHRHa-treated rats significantly increased the serum concentrations of FSH at 1700 h in proestrus. This effect of LH was blocked by the antiprogestagen RU486. The injection of RU486 alone had no effect on the serum concentrations of FSH (Fig. 1B). The administration

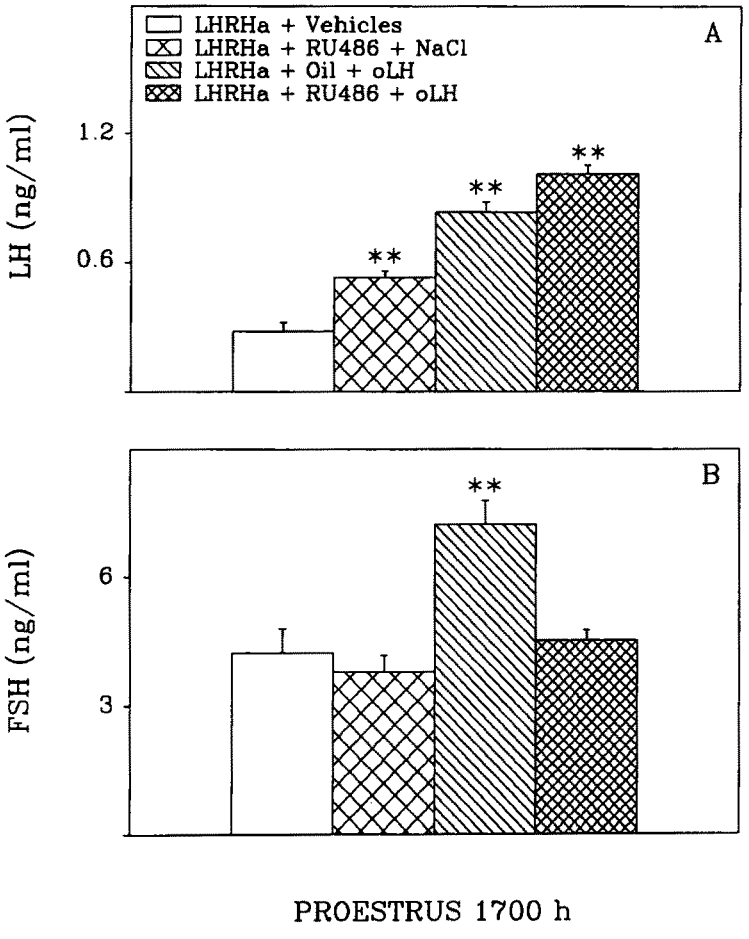

Fig. 1. Serum concentrations of LH (A) and FSH (B) at 1700 h in proestrus in LHRHa-treated rats (1 mg/200 μl) saline s.c. at 0800 h in proestrus) injected in proestrus with: vehicles (200 μl oil s.c. at 0800 h and 250 μl saline i.v. at 1200 h), RU486 (4 mg/200 μl oil s.c. at 0800 h) and/or LH (32 μg/250 μl saline i.v. at 1200 h). Values are mean ± SEM (7–9 rats). (**) $P < 0.01$ vs LHRHa-treated rats injected with vehicles (One way ANOVA and Tukey's test).

of RU486 and/or an ovulatory dose of LH to LHRHa-treated rats induced an increase in serum concentrations of LH at 1700 h in proestrus (Fig. 1A).

Serum Concentrations of Progesterone and 17ß-Estradiol (Fig. 2)

The ovulatory dose of LH increased serum concentrations of progesterone at 1700 h in proestrus in LHRHa-treated rats. The administration of RU486 had no effect on the serum concentrations of progesterone either in LH- or

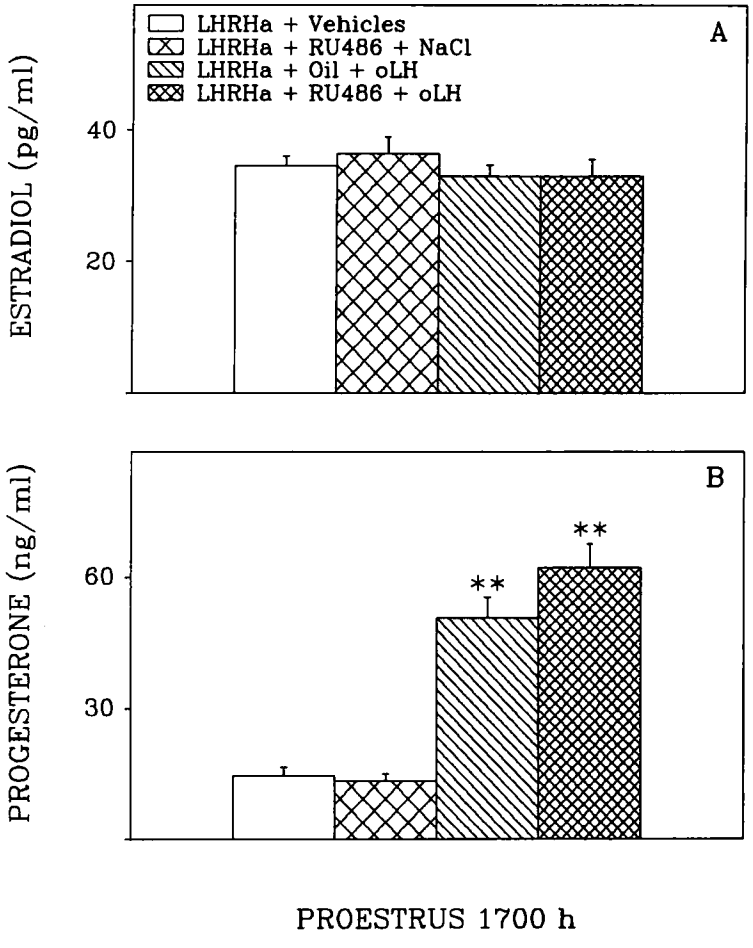

Fig. 2. Serum concentrations of 17ß-estradiol (A) and progesterone (B) at 1700 h in proestrus in LHRHa-treated rats (1 mg/200 μl saline s.c. at 0800 h in proestrus) injected in proestrus with: vehicles (200 μl oil s.c. at 0800 h and 250 μl saline i.v. at 1200 h), RU486 (4 mg/200 μl oil s.c. at 0800 h) and/or LH (32 μg/250 μl saline i.v. at 1200 h). Values are mean ± SEM (7–9 rats). (**) $P < 0.01$ vs LHRHa-treated rats injected with vehicles (One way ANOVA and Tukey's test).

vehicle-injected rats (Fig. 2B). The serum concentrations of 17ß-estradiol at 1700 h in proestrus were not different among groups of LHRHa-treated rats (Fig. 2A).

Ovarian Content and Serum Concentrations of Inhibin (Fig. 3)

The ovulatory dose of LH in LHRHa-treated rats decreased the ovarian content (Fig. 3A) and the serum concentrations of inhibin (Fig. 3B) at 1700 h in proestrus. The administration of RU486 blocked the effect of LH on the serum concentrations of inhibin (Fig. 3B) but not on the ovarian content of inhibin (Fig.

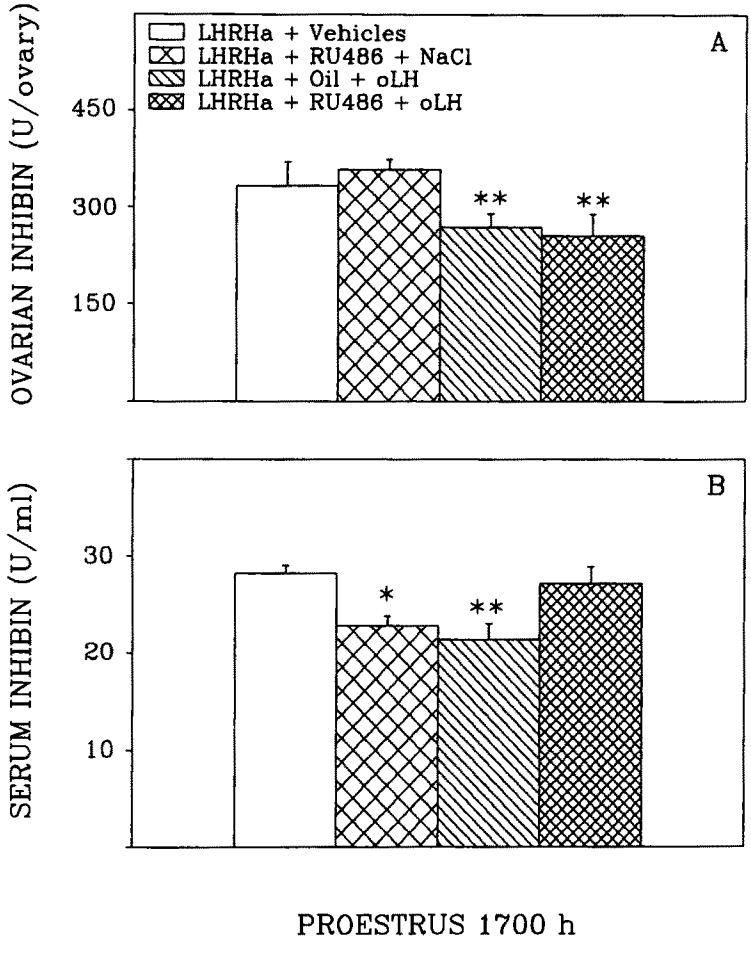

Fig. 3. Ovarian content of inhibin (A) and serum concentrations of inhibin (B) at 1700 h in proestrus in LHRHa-treated rats (1 mg/200 µl saline s.c. at 0800 h in proestrus) injected in proestrus with: vehicles (200 µl oil s.c. at 0800 h and 250 µl saline i.v. at 1200 h), RU486 (4 mg/200 µl oil s.c. at 0800 h) and/or LH (32 µg/250 µl saline i.v. at 1200 h). Values are mean ± SEM (7–9 rats). (*) $P<0.05$ and (**) $P<0.01$ vs LHRHa-treated rats injected with vehicles (One way ANOVA and Tukey's test).

3A). The injection of RU486 alone did not affect the ovarian content of inhibin (Fig. 3A) but induced a decrease in the serum concentrations of inhibin (Fig. 3B).

DISCUSSION

The effects of several maneuvers and treatments in proestrus on the secretion of FSH in estrus in the cyclic rat are shown in Table I. The abolition of the LHRH secretion through the anterior deafferentation of the medium basal hypothalamus (Rush *et al.*, 1980) or the blockade of its actions through the administration of an antiserum anti-LHRH (Blake and Kelch 1981) in the

Table I. Effect of Different Maneuvers on the Day of Proestrus on the Secretion of FSH at Early Estrus in the Cyclic Rat[a]

Reference	Treatment	Secondary surge of FSH
Ashiru & Blake 1978	Pb in the early afternoon of proestrus	Abolished
	Pb in the evening of proestrus	Unaffected
	Pb and LHRH in the afternoon of proestrus	Restored
Ashiru & Blake 1979	Pb and LH or FSH in the afternoon of proestrus	Restored
Rush *et al.* 1980	Anterior deafferentation of MBH in the early afternoon of proestrus	Abolished
	Anterior deafferentation of MBH in the evening of proestrus	Unaffected
Blake & Kelch 1981	LHRH-AS in the early afternoon of proestrus	Abolished
	LHRH-AS in the evening of proestrus	Unaffected
Elias *et al.* 1982	pFF in the evening of proestrus	Abolished
DePaolo 1988	Epostane in the morning of proestrus	Abolished
Mahesh & Brann 1992	Trilostane in the morning of proestrus	Abolished
Sánchez-Criado *et al.* 1992	RU486 from metestrus to proestrus	Abolished
Knox & Schwartx 1992	RU486 in the morning proestrus	Abolished
Knox *et al.* 1993	RU486 and LH in the afternoon of proestrus	Abolished
	RU486 and AIS in the afternoon of proestrus	Abolished
Sánchez-Criado *et al.* 1994	LHRHa in the evening of proestrus	Unaffected
Tébar *et al.* 1995a	LHRHa in the morning of proestrus	Abolished
	LHRHa and LH in the afternoon of proestrus	Restored
	APS in the afternoon of proestrus	Unaffected
	ADX in the morning of proestrus	Decreased
Tébar *et al.* 1995b	LHRHa and P in the afternoon of proestrus	Partially restored
	LHRHa and AIS in the afternoon of proestrus	Partially restored
	LHRHa and P + AIS in the afternoon of proestrus	Restored
	OVX in the morning of proestrus	Unaffected
	OVX and P in the afternoon of proestrus	Unaffected

[a] Pb: Phenobarbital; MBH: medium basal hypothalamus; LHRH-AS: anti-LHRH serum; pFF: procine follicular fluid; RU486: antiprogestagen; AIS: anti-inhibin serum; LHRHa: LHRH antagonist; APS: anti-progesterone serum; ADX: adrenalectomy; P: progesterone; OVX: ovariectomy.

afternoon but not in the evening of proestrus, prevents the rise in FSH levels in the early estrus. Ashiru and Blake (1978) demonstrated that the blockade of the primary surge of LH by the administration of phenobarbital in the early afternoon but not in the evening of proestrus, abolishes the secondary surge of FSH in estrus. When phenobarbital-treated rats are injected with LHRH (Ashiru and Blake 1978) or with LH or FSH (Ashiru and Blake 1979) in the afternoon of proestrus, the secretion of FSH in estrus is restored. The secondary surge of FSH is not affected by the administration of an antagonist of LHRH (LHRHa) in the late proestrus (Sánchez-Criado *et al.*, 1994). The treatment with the LHRHa in the morning of proestrus induces an abolition of the secondary surge of FSH which is restored when LHRHa-treated rats are injected with LH (Tébar *et al.*, 1995a). All these data clearly demonstrate that the secretion of FSH in the first hours of estrus does not depend on the LHRH secretion in this period but it does on the primary surge of gonadotropins. Since the administration of porcine follicular fluid in the evening of proestrus blocks the secondary surge of FSH (Elias *et al.*, 1982), it is concluded that the secretion of FSH in the early estrus depends on the preovulatory LH surge-dependent fall in ovarian inhibin levels (Shander *et al.*, 1980).

The administration of the antiprogesterone RU486 from metestrus to proestrus (Sánchez-Criado *et al.*, 1992b) or in the morning of proestrus (Knox and Schwartz 1992) blunts the primary surge of gonadotropins and abolishes the secondary surge of FSH. When RU486-treated rats are injected with an ovulatory dose of LH or with an anti-inhibin serum (AIS) they do not display the secondary surge of FSH (Knox *et al.*, 1993). These findings indicate that the secondary surge of FSH does not depend on the primary surge of LH itself but on its effects at ovarian level and that RU486 could affect the secretion of FSH in estrus through antagonizing the action of one or more factors which are necessary to evoke it. Since RU486 has also antiglucocorticoid activity, the secretion of corticosterone in the afternoon of proestrus could be responsible for the secretion of the secondary surge of FSH. In fact, the complete removal of corticosterone from the circulation by adrenalectomy (ADX) in the morning of proestrus decreases the levels of FSH in the early estrus (Tébar *et al.*, 1995a). It should be noted that ADX decreases but it does not abolish the secondary surge of FSH (Tébar *et al.*, 1995a). Therefore, another factor blocked by RU486, probably progesterone, also could play a role in the regulation of the secretion of FSH in estrus. This idea is in agreement with the finding that the administration of the progesterone synthesis inhibitors Epostane (DePaolo 1988) or Trilostane (Mahesh and Brann 1992) to cyclic rats in the morning of proestrus induces an abolition of the secretion of FSH in estrus. Besides, we have showed that in LHRHa-treated rats the injection of 4 mg of progesterone or an AIS in the afternoon of proestrus partially restores, while the combined treatment with progesterone and AIS completely restablishes the secondary surge of FSH (Tébar *et al.*, 1995b). These results lead us to consider that the LH-dependent secretion of progesterone in proestrous afternoon (Uchida *et al.*, 1969) could stimulate the secretion of FSH during early estrus either directly at the pituitary level or indirectly through inhibitory effects on inhibin production. The first possibility is doubtful since the

injection of progesterone in the afternoon of proestrus to OVX-rats does not modify the serum concentrations of FSH in estrus (Tébar *et al.*, 1995b). The second possibility seems to be more suitable. This is because a) granulosa cells in ovarian follicles, which are the main source of inhibin in the female rat (Erickson and Hsueh, 1978), have progesterone receptors (Schreiber and Erickson, 1979); b) RU486 readily crosses the blood-follicle barrier (Baulieu, 1989) and binds to granulosa cells progesterone receptors (Schreiber *et al.*, 1983) and c) progesterone added to granulosa cells culture decreases inhibin production (Franchimont *et al.*, 1981). Moreover, inhibin production by granulosa cells in culture decreases when these cells become luteinized (Franchimont 1987) and RU486 prevents luteinization and progesterone production by preovulatory follicles incubated with LH (Natraj and Richards 1993). Accordingly, the explanation for the full effect of the combined treatment of progesterone and AIS on the secretion of FSH at early estrus could be the existence of an additive effect between the ability of AIS to neutralize the biological activity of serum inhibin and the reported inhibitory effect of progesterone on inhibin production. Ovariectomy (OVX) in the morning of proestrus, which completely eliminates progesterone and inhibin from the circulation, does not affect the secondary surge of FSH (Tébar *et al.*, 1995b). This demonstrates that ovarian progesterone is not necessary itself to simulate the secretion of FSH in estrus and supports the hypothesis of an inhibitory effect of progesterone on the secretion of inhibin. The fact that the progesterone-anti-progesterone immune complexes do not cross the blood-follicle barrier and, therefore, they do not antagonize the local effect of progesterone on inhibin secretion could explain the lack of effect of an anti-progesterone serum on the secondary surge of FSH (Tébar *et al.*, 1995a). The discrepancies with previous data (Knox *et al.*, 1993) which report that the administration of 2.5 mg of progesterone in the afternoon of proestrus to LHRHa-injected rats does not increase the secretion of FSH at 0800 h in estrus are attributable to differences in the dose of progesterone and in the strength of the AIS used, as well as to the time of blood collection.

The results presented in this paper show that the administration of ovulatory dose of LH to LHRHa-treated rats increased the secretion of ovarian progesterone (Fig. 2B). In this endocrine milieu, which was characterized by elevated progesterone levels, there was a fall in both the serum levels and the ovarian content of inhibin (Fig. 3) and the secondary surge of FSH was restored (Fig. 1B). Both the decrease in inhibin synthesis and the fall in inhibin secretion depend on the preovulatory LH surge and they are separate phenomena (Hasegawa *et al.*, 1989). The effect of the ovulatory dose of LH on the serum concentrations of progesterone was not modified by the injection of RU486 (Fig. 2B). The administration of RU486 and LH to LHRHa-injected rats decreased the ovarian content and did not change the serum levels of inhibin with respect to LHRHa-treated rats (Fig. 3). All these results together could be interpreted as that ovarian progesterone mediates the inhibitory effect of LH on inhibin secretion but not that of LH on inhibin synthesis. It should be noted that RU486 alone negatively affected the serum levels of inhibin in LHRHa-treated rats (Fig. 3B) while it did not modify the ovarian content of inhibin (Fig. 3A). RU486 has

been reported to act as partial agonist in some tissues and under certain conditions (Jordan 1984) and to have agonistic activity in the endometrium and pituitary in the absence of progesterone (Gravanis *et al.*, 1985). Thus, a possible explanation for this effect could be that RU486 has the same inhibitory effect than progesterone on the secretion of inhibin in the absence of the preovulatory surge of LH and, in consequence, of the progesterone rise. In this group of animals with low inhibin serum levels, the secondary FSH surge was prevented because the corticosterone actions were blocked by the injection of RU486 (Fig. 1B).

In summary, the data presented in this paper demonstrate that the antiprogestagen RU486 blocks the inhibitory action of the preovulatory surge of LH on the secretion but not on the ovarian synthesis of inhibin. There are data reporting that at 0600 h in estrus the serum concentrations of inhibin are basal in rats injected with lower doses (2–6 mg/kg) of RU486 (Knox and Schwartz 1992; Knox *et al.*, 1993). Therefore, the antiprogestagen RU486 would delay, rather than prevent, the LH-dependent drop in the serum concentrations of inhibin in the afternoon of proestrus.

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