Ability of progesterone to reverse anti-androgen (hydroxyflutamide)-induced interference with the preovulatory LH surge and ovulation in PMSG-primed immature rats

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Summary. In Exp. 1, PMSG was injected to 26-day-old prepubertal rats to induce ovulations. On Day 2 (2 days later, the equivalent of the day of pro-oestrus) they received at 08:00 h 5 mg hydroxyflutamide or vehicle and at 12:00 h 2 mg progesterone or testosterone or vehicle. Animals were killed at 18:00 h on Day 2 or at 09:00 h on Day 3. Progesterone but not testosterone restored the preovulatory LH surge and ovulation in hydroxyflutamide-treated rats. In Exp. 2, 2 mg progesterone or testosterone were injected between 10:30 and 11:00 h on Day 2, to advance the pro-oestrous LH surge and ovulation in PMSG-primed prepubertal rats. Injection of hydroxyflutamide abolished the ability of progesterone to advance the LH surge or ovulation. Testosterone did not induce the advancement of LH surge or ovulation. In Exp. 3, ovariectomized prepubertal rats implanted with oestradiol-17 β showed significantly ($P < 0.01$) elevated serum LH concentrations at 18:00 h over those observed at 10:00 h. Progesterone injection to these animals further elevated the serum LH concentrations at 18:00 h, in a dose-dependent manner, with maximal values resulting from 1 mg progesterone. Hydroxyflutamide treatment significantly ($P < 0.003$) reduced the serum LH values in rats receiving 0–1 mg progesterone but 2 mg progesterone were able to overcome this inhibition. It is concluded that progesterone but not testosterone can reverse the effects of hydroxyflutamide on the preovulatory LH surge and ovulation. It appears that hydroxyflutamide may interfere with progesterone action in induction of the LH surge, suggesting a hitherto undescribed anti-progestagenic action of hydroxyflutamide.

Keywords: anti-androgen; progesterone; ovulation; anti-progestagen; LH; rat

Introduction

The anti-androgen, hydroxyflutamide, blocks ovulation in PMSG-injected immature rats through its interference with the preovulatory luteinizing hormone (LH) surge (Opavsky *et al.*, 1987; Chandrasekhar & Armstrong, 1988). Hydroxyflutamide may have a direct action at hypothalamic and/or extrahypothalamic sites involved in the generation of positive feedback signals. From the studies of Neri *et al.* (1972) and Opavsky *et al.* (1987), it appears that hydroxyflutamide is not anti-oestrogenic and it does not inhibit the production of oestradiol. Therefore, it is unlikely that the observed suppression of the LH surge in hydroxyflutamide-treated animals is due to interference with oestradiol secretion or action.

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It is well recognized that progesterone facilitates gonadotrophin secretion (Everett, 1948; Calgaris *et al.*, 1968; Odell & Swerdloff, 1968; McPherson *et al.*, 1975). Progesterone has been reported to increase luteinizing hormone-releasing hormone (LHRH) content in the mediobasal hypothalamus and median eminence preceding the onset of the LH surge in rats (Kim & Ramirez, 1982; Peduto & Mahesh, 1985). The present experiments were therefore conducted to test whether progesterone or testosterone could override the effects of hydroxyflutamide on preovulatory LH secretion in order to shed further light on the mechanism of the anti-ovulatory action of this anti-androgen.

Materials and Methods

Animals

Immature female Sprague-Dawley (Crl: CD¹ (SD)BR) rats were obtained from Charles River Canada Inc. (St Constant, Quebec, Canada) at 22 days of age. Animals were housed in a temperature-controlled (22°C) environment under a photoperiod of 14-h light:10-h dark (lights on 05:00–19:00 h EST) with free access to food and water.

Experimental procedures

Experiment 1. At 08:00 h (Day 0), 26-day-old rats received s.c. injections of 4 i.u. PMSG (Equinex, Ayerst Labs, Montreal, P.Q., Canada) in 0.2 ml saline (0.154 M-NaCl). Hydroxyflutamide (5 mg, Schering: SCH 16423, Schering Pharmaceuticals, Kenilworth, NJ, USA) suspended in 0.2 ml sesame oil or the vehicle alone was injected s.c., 48 h after PMSG injection (Day 2, the equivalent of the day of pro-oestrus). At 12:00 h, on Day 2, 2 mg progesterone or testosterone in 0.2 ml oil or oil alone were injected s.c. to control and hydroxyflutamide-treated rats. One set of rats consisting of the control group and groups injected with steroids and hydroxyflutamide or with hydroxyflutamide alone were killed on the evening of Day 2 at 18:00 h to determine serum LH and progesterone concentrations. In addition, a group of control rats was killed at 10:00 h on Day 2, for serum LH and progesterone determinations. The trunk blood was collected after decapitation and allowed to clot at room temperature for at least 4 h before collection of serum by centrifugation. Serum samples were stored at –20°C for analysis of LH and progesterone. The remaining groups of rats were killed on Day 3 between 08:00 and 10:00 h, and the numbers of corpora lutea present on both ovaries were counted.

Experiment 2. To determine whether hydroxyflutamide affects the ability of progesterone to advance the LH surge and ovulation, 26-day-old prepubertal rats were injected s.c. with 4 i.u. PMSG in 0.2 ml saline. Hydroxyflutamide (5 mg) in oil or oil alone was injected 24 h later (Day 1). Progesterone or testosterone (2 mg) in oil or oil alone was injected between 10:30 and 11:00 h on Day 1 to control and hydroxyflutamide-treated rats. Groups of rats were killed at 16:00 and 18:00 h on Day 1 and trunk blood was collected and serum stored at –20°C until assayed. The remaining animals were killed between 08:00 and 10:00 h on Day 2 and ovaries were dissected out and corpora lutea counted.

Experiment 3. At 23 days of age, prepubertal rats were ovariectomized surgically under light ether anaesthesia. Capsules containing oestradiol-17 β or empty capsules were implanted s.c. under light ether anaesthesia 2 days after ovariectomy. Silastic tubing (Dow Corning, Midland, MI, USA; i.d. 0.76 mm, o.d. 1.65 mm) was filled with crystalline oestradiol-17 β mixed with cholesterol (1:1, w/w; Sigma Chemical Co., St Louis, MO, USA) and the ends were sealed with Silastic medical adhesive type A (Dow Corning), so that the length of each capsule was about 10 mm. The capsules were incubated in 0.9% (w/v) saline for about 2 h at 37°C, before implantation. At 08:00 h, 3 days after insertion of oestradiol implants, animals received 5 mg hydroxyflutamide in 0.2 ml sesame oil or oil alone. Progesterone at doses of 0, 0.5, 1.0 or 2.0 mg in 0.2 ml oil was injected s.c. at 12:00 h and all the animals were killed by decapitation at 18:00 h on the same day. Trunk blood was collected and serum stored at –20°C until assayed.

Hormone assays

Progesterone. Serum progesterone was assayed after petroleum ether extraction by specific RIA as described by Leung & Armstrong (1979). The sensitivity of the assay was 5 pg using a volume of up to 100 μ l. Intraassay coefficient of variation was 8.1%, calculated from high and low pools of progesterone (containing 1000 and 50 pg/tube, respectively). All the samples were assayed in a single assay. The cross-reactivities of hydroxyflutamide and testosterone in the progesterone RIA were <0.001%.

LH. Serum LH concentrations were determined by double-antibody radioimmunoassay procedures using an RIA kit provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (NIADDK); the concentrations were expressed relative to NIADDK-rLH-RP-2. The intra- and inter-assay coefficients of variation were 6.0 and 9.0% when calculated from 6 replicates of a sample run in the same assay and two samples of different

concentrations (low, high) run in duplicate in each assay, respectively. The sensitivity of the assay was 10 pg using a volume of up to 100 µl serum.

Statistical analysis

Significance of difference was assessed by analysis of variance and comparisons between treatment means were by the Student–Newman–Keul's test. Significance between proportions was determined by using χ^2 analyses (Siegel, 1956; Steel & Torrie, 1960).

Results

Ability of progesterone to override the inhibitory effects of hydroxyflutamide on the pro-oestrous LH surge and ovulation in PMSG-primed prepubertal rats

All the animals in the control, progesterone- and testosterone-injected groups in Exp. 1 ovulated, with similar mean ovulation rates (Table 1). Hydroxyflutamide treatment significantly ($P < 0.05$) reduced the number of ovulations as well as the proportion of rats ovulating. Progesterone injection to hydroxyflutamide-treated rats restored ovulation number, but testosterone did not. These differences in ovulation appeared to be consequent to the changes in serum LH concentration on the day of pro-oestrus. Serum LH concentrations at 18:00 h in control, progesterone- or testosterone-treated rats were similar (Table 1) and were significantly ($P < 0.01$) higher than the values in controls at 10:00 h (1.20 ± 0.20 ng/ml). Serum LH values at 18:00 h in hydroxyflutamide-treated rats were not significantly higher than values observed in controls at 10:00 h, suggesting lack of an LH surge in hydroxyflutamide-treated rats. Progesterone treatment of hydroxyflutamide-treated rats restored the LH surge but testosterone was unable to override the inhibitory action of hydroxyflutamide on the LH surge (Table 1).

Table 1. Effect of hydroxyflutamide (HF), progesterone and testosterone on the LH surge and ovulation in PMSG-primed prepubertal rats

Treatment	Day 2, 18:00 h	Day 1, 09:00 h	
	Serum LH (ng/ml)	No. of CL/rat	Proportion of rats ovulating
Control	12.1 ± 1.8^b	8.1 ± 0.4^b	12/12 ^b
HF	2.5 ± 2.3^a	1.3 ± 1.3^a	2/12 ^a
Progesterone	10.9 ± 1.3^b	8.4 ± 0.8^b	7/7 ^b
Testosterone	11.8 ± 2.3^b	10.4 ± 0.8^b	5/5 ^b
HF + progesterone	7.9 ± 1.6^b	8.8 ± 0.3^b	5/7 ^b
HF + testosterone	1.8 ± 1.0^a	1.6 ± 1.0^a	1/5 ^a

Values are mean $\pm$ s.e. of 5 animals for LH measurements and 5–12 animals for the ovulation study.

Means within each column with different superscripts differ significantly ($P < 0.05$).

Serum progesterone concentrations were similar in control (2.8 ± 1.1 ng/ml) and hydroxyflutamide-treated (5.1 ± 2.4 ng/ml) animals at 10:00 h on Day 2. Control animals had significantly ($P < 0.01$) elevated progesterone values (12.32 ± 2.90 ng/ml) at 18:00 h on Day 2 whereas in hydroxyflutamide-treated animals progesterone concentrations (4.1 ± 0.4 ng/ml) were not elevated and were significantly ($P < 0.01$) lower than those of controls.

Effect of hydroxyflutamide on the ability of progesterone to advance the LH surge and ovulation in PMSG-primed prepubertal rats

An injection of progesterone but not testosterone at 11:00 h on Day 1 (27 h after PMSG injection) advanced the preovulatory LH surge and ovulation by 24 h in Exp. 2 (Table 2). Serum LH concentrations were not elevated at 16:00 and 18:00 h in control and hydroxyflutamide-treated rats or in hydroxyflutamide-treated rats receiving testosterone, but were elevated in progesterone-treated rats over the pre-surge levels at 10:00 h. However, progesterone was unable to advance the LH surge in hydroxyflutamide-treated rats. These differences in advancement of the LH surge were reflected in the ovulations on the following day (Table 2): 0 of the control and hydroxyflutamide-treated animals and only 1 out of 5 rats receiving testosterone with or without hydroxyflutamide ovulated. Ovulation rates similar to those expected on Day 3 (Table 1, controls) had occurred in progesterone-treated rats on Day 2. The advanced ovulation in the progesterone-treated animals was blocked by hydroxyflutamide injection.

Table 2. Effect of hydroxyflutamide (HF) on the ability of progesterone, and testosterone to advance the LH surge (Day 1) and ovulation (Day 2) in PMSG-primed prepubertal rats

	Serum LH (ng/ml) on Day 1*		Day 2	
	16:00 h	18:00 h	No. of CL/rat	Proportion of rats ovulating
Control	0.17 ± 0.03	0.16 ± 0.02	0	0/4 ^a
HF	0.19 ± 0.01	0.16 ± 0.02	0	0/4 ^a
Progesterone	2.53 ± 1.10	3.30 ± 0.31	8.2 ± 2.2 ^a	8/10 ^b
Testosterone	0.12 ± 0.05	0.14 ± 0.03	2.2 ± 2.2 ^b	1/5 ^a
HF + progesterone	0.21 ± 0.04	0.18 ± 0.02	2.2 ± 2.2 ^b	2/10 ^a
HF + testosterone	0.14 ± 0.04	0.14 ± 0.03	2.6 ± 2.6 ^b	1/5 ^a

Values are mean ± s.e. of 5 animals for LH measurements and 4–10 animals for the ovulation study.

Means within each column without a common superscript differ significantly (*P* < 0.05).

*The pre-surge LH concentrations measured at 10:00 h on Day 1 in control rats were 0.21 ± 0.05 ng/ml.

Effect of hydroxyflutamide and progesterone on LH release in ovariectomized–oestradiol-17β-implanted rats

In ovariectomized rats implanted with oestradiol-17β (Exp. 3) serum LH concentrations at 18:00 h were elevated over those observed at 10:00 h. Injection of 0.5, 1.0 and 2.0 mg progesterone at 12:00 h further increased serum LH values at 18:00 h in a dose-dependent manner, with maximal values resulting from 1 mg progesterone (Fig. 1). Hydroxyflutamide treatment significantly (*P* < 0.003) reduced the serum LH concentration at 18:00 h in rats receiving 0–1 mg progesterone, but 2 mg progesterone was able to overcome this inhibition and resulted in serum LH concentrations similar to those in rats receiving 1 or 2 mg progesterone but no hydroxyflutamide.

Discussion

This study demonstrates the ability of progesterone but not testosterone to override the inhibitory effects of the anti-androgen, hydroxyflutamide on the preovulatory LH surge and ovulation in

Fig. 1. Effect of hydroxyflutamide (HF) and progesterone on serum LH concentrations at 18:00 h in ovariectomized rats receiving oestradiol-17 β implants. Each bar represents the mean $\pm$ s.e. of 11 animals per group. * $P < 0.003$ compared with group given same dose of progesterone but no hydroxyflutamide.

PMSG-primed prepubertal rats. Administration of progesterone, but not testosterone, to such rats can advance the LH surge and ovulations by 24 h and hydroxyflutamide abolishes this effect. Inhibition of ovulation due to suppression, by hydroxyflutamide, of the LH surge was observed by Opavsky *et al.* (1987), and LHRH was able to reverse this blockade of both the LH surge and ovulation (Chandrasekhar & Armstrong, 1988). From those studies it appeared that blockade of the LH surge might be due to direct action of hydroxyflutamide at the hypothalamus and/or on the action of feedback factors involved in the release of LHRH. Since hydroxyflutamide is an anti-androgen, the inference that could be drawn from those studies was that a positive feedback system for androgens exists in the induction of the preovulatory LH surge. However, the inability of exogenous testosterone either to override the effects of hydroxyflutamide on the pro-oestrous LH surge or to advance the LH surge when administered on the day before pro-oestrus, observed in the present studies raises doubts about this interpretation.

Several lines of evidence indicate that oestradiol is the primary stimulus for the preovulatory LH surge. Administration of antibodies to oestradiol or oestradiol antagonists abolishes the LH surge in rats (Ferin *et al.*, 1969; Labhsetwar, 1970). However, hydroxyflutamide is not anti-oestrogenic (Neri *et al.*, 1972; Opavsky *et al.*, 1987) and it does not inhibit the production of oestradiol by the ovaries (Opavsky *et al.*, 1987). Therefore, it is unlikely that the observed suppression of the LH surge in hydroxyflutamide-treated animals was due to interference with the oestradiol-mediated positive feedback system.

While the significance of the role of oestradiol in triggering the preovulatory LH surge is well accepted, a possible physiological role of progesterone in the ovulatory process has received less attention, or been denied (Knobil, 1980), despite evidence supporting such a possibility (Armstrong & Kennedy, 1972). In the rat, a preovulatory rise in progesterone was observed before the rise in LH (Kalra & Kalra, 1974, 1977) while others observed no such a rise (Butcher *et al.*, 1974; Smith *et al.*, 1975). An increase in LHRH release and an increased sensitivity of the pituitary to LHRH has been observed on the day of pro-oestrus in normally cyclic rats and in ovariectomized rats treated with oestrogen and progesterone (Greeley *et al.*, 1975; Kalra & Kalra, 1977; Barr & Barraclough, 1978; Rance *et al.*, 1981). Dose-dependent effects of progesterone have been described after chronic (McPherson *et al.*, 1975) or single dose (McPherson & Mahesh, 1979) administration. Progesterone advances (by 24 h) the LH surge and ovulation in the rat, presumably by an effect mediated by

a neurohormonal mechanism (Everett, 1948; McCormack & Meyer, 1962). In immature ovariectomized rats primed with low doses of oestradiol, progesterone administration causes an increase in mediobasal hypothalamic LHRH concentrations and plasma LHRH concentrations that are associated with the progesterone-induced LH release (Kim & Ramirez, 1982; Peduto & Mahesh, 1985). That progesterone was necessary for the full expression of the gonadotrophin surge induced by oestrogens was shown by Ayer & Fink (1974).

In addition to the studies in the rat showing the effect of progesterone on LH release (McPherson *et al.*, 1975; McPherson & Mahesh, 1979), small amounts of progesterone have been shown to bring about a rapid release in LH in women (Odell & Swerdloff, 1968; Chang & Jaffe, 1978; March *et al.*, 1979). In rats and in women, progesterone augments the response of LHRH in the release of LH (Lasley *et al.*, 1975; Shaw *et al.*, 1975; McPherson & Mahesh, 1979; Kalra & Kalra, 1983). Analysis of rat (Kalra & Kalra, 1974, 1983), monkey (Helmond *et al.*, 1980) and human (Liu & Yen, 1983) ovulatory cycles suggests that progesterone may be necessary for the preovulatory LH surge to be of normal magnitude.

The progesterone antagonist, RU 486, when administered on the day of pro-oestrus as a single injection (200 µg/rat) to PMSG-primed immature rats, reduced the preovulatory LH surge and the number of ova shed per rat (Rao & Mahesh, 1986). Interference, by hydroxyflutamide, with progesterone action at the hypothalamus could possibly result in the impairment of LHRH secretion and therefore the LH surge and ovulation. It is also possible that hydroxyflutamide might have interfered with the production of progesterone, since progesterone concentrations measured at 18:00 h on the day of pro-oestrus were lower in hydroxyflutamide-treated rats than in controls.

In Exp. 1, LH concentrations in hydroxyflutamide-treated rats were 2.5 ± 2.3 ng/ml and most of the rats did not ovulate, while in Exp. 2, LH concentrations in the progesterone-treated group were 3.30 ± 0.37 (18:00) and most of the rats ovulated (Tables 1 & 2). Of the 5 hydroxyflutamide-treated rats in Exp. 1, only 1 had an LH surge whereas in Exp. 2 all the rats had an LH surge. Therefore, despite similar mean LH concentrations in Exp. 1 and 2, differences in the number of animals which had an LH surge may reflect differences in the number of animals ovulated. Serum LH concentrations of 10.9–12.1 ng/ml in Exp. 1 and 3.3 mg/ml in Exp. 2, at 18:00 h, gave comparable numbers of ovulations, suggesting an LH concentration threshold required for optimal ovulations; over and above that concentration, elevations in serum LH do not appear to enhance the number of ovulations.

The anti-androgenic activity of hydroxyflutamide and flutamide has been well established (Neri *et al.*, 1972; Katchen & Buxbaum, 1975; Neri, 1976), and flutamide has been claimed to be without detectable progestational or anti-progestational activity (Neri *et al.*, 1972). The latter conclusions were based on the inability of flutamide to elicit endometrial proliferation (no progestational action) or to prevent endometrial proliferation by progesterone (no anti-progestational action) in immature rabbits primed with oestradiol-17 β (Neri *et al.*, 1972). It is possible that species differences and differences in the relative potencies of flutamide and hydroxyflutamide may contribute, at least in part, to the differences between our observations and those of Neri *et al.* (1972). It has been demonstrated that hydroxyflutamide is approximately 1.5 times more potent than its parental compound, flutamide (Neri *et al.*, 1979). To our knowledge, the present findings represent the first report of anti-progestational activity of hydroxyflutamide, which hitherto has been claimed to lack other agonistic or antagonistic activities.

In summary, progesterone, but not testosterone, can override the inhibitory effects of the anti-androgen, hydroxyflutamide, on the preovulatory LH surge and ovulation in rats. It therefore appears that the inhibitory action of hydroxyflutamide may be due to interference with progesterone action involved in induction of the LH surge, suggesting a hitherto undescribed anti-progestagenic action of hydroxyflutamide.

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