Effect of treatment with the selective oestrogen receptor modulator LY117018-HCl on pituitary sensitivity to GnRH and subsequent ovulation

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The effects of LY117018-HCl (LY; a benzothiophene similar to raloxifene) were examined on various reproductive parameters in female rats. Four-day cyclic rats were treated (10:00 h on dioestrus) with LY (0.01, 0.1, 0.5, 1, 2, 4 or 16 mg kg⁻¹ p.o.) and assessed for ovulation at oestrus. LY inhibited ovulation at doses as low as 0.5 mg kg⁻¹, and ovulation did not occur at doses of 4 and 16 mg kg⁻¹. LY (16 mg kg⁻¹) reduced wet uterine mass and LH concentrations at the time of the expected ovulatory surge. Ovulation induced by hCG in pentobarbital-treated rats was not altered by LY treatment, indicating normal ovarian sensitivity to gonadotrophins. LY, however, completely blocked the effects of oestradiol (under either negative or positive feedback modes) on LH secretion

in ovariectomized rats. GnRH secretion into hypophyseal portal blood during pro-oestrus was not affected by treatment with LY, whereas the concentrations of serum LH remained reduced. Finally, treatment with LY markedly reduced pituitary sensitivity to GnRH during pro-oestrus, as it completely blocked GnRH-induced LH secretion. These results demonstrate that LY inhibits oestradiol action in the uterus and prevents ovulation in normal cyclic rats. LY-induced inhibition of ovulation is not caused by an alteration of the ovarian response to gonadotrophins or an impairment of GnRH secretion at the hypothalamus, but by a reduction in the sensitivity of gonadotrophs to the stimulatory effects of GnRH during pro-oestrus.

Introduction

Oestrogen plays an important role in the control of the reproductive axis by stimulating sex accessory organs and establishing a feedback loop between the gonads, the pituitary gland and the brain (Freeman, 1994). Oestrogen affects many systems, such as the sex accessory organs, skeleton, cardiovascular and central nervous systems (CNS). Because of these actions, oestrogen has been used as an important therapeutic treatment in postmenopausal women (Ettinger, 1998). However, long-term oestrogen treatment may increase the risk of breast (Zumoff, 1998) and uterine cancer (Beresford *et al.*, 1997). This has led to the development of pharmacological agents that can maintain the benefits of hormone replacement therapy in chronic oestrogen deficiency, as well as avoid the risks of long-term oestrogen treatment (Cosman and Lindsay, 1999).

Efforts to develop new drugs acting as oestrogen receptor (ER) antagonists have produced two major groups of compounds. The seven alpha-substituted derivatives of oestradiol, represented by the compounds ICI 164 384 and ICI 182 780, block oestrogen action and behave like anti-oestrogens in all tissues. Concurrently, these compounds enhance ER turnover rate, which contributes to the extinction of oestrogen signalling (Howell *et al.*, 2000). These compounds are being studied for the treatment of advanced breast cancer. A second group of compounds, known as selective oestrogen receptor modulators (SERMs), act as oestrogen agonists or antagonists depending on the tissue in which they exhibit their actions. SERM molecules are exemplified by tamoxifen, which is used for the treatment of breast cancer, and raloxifene, which is currently approved for the prevention and treatment of osteoporosis (MacGregor and Jordan, 1998).

Although much of the interest in SERMs has focused on their use in osteoporosis (López, 2000) as well as their

anti-neoplastic (Kedar *et al.*, 1994) and anti-metabolic properties (Nuttall *et al.*, 1998), these compounds are also proving to be valuable in neuroendocrine studies. Raloxifene has been shown in animals and humans to be an effective oestrogen receptor antagonist in uterine tissue, without affecting either ovulation or the duration of the ovarian cycle (Draper *et al.*, 1995; Sato *et al.*, 1996; Baker *et al.*, 1998). In contrast, studies using dispersed anterior pituitary cells in culture have shown that raloxifene (originally termed keoxifene) decreases GnRH-stimulated LH secretion (Simard and Labrie, 1987) without affecting prolactin secretion (González *et al.*, 2000). Therefore, these studies indicate that raloxifene blocks oestrogen action at the anterior pituitary gland. However, SERMs such as tamoxifen exhibit greater oestrogen-like activity: tamoxifen increases both GnRH-stimulated LH and prolactin secretion in culture (González *et al.*, 2000). The present study investigated the effects of LY117018-HCl (LY), a pyrrolidin analogue of raloxifene, on the reproductive system of female rats to understand better the relationship between specific sites of oestrogen action and the regulation of female reproductive function.

Materials and Methods

Animals

Adult cyclic female Sprague–Dawley rats obtained from the central animal facility of La Laguna University were used for experimentation. Animals (about 200 g body weight) had free access to food and tap water, were housed three per cage in a standard environment (14 h light: 10 h dark, lights on from 03:00 to 17:00 h) and constant temperature (20–23°C). All experimental procedures were performed in accordance with the European Community guidelines for the care and use of laboratory animals. Vaginal smears were examined daily, and only those animals showing at least two consecutive 4 day oestrous cycles were selected. Bilateral ovariectomy was performed on the morning of dioestrus (10:00 h) under Saffan® (6 mg kg⁻¹, i.p.) anaesthesia, as this agent does not affect ovulation (Sánchez-Criado *et al.*, 1994) or GnRH–LH secretion (Fink and Sheward, 1989) in rats.

Drugs and reagents

Oestradiol was purchased from Sigma-Aldrich Co. (Madrid), and normal sheep serum and rabbit anti-sheep IgG serum were procured from Antibodies Inc. (Davis, CA). Saffan® was obtained from Pitman-Moore (Uxbridge). Sodium pentobarbital (Euta-Lender®), hCG (Profasi HP®) and heparin (Heparina Rovi® 5%) for clinical use were used. Synthetic GnRH was purchased from Peninsula Laboratories (St Helens). Commercial LH and FSH radioimmunoassay kits, and I¹²⁵-labelled

GnRH were obtained from Amersham Life Sciences (Little Chalfont), whereas the DPC Coat-a-Count oestradiol radioimmunoassay kit was purchased from Dipsa (Madrid). LY was generously provided by Eli Lilly and Company (Indianapolis, IN).

Experimental paradigms

Experiment 1. LY was tested for its anovulatory properties in cyclic adult rats. Animals were injected s.c. on the morning of dioestrus (10:00 h) with either 0.2 ml olive oil (vehicle) or LY (0.01, 0.1, 0.5, 1, 2, 4 or 16 mg kg⁻¹) suspended in vehicle. Animals were killed by decapitation without anaesthesia on the morning of oestrus (09:00–11:00 h), and the number of ova was counted by examination of the ampullary region of the uterine horn under a stereoscopic microscope.

Experiment 2. The effects of LY on uterine wet mass, ovulation and gonadotrophin concentrations during the preovulatory surge in cyclic female rats were evaluated. Animals were injected on the morning of dioestrus (10:00 h) with vehicle or LY (16 mg kg⁻¹), as described in Expt 1. On the afternoon of pro-oestrus (15:30–17:00 h), the animals were anaesthetized with Saffan® (6 mg kg⁻¹, i.p.) and blood samples were obtained by jugular venepuncture in heparinized syringes. Samples were centrifuged at 1100 g for 15 min and plasma was collected and stored at –20°C for analysis of LH and FSH concentrations by radioimmunoassay. Animals were killed on the morning of oestrus (9:00–11:00 h) by decapitation without anaesthesia. The uterus was collected, blotted dry and weighed, and the number of ova was counted as described in Expt 1.

Experiment 3. Pro-oestrous rats were anaesthetized with sodium pentobarbital (50 mg kg⁻¹, i.p.) at 10:00 h to block the endogenous GnRH surge, the surge of gonadotrophins and consequently ovulation, to determine whether LY would alter ovarian responsiveness to gonadotrophins. The rats were then subdivided into two groups and injected s.c. with hCG (10 i.u. per rat in 0.2 ml saline) or saline (vehicle). One group of pentobarbital-anaesthetized pro-oestrous rats was used as a control, and another group was treated with LY (16 mg kg⁻¹) at dioestrus. On the morning of oestrus the animals were killed by decapitation and the number of ova was counted as described in Expt 1.

Experiment 4. The effects of LY on the negative and positive feedback actions of oestradiol on the GnRH–LH system were studied. Cyclic female rats were bilaterally ovariectomized at 10:00 h on the morning of dioestrus. Animals were injected s.c. with oil, oestradiol (2 or 10 µg per rat), or oestradiol (2 or 10 µg per rat) plus LY (16 mg kg⁻¹). This paradigm is similar to that described by Fink (1979) to recreate the negative and positive feedback effects of oestrogen on GnRH–LH secretion. Rats were killed by decapitation without anaesthesia 1 day after

treatment, at the time of the expected LH surge (15:30–17:00 h). Uteri were collected, blotted dry, and weighed. Blood samples were collected into heparinized tubes and plasma was separated by centrifugation (1100 *g* for 15 min), collected and stored at -20°C for determination of gonadotrophin concentrations by radioimmunoassay. A different group of animals was used to monitor oestradiol concentrations in castrated animals treated with 2 or 10 μg steroid per rat.

Experiment 5. The potential effects of LY on the GnRH neuronal system were evaluated by measuring GnRH concentrations in hypophyseal portal blood plasma of cyclic rats. Animals were injected s.c. on the morning of dioestrus (10:00 h) with either vehicle or LY (16 mg kg^{-1}). Blood samples from the hypothalamic–pituitary portal system were collected at the time of the preovulatory LH surge. Briefly, animals were anaesthetized under Saffan[®] (9 mg 3 α -hydroxy-5 α -pregnane-11,20 dione and 3 mg 21 acetoxy-3 α -hydroxy 5 α -pregnane-11,20 dione ml^{-1}) injected at 6 mg kg^{-1} , i.p. When portal vessels were exposed and sectioned as described by Worthington (1966), portal blood samples were collected from heparinized rats for 45 min between 15:30 and 17:00 h. A 0.5 ml sample of jugular blood was collected immediately before severing the portal vessels and immediately after portal blood collection was finished. Plasma concentrations of LH were measured in these samples to confirm the occurrence of the LH surge. Total output of GnRH into the portal blood was expressed as a secretion rate: (concentration $\times$ volume of collection)/time of collection. This allows for correction of potential dilution of the portal blood sample by cerebrospinal fluid or systemic blood (Fink and Sheward, 1989).

Experiment 6. This experiment was designed to evaluate pituitary sensitivity to GnRH after treatment with LY. Animals were injected with oil or LY (16 mg kg^{-1}) at 10:00 h on the morning of dioestrus. On the morning of pro-oestrus, rats were anaesthetized with sodium pentobarbital (11:00 h; 50 mg kg^{-1} , i.p.) to block the endogenous surge of gonadotrophins. On the afternoon of pro-oestrus (16:00 h), rats were injected in the jugular vein with a GnRH bolus (50 ng (100 g^{-1} body weight, in 0.5 ml of saline) as described by Sánchez-Criado *et al.* (1994). Samples of jugular vein blood (400 μl each) were collected from heparinized rats (1000 i.u. per rat injected i.v. 10 min before GnRH administration), 10 min before, as well as 0, 15, 30 and 60 min after the GnRH bolus was administered. Plasma concentrations of LH were measured by radioimmunoassay.

Radioimmunoassays

Plasma concentrations of LH, FSH and oestradiol were measured in 100 μl duplicate plasma samples using commercially available radioimmunoassay kits, follow-

ing the manufacturer's protocol. The assay sensitivities were 1.00 ng per tube, 1.75 ng per tube, and 8 pg ml^{-1} for LH, FSH and oestradiol, respectively. All samples from the same experiment were measured in the same radioimmunoassay to avoid interassay variability. The intra-assay variability was less than 10% for LH, FSH and oestradiol radioimmunoassays.

Samples were assayed for GnRH using a specific sheep anti-GnRH serum (FJL no. 12-3a) as described by Moretto *et al.*, (1993) with some modifications. In brief, samples and standards (200 μl) were incubated with primary anti-serum (100 μl FJL no. 12-3a at 1:75 000 initial dilution) for 48 h at room temperature ($22 \pm 1^{\circ}\text{C}$). Thereafter, 100 μl iodinated GnRH (10 000 c.p.m. per 0.1 ml) was added and incubation was continued for 24 h at room temperature. Bound and free radioactivity was separated by adding 50 μl 1:5 rabbit anti-sheep serum (Antibodies Inc.) and 50 μl 0.5% normal sheep serum, and incubating the tubes overnight at 4°C . Thereafter, 1 ml cold PBS was added and the tubes were centrifuged at 2500 r.p.m. for 30 min. Supernatants were decanted and pellets were counted in a multi-channel gamma counter. Under these conditions, the minimum detectable concentration was 3.20 pg per tube with an IC_{50} value of 17.44 pg, and the intra-assay variability was less than 10%. All samples from one experiment were measured in the same radioimmunoassay to avoid interassay variability.

Displacement curves using pooled hypophyseal portal blood plasma were fitted to the four parameter logistic equation using the method of Ghosh *et al.*, (1998) to assess parallelism of GnRH immunoreactivity detected in portal plasma samples to the synthetic GnRH standard. Curves were considered to present parallel displacement to the synthetic GnRH standard when the sample slope was similar to that of synthetic GnRH. Confidence limits (95%) of the estimated slope were used to ascertain whether there was statistical evidence to exclude parallelism. The 95% confidence limits for the portal blood sample slope included the slope of the synthetic standard; therefore, samples evoked displacement of the tracer that paralleled that of the synthetic standard.

Statistical analysis

All results except LH concentrations are expressed as means $\pm$ SEM. LH concentrations are expressed as geometric means $\pm$ SEM. Data were evaluated for statistically significant differences using Student's *t* test, one-way ANOVA, or two-way ANOVA, followed by Fisher's test when appropriate. Raw or Box-Cox-transformed data (Box and Cox, 1964; Box, 1974; specific transformations used for specific data sets are provided in the figure legends) were used depending on normality and variance homogeneity criteria. ED_{50} values were estimated by fitting dose–response curves to the four parameter logistic equation (Ghosh *et al.*, 1998). Fisher's exact probability

Table 1. Effects of LY117018-HCl (LY) administration on ovulation in cyclic rats

LY dose (mg kg ⁻¹)	Number of ovulating rats : total number (%)	Ova emitted per rat Mean $\pm$ SEM (n)	P value (Fisher's exact probability test)
0	8 : 8 (100)	11.0 $\pm$ 1.01 (8)	NA
0.01	5 : 5 (100)	10.4 $\pm$ 0.92 (5)	NS
0.1	5 : 5 (100)	12.0 $\pm$ 0.57 (5)	NS
0.5	2 : 5 (40)	9.5 $\pm$ 0.35 (2)	0.0350
1	1 : 4 (25)	15 (1)	0.0070
2	1 : 4 (25)	9 (1)	0.0070
4	0 : 5 (0)	NA	0.0008
16	0 : 4 (0)	NA	0.0020

Different doses of LY were administered s.c. at 10:00 h at dioestrus. The animals were killed between 10:00 and 12:00 h at oestrus, and the number of ova emitted was monitored. Different *P* values from two-tail Fisher's exact probability test versus the control group (no LY dose) are given for each dose. NA, not applicable; NS, not significant.

Fig. 1. Effects of LY117018-HCl (LY) administration on ovulation in cyclic rats. Different doses of LY were administered s.c. at 10:00 h at dioestrus. The animals were killed between 10:00 and 12:00 h at oestrus, and ovulation was assessed by inspection of the ampullary region. *Significant differences ($P < 0.05$; two-tailed Fisher's exact probability test) versus the control group (bar; no LY dose). Numbers between parentheses indicate the number of rats ovulating out of the total number of rats per group.

test was used to analyse ovulation incidence data. *P* values less than 0.05 only are shown.

Results

Experiment 1

LY treatment in cyclic rats prevented ovulation in a dose-dependent fashion (Fig. 1). Statistically significant effects were observed at 0.5 mg LY kg⁻¹ which inhibited ovulation in 40% of the animals (Fig. 1). Prevention of

ovulation was obtained in 100% of the rats at doses of 4 and 16 mg kg⁻¹ (Fig. 1). The estimated ED₅₀ value was about 0.4 $\pm$ 0.1 mg kg⁻¹ with 95% confidence limits of 0.3–0.7 mg kg⁻¹. The results from this experiment are summarized (Table 1). The maximal dose (16 mg kg⁻¹) was chosen for the experiments described below.

Experiment 2

Control rats at pro-oestrus exhibited nucleated epithelial cells in vaginal smears, whereas LY-treated rats showed vaginal smears predominantly infiltrated by leukocytes. Treatment with LY reduced relative uterine mass by about 50% (Fig. 2a). Furthermore, plasma LH concentrations at the time of the ovulatory surge (15:30–17:00 h) were reduced by about 60% (Fig. 2b), whereas FSH concentrations were unaffected (Fig. 2c). The reduced concentrations of LH at the time of the expected preovulatory surge may be an indication of a reduced ovulatory surge of LH. In this regard, evaluation of the number of rats ovulating indicated that treatment with LY completely prevented ovulation (Tables 1 and 2).

Experiment 3

Ovarian responsiveness to gonadotrophins was assessed in LY-treated cyclic female rats after administration of sodium pentobarbital to block the endogenous surge of gonadotrophins. Sodium pentobarbital treatment of cyclic rats on the morning of pro-oestrus resulted in anovulation in 100% of the animals (Table 2; 9 : 9 versus 0 : 7 for control and sodium pentobarbital-treated rats, respectively; $P < 0.001$). Administration of hCG resulted in 100% ovulatory response in pentobarbital-treated rats (Table 2; 7 : 7). Treatment of cyclic rats with LY resulted in anovulation (Tables 1 and 2). In animals treated with pentobarbital during the morning of pro-oestrus, inhibition of ovulation was reverted by challenging the

Fig. 2. LY117018-HCl (LY) administration during dioestrus in 4 day cyclic rats. (a) Uterine mass and (b) plasma LH concentrations were reduced by LY at pro-oestrus without affecting (c) plasma FSH concentrations. Each group consisted of 8–10 animals. Student's *t* test was conducted on transformed data before analysis and to conform with normality and variance homogeneity criteria. Uterine mass, LH and FSH data were elevated to the power –1.4, –0.4 and –1.6, respectively. **P* < 0.01 versus the control group.

ovary with hCG even after treatment with LY (Table 2). This effect was also apparent in cyclic animals treated with LY after challenge with hCG (data not shown). As shown (Table 2), the number of ova emitted was similar after hCG treatment in rats treated either with

Table 2. Effect of LY117018-HCl (LY) administration on hCG-induced ovulation in sodium pentobarbital-treated cyclic rats

Treatments	Ovulating rats : total rats (%)	Ova emitted per rat Mean $\pm$ SEM (n)
Control	9 : 9 (100)	10.0 $\pm$ 1.4 (9)
SPB	0 : 7 (0)*	NA
SPB + hCG	7 : 7 (100)	9.8 $\pm$ 1.9 (7)
LY	0 : 9 (0)*	NA
SPB + LY + hCG	7 : 7 (100)	9.2 $\pm$ 2.3 (7)

Control: vehicle-treated cyclic rats; LY: 16 mg kg⁻¹ injected s.c. at 10:00 h at dioestrus; SPB: sodium pentobarbital (50 mg kg⁻¹) injected i.p. at 12:00 h at pro-oestrus; hCG: 10 i.u. per rat injected s.c. at 15:00 h at pro-oestrus. Rats were killed between 10:00 and 12:00 h at oestrus, and the number of ova emitted was monitored. **P* < 0.001 versus all unmarked groups by two-tailed Fisher's exact probability test. NA: not applicable.

LY or vehicle. In addition, the number of ova shed during the hCG challenge was indistinguishable from that observed in normal cyclic female rats (Tables 1 and 2). These observations indicate that LY-induced anovulation does not occur by reduction of ovarian sensitivity to gonadotrophins, but rather by a decrease in the concentrations of LH.

Experiment 4

The negative and positive feedback of oestrogen on gonadotrophin secretion was affected in a different way by treatment with LY. Oestradiol concentrations were slightly increased over those of dioestrous animals after administration of 2 μ g oestradiol per rat (51.96 $\pm$ 15.61 pg ml⁻¹ and 70.55 $\pm$ 12.40 pg ml⁻¹, for dioestrous and oestradiol-treated rats, respectively). As expected, administration of 10 μ g oestradiol to castrated animals resulted in higher circulating concentrations of the hormone (115.91 $\pm$ 12.99 pg ml⁻¹).

Oestradiol treatment in a negative feedback mode (2 μ g) resulted in lower plasma LH and FSH concentrations when compared with oil-treated rats and dioestrous animals (Fig. 3b,c; left and middle panels). Treatment with LY abolished the oestradiol-induced decline in both gonadotrophins (Fig. 3b,c; middle panel), indicating that the compound blocks the negative feedback loop induced by low-dose oestradiol treatment. In contrast, oestradiol treatment in a positive feedback mode (10 μ g, high dose) increased plasma concentrations of gonadotrophins (Fig. 3b,c; right panel) resembling the stimulating effects of the endogenous oestrogen surge to trigger the ovulatory surge of gonadotrophins. The stimulatory effect of high-dose oestrogen on LH secretion was completely blocked by treatment with LY (Fig. 3b; right panel); however, the compound was not effective in altering the stimulatory positive feedback effects of

Fig. 3. Effects of LY117018-HCl (LY) on oestrogen action in ovariectomized rats. (a) The effects of oestrogen administration on uterine mass were blocked by LY. LY also blocked the negative and positive feedback actions of oestradiol on (b) LH and (c) FSH secretion. Oil denotes rats treated with vehicle. E₂ indicates oestradiol-treated groups in the negative (2 µg per rat) or positive (10 µg per rat) feedback modes. E₂ + LY represents the group of rats treated with oestradiol and LY. Each group consisted of 8–10 rats. Data represent the mean ± SEM except for LH concentrations for which the geometric mean ± SEM was used. Statistical analysis (one-way ANOVA followed by the Fisher's LSD test) was conducted on raw data or data elevated to the power -0.2 for uterine masses in negative and positive feedback modes. LH data were increased to -0.4 and -0.2 for negative and positive feedback modes, respectively. FSH data were elevated to the power -1. **P* < 0.05 versus oil, ***P* < 0.05 versus dioestrus.

Fig. 4. Administration of LY117018-HCl (LY) in normal 4 day cyclic female rats on the afternoon of pro-oestrus. (a) GnRH secretion rate from the hypophyseal portal system was not altered by LY, whereas (b) the peripheral plasma concentration of LH was reduced by LY. Peripheral blood samples were obtained by jugular venepuncture (1) before and (2) after the portal blood sample collection. Data correspond to five or six rats per group. Student's *t* test was performed on raw data (GnRH secretion rate) or data were elevated to the power -0.6 (LH concentrations). Data represent the mean $\pm$ SEM for GnRH secretion rate or the geometric mean $\pm$ SEM for LH concentrations. **P* < 0.001 versus pro-oestrus.

oestradiol on FSH secretion (Fig. 3c; right panel). Therefore, LY blocks the effects of oestradiol independently of whether oestradiol is inhibiting (negative feedback) or stimulating (positive feedback) LH secretion without affecting positive feedback on FSH secretion. Overall, these observations indicate that the ability of LY to prevent ovulation is related to an impaired positive feedback mechanism.

Experiment 5

The preovulatory GnRH secretion rate from the hypothalamic-pituitary portal system was evaluated in an attempt to identify the site at which LY inhibits the

Fig. 5. Effect of administration of LY117018-HCl (LY) on pituitary sensitivity to GnRH in adult cyclic female rats. Rats under sodium pentobarbital anaesthesia were insensitive to a GnRH challenge (50 ng (100 g)⁻¹ body weight, in 0.5 ml of saline) after administration of LY. Blood samples were collected during pro-oestrus at the time of expected endogenous surge of LH. ●: control group, treated with vehicle for GnRH and LY; ○: animals injected with GnRH; ▼: animals injected with LY plus GnRH. Values denote the geometric mean $\pm$ SEM of five or six rats per group. Two-way ANOVA with repeated measures was performed after elevating the data to the power 0.2. **P* < 0.001 versus control.

positive feedback effects of oestrogen on LH secretion in cyclic female rats. GnRH secretion rate was not altered by treatment with the compound (Fig. 4a). Similar to the results obtained in Expt 2, plasma concentrations of LH decreased in the group treated with LY (Fig. 4b). These data indicate that LY may reduce pituitary responsiveness to GnRH, as GnRH secretion rates were the same in control and LY-treated rats.

Experiment 6

The hypothesis that LY reduces pituitary sensitivity to GnRH was tested. Exogenous GnRH administration increased LH secretion in animals pre-treated with sodium pentobarbital during pro-oestrus in a time-dependent manner (Fig. 5). This effect was completely abolished in animals pre-treated with LY (Fig. 5). These findings demonstrate that the compound abolishes pituitary responsiveness to GnRH and indicate that this

mechanism is responsible for LY-induced reduction in LH concentrations during pro-oestrus, and thus, the prevention of ovulation.

Discussion

The results of the present study show that LY prevents ovulation when administered to normal cyclic rats during dioestrus, most likely as a consequence of an inhibitory action of the compound on the preovulatory surge of LH. This result is in contrast with previous reports in humans showing that raloxifene, a related compound, does not affect either ovulation or the duration of the ovarian cycle (Draper *et al.*, 1995; Baker *et al.*, 1998). However, Long *et al.* (2001) indicate that administration of toxicological doses of raloxifene for 6 months prevents ovulation in rats. These discrepancies may be related to differences in species or dose. In addition, LY antagonized the uterotrophic effect of oestradiol in the present study, confirming other reports (Black and Goode, 1980; Gordon *et al.*, 1985), and inhibited the effects of either low (negative feedback) or high (positive feedback) doses of oestradiol on LH secretion. More importantly, the present study identifies the potential site of LY to reduce LH concentrations, and therefore, prevent ovulation.

In rats, the increase in oestradiol during dioestrus is critical for the preovulatory release of GnRH and LH at pro-oestrus, which in turn induces the release of eggs during the early hours of oestrus (Freeman, 1988). Both basal and GnRH-stimulated LH secretion are dependent on oestrogen action on the anterior pituitary (Meijs-Roelofs *et al.*, 1975). The anovulatory effect of LY could be exerted at the hypothalamus, pituitary gland or ovary. Ovarian responsiveness to gonadotrophins was evaluated in normal cyclic rats injected on the morning of pro-oestrus with pentobarbital, which blocks the endogenous gonadotrophin surge and abolishes spontaneous ovulation. Administration of hCG to pentobarbital-treated rats effectively induced ovulation in all animals, irrespective of whether they had been pre-treated with LY. Furthermore, in normal cyclic rats, which were anovulatory due to treatment with LY, administration of hCG re-established normal ovulation in all animals. These findings indicate that the anovulatory effect of LY was not exerted by altering ovarian events required for spontaneous ovulation, but likely by impairing GnRH or LH secretion. However, the anovulatory action and the reduction of plasma LH concentrations induced by LY treatment were not accompanied by changes in the rate of GnRH secretion into hypophyseal portal blood. Moreover, treatment with LY completely abolished pituitary responsiveness to GnRH. These results indicate that LY blocks oestrogen-induced sensitization of the anterior pituitary to GnRH, rendering the pituitary insensitive to the decapeptide and, as a consequence, reducing LH secretion and

blocking ovulation. Whereas treatment with LY reduced the feedback effect of oestradiol on LH secretion by 50%, FSH concentrations increased by oestrogen were only slightly reduced by LY under the same conditions. As treatment with LY did not modify the GnRH secretion rate, this finding may reflect the fact that regulation of FSH secretion depends on additional regulatory systems less sensitive to the anti-oestrogenic effects of LY.

The lack of effect of LY on GnRH neurones (no changes in GnRH secretion rate into hypophyseal portal blood) and on the ovary (no alteration of ovarian sensitivity to hCG) may be related to the type of ER predominantly expressed in each region. In both mice and rats, ER β is the predominant subtype in ovarian granulosa cells (Couse *et al.*, 1997; Fitzpatrick *et al.*, 1999; O'Brien *et al.*, 1999). In addition, ER β seems to be the only ER expressed in the majority of GnRH neurones of female rats (Hrabovszky *et al.*, 2000 a,b; Herbison *et al.*, 2001). In contrast, ER α expression is high in both the uterus and the anterior pituitary of the rat (Kuiper *et al.*, 1997; Pelletier *et al.*, 2000; Schreihöfer *et al.*, 2000; Wang *et al.*, 2000). The preference of this compound for ER α similar to raloxifene (Barkhem *et al.*, 1998) may explain the sensitivity of the uterus and the pituitary to the anti-oestrogenic effects of LY, although this will depend on the tissue concentration of the compound and the dose used.

Alternatively, the lack of effect at the hypothalamus could be explained by the inability of the compound to cross the blood–brain barrier. Raloxifene shows low penetration into the blood–brain barrier-protected regions (cortex, hippocampus or hypothalamus) in humans, as only 1–2% of the concentrations observed in the pituitary gland were detected in these areas of the brain (F. Marín, personal communication). However, both raloxifene and LY alone or in combination with oestradiol have been reported to modify several neurotransmitter systems in blood–brain barrier-protected areas of the brain in rats. Raloxifene acts as an oestrogen agonist by increasing choline acetyltransferase activity in the hippocampus and other areas of the brain (Wu *et al.*, 1999). These data indicate that raloxifene crosses the rodent blood–brain barrier to elicit effects within the hippocampus. LY also exhibits oestrogen-like effects in other neuronal systems of the brain. Chronic administration of LY to intact rats decreases beta-endorphin content in the hippocampus, anterior pituitary and plasma (Genazzani *et al.*, 1999). Furthermore, LY suppresses oestrogen-induced increases in allopregnanolone in the hippocampus, hypothalamus and pituitary gland of ovariectomized rats (Genazzani *et al.*, 2000). Taken together, these data indicate that in rodents, raloxifene, and LY in particular, are able to alter various neuronal systems protected by the blood–brain barrier. Although the present experiments do not directly address whether LY reaches hypothalamic neurones (that is, crosses the blood–brain barrier), on the basis of the published observations it is likely that the compound reaches sufficient concentrations in the CNS to exhibit

an action as reported for other neurotransmitter systems (Genazzani *et al.*, 1999, 2000). Therefore, further studies need to be conducted to elucidate the mechanism(s) for the apparent selectivity of action of LY in the anterior pituitary and uterus versus the ovary and GnRH neurones.

The results from Expt 6 support the hypothesis that LY is able to decrease the sensitivity of gonadotrophs to the stimulating effects of GnRH, implying that this may be the reason behind the ability of LY to inhibit ovulation. The present data are in agreement with experiments conducted *in vitro* in which raloxifene induced desensitization of the gonadotroph to GnRH. It has been shown that raloxifene diminished the stimulatory effect of GnRH on LH release in incubated anterior pituitaries (Simard and Labrie, 1987; González *et al.*, 2000). Mechanistically, oestradiol increases the efficiency of GnRH receptors to stimulate phospholipase C and this effect is blocked by raloxifene in α T3-1 cells (Williams *et al.*, 2000). In addition, it has been shown that oestradiol increases the number of GnRH receptors in cultured bovine pituitary cells and that this effect is blocked by treatment with tamoxifen (Gregg *et al.*, 1990). Similar effects of oestradiol on GnRH receptor mRNA have been described in granulosa cells and, as in the case of the pituitary receptor counterpart, oestradiol-dependent induction is blocked by tamoxifen (Nathwani *et al.*, 2000). Therefore, treatment with raloxifene or its derivatives, that is, LY, in intact cyclic female rats could result in attenuation of oestradiol-induced sensitization of GnRH receptors by reducing efficiency of the signal transduction cascade (Williams *et al.*, 2000), reducing the number of GnRH receptors (Gregg *et al.*, 1990; Nathwani *et al.*, 2000) or both mechanisms combined.

The present results indicate that LY, at the doses used in this study, acts as an oestrogen antagonist at two different levels of the female rat reproductive axis. First, at the uterus, it inhibits the uterotrophic action of oestradiol. Second, at the pituitary gland, it reduces the effects of oestradiol on LH secretion under both negative and positive feedback modes, and blocks the stimulatory effect of GnRH on LH secretion during pro-oestrus. This effect at the pituitary gland is in agreement with the effect of raloxifene previously reported to reduce GnRH-induced LH secretion from dispersed pituitary cells *in vitro*, and probably reflects the blockage of oestrogen actions on GnRH receptors and GnRH-activated signalling in anterior pituitary cells. Therefore, LY blocks ovulation in cyclic rats by reducing pituitary sensitivity to GnRH during pro-oestrus and without altering GnRH secretion rate into hypophyseal portal blood.

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