Clomiphene citrate induces pituitary GnRH receptors in ovariectomized rats: its possible role in induction of ovulation

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Abstract. Since our previous studies have shown that clomiphene citrate (clomiphene) acts directly on the pituitary gland and exerts a facilitatory role on oestradiol-17β (E₂)‐induced LH surge in chronically ovariectomized rats, the effect of clomiphene on pituitary GnRH receptors was investigated. A single ip injection of either 5 μg E₂ or 200 μg clomiphene did not induce LH release in adult rats ovariectomized 1–2 weeks before the injection. However, a significant increase in serum LH was noted 24 h after a single injection of E₂ in the ovariectomized rats, if clomiphene was pre-injected 48 h before the E₂ injection. The content of pituitary GnRH receptors in the ovariectomized rats (62 ± 9 fmol/pituitary) remained almost unchanged until 24 h after a single injection of clomiphene but significantly increased 48 h after the injection (105 ± 13 fmol/pituitary) without any alterations in the affinity for GnRH. To determine steroid specificity for the increase in pituitary GnRH receptors, other classes of steroids were injected in the ovariectomized rats. A single dose of E₂ increased GnRH receptors, but either progesterone or 5α-dihydrotestosterone failed to show any effect on the level of GnRH receptors. These results suggest that clomiphene may augment oestrogen-induced pre‐ovulatory LH surge in anovulatory women, at least in part by increasing the number of pituitary GnRH receptors.

It has been generally accepted that clomiphene citrate (clomiphene) acts as an anti-oestrogen in hypothalamic‐pituitary system during the follicular phase and consequently increases serum levels of gonadotrophin. Studies with cultured pituitary cells, however, have suggested that clomiphene exerts an oestrogenic rather than anti-oestrogenic effect on the pituitary cells by enhancing GnRH stimulated release of gonadotrophin (Adashi et al. 1981; Huang & Miller 1983). Clomiphene, unlike oestradiol-17β (E₂), has also been shown to act as a long-acting agent in the pituitary gland (Adashi et al. 1980; Tsutsumi et al. 1983). These findings have led us to investigate how clomiphene interacts at the pituitary gland with increased ovarian oestrogen at the time of ovulation and whether clomiphene takes part in pre-ovulatory LH surge caused by E₂. We have found that E₂ elicits LH release in clomiphene-pretreated chronically ovariectomized rats but does not in non-treated chronically ovariectomized animals (Terakawa et al. 1985). These results suggest that clomiphene acts directly on the pituitary gland to augment ovarian oestrogen-induced LH surge at the pre-ovulatory stage.

It has been reported that alterations in pituitary GnRH receptors can affect the response of the gonadotrophs to GnRH and that oestrogens increase the number of GnRH receptors (Adams et al. 1981; Moss et al. 1981; Marchetti & Labrie 1982; Tang et al. 1982). In the present study, therefore, the effect of clomiphene on pituitary GnRH receptors was examined in chronically ovariectomized rats.
Materials and Methods

Animals and treatment
Adult Wistar female rats weighing about 200 g were purchased from Shizuoka Laboratory Animal Center. One to two weeks after bilateral ovariectomy, 200 μg clomiphene in 0.5 ml sesame oil was injected ip between 04.00 and 06.00 p.m. In the other group of ovariectomized rats, 5 μg F3 in 0.5 ml saline was administered 12, 24 or 48 h after injection of 200 μg clomiphene. The rats were sacrificed by decapitation 12, 24, 48 or 72 h after a single dose of clomiphene. The clomiphene pretreated rats were killed 24 h after E2 injection. To study steroid specificity for GnRH receptors, a single dose of either 5 μg F2, 50 μg progesterone or 50 μg dihydrotestosterone (DHT) was injected ip and sacrificed 12, 24, 48 or 72 h later. The trunk blood was collected in test tubes and the pituitary was removed immediately and stored at −80°C. Eight normal female rats in oestrous cycle served as the control for GnRH receptor studies.

Assay of serum LH
Serum levels of LH were determined by the double antibody radioimmunoassay (RIA) using the RIA kits kindly supplied by NIADDKD as described previously (Hayashi et al. 1979).

Estimation of GnRH receptors
Iodination of GnRH analogue was performed as described by Reeves et al. (1980), except that D-Ala6 des Gly10-GnRH ethylamide (D-Ala analogue) was used as ligand. Briefly, the labelling was carried out for 4 min, with 2.5 μg of D-Ala analogue in 30 μl of 0.5 M sodium phosphate (pH 7.2), 500 μCi of carrier-free Na[125]I and 600 ng of chloramin-T in 10 μl of 0.5 M sodium phosphate (pH 7.2). The reaction was stopped by adding 1 ml of eluant buffer [10 mM Tris, 0.1% (wt/vol) bovine serum albumin (BSA) and 0.1% (wt/vol) NaN3, pH 7.6] and the mixture was immediately layered onto a column (1 × 55 cm) of Sephadex G-25 (fine). Free 125I was eluted in the first radioactive peak and labelled D-Ala analogue in the second radioactive peak. Specific activity obtained was 120–168 μCi/μg and the labelled analogue was always used within 24 h of iodination.

The measurement of pituitary GnRH receptors was performed as reported previously (Tasaka et al. 1985) unless specified otherwise. All procedures were carried out at 0–4°C. Saturation analysis of GnRH binding sites was performed using four pituitaries in the presence of [125]I-D-Ala analogue at concentrations of 0.06–1.25 nM, and the values obtained were analyzed by Scatchard plots (Scatchard 1949). As the affinity for specific GnRH binding sites in ovariectomized rats was shown to be unchanged by clomiphene or E2 treatment, a saturating dose of [125]I-D-Ala analogue was used to determine the binding sites in this study. A single pituitary was homogenized in 1 ml of 10 mM Tris, 0.1% BSA and 1 mM diithiothreitol, pH 7.6 (TBN buffer), with a glass-Teflon homogenizer. The homogenate was centrifuged at 10,000 × g for 15 min, and the pellet was resuspended at a concentration of approximately 1 mg protein/ml in 100 μl of TBN buffer. Duplicate 50 μl aliquots of the suspension were added to tubes containing 400 μl of TBN buffer and were incubated with a saturating concentration, 0.75 nM of [125]I-D-Ala analogue, in the presence or absence of 0.1 μM unlabelled D-Ala analogue at 0–4°C. The final incubation volume was 500 μl. After incubation for 4 h, the reaction was stopped by addition of 2 ml of TBN buffer. The tubes were then centrifuged at 3000 × g for 30 min and the radioactivity in the pellet was counted in a gamma counter with efficiency of 55%. As a pituitary weight in ovariectomized rats was not changed significantly by treatment with steroids, GnRH binding sites were expressed as fmol per pituitary gland.

Statistics
All values were expressed as mean ± SEM. Student's unpaired t-test was used for the statistical analysis.

Results
Serum LH level increased to 250 ± 44 ng/ml 1–2 weeks after ovariectomy (Fig. 1). A single dose of 5 μg E2 caused a significant decrease in the LH level (94 ± 22 ng/ml) 4 h after the injection and the level remained low for 20 h. Thereafter, the LH level gradually increased and returned to the pre-administration level (203 ± 30 ng/ml) 72 h after the injection, but the levels did not exceed the pre-administration value during the period of experiment. Thus a single ip administration of 5 μg E2 was found to be ineffective in induction of LH surge in chronically ovariectomized rats. Then, the effect of a single dose of 200 μg clomiphene on LH release was examined. As shown in Fig. 1, clomiphene significantly suppressed the LH level for 72 h.

In order to examine whether E2 induces LH surge in chronically (1–2 weeks) ovariectomized rats pretreated with clomiphene, a single dose of 5 μg E2 was given after the injection of 200 μg of clomiphene. When E2 was injected 12 or 24 h after clomiphene, an increase in serum LH level was not observed during the experiment. In contrast, serum LH (581 ± 170 ng/ml) increased markedly 24 h later when E2 was injected 48 h after the clomiphene injection. This value was
Effect of clomiphene or clomiphene plus E₂ on serum LH concentrations in ovariectomized rat. The rats were sacrificed at the indicated times after ip injection of 200 µg clomiphene. In some rats, 5 µg E₂ was also given 12, 24 or 48 h after the clomiphene injection. Open circles (-----) indicate LH levels after a single injection of clomiphene. Each bar shows LH levels in clomiphene pretreated animals, which were given E₂ 24 h before sacrifice. Each value represents the mean ± SE of eight determinations. Difference between serum LH concentration in clomiphene-treated animals without E₂ injection and that with E₂ injection (P): * < 0.05 (t-test).

Fig. 1.

The effect of clomiphene on pituitary GnRH receptors was next examined. A saturation curve was plotted for specific GnRH binding sites in ovariectomized animals over a wide range of [¹²⁵I]D-Ala analogue concentrations (0.06–1.25 nM) and saturation appeared to be achieved at 0.75 nM (data not shown). Scatchard plot analysis revealed that the apparent Kd for specific GnRH binding sites was 5.8 ± 0.2 × 10⁻¹⁰M with the maximal number of binding sites of 81 ± 13 fmol/pituitary (n = 4). Since the apparent Kd was very similar to that in clomiphene-treated rats (48 h after clomiphene: 6.0 ± 0.5 × 10⁻¹⁰M, n = 4), GnRH binding sites were quantified with 0.75 nM of [¹²⁵I]D-Ala analogue in all subsequent studies. As shown in Fig. 2, the content of pituitary GnRH receptors in ovariectomized rats was found to be 62 ± 9 fmol/pituitary, which had been elevated by castration (random cycling normal female rats: 42 ± 7 fmol/pituitary). A single dose of clomiphene did not induce GnRH receptors until 24 h, but a significant (P < 0.05) increase in the content of receptors was observed 48 h after the injection (105 ± 13 fmol/pituitary). To determine the steroid specificity for the increase in GnRH receptors, a single dose of either 5 µg E₂, 50 µg progesterone or 50 µg 5α-dihydrotestosterone (DHT) was administered in ovariectomized rats. In contrast to clomiphene, an administration of E₂

Fig. 2.

Effect of a single injection of clomiphene on pituitary GnRH receptors. Ovariectomized rats were injected ip with 200 µg clomiphene and sacrificed at the times indicated. The content of GnRH receptors was measured using 0.75 nM of [¹²⁵I]D-Ala analogue. Each value represents the mean ± SE of four to five determinations. Difference from 0 h (P): * < 0.05 (t-test).
caused a rapid induction of GnRH receptors and consequently a significant increase was observed 12 and 24 h after the injection (67 ± 9 fmol/pituitary in the ovariectomized control vs 107 ± 4 fmol/pituitary at 12 h, P < 0.01 and 115 ± 9 fmol/pituitary at 24 h, P < 0.05). Thereafter, the level declined gradually and returned to the pre-injection level 72 h after the injection (79 ± 8 fmol/pituitary). However, a single dose of either progesterone or DHT did not change the content of pituitary GnRH receptors at all during the experiment. The levels of GnRH receptors obtained 24 h after the administration of various steroids are summarized in Fig. 3.

**Discussion**

The present study demonstrates that clomiphene enhances pituitary GnRH receptors and elicits E2-induced LH surge in chronically ovariectomized rats. To our knowledge, the present observation shows for the first time that an anti-oestrogen, clomiphene, increases the number of binding sites for GnRH without any alterations in affinity for GnRH. It has been well established that E2 is the primary hormone involved in triggering the pre-ovulatory LH surge. However, the injection of E2 stimulates LH release in intact or acutely ovariectomized rats but not in long-term ovariectomized animals. In these animals, pretreatment with oestrogen is required to induce LH release by E2 (McGinnins et al. 1981). In our previous studies (Terakawa et al. 1985), LH release could be induced 24 h after a single dose of E2 administration in long-term ovariectomized rats which were pretreated with clomiphene 48 h but not 12 or 24 h before the E2 injection, and the LH release was accompanied by nuclear ER processing in the pituitary. These studies imply that the biological effect of E2 on LH surge occurred when clomiphene-ER complex was substituted by E2-ER complex in the nucleus of pituitary gland. In the present study, clomiphene itself significantly increased pituitary GnRH receptors 48 h after the injection, but E2 did not induce LH release at that time. Therefore, the present results together with our previous findings suggest that the clomiphene-induced increase in pituitary GnRH receptors seems to play only a part of the facilitatory roles of clomiphene on E2-induced LH surge in the pituitary of chronically ovariectomized rats.

Although E2 as well as clomiphene increased the number of GnRH binding sites in the pituitary of ovariectomized animals, either progesterone or DHT failed to induced pituitary GnRH receptors in these animals. The induction of GnRH receptors, therefore, seems to be specific for oestrogens. These findings in the present study agree with previous results in vivo (Adams et al. 1981; Moss et al. 1981; Marchetti & Labrie 1982) and in vitro (Tang et al. 1982), indicating that E2 increases GnRH binding sites in the pituitary gland. A positive correlation between ovarian oestrogen secretion and pituitary GnRH binding sites during the rat oestrous cycle has also been documented (Savoy-Moore et al. 1980; Clayton et al. 1980). Our results imply that clomiphene acts as an oestrogen rather than an anti-oestrogen on pituitary GnRH receptors. Clomiphene has been shown to act as both oestrogen agonist and antagonist, depending on target tissues, duration of...
treatment and availability of oestrogen in the system (Clark et al. 1974). It has also been reported that clomiphene, unlike tamoxifen, sensitizes gonadotrophs to GnRH, resulting in an enhancement of release of LH from pituitary cells in culture (Adashi et al. 1981; Huang & Miller 1983). On the other hand, however, previous studies (Tsutsumi et al. 1983) have demonstrated that clomiphene administration results in the reduction of pituitary weight and the suppression of progesterone receptor synthesis in chronically oestrogenized rats. At present, however, the exact mechanisms by which clomiphene exhibits a dual effect are not known. We have found in the present and the previous (Terakawa et al. 1985) studies that in chronically ovariectomized rats, clomiphene exerts a direct oestrogen-like action on the pituitary gonadotrophs which results in an increase in GnRH receptors 48 h after the clomiphene injection in the same cells and that subsequent E₂ administration acts to promote the surge of LH secretion accompanied by nuclear processing of ER 72 h but not 48 h after the clomiphene injection by the so prepared gonadotrophs. However, the mechanism by which the administration of subsequent E₂ induces LH release by the gonadotrophs with increase in GnRH receptors remains to be elucidated.

Several studies (Clayton & Catt 1981; Clayton 1982; Loumaye & Catt 1982) have demonstrated that GnRH at physiological concentrations increases its own pituitary receptor concentration. Therefore, the increase in pituitary GnRH receptors that we observed may result from enhanced GnRH secretion from the hypothalamus. Only the direct measurement of GnRH levels in portal vessels following clomiphene treatment could bring an answer to this question.

Although the results of the present study do not rule out anti-oestrogenic effects of clomiphene on hypothalamo-pituitary system, we postulate that, in anovulatory women, clomiphene may also act as an oestrogen agonist in the pituitary and augment ovarian oestrogen-induced LH surge by enhancing GnRH receptors and modulating oestrogen receptor systems in pituitary cells.

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References


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