Increases in Ovarian GnRH Receptors by Following GnRH Treatment

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Abstract

Using a gonadotropin-releasing hormone (GnRH) analog, [D-Ser (tBu⁶)] des-Gly¹⁰-GnRH-N-ethlamide (GnRHa) as a ligand the binding capacity of the rat ovary to GnRH during sexual maturation and the mechanism regulating GnRH binding capacity were examined.

Specific high affinity binding sites for GnRH were observed in the ovary and the Kd values for the granulosa cells and the residual tissue were similar to those of whole ovary. During sexual maturation, the GnRH binding capacity of the ovary rose from 7 days of age to a peak at 28 days and declined during the prepubertal period. The treatment with PMSG decreased GnRH binding capacity in the residual tissue as well as in the whole ovary but did not change the binding capacity in the granulosa cells in diethylstilbestrol (DES) primed hypophysectomized rats. Repeated injections of GnRH caused a significant increase in the number of GnRH receptors of the ovary in PMSG treated DES primed hypophysectomized rats but not in the saline treated rat. The granulosa cells exhibited increases in GnRH binding capacity following repeated administrations of GnRH more than the residual tissue did. In GnRH treated DES primed hypophysectomized rats, increasing doses of PMSG increased the binding capacity in the granulosa cells but decreased the binding capacity in the residual tissue.

From these findings, GnRH in combination with PMSG seems to have stimulatory effects on GnRH binding capacity and to increase the sensitivity to GnRH in the granulosa cells.

Recently native GnRH and its analogs were proved to inhibit FSH-stimulated steroidogenesis in the ovary (Ying and Guillemin, 1979) and FSH induced increases in LH receptor content in the granulosa cells in vivo and in vitro (Hsueh et al., 1980). Since uptake of an iodinated GnRH analog has been reported (Mayar et al., 1979) and GnRH receptors have lately been identified in the ovary (Clayton et al., 1979a), GnRH and its analog were thought to act directly on their own receptor and inhibit the ovarian function. But the changes in the ovarian receptor under physiological conditions such as sexual maturation and the regulation mechanism of the GnRH receptor remain to be elucidated.

In this paper the binding capacity of GnRH during sexual maturation and the mechanism regulating the GnRH binding capacity in the rat were examined.
Materials and Methods

Animals

In all studies, female rats of Sprague-Dawley strain were maintained under uniform conditions of 22 ± 1.5°C and a photoperiod of 14 hr (0500 to 1900 hr). Twenty-three day-old female rats were hypophysectomized by Teikoku Hormone Mfg. Co., Ltd. and subsequently verified by observation of the sella turcica with a dissecting microscope. For the study of the sexual maturation, female Sprague-Dawley rats (7, 14, 21, 28, 35 days old and diestrous adult) were killed by decapitation.

GnRH and PMSG injections

Intact immature rats were injected with 3 ng/day native GnRH (Lutamin, Daiichi Pharmacological Co., Ltd., 1 ng in 0.4 ml saline every 8 hr i.p.) or vehicle alone for 2 days beginning at 26 days of age, and PMSG (Teikoku Hormone Mfg. Co., Ltd.; 25 IU) was injected s.c. at the time of the first GnRH injection.

In a group of hypophysectomized immature rats, diethylstilbestrol (DES, Sigma Chemical Co.; 0.5 mg/day in sesame oil) was administered s.c. every day after the hypophysectomy. Native GnRH (1 μg x 3/day) was administered for 2 days beginning at 26 days of age and 25 IU PMSG was injected on day 26. At 28 days of age, all GnRH-treated rats were decapitated 8 hr after the last injection of GnRH.

Tissue preparation

After decapitation, ovaries were quickly removed and homogenized in phosphate buffered saline (PBS; pH 7.6). Ovaries from the 7-, 14- and 21-day old groups and each of the treatment groups except the PMSG-primed groups were pooled to provide adequate amounts of ovarian tissue. For the binding analyses of the granulosa cells, fat and connective tissue were trimmed off from individual ovaries. Gentle pressure was applied to the ovaries to rupture the follicles and release the granulosa cells into PBS as described by Zelenezk et al. (1974), and the tissue was then centrifuged at 800 x g for 5 min at 4°C to isolate the granulosa cells from follicular fluid. The pellet was washed in PBS and homogenized in PBS. The residual tissue, which was the ovarian tissue remaining after expression of the granulosa cells and follicular fluid, was also homogenized in PBS.

Iodination of GnRHa and HCG

High potent and stable [D-Ser (tBu⁶)] des-Gly¹⁰-GnRH-N-ethylamide (GnRHa, Hoechst Japan Co., Ltd.) was iodinated by the modification of the Reeves’ method (Reeves et al., 1980). Briefly, the labeling was carried out with 2.5 μg of GnRHa, 500 μCi of carrier-free Na¹²⁵I and 600 ng of chloramin T in 10 μl of 0.5 M sodium phosphate (pH 7.2). The reaction was stopped by the addition of 1 ml of eluent buffer (10 mM Tris-HCl/0.1% bovine serum albumin/0.1% NaN₃, pH 7.6 at 25°C). Monoiodinated hormone was separated by Sephadex G-25 (fine) column chromatography (1 × 55 cm). The second peak was found to bind pituitary homogenates specifically as described by Reeves et al. (1980). Specific activity (800-1000 μCi/μg) was determined by equating the median effective dose (ED₅₀) value of a saturation curve with the ED₅₀ value of a displacement curve (De Lean et al., 1978). HCG (Teikoku Hormone Mfg. Co., Ltd.) was iodinated by the chloramin T method of Okumura et al. (1978) and had a specific activity of 30 μCi/μg.

GnRH receptor assay and hCG receptor assay

The radioreceptor binding assay was carried out as described by Reeves et al. (1980). The tube (12 × 75 mm) contained 300 μl of 10 mM tris-HCl/0.1% BSA/1 mM dithiothreitol (pH 7.6) buffer, 100 μl of ¹²⁵I-GnRHa (150,000 cpm) and 100 μl of homogenates of whole ovaries, granulosa cells or the residual tissue (5-10 mg of the fresh tissue or 1-5 x 10⁶ cells). Non specific binding was assessed in the presence of 50 nM unlabeled analog. The incubation was performed at 0-4°C for 120 min and the reaction was stopped by the addition of 3 ml of ice-cold pH 7.6 buffer. The tubes were then centrifuged at 2000 x g for 25 min and the radioactivity contained in the pellet was counted. For the binding study of hCG the incubation was performed at 20°C for 16 hr and non specific binding was assessed in the presence of 5 μg unlabeled hCG. Protein concentrations were estimated following the method of Lowry et al. (1951).

The serum concentration of FSH was determined by means of RIA kits provided by the NIAMDD. The amounts of FSH were expressed in terms of NIADDK-fFSH-RP-1 (AFP-180-sol). All statistical analyses were based on Student's t-test.
Result

The tissue specificity of GnRHa binding is shown in Fig. 1. $^{125}$I-GnRHa bound to various tissues of rats (28 days old) was examined. Specific binding of the analog to homogenates from the anterior pituitary and the whole ovary was observed and a small amount of binding was also seen in the frontal cortex, the uterus and the skeletal muscle.

Scatchard plots of $^{125}$I-GnRHa binding to ovarian and pituitary homogenates are shown in Fig. 2. Scatchard plots of $^{125}$I-GnRHa binding to rat tissues (28 days old). Data shown are the mean±SD (N=5).

**OVARY**

Kd = $1.92 \times 10^{-10}$ M

BINDING CAPACITY

17.9 fmol/mg protein

**PITUITARY**

Kd = $2.49 \times 10^{-10}$ M

BINDING CAPACITY

41.2 fmol/mg protein
shown in Fig. 2. Kd values of $1.92 \times 10^{-10}$ M and $2.49 \times 10^{-10}$ M were obtained for the ovary and the pituitary, respectively. Ovarian homogenates had a capacity of $17.9 \text{fmol/mg protein}$ and pituitary homogenates bound $41.2 \text{fmol/mg protein}$. Kd values of $4.28 \times 10^{-10}$ M and $8.24 \times 10^{-10}$ M were obtained from the granulosa cells and the residual tissue, respectively. Native GnRH displaced $^{125}$I-GnRHa from ovarian homogenates and a ten fold higher value of native GnRH was required to produce 50% displacement, but prolactin, TSH and hCG (1 pg-1 μg) did not displace $^{125}$I-GnRHa from ovarian homogenates. (data are not shown)

GnRH binding capacities during sexual maturation

GnRH-binding capacities in whole ovaries and serum FSH concentrations during sexual maturations are shown in Fig. 3. Serum FSH was already elevated at 7 days of age and rose further to a peak at 14 days. FSH concentrations fell rapidly to 720 ng/ml at 21 days and remained between 400–600 ng/ml thereafter. GnRH-binding capacities of the whole ovary rose from $38.7 \pm 39.8 \text{fmol/mg protein}$ (mean ± SD) at 7 days of age to a peak of $190.8 \pm 33.7 \text{fmol/mg protein}$ at 28 days ($p < 0.01$, compared to 7 days). GnRH-binding capacities then declined at 35 days of age and remained low thereafter.

Effects of PMSG and GnRH administrations in intact immature rats.

In intact immature rats, injections of GnRH (6 μg, three times a day for 2 days) decreased GnRH-binding capacities in whole ovaries from $74.5 \pm 7.3$ to $44.2 \pm 3.5 \text{fmol/mg protein}$ ($p < 0.01$). PMSG treatment decreased the binding capacities markedly from $74.5 \pm 7.3$ to $7.6 \pm 3.1 \text{fmol/mg protein}$ ($p < 0.01$), but concomitant treatment with GnRH (17.0 ± 1.7 fmol/mg protein) inhibited the PMSG-induced decrease in the binding capacities significantly ($p < 0.01$).

Effects of PMSG administrations in DES primed hypophysectomized rats

In order to exclude the effects of endogenous pituitary hormones, DES primed hypophysectomized rats were utilized. To characterize the binding capacity of GnRH to functionally active follicular tissue, DES primed immature hypophysectomized rats were treated with various concentrations of PMSG (10–200 iu). With these treatments, uniform antral follicles were microscopically observed in the ovary. The administrations of various concentrations of PMSG increased ovarian wet weight significantly ($p < 0.01$, compared to the control group) as shown in Fig. 4.

In order to determine GnRH binding capacities in different ovarian components, the whole ovary was divided into two components i.e. the granulosa cells and the residual tissue. The administration of PMSG did not change the binding capacity in the
**Fig. 4.** Effects of in vivo treatments with PMSG upon GnRH- and hCG-binding capacity of whole ovaries in DES primed hypophysectomized immature rats. Data shown are the mean±SD (N=5). * p<0.05, ** p<0.01 compared to the control group.

**Fig. 5.** Effects of in vivo treatments with GnRH upon GnRH binding capacity of whole ovaries in DES primed hypophysectomized immature rats. Data shown are the mean±SD (N=5). * p<0.05, ** p<0.01 compared to the control group.
granulosa cells but significantly (p<0.01, compared to the control group) decreased the binding capacity in the residual tissue from 68.3±3.8 to 10.5±7.6 fmol/mg protein.

Effects of GnRH administrations in DES primed hypophysectomized rat

In DES primed hypophysectomized immature rats, the administrations of increasing concentrations of GnRH caused a slight decrease in ovarian wet weight, as shown in Fig. 5, but GnRH binding capacity of the whole ovary was not influenced by increasing concentrations of GnRH. On the contrary, in PMSG-DES primed hypophysectomized rats, increasing doses of GnRH caused a dose-related increase in GnRH binding capacity from 7.2±2.4 to 75.5±8.2 fmol/mg protein. Although the ovarian wet weight decreased with the treatment with GnRH, GnRH binding capacity per ovary increased significantly (p<0.01, compared to the control group) from 10.8±3.7 to 38.4±4.2 fmol/mg protein as shown in Fig. 5.

In the granulosa cells from DES-PMSG primed rats, administrations of increasing doses of GnRH caused increases in GnRH binding capacity significantly (p<0.01, compared to the control group) from 17.9±0.8 to 53.2±2.4 fmol/mg protein, and the administrations of GnRH increased GnRH binding capacity in the residual tissue less than in the granulosa cells from 17.8±1.7 to 39.2±2.0 fmol/mg protein, as shown in Fig. 6. On the other hand, hCG binding capacity in the granulosa cells and the residual tissue decreased in the GnRH-PMSG treated rats, as shown in Fig. 7.

Effects of GnRH and PMSG administrations in DES primed hypophysectomized rats

Increasing concentrations of PMSG were administered to GnRH (1 µg every 8 hr for 2 days)-DES primed rats. Increasing doses of PMSG caused a dose related increase in ovarian weight, which was significantly smaller than that of non-treated (saline

Fig. 5. Effects of in vivo treatments with GnRH upon GnRH binding capacity of granulosa cells and residual tissue in PMSG and DES primed hypophysectomized rats. Data shown are the mean±SD (N=5). * p<0.05, ** p<0.01 compared to the control group.

Fig. 6. Effects of in vivo treatments with GnRH upon GnRH binding capacity of granulosa cells and residual tissue in PMSG and DES primed hypophysectomized rats. Data shown are the mean±SD (N=5). * p<0.05, ** p<0.01 compared to the control group.
Fig. 7. Effects of in vivo treatments with GnRH upon hCG binding capacity of granulosa cells and residual tissue in PMSG and DES primed hypophysectomized rats. Data shown are the mean±SD (N=5). * p<0.05, ** P<0.01 compared to the control group.

Fig. 8. Effects of in vivo treatments with PMSG upon GnRH binding capacity of granulosa cells and residual tissue in DES primed (non treated)- and DES and GnRH primed (GnRH treated)- hypophysectomized rats. * p<0.05, ** p<0.01 compared to the group treated without PMSG.
treated) DES primed rats as shown in Fig. 8. In the residual tissue from GnRH treated rats, PMSG induced a reduction in GnRH binding capacity, being similar to that found in non-treated rats, but in the granulosa cells increasing doses of PMSG increased GnRH binding capacity, as shown in Fig. 8. That is, in the residual tissue, PMSG had more potent effects on GnRH binding capacity than GnRH had and in the granulosa cells GnRH in combination with PMSG had stimulatory effects on GnRH binding capacity.

Discussion

GnRHa was labeled with $^{125}$I to assess ovarian GnRH receptors in this study because 90% of the native GnRH was degraded whereas only 10–20% of the analogs were metabolized during incubation with the pituitary membrane (Clayton et al., 1979b). The GnRH agonist was shown to be 50–100 times more potent than native GnRH in promoting LH release from the anterior pituitary cells (Reeves et al., 1980) and in our study on the GnRH receptor of the ovary, the GnRH agonist exhibited 10 times more potency than native GnRH in binding to the GnRH receptor.

In this study, specific high affinity binding sites for GnRH were observed in the ovary of the rat. The competition curves obtained from the ovary and the pituitary were very similar, and the affinity of the GnRH binding sites in the whole ovary was also comparable to that in the pituitary. The $K_d$ of the GnRH agonist to the whole ovarian homogenates was determined to be $1.9 \times 10^{-10} M$ which is comparable to the $K_d$ value reported by Clayton et al. (1979a) and Reeves et al. (1980). The $K_d$ values for the granulosa cells and the residual tissue ($4.2–8.2 \times 10^{-10} M$) were also similar to that for the whole ovary.

The concentration of GnRH in the portal blood system of the rat has been reported to be 50–500 pg/ml (Eskay et al., 1975). These concentrations are comparable with the affinity of the GnRH binding sites in the pituitary, but GnRH is diluted 1:500 between the portal circulation and the peripheral vein (Nett et al., 1974). Their concentrations in peripheral plasma do not seem to interact with a receptor having an affinity of $10^9 M^{-1}$. GnRH like substance has been found in ovarian follicular fluid (Ying et al., 1981), human placenta tissue (Khodr and Siler-Khodr, 1980), parasympathetic ganglion of the frog (Jan et al., 1979) and in the testis (Sharp and Fraser, 1980). This GnRH-like peptide present in the ovary may bind to the GnRH receptor and be involved in local regulation of the ovarian function.

During sexual maturation, GnRH binding capacity was very low at 7 days of age and increased thereafter. From day 28, the concentration of ovarian GnRH receptors gradually decreased during the prepubertal period to reach the adult level. Serum FSH concentrations in female rats reached on extremely high level between 10 and 15 days of age and then declined. Smith-White and Ojeda (1981) found that the ovarian steroidogenic activation that preceded the first surge of gonadotropins in the rat was associated with a marked loss in ovarian GnRH receptor content. These results suggested that the presence of high levels of ovarian GnRH receptors prevented follicular maturation around 28 days of age and that a diminished binding capacity of GnRH permitted an acceleration of ovarian sensitivity to gonadotropins just before puberty.

The mechanism of regulating GnRH binding capacity is not yet known. Marshall et al. (1981) found that pituitary GnRH receptor was increased by exposure to GnRH, and gonadotropins seem to modify the GnRH binding judging from our findings on sexual maturation. Therefore, we examined the effect of GnRH on GnRH binding capacity in intact and in DES primed hypophy-
sectomized immature rats. The treatment with PMSG was found to decrease the concentrations of ovarian GnRH receptors on the whole ovary of intact and hypophysectomized immature rats. These findings are in agreement with the results reported by Labrie et al. (1982) and Harwood et al. (1980). This inhibition of GnRH binding capacity by PMSG administration is consistent with the findings of the loss of GnRH binding capacity before puberty as ovaries stimulated by PMSG had many active antral follicles. In order to determine the degree of GnRH binding capacity in the granulosa cells, these cells were divided from the ovary as specific changes in the granulosa cells might be masked when the binding capacity of GnRH in the whole ovary was investigated. The binding capacity of GnRH after PMSG administration markedly decreased in the residual tissue as well as in whole ovaries, but the binding capacity did not change in the granulosa cells. This result indicates that the binding capacity in the whole ovary represents the binding capacity in the residual tissue rather than in the granulosa cells.

Repeated injections of GnRH caused significant increases in GnRH receptors of whole ovaries in PMSG-treated intact rats. The granulosa cells also exhibited an increase in GnRH binding capacity and a decrease in HCG binding capacity, and these cells had a greater increase in binding capacity than the residual tissue did. In GnRH-treated DES primed hypophysectomized rats, increasing doses of PMSG increased GnRH binding capacity in the granulosa cells but decreased the binding capacities in the residual tissue.

These results indicated that GnRH in combination with PMSG had stimulatory effects on GnRH binding capacity and seemed to increase the sensitivity to GnRH, that an inverse correlation might exist between the GnRH binding capacity and the HCG binding capacity, and that the mechanism regulating GnRH binding capacity in the granulosa cells seemed to be different from the mechanism in the residual tissue.

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