The Effect of Ovarian Steroid Feedback upon Radioimmunoreactive Luteinizing Hormone Releasing Hormone in the Hypothalamus

TAKUMI YANAIHARA, KIYOSHI ARAI, MOTOMI KANAZAWA, SHOICHI OKINAGA, NOBORU YANAIHARA, AND CHIZUKO YANAIHARA

Department of Obstetrics and Gynecology, Teikyo University School of Medicine, Tokyo 173
and Shizuoka College of Pharmacy, Shizuoka 420

Synopsis

A radioimmunoassay (RIA) method for luteinizing hormone (LH) releasing hormone (RH) utilizing rabbit antiserum against synthetic (Glu1)-LH-RH coupled with human serum albumin at the N-terminus, is described. This assay system for LH-RH also cross-reacted with several LH-RH analogues or fragments, but not with pituitary trophic hormones. The assay was performed on the hypothalamic extracts of adult ovariectomized rats and female immature rats which had been treated with estradiol. The FSH and LH levels in the pituitary gland and serum of the same animals were determined by RIA. The radioimmunoreactive LH-RH content of the stalk median eminence markedly increased seven days after ovariectomy. The serum levels and the pituitary contents of FSH and LH of the same rats were also significantly augmented. In immature rats, the hypothalamic content of LH-RH, as measured by RIA, was significantly increased one hour after the injection of estradiol. The FSH and LH levels in the pituitary showed a significant rise after 7 hours.

Materials and Methods

Antiserum against synthetic (Glu1)-LH-RH coupled with human serum albumin (HSA), produced in rabbits was kindly supplied to us by Dr. A. Arimura and Dr. A. V. Schally, New Orleans, Louisiana, U.S.A. This antibody was capable of binding 29% of the labelled LH-RH under the present experimental conditions.

The LH-RH analogues and fragments of LH-RH examined for crossreactivity in the LH-RH RIA system are des pGlu1-LH-RH (a), des pGlu1 des His2-LH-RH (b), LH-RH-OH (c) and (Leu3)-LH-RH (d). The syntheses of peptides a, b (Yanaihara et al., 1974a; Asai et al., 1973; Jeffcoate et al., 1974; Nett et al., 1973; Yanaihara et al., 1974), but there still remain problem to be clarified. The present investigation was undertaken to determine the LH-RH contents in the hypothalamus, and to relate these to some endocrine effects.

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iodination of the LH-RH was performed essentially by the method of Greenwood et al. (1963). Ten micrograms of synthetic LH-RH in 10 µl 0.1 M acetic acid were added to 10 µl of 0.5 M phosphate buffer, pH 7.5. This solution was mixed with 1 mCi of carrier free 125I (NEZ-033H, # I 21320) in 10 µl 0.5 M phosphate buffer, and 5 µl of freshly prepared chloramine T (6 mg/ml in 0.5 M phosphate buffer, pH 7.5) was added. After shaking for one minute, the reaction was stopped by adding 50 µl of sodium metabisulfite (2.5 mg/ml in 0.05 M phosphate buffer, pH 7.5). To the reaction tube, 100 µl DEAE-cellulose suspension (50% w/v in water) was added, mixed well and was left standing for a few minutes until most of the DEAE-cellulose precipitated. Then, the clear supernatant was applied to a CM Sephadex C-25 column (9 × 50 mm, prepared in 0.05 M phosphate buffer, pH 7.5, which had previously been eluted with 1 ml of 1% bovine serum albumin in 0.01 M phosphate buffer and 0.14 M saline solution). The column was then eluted first with 0.05 M phosphate buffer until 5 ml of effluent was collected, and was then eluted with 0.5 M sodium chloride. Effluent was collected in 0.5 ml fractions into tubes containing 0.1 ml of 0.1 M acetic acid.

RIA for LH-RH was performed according to the method described by Arimura et al. (1973). The diluent used in RIA for LH-RH was 1% bovine serum albumin made up in 0.01 M phosphate buffer, 0.14 M sodium chloride, and 0.025 M ethylene-dinitrotoacetate acid disodium, pH 7.4. To each assay tube (9 × 70 mm) was added 0.4 ml diluent, 0.1 ml standard or sample, 0.1 ml labelled hormone (approximately 5,000 cpm) and 0.1 ml diluted antiserum (the final dilution was 1 : 17,500). The contents were mixed well and were incubated at 4°C for 24 hours. Separation of the free from the conjugated fraction was performed by adding 1 ml of ice cold dextran coated charcoal suspension to the sample, which was mixed well and left at 4°C for 30 min. After centrifugation at 2,500 rpm at 4°C for 10 min, the supernatant was decanted into a counting tube and radioactivity was determined on an Aloka Auto-gamma meter (Model JDC-751).

Cross reactivity of the antiserum was checked with some LH-RH analogues and pituitary trophic hormones by its ability to replace 125I-LH-RH.

Serum and pituitary FSH and LH values were determined by the double antibody RIA method (Niswender et al., 1968; Midgley, 1967). Antigens for radioiodination, antibodies and reference standards were supplied by National Institute of Arthritis and Metabolic Diseases (NIAMD), Bethesda, Maryland, U.S.A.: NIAMD-Rat FSH-I-1, NIAMD-Anti-Rat FSH serum-6 (used at a dilution of 1 : 2,500), NIAMD-Rat LH Serum-I (used at a 1 : 37,500 dilution), and NIAMD-Rat LH-RP-1 (reference standard) were used. Detailed procedures were carried out according to instructions provided by NIAMD.

Adult female albino rats of the Wistar strain, which showed a regular 4 day cycle, were ovariectomized and they were then sacrificed on the 7th day by exsanguination from the femoral artery. As controls, 20 female rats of the same strain were used at each estrous cycle. The stalk median eminence of the hypothalamus was excised, homogenized in 0.1 N HCl, pH was adjusted to 7.4 with 1 N NaOH, the homogenate was centrifuged, and the supernatant applied to a CM Sephadex C-25 column. The column was eluted in a similar way as described previously. The elution pattern of LH-RH was checked on a CM Sephadex C-25 column while supernatant of the rat brain homogenate, which had been mixed with 3H-LH-RH, was applied. The amount of LH-RH eluted was measured by the method of Lowry et al. (1951). The radioactivity of 3H-LH-RH was measured in a Packard Tricarb liquid scintillation spectrometer (Model 3330). The same fractions were collected when the median eminence extract was purified on this column. One tenth ml aliquot of the eluate was subjected to radioimmunoassay. No corrections for extraction and purification losses were made.

Immature female albino rats of Wistar strain, 20 days old, were injected subcutaneously with an aqueous suspension of 30 μg of estradiol benzoate, and sacrificed 1 and 7 hours after the injection, respectively. The LH-RH content of the median eminence, as well as the FSH and LH levels in the pituitary gland were determined in the same manner as described above.

Results

Binding capacity of the antiserum to the 125I-LH-RH without added unlabelled hormone was 29%, under the conditions used. This B/T ratio was set as 100 (B0) and the B/B0 ratios were obtained as 10 to 5,125 pg cold LH-RH were added (Fig. 1). Almost complete inhibition was produced by 5,125 pg of LH-RH.

Of the four LH-RH analogues and fragments of LH-RH tested for crossreactivity to replace radioiodinated LH-RH in our system, two compounds (a and b) showed parallel displacement curves (Fig. 1). FSH and LH of either rat and human origin, ACTH, TRH, prolactin, oxytocin and vaso-
pression (e through m) did not crossreact with this antiserum.

The recovery of cold LH-RH added to the homogenate of the rat cerebral cortex as determined by RIA was 41% when 500 ng were used. Tritiated LH-RH (50,000 dpm) and 100 μg of cold LH-RH were applied to the CM Sephadex column; the elution patterns of non-radioactive and tritiated LH-RH were identical when the column was eluted with 0.5 M NaCl (Fig. 2).

**Fig. 1.** A standard curve of LH-RH and crossreactivities of LH-RH analogues and other pituitary trophic hormones with anti LH-RH serum. Curves of substances a & b completely overlapped with that of LH-RH.

Amounts used

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amounts (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a: His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂</td>
<td>160-1,280</td>
</tr>
<tr>
<td>b: Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂</td>
<td>160-1,280</td>
</tr>
<tr>
<td>c: pGlu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH</td>
<td>160-1,280</td>
</tr>
<tr>
<td>d: pGlu-His-Leu-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂</td>
<td>160-1,280</td>
</tr>
</tbody>
</table>

**Effect of ovariectomy upon the LH-RH and gonadotropin levels**

The overall average content of LH-RH in the stalk median eminence of 16 female rats at various stages of the estrous cycle was 923 pg per animal. Seven days after ovariectomy, the LH-RH level showed a significant rise, and reached 4,192 pg per rat (a 4.5 times increment). Serum FSH and LH levels increased significantly following castration; the former showed a 3.9 and the latter a 2.7 fold increase respectively. The pituitary contents of both FSH and LH were also significantly increased (Table 1).

**Effect of an estradiol injection in immature female rats upon the hypothalamic LH-RH and pituitary gonadotropin**

The LH-RH content of the hypothalamus in the immature female rat was, on an average, 671 pg per animal, which was slightly, but not significantly, lower than the adult values. This LH-RH value was significantly raised one hour after estradiol injection, to a value of 1,020 pg per hypothalamus. The mean LH-RH concentration was further increased at 7 hours, to a value of 1,189 pg per rat, although the difference was not statistically significant. The pitui-
Table 1. The effect of castration on hypothalamic LH-RH and gonadotropins in mature rats

<table>
<thead>
<tr>
<th></th>
<th>LH-RH (pg/hypoth.)</th>
<th>Gonadotropins</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>serum (ng/ml)</td>
<td>LH pituitary (µg/pit.)</td>
<td>serum (µg/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>923.0±139.4 (n=16)</td>
<td>4.18±0.49</td>
<td>15.14±1.57</td>
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<tr>
<td>Castration</td>
<td>4,192.0±539.4*** (n=5)</td>
<td>11.20±1.37**</td>
<td>24.16±1.20*</td>
</tr>
</tbody>
</table>

* p<0.05. ** p<0.001.

Table 2. The effect of estradiol administration on hypothalamic LH-RH and pituitary gonadotropins in immature rats

<table>
<thead>
<tr>
<th>Time after estradiol injection</th>
<th>LH-RH (pg/hypothal.)</th>
<th>LH (µg/pit.)</th>
<th>FSH (µg/pit.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>671.0±137.6 (n=10)</td>
<td>13.44±1.10</td>
<td>94.09±8.07</td>
</tr>
<tr>
<td>1 hour</td>
<td>1,020.0±46.1* (n=10)</td>
<td>14.08±1.02</td>
<td>97.09±6.22</td>
</tr>
<tr>
<td>7 hours</td>
<td>1,189.2±132.3 (n=10)</td>
<td>19.37±2.64*</td>
<td>117.20±11.01</td>
</tr>
</tbody>
</table>

* p<0.05.

The biological activity of the iodinated product was not checked in the present experiment, but the RIA system used here seems to be acceptable for the determination of LH-RH between the ranges shown in the standard curve. When assays were performed on material incubated for 24 hours, the standard curve became almost flat below 40 pg., and the paradoxical binding effect (Arimura et al., 1973; Matsukura et al., 1971) was not observed under the present experimental conditions.

Since the antiserum used in this experiment had been generated by LH-RH conjugated with HSA at the N-terminus, it showed cross-reactivity with LH-RH analogues which had the identical C-terminal heptapeptide amide sequence. Arimura et al. (1975) found that when they used the same antiserum, the last tetrapeptide fragment of the LH-RH amide showed a considerable cross-reactivity. Although none of these analogues has been found to exist in the organism, the possibility exists that RIA system measured not only the pure LH-RH, but also some of its metabolites. No specific measures have been taken to eradicate peptidases activity in the tissue which might inactivate LH-RH, under a certain experimental condition (Griffiths et al., 1973). To minimize such an effect, however, the hypothalamic tissue was extracted and processed for RIA at 4°C, immediately following exsanguination of the animals, and the control and pretreated groups were always subjected to the same experimental procedures performed simultaneously.

The negative feed back phenomenon in ovariectomized rats, particularly the castration cells in the anterior pituitary, is well known. On the other hand, only indirect evidence has been reported to support the increased hypothalamic activity following
castration (Harris, 1955; Kobayashi, 1956). Our data demonstrated an apparent increase of the radioimmunoreactive LH-RH in the hypothalamus 7 days after ovariectomy. FSH and LH both in the blood and the pituitary of the same animals also showed a significant increase. It can be argued that ablation of the effect of steroids by ovariectomy caused an increase of the hypothalamic contents of LH-RH, which induced an augmented production and the release of pituitary FSH and LH.

The effect of a relatively large dose of estrogen upon the hypothalamus is to cause ovulation in immature female rats. This has been known as the Hohlweg's phenomenon (Hohlweg, 1936). The balance of hitherto available evidence supports the view that estrogen exert its effect upon both the hypothalamus and anterior pituitary. Our present data suggest that a large dose of estradiol caused a significant increase of LH-RH content in the hypothalamus of immature rats. It should be noted that this response occurred one hour after the injection.

Igarashi (1967) reported an increase of pituitary FSH content in immature rats 60 min after the intravenous administration of estrogen, while Kobayashi (1968) observed a decrease of pituitary LH 96 hours following estrogen injection. Our results showed that the pituitary LH level increased significantly 7 hours after estrogen administration.

As has been pointed out, our RIA system has still to be improved in regard to its specificity and recoveries. With these reservations, however, the present experiments have clarified the mechanism involved in the steroid feed back process by demonstrating changes in LH-RH content of the hypothalamus.

Acknowledgements

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