Regulation of LHβ Subunit mRNA in Immature Female Rats during GnRH Agonist Treatment

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Abstract

In order to determine the changes in the expression of LHβ messenger ribonucleic acid (mRNA) during GnRH agonist (GnRHa) treatment (0.94 mg/28 days), the concentration of the mRNA of LHβ was assessed together with the serum LH concentration, pituitary LH content and LH response to GnRH at various times during long-acting GnRHa treatment in immature female rats.

The serum LH concentration was increased at hour 1, gradually decreased starting at approximately hour 3 and had returned to the control level on day 28. Pituitary LH began to decrease at hour 3. The concentrations of LHβ mRNA were not significantly different from those in the control group from hour 1 to hour 18, but were lower from day 3 to day 28. Serum LH response to native GnRH (1 µg) began to be inhibited on day 7.

These results indicate that the short term treatment with GnRHa stimulates the release of preformed LH rather than synthesis of LHβ mRNA and that the long term treatment inhibited the expression of LHβ mRNA in a time dependent manner.

Recently GnRH agonists (GnRHa) have come to be used for the treatment of endometriosis, precocious puberty, breast cancer, prostate cancer and many other human diseases, but the mechanisms involved are poorly understood. The mechanism of GnRHa inhibitory action on the gonadal axis is thought to be the down-regulation of LH secretion from the pituitary, but the loss of LH bioactivity also seems to be one of the main mechanisms of GnRHa inhibitory action (Meldrum et al., 1984).

In this study the changes in the mRNA of LHβ, which seems to be an important rate-limiting factor in the synthesis of mature gonadotropin, was assessed during GnRHa treatment in the female immature rat in vivo in order to determine the changes in the expression of LHβ mRNA during the stimulatory and down-regulatory phases of GnRHa treatment.

Materials and Methods

Animals and sample collection

Immature female Sprague-Dawley rats (21 days old) were treated s.c. with a sustained-release formulation of GnRHa (TAP-144-SR,
Takeda Chemical Ind., Ltd.) at a dose of 0.94 mg/rat and were sacrificed by decapitation 1, 2, 3, 4, 5, or 18 hours or 3, 7, 14 or 28 days after GnRHa injection. The pituitaries were harvested under sterile conditions, and were immediately quick-frozen in liquid nitrogen and stored at −70°C until subsequent RNA extraction. Trunk blood was collected, and the serum was stored at −20°C until assayed. TAP-144-SR is a depot formulation of a GnRHa (D-Leu⁶/Pro⁹-ethylamide) using copolymers composed of lactic acid/glycolic acid which allows release of the GnRHa over 4 weeks. (Ogawa et al., 1989). In the previous study this sustained GnRHa of 0.94 mg/28 days delayed vaginal opening in immature female rats (Yanagisawa et al., 1988). Therefore, we used the same dosage of GnRHa on immature female rats as in the previous study.

To monitor the pituitary response to GnRH, blood samples were obtained by heart puncture under light ether anesthesia before and 30 minutes after sc injection of 1 μg of native GnRH (Lutamin, Daiichi Pharmaceutical Co., Ltd.) on day 0, 7, 14, 21 and 28.

For RNA extraction from pituitaries obtained from day 0 to day 3, 2-3 pituitaries were combined to get a sufficient amount of RNA.

**Preparation of mRNA**

The preparation of rat pituitary mRNA for assay by dot blot hybridization was done as described by Abbot et al. (1988). Individual pituitaries were homogenized in 200 μl of homogenization buffer (0.15M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.1% (v/v) Triton). A sample of the homogenate (20 μl) was removed and diluted with phosphate-buffered saline for later determination of pituitary LH content. Nuclei were pelleted by centrifugation at 15000 g, and the supernatant was removed. Proteins in the supernatant were denatured adding 1.5% (w/v) sodium dodecylsulphate (SDS), 35 mM EDTA (pH 8.0) and 0.35 mg proteinase K/ml. After incubation at room temperature for 10 minutes, the proteins were extracted from the supernatant with phenol (250 μl) and reextracted with chloroform (220 μl). Finally, the RNA was precipitated overnight with 2.2 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate at −70°C. The RNA was recovered by centrifugation at 15000 g for 30 minutes at 4°C and dissolved in sterile distilled water. Optical densities were measured at 260 and 280 nm with a UV spectrophotometer. The optical density ratios of absorbance at 260 nm to that at 280 nm were usually between 1.8 and 2. Samples were stored at −70°C until diluted for analysis.

**Synthetic labeled oligodeoxynucleotide**

A synthetic labeled oligodeoxynucleotide was produced as described by Gharib et al. (1986).

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![Fig. 1. Determination of the size of the mRNA encoding the precursor of the rat LHβ subunit. Five micrograms (left lane) and 0.5 μg (right lane) of RNA extracted from the pituitary glands were subjected to the autoradiogram. The position of the origin and the 0.7 kb (LHβ mRNA) are shown in the figure. Pituitary RNA separated by electrophoresis in denaturing agarose gel showed a single band for the LHβ mRNA (0.7 kb) as identified by Northern blotting.](image)
In brief, an oligodeoxynucleotide (probe) corresponding to a portion of the coding region of the rat LHβ-subunit (24 nucleotides from amino acids +29 to +36) was synthesized on an automated DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The probe was 5'-end-labeled using [γ-32P]ATP (259 TBq/mmol) and polynucleotide kinase to achieve a specific activity of 1.5 × 10^6 cpm/pmolDNA (≈ 1.0 × 10^10 cpm/μgDNA).

Five micrograms of RNA (OD260) was subjected to electrophoresis on a 1.0% (wt/vol) agarose gel, transferred to a nylon filter by diffusion blotting and hybridized as described above.

Assay of mRNA levels

Portions of five and one μg of each RNA sample were applied to a genescreen hybridization membrane which was held on a 96 place manifold. The filters were baked at 80°C for 2 hours before prehybridization to block nonspecific binding of labeled cDNA. The baked filter was incubated at 65°C for 4 hours with 5 ml of prehybridization buffer before incubation with 50–100 ng of [32P]cDNA for 3 hours at 60°C. The prehybridization buffer consisted of 5×SSPE (1×SSPE=0.15 M NaCl, 10 mM NaH2PO4, 1 mM EDTA), 10×Denhardt's solution (1×Denhardt's=0.02% polyvinyl pyrrolidone, 0.02% Ficoll), 0.1% SDS and 100 μg/ml of HMRNA (Homo Mixture I RNA). The hybridization buffer consisted of 5×SSPE, 10×Denhardt's solution, 0.3% SDS, 100 μg/ml of HMRNA and 2×10^6 cpm/ml of labeled probe.

Following hybridization, excess unbound [32P]cDNA was removed by sequential washing with constant agitation in buffers as follows: 4 times in 200 ml, 6×SCC (1×SCC=0.15 M NaCl, 0.015 M Na citrate) at room temperature for 5 min and once in 200 ml 6×SCC at 60°C for 3 min. Washed filters were exposed to X-ray film and autoradiographed for 16–24 hours at −70°C. The mRNA content of each dot was determined by densitometry of autoradiograms with a densitometer (LKB, Ultrascan XL).

Hormone assays and statistical analyses

Serum LH

Serum LH concentrations were increased at hour 1 (p<0.05) and continued to rise until hour 2 (p<0.01). Thereafter, the levels decreased gradually but were higher than those measured in the intact control animals on day 3 (p<0.01). On day 28 the concentrations had returned to the control levels (Fig. 2, 3).

Serum LH responses to GnRH

Serum LH responses to GnRH (1 μg) in GnRHa treated immature rats are shown in Fig. 4. On day 7, LH responses (increase % of the preinjection values) were inhibited significantly (p<0.05) as compared with those in the control group, and this blunted response remained unchanged up to 4 weeks after GnRHa administration.

Pituitary LH content

The pituitary LH content, as shown in Fig. 2 and Fig. 3, decreased gradually after GnRHa administration. At hour 3 the LH content was significantly lower than that at hour 0, and it continued to drop slowly until day 3, after which time it remained unchanged up to day 28.

LHβ mRNA

Fig. 5 and Fig. 6 show X-ray autoradiographic visualization of the LHβ mRNA in the total RNA (5 μg) extracted from 1–3 pituitary glands of intact rats. LHβ mRNA concentrations in the GnRHa-treated group were not significantly different from those in the control group from hour 1 to hour 5 or at hour 18 as shown in Fig. 2, but the mRNA concentrations in the GnRHa-treated animals from day 3 to day 28 (p<0.05) were lower than those in the
Fig. 2. Responses of serum LH (mean±SE, n=5), pituitary LH content (n=5) and LHβ mRNA (n=3-4) at hour 1, 2, 3, 4, 5, and 18 following GnRHa administration to immature female rats. The levels of LHβ mRNA are expressed as a percentage of the control values. The hatched bars are data from GnRHa treated rats. Stars indicate statistical significance compared to control level: * p<0.05, ** p<0.01.

Fig. 3. Responses of serum LH (mean±SE, n=5), pituitary LH content (n=5) and LHβ mRNA (n=5) to GnRHa administration on day 3, 7, 14 and 28 in immature female rats. The levels of LHβ mRNA are expressed as the percentage of the control. The hatched bars are data from GnRHa treated rats. Stars indicate statistical significance compared to control level: * p<0.05, ** p<0.01.
**Fig. 4.** Serum LH responses to GnRH (1 µg/rat sc) in intact immature female rats (upper panel) and in GnRHa-treated immature female rats (lower panel). Blood samples were obtained before and 30 minutes after native GnRH administration on day 0, 7, 14, 21 and 28 (mean±SE, n=5). LH responses (increase % of the preinjection values) were inhibited significantly (p<0.05) as compared with those in the control group from day 7 to day 28.

Control

GnRH-agonist treated

0
1
2
3
4
5

Hours after GnRH agonist injection

**Fig. 5.** Rat LHβ mRNA Northern blot hybridization analysis. X-ray autoradiographic visualization of LHβ mRNA in the total RNA (5 µg) extracted from 3 pituitaries of immature female rats at 0, 1, 2, 3, 4 and 5 hours after GnRHa administration.

**Ovarian weight**

Ovarian weights of the GnRHa-treated group were 17.7±1.7 mg/ovary (mean±SE, n=5) on day 0 and 18.3±0.8 mg (control; 17.1±1.2 mg) on day 3. Thereafter, the weights decreased significantly to 15.2±0.9 mg (22.9±2.3, p<0.05) on day 7, 19.8±2.2 mg (48.4±12.7, p<0.01) on day 14, and 9.2±1.7 mg (33.2±5.8, p<0.01) on day 28 as compared with the control group.

**Discussion**

In the process of pituitary desensitization, GnRHa stimulates the gonadotropin secretion in the early stage (flare-up phenomenon) and inhibits the gonadotropic responses to GnRH and decreases the gonadotropin secretion in the late stage (desensitization). There have been few detailed studies on the changes in LHβ mRNA with time after GnRH administration and most have involved only a few time points. The
present study describes changes in LHβ mRNA together with changes in serum LH, the pituitary LH and LH responses to GnRH at various times after GnRHa administration.

The expression of the gonadotropin subunit mRNA appears to be highly dependent on the frequency and the amplitude of GnRH stimulation. Halsenleder et al. (1988) reported that frequent treatment (7.5-min intervals) and/or a higher dose (125-ng/pulse) induced a decrease in LH secretory responsiveness to GnRH and that treatment with 25 ng-pulses every 30 min maintained LH secretion throughout the 48-h study accompanied by a maximum increase in LHβ mRNA in castrated-testosterone replaced male rats. Dalkin et al. (1989) also reported that LHβ mRNA was increased by frequent stimulation with GnRHa (8- or 30-min pulse intervals, 25-ng/pulse for 24 hours) in castrated-testosterone-treated adult male rats while less frequent stimulation with GnRH (120- to 480-min pulse intervals) did not change LHβ mRNA. Papavasiliou et al. (1986) showed an increase in LHβ subunit mRNA levels within 48 hours of GnRH pulse injection (25-ng/30 min) in castrated testosterone-replaced male rats, but larger doses (75-ng or 250-ng/pulse) produced a smaller increase in LHβ mRNA.

This 25-ng dose which results in a peak serum GnRH concentration of 200 pg/ml is within the range reported to be present in the rat hypophysial-portal circulation (Garcia et al., 1984), and the GnRH pulse interval in castrated male rats is approximately 30 min (Steiner et al., 1982). Therefore, LHβ mRNA, as well as the pituitary GnRH receptor (Katt et al., 1985) appears to be selective and to respond to a narrow range of GnRH pulse frequencies and amplitudes, and this range is the same range as that of the GnRH signal in physiological situations.

There are few reports on the effects of short term treatment in vivo with long acting GnRHa on LHβ mRNA. We have shown here that the LHβ mRNA concentration do not change within 18 hours after administration of GnRHa. The present data show that the administration of GnRHa causes no increase in the concentration of

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### Fig. 6. Rat LHβ mRNA Northern blot hybridization analysis.

- **Control**
- **GnRH agonist treated**
- **Day after GnRH agonist injection**

<table>
<thead>
<tr>
<th>Day after injection</th>
<th>0</th>
<th>3</th>
<th>14</th>
<th>28</th>
</tr>
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</table>
| X-ray autoradiographic visualization of LHβ mRNA in the total RNA (5 μg) extracted from 1-2 pituitaries of immature female rats 0, 3, 14 and 28 days after GnRHa administration. | }
LHβ mRNA while serum LH increases rapidly and dramatically with a decrease in pituitary LH at hour 3 and thereafter up to hour 18. These data indicate that short term treatment with GnRHa stimulates the release of preformed LH rather than the synthesis of LH and LHβ mRNA.

As to the long term effects of GnRHa, serum LH concentrations in GnRHa-treated intact animals were higher on day 3 and day 14 and were not significantly different from those in the control animals on days 7 and 28. These data are similar to those of Kim et al. (1988), that is, serum LH is higher in GnRHa (1 µg/day)-treated intact adult male rats on day 1 and not significantly different from that in the control on days 7 and 28. Lalloz et al. (1988) also showed no change in serum LH and a significant decrease in pituitary LH after 14 and 28 days of continuous infusion of GnRH (290 µg and 14 µg/day, respectively) in intact male rats. However, in contrast to our results Kim et al. (1988) observed no difference in the intact male rats treated with GnRHa (1 µg/day sc) and those in the control group on day 1 or day 7.

An explanation for the differences may be found in the dose and mode of GnRHa administration. In our study, a higher dose of GnRHa (0.94 mg/28 days) was used and this might cause a more rapid decrease in LHβ mRNA. Furthermore, continuous administration of GnRHa has been shown to result in greater inhibition of LH secretion than intermittent administration (Akhtar et al., 1983).

In the process of gonadotrope desensitization, GnRHa did not change the expression LHβ mRNA with increasing LH release following short term treatment and inhibited expression of LHβ mRNA in a time-dependent manner during long term treatment. The availability of this LHβ mRNA may be one factor limiting LH biosynthesis, since pituitary LH content falls and pituitary responses to GnRH are suppressed in concert with LHβ mRNA, even though LHβ gene expression is not involved in the marked reduction in pituitary content during the early stage of GnRH treatment.
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References


