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Gonadotropin-releasing hormone differentially regulates expression of the genes for luteinizing hormone α and β subunits in male rats

(mRNA quantitation)

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ABSTRACT **Gonadotropin-releasing hormone (GnRH) and gonadal steroids regulate synthesis and release of luteinizing hormone (LH). GnRH is secreted intermittently by the hypothalamus, producing pulsatile LH release, and a pulsatile GnRH stimulus is required to maintain LH secretion. We report the regulatory effects of GnRH pulse injections on pituitary concentrations of LH α and β subunit mRNAs in a castrated/testosterone-replaced male rat model. Replacement with physiologic amounts of testosterone decreased concentrations of both LH subunit mRNAs. GnRH pulse injections (10–250 ng per pulse given every 30 min for 48 hr) increased both mRNA concentrations, but the dose response patterns were markedly different. α subunit mRNA was increased by all GnRH doses but not the levels seen after castration alone. In contrast, LH β subunit mRNA concentrations showed a marked dependence on GnRH dose. Maximal responses, to values similar to those in castrates, occurred after 25-ng GnRH pulses, and larger doses produced a smaller increase in LH β subunit mRNA. Both the acute LH secretory response to GnRH and the number of GnRH receptors followed a pattern similar to the LH β subunit mRNA concentration and were maximal after the 25-ng GnRH dose. These results show that GnRH can differentially regulate LH subunit mRNAs and suggest that concentrations of LH β subunit mRNA may be a limiting factor in GnRH-stimulated LH release.**

Previous studies (1–6) suggested that gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner, and simultaneous measurements of GnRH in hypothalamic-portal blood and luteinizing hormone (LH) in jugular venous blood have shown that each GnRH pulse is followed by acute secretion of LH (7). The intermittent nature of the GnRH stimulus is essential for the maintenance of LH secretion, and administration of GnRH by continuous infusion to experimental animals or humans results in desensitization of LH release (8–10). GnRH action on the gonadotroph is initiated by binding to specific membrane receptors (11), and calcium has been postulated as the second messenger involved in mediating LH release (12, 13). The results of studies in GnRH-deficient animals and humans have suggested that GnRH is essential for LH synthesis (14, 15), but little is known of the regulatory effects of GnRH on the synthesis of the LH subunits or their mRNAs. Within a species a single gene codes for the α subunit of all glycoprotein pituitary hormones, whereas different genes code for the β subunits. Recently, cDNAs coding for LH α and β subunits were isolated (16, 17), and we have used these cDNAs as hybridization probes to develop a sensitive assay for mRNAs using dot-blot hybridization of extracted cytoplasmic RNA from rat pituitaries.¶

We employed this mRNA quantitation assay to study the effects of GnRH pulse injections on expression of the genes for LH α and β subunits in gonadectomized male rats that were given testosterone implants. In this animal model endogenous GnRH secretion is markedly reduced, as judged by infrequent serum LH pulses (18). Thus assessment of the effects of exogenous GnRH pulse injections, given to mimic the intermittent physiologic stimulus, is not unduly complicated by endogenous GnRH secretion. In addition, experiments are performed in the presence of stable physiologic concentrations of testosterone, which avoids the potential effects of fluctuating serum testosterone levels in intact animals (19). This model has been previously used to study the effects of testosterone in regulating GnRH receptor and gonadotropin responses to a pulsatile GnRH stimulus (19). In the present study we examined the effects of different doses of GnRH per pulse injection on GnRH membrane receptors and LH subunit gene expression to assess their role in the regulation of gonadotropin secretion.

MATERIALS AND METHODS

Male Sprague-Dawley rats (250–300 g) were castrated and implanted with two 20-mm Silastic implants containing testosterone, to produce a serum testosterone concentration of 2.3 ± 0.12 ng/ml (mean ± SEM). Beginning immediately after recovery from anesthesia, GnRH pulse (10–250 ng per pulse) or control saline injections were given every 30 min for 48 hr through a carotid cannula (19). Blood samples were obtained immediately before and 20 min after the last GnRH pulse injection for measurement of LH and follicle-stimulating hormone (FSH). For comparison, groups of intact and castrated male rats were also given saline pulses for 48 hr. After sacrifice, anterior pituitaries were immediately removed under sterile conditions, quickly frozen, and stored at −70°C prior to measurement of GnRH receptors or mRNAs.

GnRH pituitary membrane receptors were measured by saturation analysis using a pituitary homogenate preparation and ^{125}I-labeled [D-Ala^6]GnRH ethylamide as radioligand as previously described (19, 11). Results are expressed as fmol of radioligand bound per mg of pituitary protein.

For mRNA quantification pituitaries were individually homogenized in 10 mM Tris/0.5% Nonidet P-40/1 mM EDTA, pH 7.4, and the homogenate was centrifuged (13,000 × *g* for 5 min). DNA was measured (20) in the nuclear pellet, and total RNA was extracted from the cytosol supernatant by using a phenol/chloroform/isoamyl alcohol mixture (100:100:1,

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Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

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vol/vol). RNA was measured by absorbance at 260 nm and samples were spotted onto nitrocellulose filters and fixed by heating at 80°C under reduced pressure for 90 min (21, 22). The amounts of RNA used were 3 μg per spot for intact or castrated + testosterone rats, 0.5 μg per spot for castrated rats in α subunit mRNA assays, and 8 μg per spot for all rats in LH β subunit mRNA assays. Rat LH α and β subunit cDNA inserts were purified (23), and 100–200 ng was nick-translated (24) to a specific activity of 2–5 × 10^8 cpm/μg. The labeled probes migrated as a single band on 4% polyacrylamide gel electrophoresis. In initial experiments we determined the saturating concentrations of labeled cDNA probes required for the amounts of RNA applied to the nitrocellulose filters. In subsequent studies saturating concentrations of labeled cDNA probes (40–80 ng/5 ml of hybridization buffer) were used to hybridize to mRNA (18–20 hr of hybridization), and, after exposure to film, nitrocellulose spots were excised and their radioactivities were measured. Results are expressed as pg of probe bound per 100 μg of pituitary DNA, normalized for total pituitary RNA yield, and adjusted for pituitary DNA content and radioactive decay. Prior experiments showed linear relationships of cDNA hybridized to increasing amounts of pituitary RNA (0.5–10 μg per spot). Pooled pituitary RNA from intact rats was measured in all assays and the assay coefficient of variation was 5% intraassay and 28% interassay.¶

Results were analyzed by paired *t* tests (LH responses to GnRH) or by analysis of variance and differences between treatment groups were assessed by Duncan's multiple range test.

RESULTS

The effects of GnRH pulse injections given every 30 min for 48 hr on pituitary GnRH receptors, LH α and β subunit mRNA concentrations, and acute LH and FSH responses are shown in Fig. 1.

Pituitary membrane GnRH receptors showed a biphasic pattern after GnRH pulse injections (Fig. 1 *Top*). In castrated/testosterone-replaced animals pulsatile GnRH increased the number of GnRH receptors, and maximal concentrations were seen after 25 ng per pulse. The maximal values were similar to receptor concentrations observed in male rats that received saline pulses for 48 hr after castration (600 ± 50 fmol/mg of protein, mean ± SEM). Higher doses of GnRH per pulse reduced GnRH receptors; a similar biphasic pattern of receptor regulation has been observed after GnRH pulses (19) and after continuous infusions of GnRH in intact or castrated male rats (25).

α subunit and LH β subunit mRNA concentrations are shown in Fig. 1 *Middle*. In castrated/testosterone-replaced animals given 0.9% NaCl pulse injections, α subunit mRNA was decreased by 50% compared to intact controls (α subunit mRNA in intact rats, 105 ± 5 pg of α subunit cDNA bound per 100 μg of pituitary DNA; n = 10). Administration of GnRH pulses resulted in an increase in α subunit mRNA that was maximal after 75 ng of GnRH per pulse but did not reach the levels present in castrated animals (220 ± 18 pg of cDNA bound 48 hr after castration without testosterone replacement; n = 8). The effects of GnRH pulses on LH β subunit mRNA were markedly different and showed a biphasic effect of GnRH dose per pulse. In testosterone-replaced rats given saline pulses, LH β subunit mRNA concentrations were suppressed to 50% of the amount found in intact rats (20 ± 2 pg of LH β subunit cDNA bound per 100 μg of pituitary DNA; n = 11). Administration of GnRH increased LH β subunit mRNA, with maximal concentrations (2.5-fold increase over saline controls) occurring after 25 ng of GnRH per pulse. These values were similar to those for rats castrated for 48 hr (27 ± 2.5 pg of cDNA bound; n = 12).

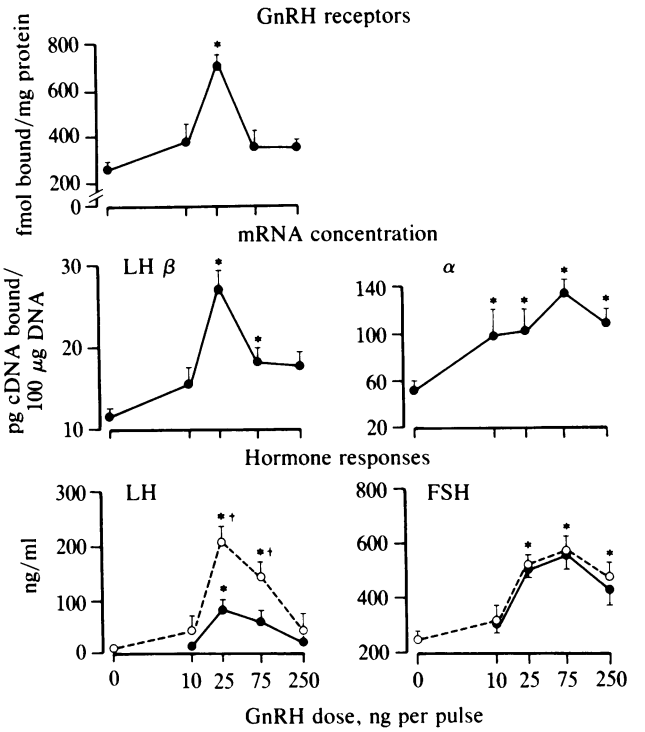

FIG. 1. Responses of pituitary GnRH receptors, α subunit and LH β subunit mRNA, and acute gonadotropin secretion to GnRH pulses given every 30 min for 48 hr to castrated/testosterone-replaced male rats. (*Top*) Pituitary GnRH receptor concentrations. (*Middle*) α subunit and LH β subunit mRNA concentrations. (*Bottom*) Acute LH and FSH responses. Serum LH and FSH were assessed in blood samples obtained immediately before (basal, ●—●) and 20 min after (peak, ○---○) the last GnRH pulse. The 0 point on the horizontal axis represents saline-pulsed controls. Each point represents mean ± SEM; n = 4–12 per group. *, $P < 0.05$ vs. saline-pulsed controls; †, $P < 0.05$ vs. basal LH. For comparison with results in this figure, the values for intact and castrated rats, respectively, receiving saline pulses for 48 hr were as follows: GnRH receptors, 305 ± 48 and 600 ± 50; LH β subunit mRNA, 20 ± 2 and 27 ± 2.5; α subunit mRNA, 105 ± 5 and 220 ± 18; basal serum LH, 15 ± 3 and 598 ± 93; and basal serum FSH, 230 ± 15 and 648 ± 42. These data are mean ± SEM and are expressed in the same units shown in the figure.

However, after higher GnRH doses LH β subunit mRNA concentrations decreased to the levels present in intact rats.

The acute gonadotropin responses to GnRH after 48 hr of GnRH pulses are shown in Fig. 1 *Bottom*. Serum LH values showed dose-dependent changes, and maximal LH responses were seen after the 25 ng per pulse dose. Both basal and stimulated LH values declined after the higher doses. Basal serum FSH was increased after GnRH pulses (25–250 ng per pulse) but tended to be lower after the highest GnRH dose. Acute FSH responses to GnRH were not seen, reflecting the fact that the half-life of FSH is longer than that of LH and serum FSH did not fall during the 30-min interval between GnRH pulses.

DISCUSSION

This study concentrated on the effects of GnRH on expression of the genes encoding LH α and β subunits in the male rat. Our data show that an excess of α subunit mRNA over LH β subunit mRNA is present in the pituitary of the intact male rat, and this relationship is maintained in castrated rats after testosterone replacement and after pulsatile stimulation by GnRH (note the different scales for α subunit and LH β subunit mRNA in Fig. 1). These findings are consistent with previous studies in ovariectomized ewes, in which an excess of α subunit mRNA over LH β subunit mRNA was observed

(26). In that study estrogen treatment resulted in a decrease in both α subunit and LH β subunit mRNAs, but α subunit mRNA was still present in excess. Additionally, *in vivo* estimates of α subunit and LH β subunit mRNAs by using other quantitation systems have also shown an excess of α subunit mRNA in the rat anterior pituitary (27). The magnitude of the quantitative differences found in the present study cannot be attributed to differences in probe sizes that enter into our calculations of mRNA concentration. Rather, the excess α subunit mRNA probably represents mRNA molecules available for the synthetic pools of LH, FSH, and thyroid-stimulating hormone (TSH) (16). The precise quantitative relations between α subunit mRNA and the specific β subunit mRNAs are uncertain and will require further clarification as cDNA probes for FSH β subunit become available. However, the observation of excess α subunit mRNA is consistent with previous *in vitro* studies using cultured rat pituitary cells (28), in which free α subunit was present in large excess over free β subunit and intact LH, and free α subunit was secreted into the culture medium whereas virtually no free LH β subunit was secreted.

The correlation between LH β subunit mRNA concentrations and acute LH secretion is intriguing and suggests that LH β subunit mRNA is regulated by GnRH and may be the limiting step in the synthesis and release of LH. This is supported by the studies in cultured pituitary cells (28), which showed only small amounts of free cytoplasmic LH β subunit and minimal secretion of free β subunit into the culture medium. Moreover, other investigators (29) have reported that coupling of the α and LH β subunits to form intact LH occurs early in the synthetic process, before glycosylation and transport of the newly synthesized subunits to secretory granules has occurred. In these circumstances, the availability of LH β subunit mRNA at the time of stimulation by GnRH would be expected to be limiting in the synthesis and subsequent release of LH.

The precise mechanisms of the observed differential regulation of the α subunit and LH β subunit mRNAs by GnRH are unknown and may involve differences in transcription rates, mRNA splicing or other processing, or differential stability of these mRNAs in the cytoplasm. However, our data suggest that LH secretory capacity is strongly dependent on the availability of LH β subunit mRNA. The observation that GnRH membrane receptors also varied in a similar manner to LH β subunit mRNA is also of interest and suggests that GnRH receptor regulation by GnRH may be an important step in the mechanisms leading to expression of LH β subunit mRNA. Previous *in vivo* studies have demonstrated a positive relationship between the number of GnRH receptors and LH secretion. Both GnRH receptors and serum LH increase in parallel during sexual maturation (30, 31), and receptor concentrations and LH responses to GnRH are maximal on the morning of proestrus in rats (32, 33). These data support the suggestion that the number of GnRH receptors may be an important determinant of LH β subunit mRNA concentrations and LH release. Here again the exact mechanisms involved remain uncertain and await identification of the second messenger systems by which GnRH–membrane receptor interactions regulate LH subunit gene expression.

LH secretion and GnRH receptor number do not always change in parallel. In pituitary cell culture systems, where cells are continuously exposed to GnRH for 10 hr, LH secretion is maintained while a biphasic receptor response is seen (34). The number of receptors initially falls but after 6 hr is increased above baseline and the increase in receptors is dependent both upon the presence of calcium and upon protein synthesis. The reasons for the differences between these results and those reported here are uncertain, but they may be related to our use of a pulsatile GnRH stimulus to mimic normal physiological secretion. Our results with a pulsatile stimulus suggest that regulation of both GnRH membrane receptors and expression of LH β subunit mRNA are markedly dependent upon the dose of GnRH, though the nature of the regulatory mechanisms remains unknown and awaits further studies. However, the present results provide evidence that GnRH can differentially increase mRNAs for the LH subunits and suggest that the densensitization of LH secretion observed after high doses of GnRH may be due, at least in part, to a reduction in the availability of the specific LH β subunit mRNA. In this regard, the effects of GnRH pulse frequency on LH β subunit mRNA may also be important. The frequency of GnRH pulses varies during the estrous and menstrual cycles (6, 35). We have recently shown marked effects of GnRH pulse frequency on both GnRH receptors and LH release, with a 30-min interval being optimal for induction of receptors and acute LH secretion (36). Higher or lower frequencies did not result in the same increase in receptors and were associated with absence of acute LH release. By comparison to the data in the present paper, this suggests that frequency may also regulate LH β subunit mRNA concentrations. The pituitary gonadotrope cell may recognize both the amount and the frequency of the GnRH stimulus, responding by alterations in both GnRH receptor and LH β subunit mRNA concentrations, and the latter determine the availability of LH for secretion.

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