Differential Effects of Gonadotropin-Releasing Hormone (GnRH) Pulse Frequency on Gonadotropin Subunit and GnRH Receptor Messenger Ribonucleic Acid Levels in Vitro*

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ABSTRACT

The hypothalamic hormone, GnRH, is released and transported to the anterior pituitary in a pulsatile manner, where it binds to specific high-affinity receptors and regulates gonadotropin biosynthesis and secretion. The frequency of GnRH pulses changes under various physiological conditions, and varying GnRH pulse frequencies have been shown to regulate differentially the secretion of LH and FSH and the expression of the gonadotropin α, LHβ, and FSHβ subunit genes in vivo. We demonstrate differential effects of varying GnRH pulse frequency in vitro in superfused primary monolayer cultures of rat pituitary cells. Cells were treated with 10 nM GnRH pulses for 24 h at a frequency of every 0.5, 1, 2, or 4 h. α, LHβ, and FSHβ messenger RNA (mRNA) levels were increased by GnRH at all pulse frequencies. α and LHβ mRNA levels and LH secretion were stimulated to the greatest extent at a GnRH pulse frequency of every 30 min, whereas FSHβ mRNA levels and FSH secretion were stimulated maximally at a lower GnRH pulse frequency, every 2 h. GnRH receptor (GnRHR) mRNA levels also were increased by GnRH at all pulse frequencies and were stimulated maximally at a GnRH pulse frequency of every 30 min. Similar results were obtained when the dose of each pulse of GnRH was adjusted to maintain a constant total cumulative dose of GnRH over 24 h. These data show that gonadotropin subunit gene expression is regulated differentially by varying GnRH pulse frequencies in vitro, suggesting that the differential effects of varying GnRH pulse frequencies on gonadotropin subunit gene expression occur directly at the level of the pituitary. The pattern of regulation of GnRHR mRNA levels correlated with that of α and LHβ but was different from that of FSHβ. This suggests that α and LHβ mRNA levels are maximally stimulated when GnRHR levels are relatively high, whereas FSHβ mRNA levels are maximally stimulated at lower levels of GnRHR expression, and that the mechanism for differential regulation of the gonadotropins by varying pulse frequencies of GnRH may involve levels of GnRHR. Furthermore, these data suggest that the mechanisms whereby varying GnRH pulse frequencies stimulate α, LHβ, and GnRHR gene expression are similar, whereas the stimulation of FSHβ mRNA levels may be different. (Endocrinology 138: 1224–1231, 1997)

THE REGULATION of the biosynthesis and secretion of the gonadotropins, LH and FSH, is critical for normal reproductive function. The synthesis and release of these two pituitary glycoproteins are controlled by the complex interaction of multiple factors, one of the most important of which is GnRH, a hypothalamic decapetide. GnRH is released into the hypophysial portal circulation and transported to the anterior pituitary in a pulsatile manner, where it binds to specific high-affinity receptors (1, 2).

GnRH stimulates the secretion of LH and FSH, as well as the biosynthesis of the gonadotropin subunits, α, LHβ, and FSHβ. The stimulation of GnRH biosynthesis and secretion by GnRH is dependent on the pulsatile nature of GnRH delivery to the anterior pituitary. Administration of exogenous GnRH in a continuous fashion results in the down-regulation of gonadotropin subunit messenger RNA (mRNA) levels and of LH and FSH secretion, whereas pulsatile GnRH stimulates mRNA levels and secretion (3–6). This variability in GnRH responsiveness seems to correlate, at least partially, with the concentration of GnRH receptor (GnRHR) on the cell surface (7, 8).

The frequency and amplitude of GnRH pulses secreted by the hypothalamus vary under different physiological conditions (9). It has been postulated that the frequency and amplitude of GnRH stimulation provide signals for the differential regulation of LH and FSH secretion (10). At higher GnRH pulse frequencies, LH secretion increases disproportionately more than FSH secretion, whereas, at lower GnRH pulse frequencies, FSH secretion is favored (11, 12). In recent years, several investigators have provided further support for this hypothesis using in vivo models by showing that the frequency and amplitude of GnRH pulses also determine α, LHβ, and FSHβ mRNA levels.
levels (13–16). This regulation seems to occur at the level of gene transcription (17, 18).

Although the data from most in vivo studies provide evidence in support of frequency-dependent gonadotropin regulation by GnRH, the concept is not supported by other studies (19), and the mechanism of such regulation is not clear. Many of the in vivo models have relied on orchidectomized male rats treated with testosterone to suppress endogenous GnRH release. Testosterone, as well as other steroids and other endogenous factors, may modulate GnRH actions. Indeed, studies in ovariectomized female rats treated with phenoxybenzamine to suppress endogenous GnRH secretion have not produced the same results (20). It also has been suggested that the presence of testosterone is necessary for stimulation of LHβ mRNA levels by pulsatile GnRH (21).

To provide direct evidence of differential regulation of gonadotropin subunit gene expression and gonadotropin secretion by varying GnRH pulse frequencies at the level of pituitary cells, we have used an in vitro model of superfused primary rat pituitary cultures. This model allows for controlled conditions, thereby eliminating the possible effects of neuroendocrine, gonadal, or other extrapituitary factors. We have previously demonstrated the suitability of this system for such studies by showing that superfused pituitary cells maintain prolonged secretory responsiveness to GnRH pulses and that all gonadotropin subunit mRNAs could be upregulated under these conditions (22).

In addition, to investigate whether frequency-dependent regulation of LH and FSH secretion could involve regulation of GnRHR synthesis, we have included determinations of GnRHR mRNA expression in our study. It has been shown previously that GnRH binding and GnRHR mRNA levels are regulated by gonadectomy and sex steroid hormone replacement, through the estrous cycle, and by pulsatile GnRH in vivo (24–26). In this report, we demonstrate the correlation between GnRHR and gonadotropin subunit gene expression in response to varying GnRH pulse frequencies, suggesting a possible mechanism for these differential effects of GnRH.

Materials and Methods

In vitro study protocol

Studies were performed using superfused primary monolayer cultures of rat pituitary cells as previously described (27). Briefly, pituitary cells isolated from 18-day-old male rats were plated in Matrigel-coated superfusion chambers in chemically defined medium [DMEM:Ham’s F-12 (1:1) supplemented with ITS+ Premix, vitamins A and E (200 ng/ml), and EGF (10 ng/ml)]. After 4 days of stationary culture, the chambers were superfused at a constant flow rate of 0.25 ml/min. During the superfusion, GnRH was delivered in pulses (5 min/pulse) for 24 h at the concentrations and frequencies indicated. All cells were harvested 20 min after the last pulse of GnRH. Experimental procedures were approved by the University of Texas Medical School animal research committee.

Preparation of RNA

Total RNA was prepared from cultured cells by the acid guanidinium thiocyanate-phenol-chloroform procedure (28) using RNAzol B (Biotec Laboratories, Inc., Houston, TX). The RNA concentrations were estimated by measuring the A260.

mRNA determinations

Five micrograms of total RNA (A260) from each sample were denatured and subjected to electrophoresis and diffusion blotting onto a Duralon membrane (Stratagene, La Jolla, CA) (29). Each blot was sequenentially hybridized with rat α-subunit, LHβ, FSHβ, GnRHR, and cyclophilin cDNA probes (30). Blots were washed and subjected to phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA), and band densities were quantitated. The amount of total RNA in each sample was internally standardized within each blot by correcting the gonadotropin subunit and GnRHR mRNA levels according to the levels of cyclophilin mRNA.

RIA of LH and FSH

Media samples were collected at 6-min intervals during the final 4 h of superfusion for LH and FSH RIAs. The LH and FSH contents were determined as previously described (27). Specific RIAs were performed according to the procedure recommended by the NIH. The results are expressed in terms of LH and FSH RP-2 standards. The intra- and interassay variations were 8% and 9% for LH and 7% and 9% for FSH, respectively. Total LH and FSH secretion over the 4-h time period of collection was then determined and compared with secretion in control cells not treated with GnRH.

Statistical analysis

Each experiment was repeated at least 4 times. Cumulative data from individual experiments were combined, with the mRNA levels at a GnRH pulse frequency of every 1 h used as a standard for comparison between experiments. Results are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by post hoc comparisons with Fisher’s protected least significant-difference test. In all cases, differences were considered significant if P < 0.05. The errors in the ratios were calculated by standard methods of propagation of errors in computation (31).

Results

Effects of varying GnRH pulse frequencies on gonadotropin subunit mRNA levels in superfused primary pituitary cultures

Primary monolayer cultures of rat pituitary cells were treated with pulses of 10 nm GnRH (5 min/pulse) at varying pulse frequencies (every 0.5, 1, 2, or 4 h) for 24 h. α, LHβ, and FSHβ subunit mRNA levels, corrected for cyclophilin mRNA levels, are shown for each frequency of pulsatile GnRH tested (Fig. 1). Values are the combined results of four independent experiments expressed as a percentage relative to levels at an hourly GnRH pulse frequency. Pulsatile GnRH significantly stimulated α, LHβ, and FSHβ subunit mRNA levels at all pulse frequencies tested. LHβ subunit mRNA levels were stimulated to the greatest extent at a GnRH pulse frequency of every 30 min. At lower frequencies, levels also were increased but to a lesser extent. In contrast, FSHβ subunit mRNA levels were stimulated to the greatest extent at lower GnRH pulse frequencies, such as every 2 h. α subunit mRNA levels were less dependent on GnRH pulse frequency, but seem to follow a pattern similar to that of LHβ; that is, they were stimulated to the greatest extent at GnRH pulse frequencies of every 30 min. The responses of all three (α, LHβ, and FSHβ) mRNA levels to GnRH pulses every 2 h are significantly different from those in response to pulses every 30 min (LHβ - GnRH every 30 min: 116.2 ± 8.6% vs. GnRH every 2 h: 68.8 ± 8.7%, P < 0.0005; FSHβ - GnRH every 30 min: 80.5 ± 4.5% vs. GnRH every 2 h: 101.8 ± 3.1%, P < 0.0005;
Effects of varying GnRH pulse frequencies on GnRHR mRNA levels in superfused primary pituitary cultures

Similarly, GnRHR mRNA levels were significantly increased by pulsatile GnRH at all pulse frequencies tested (Fig. 2). Like \( \alpha \) and LH\( \beta \), GnRHR mRNA levels were increased to the greatest extent at a GnRH pulse frequency of every 30 min; at a GnRH pulse frequency of every 2 h, GnRHR mRNA levels were increased to a significantly lesser degree (GnRH every 30 min: 101.8 ± 9.7\% vs. GnRH every 2 h: 74.2 ± 5.2\%, \( P < 0.05 \)).

Effects of varying GnRH pulse frequencies on LH and FSH secretion in superfused primary pituitary cultures

LH and FSH secretion from the cultured cells into the media were stimulated by pulsatile GnRH at all pulse frequencies tested (Fig. 3). LH secretion was stimulated to the greatest extent at a GnRH pulse frequency of every 30–60 min, whereas FSH secretion was stimulated to the greatest extent at a GnRH pulse frequency of every 1–2 h. LH and FSH secretion were significantly different at a GnRH pulse frequency of every 2 h, compared with every 30 min (LH - GnRH every 30 min: 621 ± 107\% vs. GnRH every 2 h: 394 ± 65\%, \( P < 0.005 \); FSH - GnRH every 30 min: 217 ± 52\% vs. GnRH every 2 h: 412 ± 51\%, \( P < 0.0005 \)). As a result, the ratio of LH:FSH secreted was 3.0 ± 0.6 at a GnRH pulse frequency of every 30 min but only 0.9 ± 0.2 at a pulse frequency of every 2 h.

Effects of varying GnRH pulse frequencies with correction for cumulative dose of GnRH on gonadotropin subunit mRNA levels in superfused primary pituitary cultures

It is possible that the differential effects of varying GnRH pulse frequencies may be caused by differences in total cumulative GnRH exposure rather than to the pulse frequency itself. Therefore, superfused primary monolayer cultures of rat pituitary cells were treated again with pulses of GnRH delivered every 30 min or every 1, 2, or 4 h but with the concentration of GnRH adjusted so that the total cumulative amount of GnRH administered to the cells over 24 h was the same for all frequencies studied. Therefore, cells were treated with 5 nM GnRH every 30 min, 10 nM GnRH every 1 h, 20 nM GnRH every 2 h, or 40 nM GnRH every 4 h. As in the first set of experiments, values represent the combined results of four independent experiments, normalized to cyclophilin mRNA.
levels, and expressed as a percentage relative to levels at an hourly pulse frequency. The results are essentially the same as those observed using a fixed concentration of pulsatile GnRH (Fig. 4). That is, pulsatile GnRH again significantly stimulated $\alpha$, LH$\beta$, and FSH$\beta$ subunit mRNA levels at all pulse frequencies and concentrations tested. $\alpha$ and LH$\beta$ sub-
unit mRNA levels were stimulated to the greatest extent at a GnRH pulse concentration of 5 nm and frequency of every 30 min, whereas FSHβ subunit mRNA levels were stimulated to the greatest extent at a GnRH pulse concentration of 20 nm and frequency of every 2 h (LHβ-GnRH every 30 min: 109.8 ± 8.5% vs. GnRH every 2 h: 65.3 ± 18.2%, P < 0.005; FSHβ-GnRH every 30 min: 79.0 ± 6.1% vs. GnRH every 2 h: 108.3 ± 8.5%, P < 0.005; α - GnRH every 30 min: 107.2 ± 7.2% vs. GnRH every 2 h: 83.3 ± 13.3%, P < 0.05). LH and FSH secretion was again stimulated by pulsatile GnRH at all pulse frequencies and amplitudes tested, with similar results to those observed when a fixed GnRH concentration was used (data not shown).

Effects of varying GnRH pulse frequencies with correction for cumulative dose of GnRH on GnRHR mRNA levels in superfused primary pituitary cultures

Similarly, GnRHR mRNA levels also were regulated by GnRH pulse frequency with an adjusted concentration of GnRH per pulse (Fig. 5). GnRHR mRNA levels were increased to the greatest extent at a GnRH pulse concentration of 5 nm and frequency of every 30 min; at a GnRH pulse concentration of 20 nm and frequency of every 2 h, GnRHR mRNA levels were increased to a significantly lesser degree (GnRH every 30 min: 105.7 ± 8.4% vs. GnRH every 2 h: 78.3 ± 17.5%, P < 0.005).

Discussion

The stimulation of the gonadotropin subunit mRNA levels by pulsatile GnRH has been observed in vivo in rats, as well as sheep and monkeys (3, 4, 6). However, the results of previous in vitro studies have been inconsistent. Haisenleder et al. (32) observed an increase in all three gonadotropin subunit mRNA levels in response to 24 h of pulsatile GnRH in vitro, although the effects were smaller in magnitude than that observed in vivo. A study of the effects of GnRH on LHβ gene transcription in vitro in anterior pituitary fragments in culture showed that LHβ mRNA synthesis was stimulated by pulsatile GnRH (17). In contrast, other studies have failed to see an increase in LHβ mRNA levels after 8–12 h of pulsatile GnRH treatment, although α and FSHβ mRNA levels were increased (5, 33). In our experimental paradigm, α, LHβ, and FSHβ subunit mRNA levels were increased significantly by GnRH at all pulse frequencies tested. This is consistent with our previous report of the stimulation of α, FSHβ, and LHβ mRNA by pulsatile GnRH. α subunit mRNA
levels were stimulated after 10 and 20 h of pulsatile GnRH. FSHβ mRNA levels were stimulated after 4, 10, and 20 h of pulsatile GnRH, whereas LHβ mRNA levels were increased only after 20 h of pulsatile GnRH treatment (22). The differences between our studies and previous in vitro studies may be caused by the different durations of exposure to pulsatile GnRH. Many of the in vitro studies that showed an increase in LHβ mRNA levels by pulsatile GnRH were performed using 48 h of pulsatile GnRH treatment (13, 34), and the maximal stimulation of LHβ gene transcription by pulsatile GnRH in vivo was shown to be after 24 h (18). Alternatively, the difference may be caused by the use of male rats in our studies but female rats in other studies (5). Pulsatile GnRH has been shown to increase LHβ subunit mRNA levels in vivo in male rats, but not in females, unless the females are exposed to testosterone (20, 21, 34, 35).

In this study, GnRH pulse frequency differentially affected the magnitude of the stimulatory response of the gonadotropin subunit mRNA levels to GnRH. Focusing on the GnRH pulse intervals of 30 vs. 120 min (which are the intervals at which the greatest differences were observed in vivo), LHβ subunit mRNA levels were stimulated to a greater extent by a GnRH pulse frequency of every 30 min. In contrast, FSHβ subunit mRNA levels were stimulated to a greater extent by the lower GnRH pulse frequency, every 120 min. This was true both with a fixed concentration of 10 nM GnRH per pulse and with an adjusted GnRH concentration per pulse, so that the total cumulative GnRH administered over 24 h was constant. α subunit mRNA levels were less stringently regulated by GnRH pulse frequency but also were greater at a GnRH pulse frequency of every 30 min. These data are consistent with observations in vivo (13, 15, 16, 34, 35). Thus, gonadotropin subunit gene expression is regulated differentially by varying GnRH pulse frequencies in vitro in a parallel fashion to that observed in vivo. This suggests that the differential effects of varying GnRH pulse frequencies on gonadotropin subunit gene expression occur directly at the level of the pituitary, not involving extrapituitary steroid or neuroendocrine factors. We have not, however, excluded the possibility of an indirect effect involving other nongonadotrope pituitary cell types.

Our data differ somewhat from those of another study, which failed to show that slow frequency GnRH pulses preferentially increased FSHβ mRNA levels or FSH secretion in vitro (33). However, it compared a GnRH pulse frequency of every h with every 4 h. In fact, our data are consistent with theirs, as we saw a maximal stimulation of FSHβ mRNA levels and FSH secretion at a GnRH pulse frequency of every 2 h; the stimulation of FSHβ mRNA levels and FSH secretion when GnRH was delivered at 4 h intervals was significantly lower, even when the cumulative GnRH dose was controlled.

LH and FSH secretion were stimulated differentially by varying GnRH pulse frequencies, paralleling the changes observed in LHβ and FSHβ mRNA levels. LH secretion was maximally stimulated at a GnRH pulse frequency of every 30 min, whereas FSH secretion was maximally stimulated when the cells were treated with pulsatile GnRH every 2 h. Thus, gonadotropin subunit gene expression and LH and FSH secretion seem to be coordinately regulated in vitro. Moreover, the pattern of regulation of secretion mirrors that observed in vivo, confirming that our in vitro model of superfused primary rat pituitary cell cultures reflects the in vivo physiological situation (11, 12).

Like LHβ, GnRHR mRNA levels were stimulated to the greatest extent by a GnRH pulse frequency of every 30 min. Thus, GnRHR mRNA levels are regulated differentially by varying GnRH pulse frequencies, with the pattern of regulation correlating with that of LHβ mRNA but different from that of FSHβ. This suggests that LHβ mRNA levels are maximally stimulated when GnRHR concentrations are relatively high, whereas FSHβ mRNA levels are maximally stimulated at lower concentrations of GnRHR. These data are consistent with observations in vivo that GnRH pulse frequency determines the number of pituitary GnRHR, as determined by GnRH binding studies, with maximal receptor numbers occurring after 30-min pulses for 48 h (23), although studies of GnRHR mRNA levels in vivo did not show a significant difference between GnRHR mRNA levels after 12 h of pulsatile GnRH given at 30-min vs. 2-h intervals (26). It may be necessary to expose the animals to the pulsatile GnRH paradigms for longer times, such as 24 or 48 h, to elicit a significant difference. Thus, it seems that GnRHR mRNA levels and receptor number are regulated in a coordinate fashion both in vivo and in vitro.

These data suggest that the mechanisms whereby varying GnRH pulse frequencies stimulate LHβ and GnRHR gene expression are similar, whereas the mechanism of stimulation of FSHβ gene expression may be different. We hypothesize that when GnRH is released from the hypothalamus at a pulse frequency of every 30 min, expression of the α, LHβ, and GnRHR genes are stimulated to a relatively greater extent compared with lower GnRH pulse frequencies. This results in gonadotropes expressing relatively high numbers of GnRHR, hence being more responsive to GnRH. When GnRH is released from the hypothalamus at a lower pulse frequency, such as every 2 h, expression of the α, LHβ, and GnRHR genes is stimulated to a lesser degree. In contrast, expression of the FSHβ subunit gene is relatively more stimulated. These results suggest in gonadotropes that express lower numbers of GnRHR and are less responsive to GnRH, yet have higher levels of FSHβ mRNA. These data support our hypothesis that varying GnRH pulse frequencies regulate differentially LH and FSH subunit gene expression by regulating pituitary GnRHR concentrations. Using a heterologous pituitary cell line, GH3 cells, we have shown previously that different cell surface densities of GnRHR result in the differential regulation of LH and FSH subunit gene expression by GnRH. The expression of the α and LHβ subunit genes is optimally stimulated at relatively high cell surface concentrations of GnRHR, whereas FSHβ gene expression is optimally stimulated at lower cell surface concentrations of the receptor (36).

These data add to the accumulating evidence that there are disparate mechanisms for the regulation of LHβ and FSHβ subunit gene expression and hence, by inference, for LH and FSH biosynthesis. In addition to the mounting evidence for differential regulation of these genes by GnRH, there is now evidence that the nuclear transcription factor, steroidogenic factor 1, is important for expression of the LHβ subunit gene, whereas a role for this factor in FSHβ subunit gene expres-
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We thank Elena Sallagh and Sakina Hakim for their technical assistance and Dr. Christoph Meier for his assistance with statistical analysis of the data.

References


