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ORIGINAL ARTICLE

FSH β-subunit gene expression in long-term ovariectomized rat after pulsatile intracerebroventricular microinjections of GnRH

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Abstract

OBJECTIVES: The purpose of this study was to examine whether in long-term ovariectomized rats direct pulsatile intracerebroventricular microinfusions of GnRH would result in frequency dependent biosynthesis of pituitary FSH β-subunit mRNA. METHODS: Stainless steel cannula was stereotaxically implanted into the third ventricle of ovariectomized rats. 1 nM of GnRH microinjections were given at frequency 1, 2, 4 pulses/hour during 5 hours. Pituitary FSH β-subunit mRNA level was determined by Northern-blot and serum FSH concentration was examined by RIA. RESULTS: Exogenous GnRH (1nM) induced a significant increase of pituitary content of FSH β-subunit mRNA when administered 30 or 60 min intervals over 5 hours to ovariectomized rats. RESULTS: GnRH microinjections given at frequency of 1 pulse/hour were mostly effective resulting both in 85% increase of FSH β-subunit mRNA level as compared to control as well as a significant (78%) stimulation of FSH release. CONCLUSIONS: our data show that in a long term ovariectomized rats (without steroid and gonadal peptides supplementation) a direct pulsatile intracerebroventricular microinjections of GnRH induce frequency-dependent pituitary FSH β-subunit mRNA biosynthesis in the same mode as it was reported for gonadectomized and steroid replaced rats. It seems therefore that this method would represent an interesting alternative to the use of classical agents to disconnect, *in vivo*, the pituitary from hypothalamic GnRH influence. It may also provide a physiological data concerning regulatory aspects of gonadotropin subunits biosynthesis.

Introduction

The hypothalamic decapeptide GnRH plays a key role in the neurohormonal control of reproduction by stimulating the release of pituitary gonadotropins, LH and FSH, which, in turn, promotes the development of gonadal functions such as steroid production / secretion and gametogenesis. Several lines of evidence reveal that GnRH is a determinant also for the synthesis of gonadotropins and acts at least in part by regulating the expression of genes coding for constitutive gonadotropin subunits a, LHβ, FSHβ. The three subunits are encoded by separate genes [1, 2, 3] and gonadotropin synthesis/ secretion have been shown to occur in both parallel and divergent fashions [4, 5]. Thus in hypothalamus-pituitary disconnected animals, gonadotropin mRNAs are dramatically depressed to nearly indetectable levels and can be restored to near normal levels by the administration of pulsatile GnRH [6, 7]. GnRH antagonists or GnRH immunoneutralization also reduced gonadotropin mRNA levels in intact animals and prevented their gonadotropin mRNA levels in intact animals, implying a tonic stimulation of gonadotropin gene expression and the mediation by GnRH of gonadectomy induced increase in subunit mRNAs. Using the castrate testosteroid-replaced rat model which depressed endogenous GnRH secretion and thus allowed the manipulation of GnRH pulse frequency [8] or animals treated permanently with GnRH superagonists [9], the effect of GnRH on gonadotropic gene expression was shown to be critically dependent on the mode of presentation at the level of gonadotrope cells. Some studies indicated [10, 11] that a and LHß respond best to more frequent while FSHβ to slow pulse frequencies, whereas continuous exposure to GnRH leads to more or less rapid depletion of both LHβ and FSHβ with the a remaining elevated. Gonadal steroids are well known as potent regulators of gonadotropin subunit gene expression acting both at hypothalamic and pituitary levels. Additionally, selective regulation of FSH β gene expression is related to the secretion of gonadal peptide [12] and the pituitary FSH β-subunit mRNA level is the resultant of regulatory influences reflecting GnRH, gonadal steroids and gonadal peptides impact on gonadotropic cells.

The purpose of this study was to examine whether in long-term ovariectomized rats (without gonadal—both steroid and peptide—input) direct pulsatile intracerebroventricular microinfusions of GnRH would result in frequency dependent biosynthesis of pituitary FSH β-subunit mRNA.

Materials and Methods

Animals

Adult, cycling female Wistar rats (240–300 g) laboratory strain were kept under controlled light (LD 12:12) and temperature (22°C) conditions with free access to pelleted food and tap water. Animals were ovariectomized and two weeks post-operative a stainless steel cannula was stereotaxically implanted into the third ventricle according to the atlas of [13]. The cannula was cement ed in place (its location was confirmed with the flow of cerebrospinal fluid), and a removable stylette was inserted so that its tip was flush with the tip of the guide cannula. Ten days after the cannula was implanted, freely moving rats were connected to an automatic pump (CMA/100, Sweden) and received intracerebroventricular (i.c.v.) microinjections of 0.9% NaCl (control group) or 1nmol of GnRH (experimental group). The flow rate was carefully balanced and set to deliver 10 μl/5min. Microinjections were given with the frequency of 4, 2 and 1 pulses per hour during 5 hours. Fifteen minutes after the last microinjection the rats were killed by decapitation, the anterior pituitary was excised and immediately frozen in liquid nitrogen. Blood was collected, the serum prepared and kept in –20°C until RIA assay.

RNA preparation and hybridization

RNA was prepared from individual anterior pituitaries according to a scale-adapted guanidinium method [14]. Northern analysis of FSH beta subunit mRNA was achieved as previously described [10] with the β-actin mRNA, probed with 1150bp mouse cDNA [15] used as internal reference to correct for the amount of mRNA loaded. cDNA was labelled with Amersham Megaprime labeling kit (Amersham, Arlington Heights, IL). Radioactive hybrids on filter were revealed by autoradiography and quantified by laser densitometry of the entire surface of the spot using a computer image processing system (COHU high performance CDD camera and One-Dscan software, Scanalytics, Billeria, MA). Data were expressed in arbitrary densitometric units.

Radioimmunoassay

Rat serum FSH was measured by RIA using antibodies and FSH preparations gifted by NIDDK. Values were expressed in terms of rat FSH (RP-2) reference preparations. Intra- and interassay variations were 7.5% and 10.5%, respectively.

Statistical analysis

Statistical evaluations were made by analysis of variance and then by Duncan Multiple Comparison Test and Student t-test. Differences resulting in $P < 0.05$ were considered significant.

Results

Effect of direct intracerebroventricular microinjections of GnRH on pituitary FSH β-subunit mRNA was examined in female ovariectomized rats. Altered levels of pituitary FSH β-subunit mRNA were detectable after a 5-hour treatment with GnRH in a manner depending on pulse frequency (Fig.1) In particular a net, significant increase in the FSHß mRNA levels was observed in response to exogenous GnRH. The most effective were GnRH microinjections given at a frequency of 1 pulse/hour which resulted in an 85% increase of FSH beta mRNA level as compared to controls. In case of animals receiving more frequent (2 pulses/hour) pulses of GnRH, FSH β-subunit gene expression was stimulated less abundantly but still significantly (25%), whereas the most rapid 4 pulses/hour were inoperative.

The effect of pulsatile intracerebroventricular GnRH microinfusions on FSH plasma release is shown on Fig. 2. Exogenous GnRH administered into the third ventricle also induced an increased (78%) release FSH from the pituitary, however only in response to 1 pulse/hour.

Discussion

In our experiment we have applied an *in vivo* system to establish the effect of intracerebroventricular pulsatile GnRH microinjections on pituitary FSH β-subunit mRNA level in long-term ovariectomized rats. This approach offers several advantages. Firstly, in the presence of anatomical and regulatory relationships existing between the hypothalamus and pituitary, data reflecting physiological aspects of gonadotropin biosynthesis regulation can be obtained. Secondly, by applying exogenous GnRH directly into the third cerebral ventricle, thus in the vicinity of the hypothalamus and pituitary gland, blood circulation effects (rapid degradation of neuropetide which requires repeated administration of low doses to avoid GnRH receptor desensitization) can be excluded. Thirdly, ovariectomy results in significant consequences affecting FSH biosynthesis *in vivo*: lack of regulatory input on hypothalamus and pituitary gland both from gonadal steroids (estradiol/progesterone) and peptides (inhibin/activin).

Existing data provide some information concerning an effect of pulsatile GnRH microinjections on subunit α and LHβ mRNA expression in the pituitary gland of ovariectomized, estradiol-treated rats [10] as well as in orchidectomized, testosterone-implanted rats [10, 11]. Gonadal steroids may regulate gonadotropin subunit gene expression by effects at the level of the hypothalamus and directly at the pituitary. It was reported [16] that in females, estradiol administration at the time of ovariectomy acts via GnRH to prevent the increase in LH secretion and LHβ mRNA levels, but does not prevent the increase in FSH β gene expression *in vivo*. Moreover,

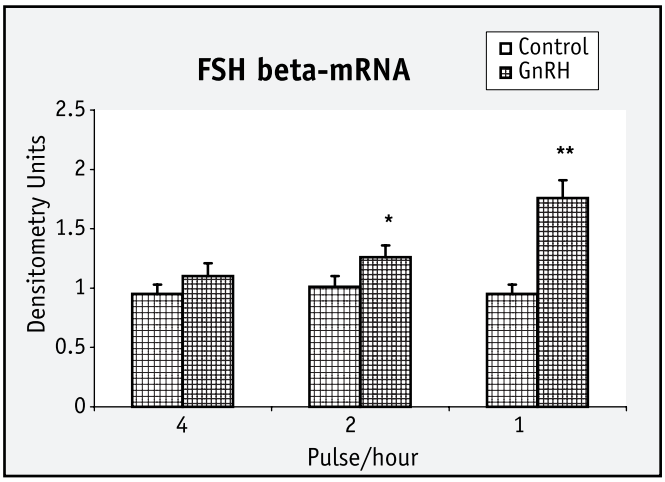

Fig. 1. Effects of pulsatile microinjection of GnRH directly into the third cerebral ventricle of ovariectomized rats on pituitary content of FSHβ mRNA. Rats were cannula-implanted two weeks after castration and were used ten days later. GnRH (1nM) or saline (control) was infused for 5 min every 15, 30 or 60 min (frequencies 4, 2, and 1 pulses/h, respectively). Total RNA was prepared from individual pituitaries and analyzed for FSH β-subunit mRNA content by Northern blot hybridization. Obtained values were standarized with β-actin mRNA. Data are expressed as the mean ± SEM of 5 separate experiments. *P ≤ 0.05; **P ≤ 0.01 (vs. control group).

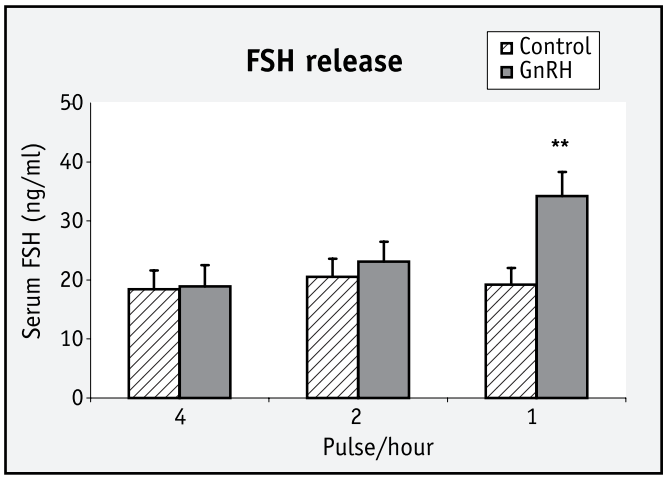

Fig. 2. Effects of intracerebroventricular pulsatile microinjection of GnRH on FSH release in ovariectomized rats. Microinjections of GnRH (1 nM) were given with the frequency of 4, 2, and 1 pulses per hour during 5 hours. Serum FSH was assayed in triplicate by RIA. Data represent the mean ± SEM of 5 separate experiments. **P ≤ 0.01 (vs. control group).

progesterone in conjunction with estradiol appears to inhibit GnRH secretion *in vivo* in the rat and to reduce gonadotropin secretion. Progesterone may also have an direct action at the pituitary to exert selective increase of FSH β mRNA levels [16]. Taken together, gonadal steroids effect preferential increases in FSH biosynthesis and secretion, although the mode of steroid actions directly at the gonadotrope cells are still unclear.

The selective regulation of FSH β gene expression and FSH secretion is related not only to GnRH and gonadal steroid input but additionally is influenced by the secretion of inhibitory (inhibin) and activatory (activin) gonadal peptides) [17]. In female rats both ovariectomy and inhibin immunoneutralization produced a similar rapid and selective increase in FSH β mRNA level and secretion as soon as 2 hours after ovariectomy [18], but this effect was not associated with altered mRNA synthesis (the FSH β-gene transcription rate was unchanged) and might reflect nontranscriptional mechanism through inhibin could regulate FSH β gene expression.

To avoid confounding effects of gonadal steroids and peptides on pituitary FSH β-subunit mRNA level, we have chosen ovariectomized rats as a model suitable for studying GnRH frequency dependent FSH beta subunit mRNA biosynthesis. Nevertheless an important question concerning endogenous GnRH input on pituitary gland stimulation in ovariectomized and without steroid supplementation rats still exist. It is well known that gonadectomy results in an increase of pituitary GnRH receptor density and this effect can be abolished by the manipulations which prevent the post-castration rise in GnRH secretion such as hypothalamic lesions, administration of anti-GnRH sera or steroid replacement [19, 20]. In the case of our experiment we did not use any of these method to disconnect, *in vivo*, the pituitary from hypothalamic influence. It might be supposed that in ovariectomized rats an enhanced endogenous GnRH would be a potent stimulatory agent affecting pituitary gland and responsible for FSH β-subunit mRNA biosynthesis. Pulsatile secretion of GnRH from the hypothalamus is very well established [21, 8]. Taking into account that GnRH agonist (buserelin) was shown to decrease GnRH release via GABAergic and taurinergic neurons in explanted superfused hypothalami [22], it can be supposed that microinjected GnRH could similarly inhibit the release of endogenous GnRH in this study. Though in our experimental model there was no possibility to evaluate an exact amount of GnRH reaching pituitary gland (no access to portal blood), we could conclude about endogenous GnRH efficiency to stimulate pituitary FSH β-subunit mRNA level by comparison levels of mRNAs after pysiological saline microinjections with that observed after pulsatile exogenous GnRH application. In control group group FSH β-subunit mRNA was markedly lower than the level of mRNA observed after decapeptide microinjections. If so, we can assume that in our *in vivo* model, exogenous GnRH rather substituted to than cumulated with endogenous portal GnRH and in consequence we have not observed desensitization of pituitary GnRH receptor but could get the results reflecting exogenous GnRH stimulating potency on FSH β-subunit mRNA biosynthesis. In that case this method offers an interesting alternative to the use of classical agents functionally disconnecting the pituitary from the hypothalamic input. In our experimental model an exogenous GnRH was capable of inducing an increase in FSH β-subunit mRNA level when administered with lower frequencies (1 or 2 pulses per hour). Also mean serum FSH concentration was stimulated by low frequency (1 pulse/per hour) of GnRH and it seems that lower frequencies of the neuropeptide are responsible for activating FSH release and biosynthesis. It was shown [23] that selective rise in FSH secretion can be achieved by slowing the frequency of GnRH pulses. In contrast, the α-subunit mRNA increased in responses to GnRH stimulation at any frequency tested (data not shown).

Relatively little is known about regulatory mechanisms involved in frequency-dependent regulation of gonadotropin subunit mRNA expression. In male rats FSH β mRNA gene stimulation by low frequency pulses of GnRH was reported [24] and this process may be mediated by pituitary expression of activin, which stimulates FSH β mRNA and follistatin and which blocks activin. Some data [25] suggest that PKC-dependent pathways play a larger role in GnRH -mediated trancriptional control of the LHβ and FSH β genes than the α-subunit gene, however all three gonadotropin subunit gene promoters are responsive to PKC-dependent pathways. Alterations in the frequency of calcium signals can regulate gonadotropin subunit gene expression [26]. It seems that more frequent signals more effective in stimulating α and LHβ mRNA pituitary levels.

In conclusion, our data show that in long-term ovariectomized rats (without steroid and gonadal peptides supplementation) direct pulsatile intracerebroventricular microinjections of GnRH induce frequency-dependent pituitary FSH β-subunit mRNA biosynthesis in the same mode as it was reported for gonadectomized and steroid replaced rats. If so, it seems that this method would represent an interesting alternative to the use of classical agents to disconnect, *in vivo*, the pituitary from hypothalamic GnRH influence. It may also provide a physiological data

concerning regulatory aspects of gonadotropin subunit biosynthesis.

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