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INVITED NEL REVIEW

GnRH Pulsality and the Differential Activation of the Rat Luteinizing Hormone Subunit Genes in the Anterior Pituitary Gland

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Abstract Detailed studies have been focused on the mechanisms by which the rat α and LHβ genes are differentially regulated by GnRH and indicate that differential sensitivity to the second messenger exists in a physiological context. Differential signaling from the GnRH receptor may be a mechanism for preferential regulation of luteinizing hormone subunit gene transcription; however which of these genes are specifically regulated by PKC or calcium and how GnRH pulsatility could preferentially activate individual pathways of second messengers within gonadotrope cells remain unclear. Several transcription factors that have profound effects on basal and/or GnRH-stimulated LHβ gene promoter activity have been identified: SF-1, Egr-1, Sp-1. A model explaining possible interactions among them in mediating GnRH responsiveness of the LHβ gene has been proposed: Sp1, SF-1 and Egr-1 form a tripartite GnRH response element which is sensitive to the spacing changes between the upstream Sp1 binding sites and the downstream SF-1/Egr-1 binding elements and SF-1 plays a critical role in integrating the effects of Sp1 and Egr-1. GnRH responsive element located on LHβ gene promoter in position between –495 to –342 has been identified. At 3'-end of the promoter three Sp-1 binding sites have been identified: position –416, sequence: GGGGGCTGGG and two sites almost completely overlapping, position –403, sequence; GGGGCGGCGCCCA while at the 5'region of the promoter one Sp-1 binding site exists: position –450, sequence: ACCACACCCATTTTTGG. The 5'Sp1 site overlaps a CArG box (at -443 to –434, sequence: CCATTTTTGG) which seems to be essential in LHβ gene sensitivity for pulsatile GnRH stimulation.

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Introduction

Gonadotropin-releasing hormone (GnRH) is the decapeptide released from specialized neurons scattered throughout the hypothalamus that governs the central nervous system control of reproduction. It is released into the pituitary portal vessels in a pulsatile fashion and these pulses, the frequency and amplitude of which vary physiologically, are essential for the maintenance of normal reproductive functions in mammals. Essentially, two patterns of GnRH secretion have been identified in the rat. The basal pattern is characterized by low-amplitude and -frequency episodic GnRH secretion. The second occurs in the afternoon of proestrus where acceleration of the GnRH pulse generator activity interrupts intermittent basal pattern of GnRH secretion and results in a dramatic increase in GnRH pulse amplitude and frequency. Finally, this leads to the onset of estrous behavior and a preovulatory luteinizing hormone (LH) surge. [1]. Upon binding to specific, high-affinity receptors on gonadotrope cells of the anterior pituitary gland, GnRH activates two primary signaling pathways, calcium and protein kinase C (PKC). GnRH acts via seven transmembrane region G protein coupled receptors and activates $G_{q/11}\alpha$ proteins [2] to stimulate phospholipase Cβ which hydrolyzes membrane phosphoinositides and generates diacylogliceroIs and inositol phosphates, including inositol 1,4,5 trisphosphate. Activation of diacylogliceroIs results in the activation of several isozymes of protein kinase C [3] and thus mitogen-activated protein kinase (MAPK) [4, 5] while inositol 1,4,5 trisphosphate is responsible for Ca^{2+} mobilization from intracellular stores [6]. It is now well established that receptor activation induces a biphasic effect of GnRH on cytosolic Ca^{2+}, with an initial spike dependent on internal calcium stores, and a sustained plateau that is dependent on increased Ca^{2+} influx through L-type voltage-gated channels [7, 8]. GnRH is also a determinant factor in stimulating the transcription of three different genes of the gonadotropin (LH and FSH) subunit encoding the common α-subunit and unique LHβ and FSHβ-subunit that confer biological specificity of these hormones [9].

GnRH pulsality and differential signal transduction pathways in the specific LH subunit genes expression

The hypothalamic pulse generator which regulates intermittent discharges of GnRH into the pituitary portal circulation, thereby modulating the pulsatile pattern of gonadotropin release, is also essential for the specific regulation of the gonadotropin subunit gene expression. Transcriptional activation of gonadotropin subunit genes is differentially regulated by GnRH pulse frequency *in vivo* and *in vitro* [10, 11]]. Models such as castrated steroid-replaced rat [12], ovariectomized rat [13, 14] or animals chronically treated with GnRH superagonists [15] have shown that the effect of GnRH on gonadotropin gene expression is critically dependent upon the mode of presentation to the gonadotrope cells and GnRH pulses regulate gonadotropin subunit gene transcription in a frequency-dependent and a subunit-specific manner. Transcription from the rat α-subunit gene is stimulated by pulses at short or intermediate intervals whereas LHβ gene responds better to intermediate and slow pulse frequencies. Moreover, continuous exposure to GnRH leads to a more or less rapid depletion of LHβ gene, with the α-subunit gene remaining elevated.

Since GnRH pulses have been shown to increase GnRH receptor levels [16], thus amplifying the signal, this may be one mechanism responsible for the specific LHβ *vs* α-subunit gene transcription. Indeed, the results obtained in a somatomammotrope cell line stably transfected with the GnRH receptor and transiently transfected with the human α, rat LHβ and rat FSHβ promoter receptor constructs have shown [17] that the GnRH receptor density is correlated with preferential gonadotrope subunit promoter activation. Moreover, the high GnRH receptor density favours stimulation of the LHβ gene. In contrast, GnRH receptor mRNA was not increased in αT3-1 cells in response to GnRH [18].

More detailed studies have been focused on the mechanisms by which the rat α and LHβ genes are differentially regulated by GnRH. Since the intracellular messenger cascades mediating the stimulation of gonadotropin secretion by GnRH are well recognized and a major role of calcium in this process is well established, the research efforts have been made to determine whether these distinct signaling cascades (PKC and calcium) may be also responsible for the GnRH stimulation of transcriptional activity in the α *vs* the β-subunit genes. Studies on endogenous gene transcription and expression of GnRH-responsive areas in transfected αT3 cells and LHβ promoter activity in transgenic mice [19] indicate that differential sensitivity to the second messenger exists in a physiological context. In this study, both α and LH β-subunit genes were significantly stimulated by GnRH, nonetheless they responded differentially to the intracellular modulators of GnRH signaling: α-subunit gene was more sensitive for PKC stimulation whereas a calcium influx preferentially activated LHβ gene transcription. Moreover, PKC stimulation of the α-subunit gene promotor observed both in the primary cell culture and in α - T3 cells was dependent on MAP kinases activation while an activation of this

intracellular pathway was not indispensable for LHβ gene promoter stimulation. However, these results are in apparent contradiction with those obtained by Saunders *et al*. [20]. Using a GH_3-1' somatolactotropic cell line stably transfected with the rat GnRH receptor cDNA and co-transfected with a 5'-flanking region of the promoter α (–846/0), LHβ (–791/+5) and FSHβ (–2000/+1709) they investigated the effect of PKC-dependent pathway(s) and calcium channels agonist/ antagonist on the specific α and both β-subunits gene promoters regulation. Their results demonstrate that the rise in the intracellular calcium concentration is mainly responsible for the α-subunit gene promoter activation whereas GnRH-induced expression of the LHβ and FSHβ-subunit genes occurs primarily via a PKC-dependent pathway. Moreover, PMA-dependent LHβ mRNA synthesis in rat pituitary cells was reported by Park *et al*. [21] whereas Call and Wolfe demonstrated that the equine LHβ promoter gene activation by GnRH requires PKC/MAPK cascade stimulation while calcium is not involved in the induction of this promoter activity [22]

In brief, existing data support the hypothesis that differential signaling from the GnRH receptor may be a mechanism for preferential regulation of luteinizing hormone subunits gene transcription; however which of these genes are specifically regulated by PKC or calcium and how GnRH pulsality could preferentially activate individual pathways of second messengers within gonadotrope cell still remain unclear.

Trans and *cis* acting factors

Recent studies have identified several transcription factors that have profound effects on basal and/or GnRH-stimulated LHβ gene promoter activity. Steroidogenic factor 1 (SF-1), an orphan nuclear receptor, is a member of the nuclear hormone receptor family of transcription factors which is selectively expressed in the gonadotrope cells population as well as in the adrenal gland and the gonads [23]. SF-1 binding elements have been identified in a range of genes responsible for the regulation of steroidogenesis, sexual differentiation and reproductive functions [24]. SF-1 binds as a monomer to a DNA promoter region, called the gonadotrope-specific element (GSE), to a sequence element comprised of an estrogen receptor half-site and three specific 5'-adjacent nucleotides [25]. Two functional GSE sites located at positions –127 (sequence: TGACCTTG) and –59 (sequence: CGGCCTTG) both binding SF-1 protein have been identified on the rat LHβ gene promoter [26, 27] and studies *in vivo* and *in vitro* have established the functional role of SF-1 in *trans*-activation of the LHβ gene [28, 29]. In mice lacking *FTZ-F1* gene which encodes sterodogenic factor-1, mRNA level for LHβ-subunit was undetectable [23], whereas a mutation of the 5'GSE site of the bovine LHβ gene promoter resulted in a significant down-regulation of the reporter gene expression relative to the expression levels in the presence of wild-type promoter [28].

The second *trans*-acting protein actively involved in the transcriptional regulation of LHβ gene expression is the early growth response protein 1, Egr-1, known also as zif/268, Krox-24 and NGFI-A [27, 30, 31] and whose expression within the pituitary gland is limited to the gonadotrope and somatotrope cells. It belongs to the immediate early genes family whose members contain a zinc finger domain with a Cys_2-His_2 motif that recognizes GC-rich nucleotide sequence [32]. Experiments on transgenic models have demonstrated specific loss of LHβ gene expression as well as inability to respond to GnRH stimuli in Egr-1 deficient mice, whereas FSHβ and α-subunit gene expression in these animals were maintained at the normal level [30, 31]. Moreover, in transient transfection studies Egr-1 has been reported to stimulate rat LHβ gene promoter activity and this effect is attributed to two highly conserved Egr-1 binding sites located at positions –112 (sequence: CGCCCCCG) and –50 (sequence: CGCCCCCAC) [27]. Mutation of the Egr-1 sites within the equine LHβ promoter sites completely attenuated the ability of Egr-1 to transactivate the promoter [33]. In this study Egr1 has been also shown to induce the activity of the promoter and endogenous expression of Egr-1 was not detectable in the basal condition but was rapidly induced after GnRH stimulation.

Egr-1 contains an internal inhibitory domain consisting of 34 amino acids, and even a single-point mutation within this domain results in a 15-fold increase of Egr-1 transcriptional activity [34]. More detailed studies have identified two Egr-1 binding proteins, Nab1 and Nab 2 which repress the activity of Egr-1 [34, 35]. Since GnRH was reported to induce not only Egr-1 expression, leading to the subsequent activation of the eLHβ promoter, but also Nab1 protein which can repress Egr-1-induced transcription [33], it cannot be ruled out that Nab-1 could be an important factor involved in the regulation of LHβ promoter activity in the pituitary gland. Whether GnRH pulsality can specifically stimulate LHβ gene activity through a frequency-dependent Egr-1 and Nab1 pattern of expression, thus determining whether transcription of Egr-responsive genes is induced or repressed, is still an open question.

The third *trans*-acting protein involved in the GnRH-dependent rat LHβ-subunit gene expression is Sp1 protein which binds to the DNA sequences between –490/-352, in the rat LHβ gene promoter. Several Sp1 binding sites were identified within this

region and mutations of these elements which block binding of Sp1, reduce both basal LHβ gene promoter activity and the level of stimulation by GnRH [36]. Like Egr-1, Sp1 is a member of the Cys_2-His_2 zinc finger family of transcription factors and bind to similar but distinct GC-rich sequences [37].

Recently, a model explaining possible interactions among these three transcription factors, SF-1, Egr-1 and Sp1, in mediating GnRH responsiveness of the LHβ gene has been proposed by Kaiser *et al* [38. Their studies on GGH_3-1' cells and LβT2 cells transfected with the construct-containing region –797 to +5 of the rat LHβ have indicated that SF-1, Sp-1 and Egr-1 act and interact to contribute to GnRH responsiveness. SF-1 and Egr-1 proteins form two composite regulatory elements located at the proximal part of the promoter (positions –127 and –59), whereas Sp1 protein binds to the more distal sequences (positions between –451 and –386) and such localization requires, consequently, the presence of an additional integrating transcriptional cofactor enabling interactions among SF-1, Sp1 and Egr-1. According to this model, Sp1, SF-1 and Egr-1 proteins form a tripartite GnRH response element in the rat LHβ gene promoter. This regulatory response element is sensitive to the spacing changes between the upstream Sp1 binding sites and the downstream SF-1/Egr-1 binding elements, leading to the reduction of the promoter response to GnRH. SF-1 plays a critical role in integrating the effects of Sp1 and Egr-1, and only in the presence of SF-1 all three transcriptional factors interact with the transcriptional cofactor to mediate the full response to GnRH. The efforts undertaken to identify this cofactor have not been successful yet nevertheless transcription factors coactivators SRC-1 and CBP/p300 protein, known to interact with SF-1, Egr-1 and Sp-1 [39, 40,41] seems to be not involved in this regulation. Among the candidates which might be, at least partially, responsible for SF-1, Egr-1 and Sp-1 interaction is transcription factor Ptx1, a member of the *bicoid*-related subclass of homeobox genes. Nested between the pairs of Egr-1 and SF-1 binding elements, in the proximal domain of the LHβ promoter (at the position –100; sequence:AGATTA) is a single binding site for transcription factor Ptx1 (pituitary homeobox 1) [42] which has been shown to enhance LHβ gene expression through synergistic direct interaction with SF-1 and Egr-1 proteins [43]. This cooperative interaction seems to occur independently of Ptx1 binding to DNA [44] and Ptx1 may serve as a physiological ligand for SF-1 and Egr-1. Indeed, recent studies on transgenic mice have established that LHβ promoter activity is strongly dependent on the presence of the functional Pitx1 binding site whereas a functional synergism among Pitx1, SF-1 and Egr-1 leads to the formation of higher-order transcriptional complex that governs the spatial expression and GnRH responsiveness of the LHβ gene *in vivo* [45].

Studies on *cis*-acting regulatory elements within the rat LHβ gene promoter have brought to the identification of the two regions: region A (–490/–352bp) and region B (–201/–82) which may act as a compound element involved in mediating the response to GnRH (GnRH-RE) [46]. Using rat pituitary GH_3 cell line transfected with the rat GnRH receptor cDNA and cotransfected with regulatory regions of the LHβ genes they have shown that region A conferred GnRH responsiveness to the promoter whereas region B did not. However, the presence of both regions A and B conferred a greater GnRH response than region A alone. Comparison of the rat LHβ gene region A with known DNA consensus sequences have revealed an interesting homologies with the sequences identified on prolactin promoter (100% homology with the prolactin conserved motif (position –419 / –412), 75% homology with the GnRH response element both in the murine α-subunit gene (position –421/–414) and in the human α-subunit gene (position –375/–361). Moreover, sequence analysis of the B region have shown homologies with the sequences known for binding several transcription factors: CRE, (cAMP-responsive element, 75% of homology; position–171/–164), AP-1 (activating protein-1, 85% of homology, position –157/–151) and GSE (gonadotrope specific element highly conserved on the human, bovine, mouse, equine and rat α-subunit gene promoter, 100% of homology, position –125/–117). Recently, Weck *et al.*, [47] using deletion/mutation analysis in LβT2 cell line transfected with –617/+44 bp rat LHβ promoter gene, have defined its GnRH responsive element located in position between –495 to –342. At 3'-end of the promoter three Sp-1 binding sites have been identified: position –416, sequence: GGGGGCTGGG and two sites almost completely overlapping, position –403, sequence; GGGGCGGCGCCCA while at the 5'region of the promoter one Sp-1 binding site exists: position –450, sequence: ACCACACCCATTTTTGG. Moreover, the 5'Sp1 site overlaps a CArG box (at –443 to –434, sequence: CCATTTTTGG) which seems to be essential in LHβ gene sensitivity for pulsatile GnRH stimulation as mutating the CArG element specifically eliminates the response to pulsatile GnRH. Instead, single high dose GnRH stimulation of LHβ gene has been blocked by the mutations within 5'Sp-1 and 3'Sp-1 sites while CArG box mutations have not prevented static GnRH LHβ gene activation.

GnRH-dependent regulatory elements on the α-subunit gene differ from those identified on LHβ gene. Using the same Lβ T2 cell line transfected with –479/+77 bp rat α-subunit gene Weck *et al.*, [47]

have demonstrated that the α-subunit gene GnRH-responsive region lies between –411 and –375 bp. The region contains two Ets-domain protein binding sites (sequences GGAA localized on both DNA strands) and mutations of either the distal 5' or proximal 3' or both Ets sites have eliminated static GnRH stimulation of α gene promoter as well as suppressed basal expression of this gene. Since Ets proteins have been shown to be phosphorylated by MAPK pathway [48] and inhibition of the MAPK pathway has been reported to suppress both basal and stimulated α-subunit promoter activity [19] as is observed with mutation of the putative Ets protein binding sites, they may directly, through the MAPK pathway, modulate static stimulatory GnRH action on α-subunit gene expression.

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