PITUITARY GONADOTROPINS AND RECEPTORS FOR ESTROGEN AND GnRH IN FASTED DOES

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ABSTRACT

The present work examined the expression for both estradiol-17β subtype α (ERα) and GnRH receptors (GnRH-R) and that for FSHβ as well as their regulation after two-days of fasting in the anterior pituitary gland of control and estrogen-primed rabbits, together with the LH dynamic secretion following GnRH stimulation. Sexually mature unmated NZW female rabbits were randomly assigned to the following groups: control does fed *ad libitum* (AL), AL implanted with 2.0 mg estradiol-17β (AL-E), 48 h fasted does (F), and F implanted with estrogens (F-E). Whereas control does were fed *ad libitum*, treated does were fasted for 48 h before killing or i.m. GnRH injection. Immediately after GnRH treatment, fasted does were re-fed *ad libitum*. ERα immunoreactivity was evidenced in the nucleus of most pituitary cells in both control and treated does. In F-E rabbits, the intensity of ERα signal and the number of positive cells were markedly reduced. Positive staining for GnRH-R was evidenced in the nucleus and in the cytoplasm, although with weaker signal, of many pituitary cells of both control and treated does. In pituitaries of F-E does, GnRH-R signal intensity was markedly reduced. Fasting and oestrogen did not affect basal plasma LH concentrations. In all the groups, the LH peak surge was observed 30 min after GnRH injection. The LH magnitude was lower ($P \leq 0.01$) in F than in AL rabbits, but higher ($P \leq 0.01$) in F-E than in AL-E does. In all the groups, plasma LH levels declined close to basal values 4 h after GnRH. Fasting as well as oestrogen priming of both AL and F rabbits reduced ($P < 0.01$) the levels of pituitary ERα mRNA expression. The lowest ERα gene expression was found in F-E does. GnRH-R mRNA relative abundance was down regulated ($P < 0.01$) four-fold in F-E does. FSH mRNA expression was down-regulated ($P < 0.01$) only in F-E rabbits. In conclusion, the rabbit anterior pituitary responds to changes in nutritional status, as provoked by 48 h fasting, and to gonadal steroid through adjustments of ERα, GnRH-R, and FSHβ at the level of gene expression as well as through regulations of LH release into the blood stream in order to adjust the reproductive system to metabolic condition. Complete deprivation of food for a short period of time could be a useful model for analysing the interrelationships between nutritional factors and reproductive function in rabbits.

Key words: ERα, GnRH-R, Gonadotropins, Fasting, Pituitary.

INTRODUCTION

In different animal species, prolonged caloric restriction inhibits pulsatile LH secretion and induces a condition of anoestrous by depressing the gonadotropin releasing hormone (GnRH) pulse generator within the hypothalamus (Wade *et al*., 1986). The nutritional status of rabbit does decreases both fertility and sexual receptivity with mechanisms not yet well understood (Brecchia et al., 2006). Peripheral plasma oestradiol-17β and GnRH-induced LH secretion were much lower in 48 h fasted does than in rabbits fed *ad libitum* (Brecchia *et al*., 2006). In principle, the fault LH surge may be due

to a decreased synthesis of LH by the pituitary gonadotroph cells and/or to a reduced sensitivity for GnRH caused by a scarce density of GnRH receptors (GnRH-R) on their plasma membrane.

An increasing body of evidence indicates that the expression of GnRH-R in the pituitary is regulated by GnRH itself and several other factors, including gonadal hormones (Rispoli and Nett, 2005; Hapgood *et al*., 2005). Among them, estradiol-17β, acting via its receptor (ER), is a likely candidate for targeting at different hypothalamic loci both local pre-motor and GnRH neurons to modulate the GnRH pulse pattern (Bauer-Dantoin *et al*., 1995; Pau and Spies, 1997) as well as pituitary gonadotropes to modify their responsiveness to GnRH through modulation of its cognate receptor.

In the present work we examined in the anterior pituitary of rabbits the expression for both estradiol-17β subtype α (ERα) and GnRH receptors and that for FSH as well as their regulation after two-days of fasting in rabbits, either control or estrogen primed. In addition, in the same animal model, we further studied the LH dynamic secretion following GnRH stimulation.

MATERIALS AND METHODS

Sexually mature unmated NZW female rabbits were housed individually under controlled conditions of light (14 h L/10h D) and temperature (20°C). The rabbits were fed a commercial pellet diet containing 11.0 MJ DE/kg dry matter and 18.3% crude protein. Rabbits were randomly assigned to the following four groups: control does fed *ad libitum* (AL), AL implanted with estradiol-17β (2.0 mg) in a silastic tube (1x13 mm, provided by Merck) (AL-E), 48 h fasted does (F), and F implanted with estrogens (F-E). Whereas control does were fed *ad libitum*, treated does were fasted for 48 h before killing (Exp. 1) or GnRH injection (Exp. 2). Immediately after GnRH treatment, fasted does were re-fed *ad libitum*.

Experiment 1

To localize the cell type distribution of ERα and GnRH-R in the anterior pituitary and to quantify gene expression for both receptors and FSH, three rabbits/group were euthanized. Upon collection, pituitaries were halved and processed in parallel for IHC and RT-PCR analysis.

Experiment 2

To evaluate the role of estrogens during fasting on the dynamic response of LH to GnRH (0.8 μg, Receptal, Hoechst-Roussel), blood samples were drawn at 30 min intervals from five rabbits per group, during the last 3 h of fasting and the next 6 h following re-feeding. Each plasma sample was assayed for LH as previously reported (Brecchia *et al*., 2006).

ERα and GnRH-R were identified in serial, 5 μm-thick pituitary sections using monoclonal mouse anti-ER (1:80, Zymed Laboratories Inc., San Francisco, CA, USA) and anti-GnRH-R (1:100, Lab Vision Corp., Fremont, CA, USA) as primary antibodies at the specified dilutions. The sections, processed as previously described (Boiti *et al*., 2007) were incubated for 30 min with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), exposed to the avidin-biotin complex (Vector Elite Kit, Vector Lab) for 30 min and, after rinsing with PBS, developed with a 0.05% chromogen 3,3'-diaminobenzidine tetrachloride (Vector Lab). Total RNA was extracted from the anterior pituitary using TRIzol as previously described (Boiti *et al*., 2007). Total RNA (5 μg, 1μg/μL) was reverse transcribed into first strand cDNA using random hexamers according to instructions (iSCRIPT cDNA Synthesis Kit, Bio-Rad). An aliquot (1.5 μl) of cDNA was used as a template for the subsequent semi-quantitative PCR amplification reaction. This (25.0 μl) was performed with 0.1 μl Taq (Platinum) DNA Polymerase (5 U/μl), 0.5 μl dNTPs (10 mM), 2.5 μl Taq buffer 10x, 1.0 μl (10 μM) of both forward and reverse primers (Table 1). The target genes were co-amplified with housekeeping 18S primers at the same PCR cycle. The PCR reactions consisted of a first denaturing

cycle at 94°C for 75 s, followed by a different number of amplification cycles (Table 1) at 94°C for 15 s, primers annealing temperature (T_a) (Table 1) for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 10 min.

Table 1: Primers for ER-α, GnRH-R, FSHβ, and 18S used as internal standard

Gene		Bp	Primers	T_a Primers	N° cycles
ERα	Sense antisense	147	5'- AGATCCAAGGGAATGAGCTG- 3' 5'- CTGCGGCGTTGAACTCATA- 3'	60°C	35
GnRH-R	Sense antisense	247	5'- CCTTGCCTGGATCCTCAGTA- 3' 5'- ATGAAGGACCCGTGTCAGAG- 3'	60°C	30
FSHβ	Sense antisense	202	5'-CTGACCAACATCACCATTGC -3' 5'-AGTCTGCATGGTGAGCACAG -3'	58°C	28
18S	Sense antisense	489	5'- TCAAGAACGAAAGTCGGAGGTT-3' 5'- GGACATCTAAGGGCATCA-3'		

Statistical analysis

The ratios of each PCR product for target ERα, GnRH-R, and FSH genes, normalized against the 18S co-amplified product, were analyzed by two way ANOVA and Newman-Keuls multi comparison post-test. Data relative to overall treatment effects on LH were analysed by ANOVA for repeated measurements. Comparison between effects was performed by Student's t-test.

RESULTS AND DISCUSSION

Immunolocalization. ERα immunoreactivity was evidenced with a very strong intensity in the nucleus of most pituitary cells of both control and treated does. In fasted, oestrogen primed rabbits, the intensity of the signal for ERα as well as the number of positive cells were markedly reduced. Positive staining for GnRH-R was evidenced in the nucleus and cytoplasm, although weaker, of many pituitary cells of both control and treated does consistently with data reported for other species (La Rosa *et al*., 2000; Choi *et al.*, 2006). In pituitaries of oestrogen primed fasted does, both number of GnRH-R positive cells and signal intensity were markedly reduced compared to control fed animals.

Pituitary gene expression. The corresponding bp amplification products matched the expected sizes for target genes. Sequence analysis showed that the corresponding PCR products were homologues to the published sequences for ERα, GnRH-R, and FSHβ cDNA of rabbits. Fasting as well as oestrogen priming of both fed and fasted rabbits reduced ($P<0.01$) the levels of pituitary ERα mRNA expression (Figure 1, A). The lowest ERα gene expression was found in oestrogen-primed fasted does. GnRH-R mRNA relative abundance was down regulated ($P<0.01$) four-fold in fasted does primed with oestrogen (Figure 1, B). FSH mRNA expression was down-regulated ($P<0.01$) only in fasted rabbits primed with oestrogen (Figure 1, C).

Plasma LH. Prior to GnRH challenge, fasting and oestrogen did not affect plasma LH concentrations which remained low and comparable to those of controls. In all the does, independently of treatment, the LH peak surge was observed 30 min after GnRH injection (Figure 2). The LH magnitude was lower ($P\leq0.01$) in fasted than in fed rabbits, but higher ($P\leq0.01$) in fasted than in fed does primed with estrogens (Figure 2). In all the groups, plasma LH levels declined close to basal values 4 h after GnRH (Figure 2).

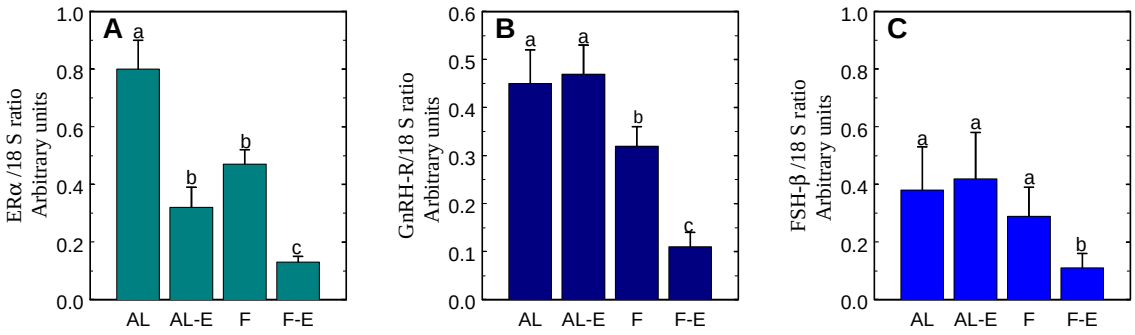

Figure 1: Relative mRNA abundances for ERα (A), GnRH (B), and FSH (C) genes in control and estrogen (E) primed rabbits, either fed (AL) or fasted (F). Different letters above bars indicate a significantly different value (P<0.05) among treatments

In a previous study (Brecchia *et al*., 2006), we hypothesised that estradiol-17β may have a key role in the maintenance of a functional hypothalamic-pituitary axis capable of responding appropriately to GnRH, given that fasted rabbits had very low plasma oestrogen concentrations with lower pulse frequency than control does fed ad libitum. In the present study, the role of estradiol-17β is further supported by the findings that oestrogen replacement to fasted does counteracts the small GnRH-induced LH release despite the scarce density of pituitary GnRH-R at both gene and protein levels. Present data, together with the low levels of circulating estradiol-17β previously reported may explain the fasting-induced decrease of sexual receptivity (Brecchia *et al*., 2006) as well as the reduced GnRH-dependent LH secretory profile. The differences in gene and protein expression for ERα in the pituitary tissue likely reflect the different hormonal and nutritional status of the rabbits after fasting when circulating estrogen levels are reduced and/or estrogens replacement. These findings suggest a direct estrogenic action which may regulate, via ERE, several genes present in the target pituitary cells of rabbits.

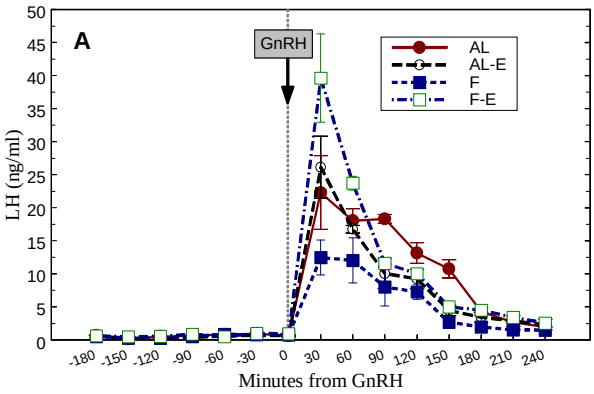

Figure 2: LH profiles in control and estrogen-primed (E) rabbits either fed ad libitum (AL) or fasted (F) following GnRH challenge (arrow)

The expression of GnRH-R in the anterior pituitary, at both gene and protein levels, was found to be down-regulated by the negative energy balance of fasting and by estrogens as well. Interestingly, the GnRH-R was prominently localized in the nuclei of positive pituitary cells suggesting that the G protein-coupled transmembrane receptor is internalized into the nuclei (Choi *et al*., 2006). Thus, the reduction of the GnRH-dependent LH secretion in metabolically stressed rabbits could be the consequence of the concurrent inhibition of the endogenous GnRH release from the neuronal tonic GnRH secretory subsystem and of the decreased density of GnRH-R in pituitary gonadotrophes. This hypothesis is supported, although indirectly, by the findings of Carlson and Perrin (1979) who

observed a decreased pituitary responsiveness in GnRH-treated ovariectomized does. Abundance of mRNA for FSHβ subunit was not affected by estrogen replacement in fed rabbits. Comparable results were obtained by Looper *et al*. (2003) in pituitary glands of nutritionally-induced anovulatory beef cows that were ovariectomized. Contents for FSHβ encoding gene decreased after two days of fasting especially in does under estrogen replacement treatment in good agreement with what reported by Kobayashy and Ishii (2002) in starved Japanese quail.

CONCLUSIONS

The results here obtained confirm that complete deprivation of food for a short period of time could be a useful model for analysing the interrelationships between nutritional factors and reproductive function in rabbits. The rabbit anterior pituitary responds to changes in nutritional status, as provoked by 48 h fasting, and to gonadal steroid through adjustments of ERα, GnRH-R, and FSHβ at the level of gene expression as well as through regulations of LH release into the blood stream in order to adjust the reproductive system to metabolic condition.

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REFERENCES

Bauer-Dantoin A.C., Weiss J., Jameson J.L. 1995. Roles of estrogen, progesterone, and gonadotropin-releasing hormone (GnRH) in the control of pituitary GnRH receptor gene expression at the time of the preovulatory gonadotropin surges. *Endocrinology, 136, 1014-1019.*

Boiti C., Maranesi M., Dall'Aglio C., Pascucci L., Brecchia G., Gobbetti A., Zerani M. 2007. Vasoactive peptides in the luteolytic process activated by PGF2alpha in pseudopregnant rabbits at different luteal stages. *Biol. Reprod., 77, 156-164.*

Brecchia G., Bonanno A., Galeati G., Federici C., Maranesi M., Gobbetti A., Gerani M., Boiti C. 2006. Hormonal and metabolic adaptation to fasting: effects on the hypothalamic-pituitary-ovarian axis and reproductive performance of rabbit does. *Domest. Anim. Endocrinol., 31, 105-122.*

Carlson J.C., Perrin D.G. 1979. The effect of LH-RH administration on LH release in the female rabbit. *J. Reprod. Fertil., 56, 175-180*.

Choi J.H., Gilks C.B., Auersperg N., Leung P.C.K. 2006. Immunolocalization of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and type I GnRH receptor during follicular development in the human ovary. *J. Clin. Endocrinol. Metab., 91, 4562-4570*.

Hapgood J.P., Sadie H, van Biljon W., Ronacher K. 2005. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *J. Neuroendocr., 17, 619-638.*

Kobayashy M., Ishii S. 2002. Effects of starvation on gonadotropin and thyrotropin subunit mRNA levels and plasma hormone levels in the male Japanese quail (Coturnix coturnix japonica). *Zoolog. Sci., 19, 331-342.*

La Rosa S., Celato N., Uccella S., Capella C. 2000. Detection of gonadotropin-releasing hormone receptor in normal human pituitary cells and pituitary adenomas using immunohistochemistry. *Virchows Arch., 437, 264-269.*

Limonta P., Ladizhenskaya A., Gunsalus G.L., Bardin C.W., Thau R.B. 1986. Regulation of pituitary gonadotropin-releasing hormone receptors by androgens in the male rabbit. *Endocrinology, 118, 340-347*.

Looper M.L., Vizcarra J.A., Wettemann R.P., Malayer J.R., Braden T.D., Geisert R.D., Morgan G.L. 2003. Influence of estradiol, progesterone, and nutrition on concentrations of gonadotropins and GnRH receptors, and abundance of mRNA for GnRH receptors and gonadotropin subunits in pituitary glands of beef cows. *J. Anim. Sci., 81, 269-278.*

Pau K.Y., Spies H.G. 1997. Neuroendocrine signals in the regulation of gonadotropin-releasing hormone secretion. *Chin. J. Physiol., 40, 181-196*.

Rispoli L.A., Nett T.M. 2005. Pituitary gonadotropin-releasing hormone (GnRH) receptor: structure, distribution and regulation of expression. *Anim. Reprod. Sci., 88, 57-74*.

Wade G.N., Jones J.E. 2004. Neuroendocrinology of nutritional infertility. *Am. J. Physiol. Regul. Integr. Com. Physiol., 25, R1277-1296.*

Younglai E.V., Todoroff E.C. 1992. The pituitary gonadotropin-releasing hormone (GnRH) receptor of the female rabbit: characterization and developmental aspects. *Can. J. Physiol. Pharmacol., 70, 1639-1646.*