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RESEARCH ARTICLE

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A role for GABA agonist in controlling the reproduction of female rats via hypothalamic ghrelin, kisspeptin, and RFRP-3 gene expression

Elaheh Rahimi Rick[a], Fariba Mahmoudi[a], Homayoun Khazali[b], Asadollah Asadi[a], Mahnaz Ghowsi[c]

[a] Faculty of Sciences, University of Mohaghegh Ardabili, Ardabil, Iran.
[b] Department of Animal Science and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.
[c] Department of Biology, Faculty of sciences, Razi University, Kermanshah. Iran.

ABSTRACT

Kisspeptin stimulates gonadotropin releasing hormone (GnRH). The GnRH neurons receive inhibitory inputs from ghrelin, RFamide related peptide-3 (RFRP-3), and gamma-aminobutyric acid (GABA) neurons. Polycystic ovary syndrome (PCOS) is associated with increased levels of GnRH/LH and kisspeptin, and decreased release of GABA, ghrelin, and RFRP-3. In the present study, the effects of GABAB receptor agonist, baclofen, were investigated on *GnRH*, *KiSS1*, RFRP-3, and *ghrelin* gene expression in the hypothalamus of PCOS model rats. For induction of PCOS, female Wistar rats weighing 180-200g received intra-muscular injection of estradiol valerate. Fifteen PCOS rats in three groups received intraperitoneal injections of saline, 5, or 10 mg/kg baclofen for two weeks. The hypothalamic samples were dissected. Gene expression levels of *GnRH*, *KiSS1*, *RFRP-3*, and *ghrelin* were determined by real time qPCR method. Results revealed that baclofen significantly decreased the mean relative *KiSS1* gene expression compared to PCOS group. Also, the mean relative *RFRP-3* gene expression significantly increased in the baclofen-receiving rats in comparison to PCOS group. Furthermore, baclofen did not change GnRH or ghrelin mRNA levels in comparison to PCOS group. According to these results it can be concluded that in PCOS condition the GABAergic signaling pathway may suppress GnRH neural activity via down or up regulation of the intra-hypothalamic neuropeptides upstream of GnRH neurons.

Keywords

Baclofen, GnRH, kisspeptin, ghrelin, RFRP-3.

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Abbreviations

GnRH: Gonadotropin releasing hormone
RFRP-3: RFamide related peptide-3
GABA: Gamma-aminobutyric acid
PCOS: Polycystic ovary syndrome
LH: Luteinizing hormone
HPG: Hypothalamus - pituitary- gonadal
ARC: Arcuate nucleus
AVPV: Antero-ventral periventricular nucleus
POA: Preoptic area

Corresponding authors: *Fariba Mahmoudi*
Emails: *f.mahmoudi@uma.ac.ir*
Tel: *+96(45)3351-4701*

Introduction

Metabolic hormones regulate the normal release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH). In addition to insulin resistance, the defects of hypothalamus - pituitary- gonadal (HPG) axis play a crucial role in the pathogenesis of polycystic ovary syndrome (PCOS) [1, 2].

Gamma-aminobutyric acid (GABA) suppresses the activity of GnRH neurons. Increased synthesis of GnRH has been shown in GABAB receptor knock-out female and male mice [3]. Baclofen inhibits the firing rate of GnRH neurons via hyper-polarization of them [4]. Using GABAB receptor antagonists completely neutralizes the inhibitory effects of baclofen on the firing rate of GnRH neurons [4]. It has been indicated that baclofen; GABAB receptor agonist, inhibits LH secretion [5]. The release of inhibitory neurotransmitters upstream of GnRH neurons such as dopamine and GABA are decreased in PCOS patients [6, 7].

Kisspeptin is a hypothalamic neuropeptide that is located in the arcuate nucleus (ARC) and antero-ventral periventricular nucleus (AVPV) of the hypothalamus. Kisspeptin acts upstream of GnRH neurons and conveys metabolic information to GnRH neurons [8, 9]. Kisspeptin/GPR54 signaling system regulates the HPG axis. Central or peripheral injection of the GPR54 receptor antagonist, peptide 234 blocks the stimulatory effect of kisspeptin on HPG axis activity [9]. The kisspeptin/GPR54 signaling system is one of the most important therapeutic targets to stimulate GnRH/LH release [8, 9].

RFamide related peptide-3 (RFRP-3) is a hypothalamic neuropeptide whose neuron cell bodies are located mainly in the dorsomedial hypothalamic nucleus (DMN). The fibers of RFRP-3 neurons project to other hypothalamic nuclei especially the preoptic area (POA), antero-ventral periventricular nucleus (AVPV), and arcuate nucleus (ARC) [10]. It has been revealed that RFRP-3 hyperpolarizes GnRH neurons and inhibits GnRH and LH secretion [10, 11].

Ghrelin, is an orexigenic peptide that is produced in the hypothalamus, stomach, and other peripheral organs [12, 13]. Ghrelin inhibits GnRH/LH and testosterone secretion [12, 13]. The GnRH neurons receive direct or indirect inputs from ghrelin neurons [13]. *Ghrelin* down-regulates *KiSS1* gene expression and decreases the stimulatory effects of kisspeptin on GnRH/LH release [14, 15]. In the present study, the effects of baclofen were investigated on hypothalamic *GnRH, KiSS1, RFRP3* and *ghrelin gene* expression in a rat model of PCOS.

Results

Mean relative *GnRH* gene expression did not significantly increase in the hypothalamus of PCOS rats in comparison to the control group (Figure 1). In PCOS rats that received 5 or 10mg/kg of baclofen, the mean relative *GnRH* gene expression did not significantly decrease in comparison to PCOS control group ($p \leq 0.05$, Figures 2 and 3).

The mean relative *KiSS1* gene expression increased significantly in the hypothalamus of PCOS rats compared to the control group ($p \leq 0.05$, Figure 1). In PCOS rats that received 5 or 10mg/kg of baclofen, the mean relative *KiSS1* gene expression significantly decreased in comparison to the PCOS control group ($p \leq 0.05$, Figures 2 and 3).

Induction of PCOS did not significantly decrease the mean relative hypothalamic *RFRP-3* gene expression compared to the control group (Figure 1). The mean relative *RFRP-3* gene expression significantly increased in PCOS rats that received 5 or 10mg/kg baclofen in comparison to PCOS control group ($p \leq 0.05$, Figures 2 and 3).

The mean relative *ghrelin* gene expression significantly decreased in PCOS rats in comparison to the control group ($p \leq 0.05$, Figure 1). The mean relative ghrelin gene expression did not significantly increase in PCOS rats that received 5 or 10mg/kg baclofen in comparison to the PCOS control group ($p \leq 0.05$, Figures 2 and 3).

Discussion

The obtained results showed that *GnRH* and *KiSS1* gene expression increased in the hypothalamus of PCOS rats. The results are in accordance with the literature and demonstrate that the higher GnRH/LH and kisspeptin levels are involved in the pathogenesis of PCOS [1, 16]. The increased kisspeptin neuronal activity leads to higher GnRH neuronal activity which results into excessive androgen secretion in PCOS patients [1, 16].

The present results showed that injection of baclofen significantly decreased the hypothalamic KiSS1 mRNA levels in PCOS model rats. Here we show the effects of baclofen on kisspeptin gene expression for the first time in PCOS condition. However, the present data are consistent with the previous studies that established an interaction between kisspeptin and GABAergic systems to control LH secretion. Both GABAA and GABAB receptor subtypes are expressed in kisspeptin neurons. Injection of baclofen hyperpolarizes the kisspeptin neurons and disturbs the surge secretion of GnRH/LH [17, 18]. Also, GABA release decreases in the PCOS conditions [6, 7] and the GABA- transaminase enzyme that degrades GABA,

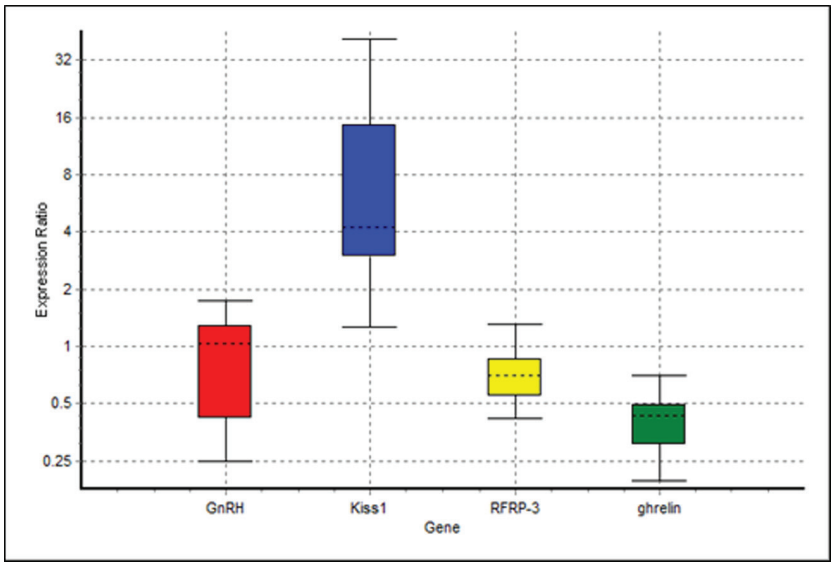

Figure 1.
The mRNA fold change of *GnRH*, *Kiss1*, *RFRP-3*, and *ghrelin* genes in PCOS rats in comparison to intact control rats. The cDNA amplified from GAPDH mRNA (as reference gene) was used to normalize the data. The significance difference was defined by $p \leq 0.05$.

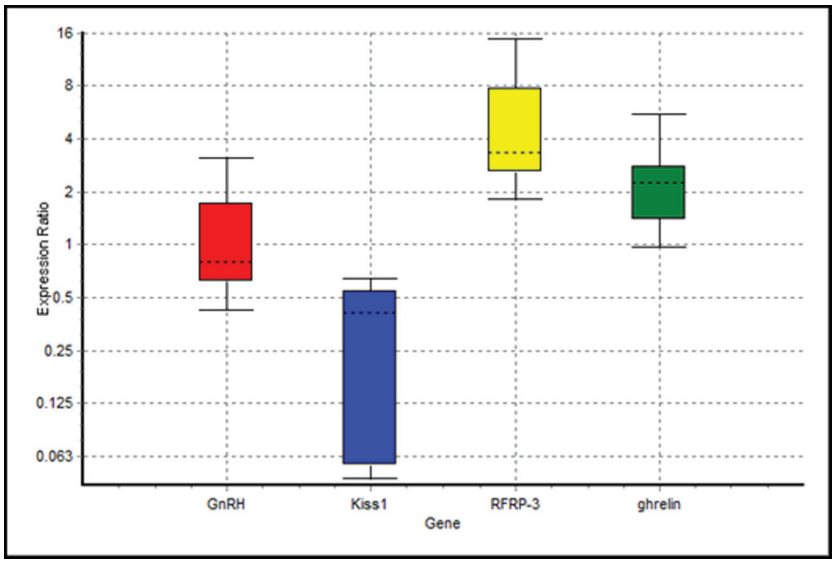

Figure 2.
The mRNA fold change of *GnRH*, Kiss1, *RFRP-3*, and *ghrelin* genes in PCOS rats receiving 5mg/kg baclofen in comparison to PCOS rats. The cDNA amplified from GAPDH mRNA (as reference gene) was used to normalize the data. The significance difference was defined by $p \leq 0.05$.

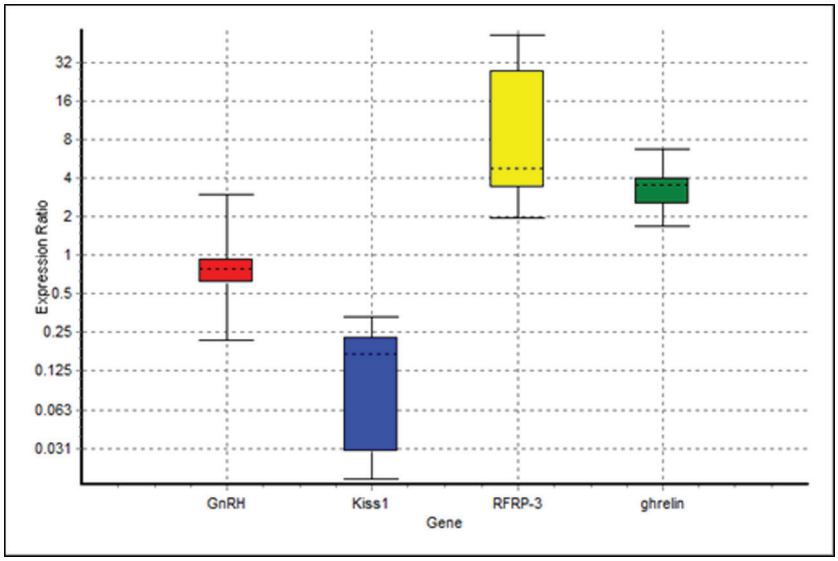

Figure 3.
The mRNA fold change of *GnRH*, *Kiss1*, *RFRP-3*, and *ghrelin* genes in PCOS rats receiving 10mg/kg baclofen in comparison to PCOS rats. The cDNA amplified from GAPDH mRNA (as reference gene) was used to normalize the data. The significance difference was defined by $p \leq 0.05$.

is significantly increased in the hypothalamus and pituitary of PCOS rats in comparison to the control group while glutamic acid decarboxylase enzyme that converts glutamate into GABA, is decreased in the hypothalamus and pituitary of PCOS rats [2]. Previous studies indicated that kisspeptin abolishes the inhibitory effects of baclofen on GnRH neurons [4]. Also, the administration of baclofen following kisspeptin attenuates the excitatory influences of kisspeptin on the depolarization of GnRH neurons [5]. Increased KiSS1 mRNA levels were observed in the arcuate nucleus (ARC) of GABAB receptor knock-out mice [3]. So, in this study, the decreased hypothalamic KiSS1 mRNA levels by baclofen may be a possible mechanism for the decline of GnRH synthesis in PCOS rats.

To find mechanisms involved in the regulatory effects of baclofen on *kisspeptin* gene expression, this study investigates the effects of baclofen on intra hypothalamic neuropeptides such as ghrelin and RFRP-3, both acting upstream of kisspeptin and GnRH neurons. The obtained results revealed that baclofen exerts a stimulatory effect on ghrelin mRNA levels in PCOS conditions. The results are in line with the previous studies and demonstrate an interaction between GABAergic, ghrelin, and kisspeptin signaling pathways. According to the previous results, ghrelin decreases GnRH/LH secretion and KiSS1 mRNA levels in the hypothalamus and pancreas [14, 15] while baclofen increases the plasma ghrelin concentration [19]. So, increasing hypothalamic ghrelin mRNA levels may be a contributing factor for baclofen to decrease *KiSS1* gene expression in PCOS rats.

Our results demonstrated that the PCOS condition did not cause a significant decrease in hypothalamic *RFRP-3* gene expression in comparison to control rats. This is in contrast to the findings of Shaaban et al. that demonstrated a significant decrease of RFRP-3 mRNA levels in dorsomedial hypothalamic nucleus [20]. Maybe this conflict could be to the used method for induction of PCOS model rats. Herein estradiol valerate was used for induction of PCOS, while Shaaban et al. used constant light induction to generate PCOS model [20]. However, further studies are needed for evaluation of the *RFRP-3* gene expression in PCOS conditions. Interestingly, our results indicate the stimulatory effects of baclofen on *RFRP-3* gene expression in PCOS rats. As previously shown, the RFRP-3 suppresses the GnRH/LH secretion [21] and there is a reverse relationship between RFRP-3 and kisspeptin function [21]. The RFRP-3 receptor (GPR147) is expressed in kisspeptin neurons located in ARC and AVPV nuclei of hypothalamus and RFRP-3 fibers project to kisspeptin neurons [22, 23]. For interpretation of the obtained results, it can be suggested that the increase of RFRP-3 mRNA levels after baclofen injections might play an important role in suppressing kisspeptin and GnRH neural activity. To better understand the action of GABAergic system on controlling HPG axis activity in PCOS conditions, it is suggested that further studies should try to investigate the effects of intra cerebral ventricular injection of baclofen or other GABA agonists on gene expression levels of ovarian or intra hypothalamic peptides upstream of GnRH neurons.

In conclusion, polycystic ovary syndrome (PCOS) is associated with increased mRNA levels of hypothalamic kisspeptin which stimulate the activity of hypothalamus- pituitary- gonad (HPG) axis. However, mRNA levels of inhibitory neuropeptides upstream of GnRH neurons such as ghrelin decreased in the hypothalamus of PCOS rats. Our results demonstrated that the intraperitoneal injections of baclofen, significantly decreased KiSS1 mRNA levels in the hypothalamus of PCOS rats. Baclofen exerts stimulatory effects on hypothalamic ghrelin and RFRP-3 mRNA levels in the hypothalamus of PCOS rats. The obtained results suggest that GABAergic signaling pathway is involved in the controlling of HPG axis activity to some extent by down- or up-regulation of the hypothalamic stimulatory and inhibitory neuropeptides such as kisspeptin, ghrelin, or RFRP-3 in PCOS patients.

Materials & Methods

Animals

In this study, 20 female Wistar rats weighing 180-200 g (provided by the Iran University of Medical Sciences) were housed in the cages under controlled temperature (22 ± 2 °C) and light (12h light/ dark cycle). All procedures for the maintenance and the use of experimental animals were approved by the research and ethical committee of Ardabil University of Medical Sciences (code: IR.ARUMS.REC.1398.511).

Induction of polycystic ovary syndrome

The vaginal smear was performed for two consecutive weeks to select the rats with the normal estrus cycle. In the estrus stage which was characterized by cornfield epithelial cells, 15 rats received an intramuscular single dose of 2 mg/rat estradiol valerate (Aburayhan Co., Iran) dissolved in 0.2 ml sesame oil (Barij Essence Co., Iran). Five rats in the estrus stage received a single intramuscular injection of 0.2 ml sesame oil as an intact control group. Sixty days after the estradiol valerate injection, the polycystic status was confirmed by observation of persist cornfield epithelium cells with vaginal smear.

Intraperitoneal injections

Fifteen PCOS rats in three groups received intraperitoneal injections of saline, 5, or 10 mg/kg baclofen (Zahravi Co., Iran) in a volume of 0.2 ml at 9:00-9:30 for two weeks. Also, five intact rats received 0.2ml saline as a control group for two weeks.

Microdissections and real-time polymerase

chain reaction (RT-PCR)

One day following the last injection, animals were anesthetized by injection of ketamine and xylazine. The hypothalamic samples were dissected. According to coordinates of the Paxinos and Watson Atlas, the brains were placed ventral side up, and anterior coronal slices were cut from 1 mm anterior to optic chiasm. The slices were dissected laterally up to the hypothalamic sulci, and posterior coronal slices were cut posterior to the mammillary bodies [24, 25]. Hypothalamic samples were stored at -80 °C. Total RNA was isolated from individual frozen samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method.

To synthesize the first-strand cDNA, 5µg total RNA, 1µl of 100 µM Oligo(dT)$_{18}$ primer, 4µl of 5X Reaction Buffer, 1µl of RiboLock RNase Inhibitor (20 U/µl), 2µl of 10 mM dNTP Mix, 1µl of RevertAid RT (200 U/µl), and nuclease free water in a volume of 20 µl were incubated at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 5min (Thermo Scientific RevertAid RT reverse transcription kit, USA). Changes in gene expression levels were determined by using Rotor Gene 6000 (Corbette, Germany) and SYBR Green I kit (Takara Bio Inc., Japan). The PCR cycling conditions were as following: first denaturation 95 ºC for 2 min, followed by 40 cycles of denaturation at 95 ºC for 5 sec, annealing at 60 ºC for 20sec (*KiSS1, RFRP-3, GnRH* or *GAPDH*), annealing at 54 ºC for 20 sec for *ghrelin* and extension at 60 ºC for 25 sec. Specific oligonucleotide sequences for forward and reverse primers are shown in Table 1. The GnRH, KiSS1, RFRP-3, ghrelin and GAPDH amplified products were 133, 98, 93, 132, and 120 base pairs, respectively. In this study PCR efficiency of each gene was calculated using LinRegPCR software. Based on the outputs derived from LinReg PCR software, the PCR efficiency for GAPDH, ghrelin, GnRH, Kiss1 and RFRP-3 were 2.06, 1.762, 2.041, 1.78 and 1.846, respectively.

Table 1.
Sequences of forward and reverse primers used in this study.

Gene	primers sequences
GnRH (NM_012767)	F: 5'-GCCGCTGTTGTTCTGTTGACTG-3'
	R: 5'- CCTCCTCCTTGCCCATCTCTTG-3'
KiSS1 (NM_181692)	F: 5'- TGATCTCGCTGGCTTCTTGGC -3'
	R: 5'- GGGTTCAGGGTTCACCACAGG-3'
RFRP3 (NM_023952)	F: 5'- GAGTCCTGGTCAAGAGCAAC-3'
	R: 5'-ACTGGCTGGAGGTTTCCTAT -3'
Ghrelin (NM_021669)	F: 5'- AATGCTCCCTTCGATGTTGG -3'
	R: 5'-CAGTGGTTACTTGTTAGCTGG -3'
GAPDH (XM_039103945)	F: 5'- AAGAAGGTGGTGAAGCAGGCATC -3'
	R: 5'-CGAAGGTGGAAGAGTGGGAGTTG-3'.

Statistical analysis

The data were analyzed by using REST 2009 software. In all cases, the significance was defined by $p \leq 0.05$.

Authors' Contributions

F.M. and H. KH. conceived and planned the experiments. E.R.R. and F.M. carried out the experiments. F.M., H.KH., E. R.R., A.A., and M.GH. contributed to the interpretation of the results. F.M. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, data analyses, and the manuscript.

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Competing Interests

The authors declare no conflict of interest.

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