Kisspeptin mRNA expression is increased in the posterior hypothalamus in the rat model of polycystic ovary syndrome

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Abstract. Hypersecretion of luteinizing hormone (LH) is a common endocrinological finding of polycystic ovary syndrome (PCOS). This derangement might have a close relationship with hypothalamic kisspeptin expression that is thought to be a key regulator of gonadotropin-releasing hormone (GnRH). We evaluated the relationship between the hypothalamic-pituitary-gonadal axis (HPG axis) and kisspeptin using a rat model of PCOS induced by letrozole. Letrozole pellets (0.4 mg/day) and control pellets were placed subcutaneously onto the backs of 3-week-old female Wistar rats. Body weight, vaginal opening and vaginal smear were checked daily. Blood and tissues of ovary, uterus and brain were collected at 12-weeks of age. An hypothalamic block was cut into anterior and posterior blocks, which included the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC), respectively, in order to estimate hypothalamic kisspeptin expression in each area. The letrozole group showed a similar phenotype to human PCOS such as heavier body weight, heavier ovary, persistent anovulatory state, multiple enlarged follicles with no corpus luteum and higher LH and testosterone (T) levels compared to the control group. Kisspeptin mRNA expression in the posterior hypothalamic block including ARC was higher in the letrozole group than in the control group although its expression in the anterior hypothalamic block was similar between groups. These results suggest that enhanced KNDy neuron activity in ARC contributes to hypersecretion of LH in PCOS and might be a therapeutic target to rescue ovulatory disorder of PCOS in the future.

Key words: PCOS, KNDy neuron, Kisspeptin, LH, Letrozole

POLYCYSTIC OVARY SYNDROME (PCOS) is an ovulatory disorder that is characterized by hyperandrogenism, infertility and anovulation, and is seen in 6%-10% of women of reproductive age [1-3]. Abnormal gonadotropin-releasing hormone (GnRH) secretion, insulin resistance and obesity are common findings in PCOS patients. Compensatory hyperinsulinemia further stimulates testosterone (T) and androstenedione synthesis in theca cells and reduces sex hormone binding globulin (SHBG) [4, 5]. Reduced SHBG level leads to increased free androgen and estrogen levels. Many studies have reported abnormal gonadotropin secretion including high luteinizing hormone (LH) pulse frequency/amplitude, elevated serum LH and relatively lower follicle stimulating hormone (FSH) levels in PCOS women [6-9]. However, the mechanism by which LH is elevated in PCOS has not been fully determined because of the difficulty of studying the hypothalamus of PCOS patients.

There are several animal models of PCOS. Although a fully convincing animal model representing all features associated with human PCOS has not been established, several elements of PCOS-associated endocrine and metabolic abnormalities are reproduced in animal models of PCOS. Rodent models have often been used due to advantages such as in handling because of their smaller size, a short reproductive lifespan and a short estrous cycle. These rodent PCOS models have some differences due to the induction methods used. The estrogen-induced rat model shows lack of hyperandrogenemia, whereas the androgen-induced rat model does not display an irregular cycle, polycystic ovarian morphology or hormonal...
abnormality but does display metabolic features of PCOS [10]. However, the rat model induced by letrozole, a non-steroidal aromatase inhibitor, shows elevated LH and T levels, anovulation, absence of corpus luteum, hyperplasia of the theca cell layer, diminished granulosa cells and increased ovarian size, which are similar findings to human PCOS [11-13]. Therefore, the letrozole-induced PCOS model rat may be suitable for study of the mechanism of anovulation, including hypersecretion of LH originated from the hypothalamus, which cannot be tested in the hypothalamus of human PCOS.

Kisspeptin and its receptor GPR54 signaling have been identified as essential factors for GnRH secretion and onset of puberty [14]. Kisspeptin also has a key role in ovulation and fertility regulation through GnRH neuron activation [14-16]. Although kisspeptin might have a close relationship with hypersecretion of LH in PCOS, the features of such a relationship have not yet been clearly explained. In the present study we studied the hypothalamic-pituitary-gonadal axis (HPG axis) and kisspeptin expression using a rat model of PCOS induced by letrozole.

**Materials and Methods**

**Animals**

Nineteen immature female Wistar rats were purchased from Charles River Laboratories, Inc. (Tokyo, Japan). The rats were housed in a temperature-controlled room (24 °C) under a daily photoperiod of 14 h light: 10 h darkness (lights on at 0700 h) and were given food and tap water *ad libitum*. This study was approved by Institutional Animal Care and Use Committee, and all experiments were conducted in accordance with the ethical standards of the Institutional Animal Care and Use Committee of the University of Tokushima.

**Study procedure**

At the age of 3-weeks, the rats were randomly divided into two experimental groups, control (n=10) and letrozole (n=9), into which control pellets and letrozole releasing pellets (0.4 mg/day; Novartis Pharma AG, Basel, Switzerland), respectively, were implanted subcutaneously onto the back. The dose of letrozole was chosen according to the literature [13]. Control rats received pellets with saline. Body weight was measured daily during the study. The estrous cycle was estimated by daily vaginal smear after vaginal opening. Blood and tissues of ovary, uterus and hypothalamus were collected at 12 weeks of age in letrozole rats. These sampling were done on diestrus day in control rats during 12 weeks of age. Anterior and posterior hypothalamic tissue blocks were cut as reported previously and based on rat brain atlas [17, 18]. In this cutting method, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) are included in the anterior and posterior hypothalamic blocks, respectively. The hypothalamic tissues were snap frozen and stored -80 °C. Serum was isolated by centrifugation and stored at -20 °C.

**Real time reverse-transcription PCR analysis**

Total RNA was extracted from the anterior and posterior hypothalamic tissue blocks using the TRIzol® reagent kit (Invitrogen Co., Carlsbad, CA, USA) and an RNeasy® Mini kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized with an Oligo (deoxymyridine) primer at 50 °C using the SuperScript III First-Standard Synthesis System and the real-time polymerase chain reaction (RT-PCR) (Invitrogen™, Thermo Fisher K.K., Tokyo, Japan). RT-PCR analysis was performed by using the StepOnePlus™ RT-PCR system (PE Applied Biosystems, Foster City, CA, USA) and the Fast SYBR® Green Master Mix (Thermo Fisher K.K., Tokyo, Japan). Expression levels were normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level. The forward and reverse primers used were as follows: Kiss1: F: 5′-ATG ATC TCG CTG GCT TCT TGG-3′, R: 5′-GGT TCA CCA CAG GTG CCA TTT T-3′; GAPDH: F: 5′-ATG CCA CAG TGA GGT CCA TCT TTT T-3′, R: 5′-CCC TGA CCA GAG TGA ATG ATG AT-3′. The PCR conditions were as follows: initial denaturation and enzyme activation at 95 °C for 20 s, followed by 45 cycles of denaturation at 95 °C for 3 s and annealing at 65 °C for 30 s (Kiss1) or 64 °C for 30 s (GAPDH), and then extension at 72 °C for 1 min. The copy numbers of the transcripts were normalized against those of GAPDH transcripts for each sample [18, 19].

**Hormone assays**

Serum LH and FSH concentrations were determined using an I-125 radioimmunoassay kit (Rat LH and FSH [I-125] RIA kit; Institute of Isotopes Co., Ltd., Tokyo, Japan). The analytical sensitivity of LH and FSH assays were 0.1 mU/mL and 0.1 mU/mL, respectively, and intra-assays of LH and FSH were 6.5% and...
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4.2%, respectively. Serum T concentration was measured using the electrochemiluminiscense immunoassay kit ECLusys TESTO II (Roche Diagnostics, K.K., Tokyo, Japan). The analytical sensitivity of T assay was 0.45 ng/mL, and inter- and intra-assay coefficients of variation of the specimen whose T concentration was close to values in our experiment were 2.5% and 2.1%, respectively [20]. Serum Estradiol (E2) concentration was measured using the electrochemiluminiscense immunoassay kit ECLusys Estradiol II (Roche Diagnostics). The analytical sensitivity of E2 assay was 143 ng/mL, and inter- and intra-assay coefficients of variation of the specimen whose E2 concentration was close to values in our experiment were 7% and 6.1%, respectively [21].

**Uterine and ovarian weight and morphology**

Uteri and ovaries were weighed to evaluate the effect of letrozole. The ovaries were cut through longitudinally, fixed with 10% neutral formalin, and cut in 4 µm-thick sections. The sections were placed on a glass slide, stained with hematoxylin and eosin, and analyzed under a microscope.

**Statistical analysis**

Body weight, ovarian and uterine weights, serum hormone concentrations and hypothalamic Kiss1 mRNA expression were analyzed by one-way analysis of variance. All data are presented as mean±SE values. $p<0.05$ and $p<0.01$ were considered significant in all analyses.

**Results**

**Effect of letrozole on body weight**

The body weight of the letrozole group was heavier than that of the control group from 3 weeks after pellet implantation. Body weight was significantly heavier in the letrozole group than in the control group in rats from 6- to 12-weeks of age ($p<0.05$) (Fig. 1). This finding was identical as previous report [13].

**Ovarian morphology, ovarian and uterine weight**

The letrozole group showed a persistent anovulatory state in the estrous cycle, while the control group showed a regular 4-5 day estrous cycle (data not shown). The letrozole group had a significantly heavier ovary than the control group at the end of letrozole administration, at which time the rats were 12 weeks old ($p<0.05$, Fig. 2). Conversely, uterine weight was significantly lighter in the letrozole group than in the control group at 12-weeks of age ($p<0.05$, Fig. 2). Multiple enlarged follicles were observed in the ovary of the letrozole group (Fig. 3). These cystic follicles were filled with clear fluid. The follicle wall was stiffened due to decreased granulosa cells, a smaller granulosa cell layer and a thickened theca interna cell layer. The ovaries of the letrozole group had no corpus luteum and displayed higher numbers of follicles than the control group. The corpus luteum and follicle growth in each stage were observed in the control group. These findings were identical as previous reports [11-13].

![Graph showing body weight changes](image)

**Fig. 1** Body weight was measured over 12 weeks and is expressed as a percentage of the body weight at 3 weeks of age. Values are means±SE. * $p<0.05$ vs. control group.
Effect of letrozole on the serum steroid concentrations

Serum LH and T concentrations of the letrozole group were significantly higher than those of the control group ($p<0.05$ for both) (Fig. 4). In contrast the serum $E_2$ concentration of the letrozole group was significantly lower than that of the control group ($p<0.05$, Fig. 4). Serum FSH was not significantly different between the groups. These findings were identical as previous reports [11-13].

Hypothalamic Kiss1 mRNA expression levels

At 12 weeks of age, i.e., at 9 weeks after pellet implantation, the hypothalamic Kiss1 mRNA expression level of the letrozole group was significantly higher than that of the control group in the posterior hypothalamic tissue block that includes ARC ($p<0.05$) (Fig. 5). In contrast, the Kiss1 mRNA expression level in the anterior hypothalamic tissue block that includes AVPV was not significantly different between the groups.

Discussion

In this study, a rat model of PCOS induced by letrozole showed similar features to human PCOS including increased body fat, polycystic ovaries, enlarged ovaries, a thickened theca cell layer, a diminished granulosa cell layer, irregular cycle, anovulation and elevated serum LH and T levels. Using this model, we found that hypothalamic Kiss1 mRNA expression was increased in the posterior hypothalamic tissue block that included ARC but was not increased in the anterior tissue block that included AVPV.
In the past decade, a range of animal models such as rodents, sheep and primates have been developed and investigated to determine the etiology and pathology of PCOS. The rodent models of PCOS exhibit the majority of reproductive and endocrine findings that are associated with human PCOS including hyperandrogenism, hypersecretion of LH, disrupted cyclicity and polycystic ovary. For example, E2-induced rat models showed polycystic ovaries as well as hyperthecosis and lack of hyperandrogenemia [22, 23]. Prenatal dihydrotestosterone (DHT) treated rats showed high serum T, E2, progesterone (P4), LH and elevated LH pulse frequency [24]. Androgen-induced rat models of PCOS are mostly used to study pathophysiological mechanisms of PCOS. However, these models might not be suitable for the study of GnRH secretion, because hypothalamic Kiss1 mRNA expression is decreased in DHT treated rats [25]. For the present study, we selected letrozole-induced PCOS model rats, which show elevated serum T and LH levels, polycystic ovarian morphology and enlarged ovaries [11-13], because these hormonal and histologic findings are similar findings to human PCOS except for...
the relatively lower serum E₂ level. Letrozole blocks conversion of androgen production to estrogen, resulting in endogenous androgen synthesis accumulation in ovary, which contributes to polycystic ovarian morphology without follicular maturation, ovulation and also induces abnormal feedback signal to hypothalamus. Dose of letrozole used to induce PCOS model rats (approximately 1.6-8.0 mg/kg/day continuously for 10 weeks) is extremely higher than therapeutic dose sometimes used in human PCOS (approximately 0.05 mg/kg/day for only 5 days in one cycle).

In recent years, kisspeptin has been shown to be a key essential regulator of brain sex differentiation, puberty onset, gonadotropin secretion, ovulation and fertility. In rodents, two major kisspeptin neuron populations have been described, which are located in the hypothalamic ARC and AVPV nuclei. ARC kisspeptin is thought to be a key regulator that functions as a GnRH pulse generator that regulates LH pulse frequency [26]. Kisspeptin is co-expressed with other neuropeptides including neurokinin B (NKB) and dynorphin (Dyn), leading to the name the “KNDy neuron” [27, 28]. NKB also plays a stimulatory role in GnRH/LH pulsatile secretion [29-31], whereas Dyn and its kappa-opioid receptor (KOR) suppress LH secretion [32, 33] by modulating the activity of the KNDy neuron itself. These neuropeptides are regulated by estrogen and progesterone [34]. We found high expression of Kiss1 mRNA in the ARC-containing hypothalamic tissue block in PCOS model rats. It is assumed that enhanced KNDy neuron activity stimulates LH secretion via impaired GnRH/LH pulsatile secretion and that NKB/NK3R and Dyn/KOR signaling in the ARC KNDy neurons would be influenced by the chronic estrogen effect with absent P₄.

Theoretically, sex hormones consistently inhibit GnRH/LH pulsatile secretion by a negative feedback mechanism at the KNDy neuron, which is the ARC kisspeptin neuron in rodents [35]. On the other hand, in humans who have a longer cycle than rodents, the LH pulse frequency is the shortest in the mid and late follicular phase (around 70 min) when the estrogen level is high, while it is longer in the early follicular phase (90-100 min) and in the luteal phase (120-200 min) [36]. The tone or strength of the estrogen negative feedback on gonadotropin secretion appears to be transient according to follicular development in humans [37]; the tone of the negative feedback to the hypothalamus is weaker in the late follicular phase than in the early follicular phase. This hypothesis could explain the phenomenon that pulsatile secretion of GnRH/LH is more frequent in the late follicular phase than in the early follicular phase. Pulsatile secretion of LH is generated by pulsatile release of GnRH, which would be driven by pulsatile neuronal excitation of the ARC kisspeptin neuron. PCOS women show frequent pulsatile LH secretion although they do not have developing or mature follicles. In fact, PCOS women who have constant estrogen activity due to multiple antral follicles show high LH pulse frequency that is similar to mid or late follicular phase women and is different from the early follicular phase [8]. Constant estrogen feedback from a lot of antral follicles would stimulate the hypothalamus in PCOS, in which the estrogen effect is similar to the late follicular phase in normal cyclic women. Our rat model of PCOS induced by letrozole showed anovulation and a continuous serum E₂ level, even though this level is somewhat low due to aromatase inhibition. The combined data suggest that constant estrogen activity in PCOS just like that in the late follicular phase seemed to decrease the negative feedback tone and resulted in frequent excitation of the ARC kisspeptin neuron (KNDy neuron) and frequent pulsatile GnRH/LH secretion.

Progesterone administration inhibited the LH pulse frequency in normal women but not in PCOS women [38]. Similarly, E₂ and P₄ co-administration reduced and normalized GnRH/LH pulse frequency and LH/FSH ratio in PCOS women [39]. P₄ treatment increased Dyn concentrations and decreased LH pulse frequency in cerebrospinal fluid and restored preprodynorphin mRNA expression in the hypothalamus in an ovariectomized ewe [40], suggesting that Dyn in the KNDy neuron inhibited kisspeptin release and GnRH/LH pulse frequency via P-mediated negative feedback. Recently, Kondo et al. reported that a PCOS model rat induced by the anti-progesterin RU486 showed increased kisspeptin immunoreactivity in ARC, which might be involved in the pathophysiology of PCOS women [41]. Their finding is similar to our finding, even though we used a different induction method, suggesting the importance of enhanced KNDy neuron activity in PCOS pathophysiology. Furthermore, loss of P₄ inhibition might also play an important role in GnRH/LH secretion that leads to increase LH pulsatility in PCOS.

In conclusion, a rat model of PCOS induced by letrozole showed elevated expression of kisspeptin in the posterior hypothalamic area including ARC where
KNDy neurons are located. Enhanced KNDy neuron activity would induce a GnRH/LH pulse frequency through decreased or impaired negative feedback tone. Such derangement might be involved in human PCOS pathophysiology and could be a therapeutic target of future treatment for PCOS women.

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References


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Disclosure

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