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—Original Article—

Differential Effects of Continuous Exposure to the Investigational Metastin/Kisspeptin Analog TAK-683 on Pulsatile and Surge Mode Secretion of Luteinizing Hormone in Ovariectomized Goats

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Abstract. The aim of the present study was to determine if the estradiol-induced luteinizing hormone (LH) surge is influenced by the constant exposure to TAK-683, an investigational metastin/kisspeptin analog, that had been established to depress the pulsatile gonadotropin-releasing hormone (GnRH) and LH secretion in goats. Ovariectomized goats subcutaneously received TAK-683 (TAK-683 group, n=6) or vehicle (control group, n=6) constantly via subcutaneous implantation of an osmotic pump. Five days after the start of the treatment, estradiol was infused intravenously in both groups to evaluate the effects on the LH surge. Blood samples were collected at 6-min intervals for 4 h prior to the initiation of either the TAK-683 treatment or the estradiol infusion, to determine the profiles of pulsatile LH secretion. They were also collected at 2-h intervals from –4 h to 32 h after the start of estradiol infusion for analysis of LH surges. The frequency and mean concentrations of LH pulses in the TAK-683 group were remarkably suppressed 5 days after the start of TAK-683 treatment compared with those of the control group ($P<0.05$). On the other hand, a clear LH surge was observed in all animals of both groups. There were no significant differences in the LH concentrations for surge peak and the peak time of the LH surge between the TAK-683 and control groups. These findings suggest that the effects of continuous exposure to kisspeptin or its analog on the mechanism(s) that regulates the pulsatile and surge mode secretion of GnRH/LH are different in goats.
Key words: Estradiol, Goats, Kisspeptin analog, LH surge

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Kisspeptin (also known as metastin) was first discovered as a ligand for G protein-coupled receptor 54 (GPR54) in 2001 [1]. The majority of research on kisspeptin to date has focused on its regulatory role in reproductive function. A number of studies have reported that exogenous administration of kisspeptin or kisspeptin-10 (kp-10), the C-terminal amidated 10-amino-acid sequence necessary for GPR54 activation, induces a rise of peripheral luteinizing hormone (LH) concentration in many mammalian species [2–4]. Recent studies showed that kp-10 directly stimulated gonadotropin-releasing hormone (GnRH) neurosecretion into the hypophyseal portal circulation accompanied by increases in the peripheral concentrations of LH in sheep [5] and goats [6]. In rodents, kisspeptin-induced LH release is blocked by pretreatment with the GnRH antagonist [7, 8]. These lines of evidence suggest that kisspeptin influences LH secretion by regulating hypothalamic GnRH secretion, which gives rise to the modulation of reproductive function.

TAK-683 is an investigational metastin/kisspeptin analog evaluated by Takeda Pharmaceutical Company Limited, Osaka, Japan [9–11]. Our previous study demonstrated that a bolus injection of TAK-683 stimulates GnRH secretion into the hypophyseal portal circulation and peripheral LH secretion in castrated goats [10]. Moreover, remarkable suppression of the testicular size and the peripheral LH secretion was observed in male rats when TAK-683 or TAK-448, another investigational metastin/kisspeptin analog, was chronically administered [9, 12]. Several studies have shown that chronic or repeated administration of human kisspeptin-54 or kp-10 results in a reduction of the pituitary and/or gonadal function together with inhibition of the peripheral LH levels in rats [13], monkeys [14, 15] and women [16]. Our recent study demonstrated in castrated goats that the suppressive action of chronic TAK-683 treatment on LH was attributable to complete suppression of pulsatile GnRH secretion [10]. These studies indicate that chronic administration of kisspeptin or its analog suppresses pulsatile GnRH/LH secretion after an initial stimulatory action.

An important endocrine event of the estrous cycle is a large continuous increase in GnRH release, namely, the GnRH surge. The GnRH surge is induced by a high peripheral level of estradiol from the preovulatory follicle, and causes an LH surge for ovulation. The relationship of kisspeptin signaling with the GnRH/LH surge has been demonstrated. Similarly to the hypothalamic location of GnRH

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neurons, kisspeptin neurons have been identified in the hypothalamic anteroventral periventricular area (AVPV) and preoptic area (POA) in mammals [17]. POA infusion of the anti-rat kisspeptin monoclonal antibody blocks the estrogen-induced [18] and the proestrous LH surges [19] in rats. Intracerebroventricular infusion of kisspeptin antagonist reduced the magnitude of the estradiol-stimulated LH surge in ewes during the anestrous season [5]. Increased cFos and *Kiss-1* mRNA expressions in the AVPV or POA at the time of the LH surge have been observed in ovariectomized rats [18, 20] and mice [21], and in sheep [22, 23] treated with exogenous estradiol. While the importance of hypothalamic kisspeptin on surge mode secretion of GnRH/LH has been shown in many species, the effect of long-term exposure to kisspeptin or its analog on an estradiol-induced GnRH/LH surge is still unclear. The present study aimed to determine if the estradiol-induced LH surge is also influenced by the chronic administration of TAK-683, which has been established to suppress the pulsatile GnRH and LH secretion in goats [10].

Materials and Methods

Eight long-term (>10 months) ovariectomized Shiba goats were used, and 4 of these animals were assigned to both groups. There was at least 5 months between treatment of goats with TAK-683 in the treatment group and reassignment of the goats to the control group. The goats were fed a standard pelleted diet and dry hay or hay cubes with *ad libitum* access to water and supplemental minerals. They were kept individually in cages temporarily when they were subjected to treatment and frequent blood sampling. All procedures were approved by the Committee for the Care and Use of Experimental Animals at the National Institute of Agrobiological Sciences (#22-67).

The chemical structure of TAK-683 was described previously [24], and chronic treatment with TAK-683 (50 nmol/kg BW/week) for 5 days was confirmed to cause severe suppression of pulsatile GnRH and LH secretion in our previous study [10]. In the present study, TAK-683 was subcutaneously administered at a concentration ten times higher than in the previous study to induce more profound suppression of pulsatile LH secretion. The animals were divided into two groups, and the TAK-683 group (n=6) constantly received TAK-683 at a rate of 500 nmol/kg BW/week via an osmotic pump (ALZET model 2ML1, DURECT, Cupertino, CA, USA) until the end of the experiment. The control group (n=6) received vehicle (50% DMSO). The osmotic pump was subcutaneously implanted under brief anesthesia using ketamine chloride, and the flow rate for the administration was 10 µl/h. Five days after the start of TAK-683 or vehicle treatment, estradiol (Sigma Chemical, St. Louis, MO, USA) dissolved in 0.3% ethanol saline (0.6 µg/ml) was infused with a peristaltic mini pump into the jugular vein for 16 h at a rate of 6 µg/h through one of the catheters (18 gauge, Medicut; Nippon Sherwood Medical Industries, Tokyo, Japan) fitted bilaterally into the jugular vein in both groups, as described previously to evaluate the effects on the LH surge [25].

Blood samples (1 ml) were collected via a jugular catheter into heparinized tubes at 6-min intervals for 4 h prior to the initiation of either the TAK-683 (or vehicle) treatment (Day 0) or the estradiol infusion (Day 5), to determine the profiles of pulsatile LH secretion. They were also collected at 2-h (2 ml) and 6-h (4 ml) intervals from –4 h to 32 h after the onset of estradiol infusion for analyses of the LH surge and of the plasma estradiol concentration, respectively. Blood samples were immediately stored on ice and centrifuged at 3,000 rpm for 20 min, and the plasma was kept at –30 C until assayed for plasma LH and estradiol concentrations.

Plasma LH concentrations were measured in duplicate by a specific radioimmunoassay (RIA) [26] using rabbit anti-ovine LH serum (YM #18) [27] and expressed in terms of ovine LH standard (NIDDK-oLH-I-4). The sensitivity of the assay was 9.5 pg/tube, and the intra- and inter-assay coefficients of variation were 5.1% and 6.5%, respectively. Plasma concentrations of estradiol were assayed by a previously described method [28]. The sensitivity of the assays was 0.1 pg/tube, and the intra- and inter-assay coefficients of variation were 5.7 and 12.0%, respectively.

Data are expressed as means ± SD and statistical differences were determined by Student's *t*-test or ANOVA. A confidence level of $P<0.05$ was considered to be statistically significant. For the identification of LH pulses, the cluster analysis program developed by Veldhuis and Johnson [29] was used. The nadir and peak clusters for LH pulse detection were 2/2 points, and the *t* statistics for significant increase and decrease were 2/2. The LH surge was defined as the point when a sustained rise (for at least two consecutive points of blood sampling) in the plasma LH concentration exceeded twice the average baseline level during the pretreatment period before the estradiol infusion, as described previously [25].

Results

Representative patterns of pulsatile LH secretion before (Day 0) and after (Day 5) TAK-683 treatment in the control and TAK-683 groups are shown in Fig. 1. The effects of chronic treatment of TAK-683 on LH pulses are summarized in Table 1. There was no significant difference in the profiles of the pulsatile LH secretion on Day 0 between the TAK-683 and control groups, whereas continuous exposure to TAK-683 for 5 days remarkably suppressed both the pulse frequency and amplitude of LH secretion. The frequency and mean concentrations of LH pulses in the TAK-683 group were significantly decreased on Day 5 compared with those of the control group.

The mean plasma concentrations of estradiol during estradiol infusion ranged from 21.7 to 67.0 pg/ml; there was no significant difference between the two groups (data not shown). The changes in the LH concentration after estradiol infusion are shown in Fig. 2 and Fig. 3. Clear sustained rises in LH concentrations detected as an LH surge were observed in all animals of both groups (Fig. 2). LH concentrations in the TAK-683 group were significantly lower than those of the control group from –4 to 8 h after the start of estradiol infusion (Fig. 3). However, no significant difference in the LH concentration during the LH surge (the period from 12 to 20 h after the start of estradiol infusion) was detected between the two groups. The profiles of the estradiol-induced LH surge in both groups are summarized in Table 1. There was no significant difference in the peak time and peak concentration of the LH surge between the two groups.

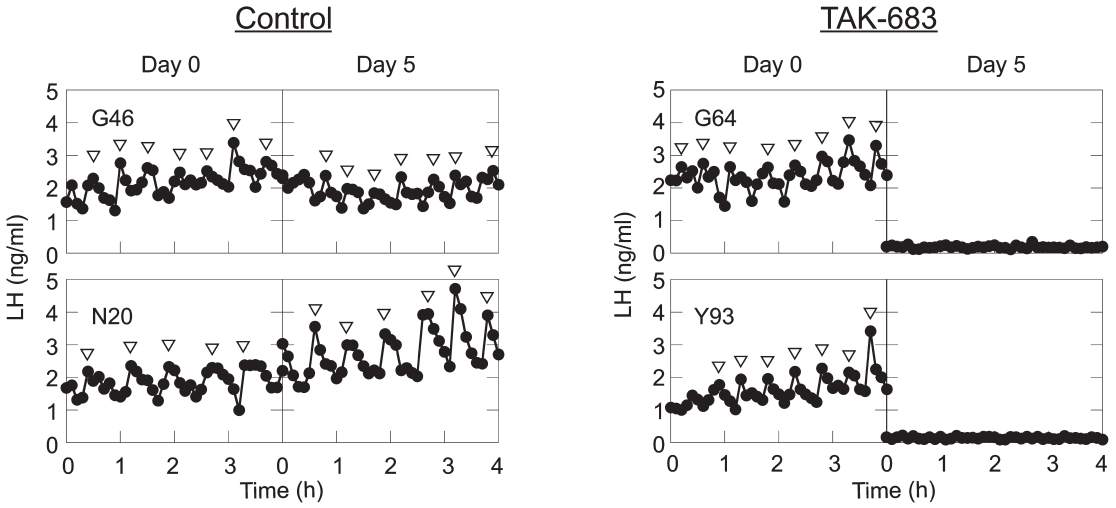

Fig. 1. Representative profiles of pulsatile LH secretion on Day 0 and Day 5 in the control (left) and TAK-683 (right) groups. Arrowheads represent LH pulses identified by cluster analysis.

Table 1. The effects of chronic treatment with TAK-683 on LH pulses and the estradiol-induced LH surge

			Control (n=6)	TAK-683 (n=6)
LH Pulse	Pulse frequency (pulses/4 h)	Day 0	6.5 ± 1.0	6.2 ± 1.3
		Day 5	5.8 ± 1.6	0.8 ± 1.3*
	Mean concentration (ng/ml)	Day 0	1.6 ± 0.7	1.5 ± 0.4
		Day 5	1.7 ± 0.9	0.3 ± 0.2*
LH Surge	Peak time of LH surge (h[1)])		14.7 ± 1.0	15.3 ± 1.0
	Peak concentration of LH surge (ng/ml)		34.2 ± 11.3	23.8 ± 18.4

Mean ± SD. * $P<0.05$ *vs.* control. [1)] Time after the start of estradiol infusion.

Discussion

The present study reconfirmed in ovariectomized goats that pulsatile LH secretion was remarkably suppressed by continuous exposure to TAK-683. Under this condition, a large sustained rise in LH secretion was observed after the start of estradiol infusion. The timing of its peak and the peak level of LH concentrations were accordance with the previous findings reported in ovariectomized goats given estradiol [25, 30]. The present findings indicate that the LH surge was induced by estradiol treatment in all animals under the chronic administration of TAK-683 that remarkably suppressed the pulsatile LH secretion.

Several studies have clearly demonstrated that hypothalamic input of estradiol is necessary for induction of the LH surge [31, 32]. GnRH secretion is substantially increased during the LH surge in estradiol-treated ovariectomized goats [33] and ewes [34, 35]. Our previous study demonstrated in castrated goats that the suppressive effect of chronic treatment of TAK-683 on the pulsatile LH secretion was due to complete suppression of pulsatile GnRH secretion without an influence on the responsiveness of pituitary gonadotrophs to a GnRH analog [10]. Taken together, the present results suggest that the occurrence of an estradiol-induced GnRH surge is not interfered with by the continuous action of TAK-683 negatively influencing pulsatile GnRH secretion.

The pulsatile and surge mode secretion of GnRH is considered to be regulated by two independent hypothalamic neural generators in females [36]. The present results suggest that the effects of continuous exposure to TAK-683 on these mechanisms are different. A different reaction of the estradiol-induced LH surge compared with that of pulsatile LH secretion has been reported under several physiological conditions. In ovariectomized lactating rats, the suckling stimulus strongly suppressed pulsatile LH secretion, whereas it did not prevent the occurrence of the LH surge after estradiol treatment [37]. On the other hand, it has been clinically reported in female goat that a subnormal level of progesterone in the peripheral circulation blocks the estradiol-induced LH surge without a suppressive influence on pulsatile LH secretion [25]. The present results are similar to the former phenomenon; it is likely that the hypothalamic generator for the GnRH surge functions in the case of the continuous stimulation of kisspeptin or its analogs.

In this case, what role does kisspeptin have in the estradiol-induced GnRH/LH surge? One possible interpretation is that kis-

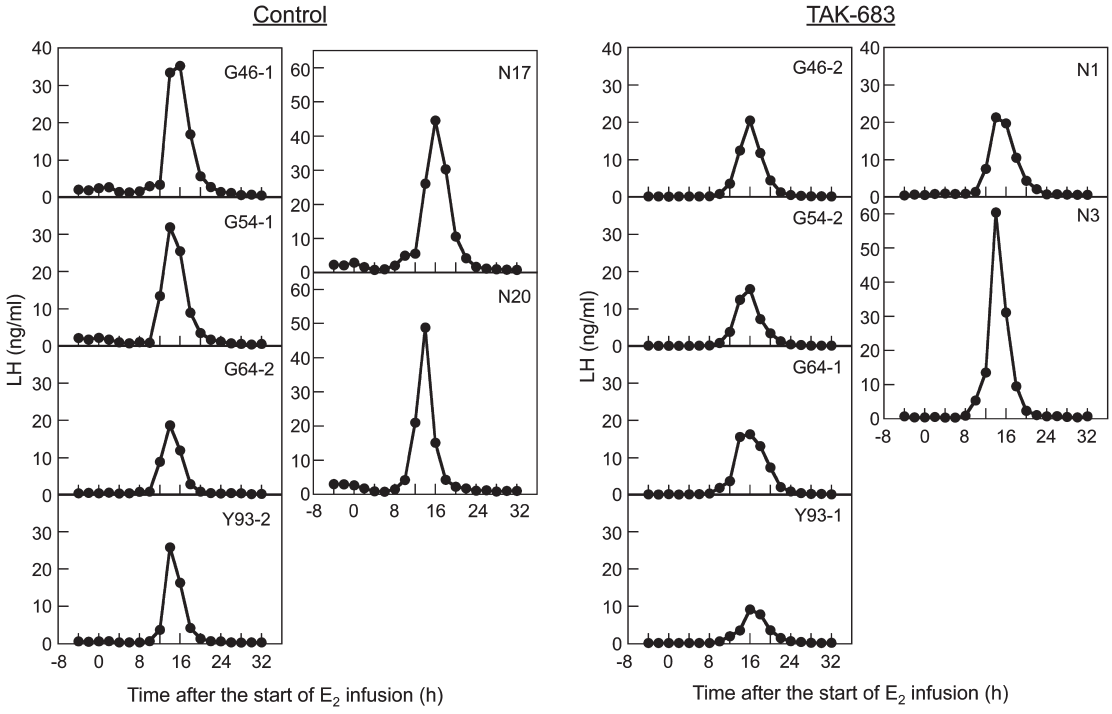

Fig. 2. Effects of the continuous administration of TAK-683 for 5 days on the estradiol-induced LH surge in all goats of the control (left 6 panels) and TAK-683 (right 6 panels) groups. A clear LH surge was observed in all animals of both groups.

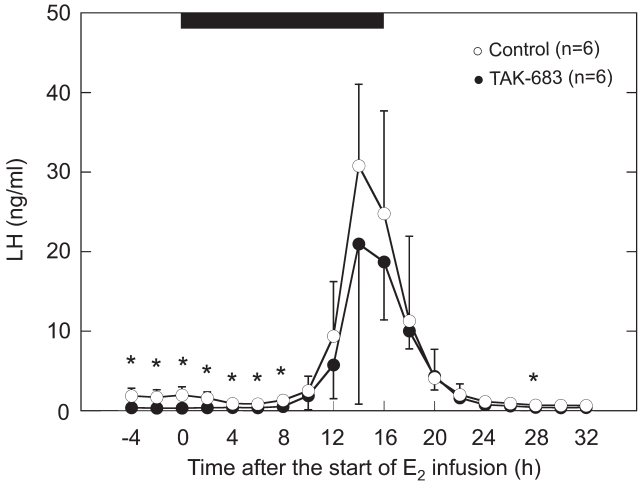

Fig. 3. Changes in the plasma concentrations of LH after the start of estradiol infusion in the TAK-683 (closed circles) and vehicle (open circles) groups. The horizontal black bar indicates the period of estradiol infusion. Mean ± SD. * $P<0.05$ *vs.* control.

speptin action is not involved in the induction of the GnRH surge. However, a large number of studies do not support this hypothesis. Immunoneutralization of kisspeptin in the POA [18] and central infusion of kisspeptin antagonist [5] suppressed the estradiol-induced LH surge. Expression of *kiss1* mRNA and cFos expression increased concomitantly with the preovulatory LH surge [18, 20], suggesting that the kisspeptin neurons participate in the induction of the GnRH surge. It seems to be plausible that a synergistic action of estradiol and endogenous kisspeptin (and/or TAK-683) can drive the GnRH surge-generating system to release a large amount of GnRH from GnRH-producing neurons that have lost the ability for pulsatile GnRH release in response to chronic administration of TAK-683.

Two hypotheses concerning the mechanism regulating the suppressive effects of long-term treatment of kisspeptin or TAK-683 on the pulsatile GnRH/LH secretion have been drawn from the several studies. Firstly, the involvement of the desensitization of GPR54 on the GnRH neurons after continuous or repeated kisspeptin treatment was proposed in previous studies on rats [12, 13] and rhesus monkeys [14]. This is similar to the fact that chronic administration of GnRH agonists suppresses the LH secretion due to the desensitization to GnRH on gonadotrophs of the pituitary after initial agonistic stimulation [38]. If this is the case, a possible explanation for the present findings is that GPR54 and its intracellular signal transduction relating to the GnRH surge were selectively prevented from undergoing desensitization. A previous study showed that kisspeptin cells are located in the POA and arcuate nucleus (ARC) in sheep [39]. The POA kisspeptin cells provide substantial input to GnRH cells in the POA [39], whereas the site of action of ARC kisspeptin cells is suggested to be on the GnRH axon terminal located in the hypothalamic median eminence [40, 41], which is a circumventricular organ lacking a blood-brain barrier [42]. Recent studies have suggested that pulsatile GnRH secretion is generated by the pulsatile release of kisspeptin at the ARC adjacent to the

median eminence [3, 43, 44]. In contrast to the median eminence, the blood-brain barrier of the POA appears to restrict the entry of large molecules, for example, kp-10 or TAK-683, into the nervous system. The specific anatomical component such as the blood-brain barrier might play a role in prevention of the desensitization of GPR54 located in the POA resulting from continuous exposure to TAK-683 circulating in the bloodstream. Another possibility is that the estradiol-induced GnRH surge might be modulated by endogenous kisspeptin through a different pathway from GPR54. Although the presence of a kisspeptin receptor(s) besides GPR54 in the regulation of GnRH secretion has not been clearly identified, in a recent study supporting this hypothesis, kp-10 exhibited potent binding and activation of GPR147 and GPR74 using a binding inhibition assay *in vitro* [45].

Secondly, Matsui *et al.* [9, 12] hypothesized that depression of LH pulses after the chronic treatment with kisspeptin analogs is associated with the severe attenuation of GnRH storage due to the continuous release of GnRH by receiving continuous stimulatory signals. In their study, a single injection of kisspeptin analogs after chronic administration of the analog clearly induced cFos expression in the majority of GnRH neurons without inducing LH release, and GPR54 mRNA levels were not downregulated after chronic administration in male rats. In this case, the present results imply that estradiol under the continuous activation of GPR54 might induce the initiation of supplemental GnRH production to stock GnRH molecules for the surge on the GnRH neurons. Then, the hypothalamic GnRH content to be released for induction of the LH surge might be restored by their actions during the period between the start of estradiol infusion and the onset of the LH surge.

In conclusion, the present study revealed that, while continuous exposure to TAK-683 strongly suppresses pulsatile LH secretion, it does not suppress the occurrence of the LH surge in ovariectomized goats given estradiol in the current experimental settings. These findings suggest that the effects of continuous exposure to metastin/kisspeptin or its analog on the reaction of the mechanism(s) that regulates the pulsatile and surge mode secretion of GnRH/LH are different. They also suggest that GnRH surge generator activity is not influenced by the chronic administration of TAK-683 that suppresses the pulsatile GnRH secretion in goats.

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