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Inhibition of Metastin (Kisspeptin-54)-GPR54 Signaling in the Arcuate Nucleus-Median Eminence Region during Lactation in Rats

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Follicular development and ovulation are suppressed during lactation in various mammalian species, mainly due to the suppression of pulsatile GnRH/LH secretion. Metastin (kisspeptin-54), a KiSS-1 gene product, is an endogenous ligand for GPR54, a G-protein-coupled receptor, and suggested to play a critical role in regulating the gonadal axis. The present study therefore aims to determine whether metastin (kisspeptin-54)-GPR54 signaling in discrete brain areas is inhibited by the suckling stimulus that causes suppression of LH secretion in lactating rats. Quantitative RT-PCR revealed that the *KiSS-1* mRNA level was significantly lower in the arcuate nucleus (ARC)-median eminence region in lactating ovariectomized (OVX) and estrogen-treated OVX rats than in nonlactating controls. *KiSS-1* mRNA in the anteroventral periventricular nucleus was kept at a low level in both lactating and nonlactating rats despite estrogen treatment. GPR54 mRNA levels were significantly lower in lactating than nonlactating rats in the anteroventral periventricular nucleus, but the levels in lactating mothers of the preoptic area and ARC-median eminence were comparable with nonlactating controls. Although *KiSS-1* mRNA-expressing cells or metastin (kisspeptin-54) immunoreactivities were densely located in the ARC of nonlactating controls, few were found in the ARC of lactating OVX animals. Various doses of metastin (kisspeptin-54) (0.02, 0.2, and 2 nmol) injected into the third ventricle caused a significant increase in LH secretion in both lactating and nonlactating OVX rats, suggesting that lactating rats are responsive to metastin (kisspeptin-54) stimulus. Thus, the present study demonstrated that *KiSS-1* mRNA/metastin (kisspeptin-54) expression is inhibited in the ARC by the suckling stimulus, suggesting that the inhibition is most probably involved in suppressing LH secretion in lactating rats. (*Endocrinology* 148: 2226–2232, 2007)

FOLLICULAR DEVELOPMENT and ovulation are suppressed during lactation in various mammalian species including cows (1) and women (2). The inhibition of estrous cyclicity is mainly due to the suppression of pulsatile GnRH/LH secretion in lactating rats (3–5) but not the suppression of LH surges because the positive feedback effect of exogenous estrogen on LH secretion is left intact during lactation in rats (6). We previously revealed that the suckling stimulus plays a critical role in suppression of pulsatile LH secretion in lactating rats because this secretion is strongly inhibited, even in ovariectomized (OVX) lactating rats on d 8 of lactation, and removal of the litter restores LH pulses within 24 h (7). Furthermore, administration of bromocriptin, a dopamine agonist, did not restore the suckling-induced pulsatile LH suppression during the first half of lactation (8–10), suggesting that the contribution of ovarian steroids and prolactin to the LH suppression during early to mid-lactation is less important. However, the neuroendocrine mechanism mediating this LH suppression has not yet been fully understood (4, 11, 12).

Metastin (kisspeptin-54) was first isolated from the human placenta and proposed to be the natural ligand for GPR54, a G protein-coupled receptor (13). Metastin (kisspeptin-54) and *KiSS-1* mRNA, a gene encoding metastin, was found mainly in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) in the rodent brain (14–16). On the other hand, GPR54 mRNA is highly expressed in the preoptic area (POA) and fairly expressed in both the AVPV and ARC but not in the anterior pituitary in the rat (14, 15). The metastin (kisspeptin-54)-GPR54 system has attracted the interest of reproductive scientists because of its key role in puberty (17) and potent stimulatory effect on GnRH/LH release in rats (18, 19), mice (20), and monkeys (21). Metastin (kisspeptin-54) may directly act on GnRH neurons to stimulate GnRH and LH release because GnRH neurons have been reported to express GPR54 mRNA (22). In addition, it has been reported that diminished expression of hypothalamic *KiSS-1* mRNA may be associated with suppression of reproductive function in food-deprived prepubertal rats (23) or *ob/ob* mouse (24).

The present study therefore aims to test whether the in-

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Abbreviations: ARC, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; DIG, digoxigenin; E2, estradiol-17β; GPR, G protein-coupled receptor; MBH, mediobasal hypothalamus; ME, median eminence; OVX, ovariectomized; POA, preoptic area; SSC, saline sodium citrate; UPW, ultrapure water; 3V, third ventricle.

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hibition of metastin (kisspeptin-54) synthesis, its release, and/or its receptor expression by the suckling stimulus causes the suppression of LH secretion in lactating rats. First, we examined the effect of suckling stimulus and estrogen on expression of *KiSS-1* and GPR54 gene and on metastin (kisspeptin-54) immunoreactivity in the AVPV, POA, and ARC. Second, various doses of metastin (kisspeptin-54) were exogenously challenged in the third ventricle (3V) to determine whether lack of ligand (metastin) and/or responsiveness via GPR54 is involved in the suppression of LH pulses in lactating OVX rats.

Materials and Methods

Animals and treatments

Wistar-Imamichi strain female rats were kept under a condition of 14 h light, 10 h darkness (lights on at 0500 h) and 22 ± 2 C with free access to food (CE-2; Clea Japan Inc., Tokyo, Japan) and water. Animals having shown two consecutive estrous cycles were mated with males overnight on the day of proestrus and then pregnant females were housed individually. The day of parturition was designated d 0 postpartum. The litter size was adjusted to eight (four males and four females) on d 1. Some mother rats were deprived of their litters on d 1 to serve as nonlactating controls. All animals were bilaterally OVX on d 2. Some nonlactating and lactating rats were OVX and sc implanted with a SILASTIC brand silicon tubing (1.5 mm inner diameter; 3.0 mm outer diameter; 25 mm in length; Dow Corning, Midland, MI) containing estradiol-17β (E2, Sigma, St. Louis, MO) dissolved in peanut oil at 20 μg/ml to serve as the OVX+E2 group. This estrogen treatment has been demonstrated to produce a plasma estradiol level at around 30 pg/ml and shows a negative feedback effect on LH secretion (25). The entire surgical procedure was performed under anesthesia with ketamine-xylazine solution.

The present study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Real-time RT-PCR for discrete brain areas

On d 8 postpartum, lactating and nonlactating OVX or OVX+E2 rats were decapitated between 1300 and 1400 h. After removing the brain, the AVPV and POA were punched out with an 18-gauge stainless-steel tubing from the coronal section of the brain according to the following coordinates of a brain atlas (26): anterior and posterior ends are 0.6 mm anterior to and 0.6 mm posterior to the bregma, respectively. The ARC-median eminence (ME) region was dissected out with a microknife from the brain according to the following coordinates of the brain atlas: anterior and posterior ends are 1.8 and 3.96 mm posterior to the bregma, respectively. The rest of the brain was fixed with 10% formalin, and 50-μm coronal sections were made by a cryostat. Sections were stained with thionin and observed under a microscope to verify whether the AVPV, POA, or the whole ARC-ME region had been correctly dissected out.

Expression of *KiSS-1* and GPR54 mRNA was determined by quantitative RT-PCR in the AVPV, POA, and ARC-ME regions. Real-time RT-PCR analysis (TaqMan) was performed using ABI PRISM 7900HT (PE Applied Biosystems, Foster City, CA) as previously described (15). Briefly, DNA-free total RNA was purified from each of the brain tissues consisting of the AVPV, POA, or ARC-ME using RNeasy Mini kit and ribonuclease-free deoxyribonuclease Set (QIAGEN, Valencia, CA) following the manufacturer's instructions. cDNA from each RNA sample was synthesized with oligo (deoxythymidine) primer at 50 C using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Forward primer, reverse primer, and TaqMan probe for *rKiSS-1*, *rGPR54*, and *β-actin* were described elsewhere (15, 27). The copy number of *rKiSS-1* and *rGPR54* transcript was normalized to the ratio to the copy number of *β-actin* transcript for each sample.

In situ hybridization

We used only the lactating OVX model in the following study because the low level of estrogen did not affect *KiSS-1* and GPR54 mRNA expressions in lactating rats. OVX animals were deeply anesthetized with sodium pentobarbital and perfused with 0.05 M PBS followed by 4% paraformaldehyde in 0.05 M PB on d 8 postpartum. The brain was immediately removed from the skull, postfixed with the same fixative for 6–7 h at 4 C, and then kept in 30% sucrose in 0.05 M PB for 3–4 d at 4 C under RNase-free condition. Serial coronal sections (50 μm in thickness) containing AVPV or ARC were obtained using a cryostat on the day before *in situ* hybridization and then stored at 4 C in the PBS until the next day. To detect *KiSS-1* mRNA, we made a KiSS-1-specific digoxigenin (DIG)-labeled probe and performed nonradioactive free-floating *in situ* hybridization as previously described (14). Briefly, every second section through the AVPV (eight sections, from 0.36 mm anterior to 0.48 mm posterior to the bregma) or every fourth section through the ARC (11 sections, from 1.8 to 3.96 mm posterior to the bregma) taken from each rat, according to the rat brain atlas (26), was washed with PBS and treated with 1 μg/ml Protease K for 15 min at 37 C and then incubated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Finally, the sections were hybridized with 1 μg/μl DIG-labeled antisense cRNA probes (position 33–348; GenBank accession no. AY196983) synthesized from the rat hypothalamus using a labeling kit (Roche Molecular Biochemicals GmbH, Mannheim, Germany) overnight at 60 C. A sense RNA probe was used as a negative control. After hybridization, the sections were washed twice with 2× saline sodium citrate (SSC) containing 50% formamide for 15 min at 60 C. The sections were then treated with 20 μg/ml RNase A for 30 min at 37 C and immersed sequentially with 2× SSC, 0.5× SSC, and DIG-1 buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.01% Tween 20] for 15 min, twice each. Then the sections were immersed with 1.5% blocking reagent (Roche Molecular Biochemicals) in DIG-1 buffer for 1 h at 37 C and incubated with an alkaline phosphatase-conjugated anti-DIG antibody (1:1000, Roche Diagnostics Corp., Indianapolis, IN) for 2 h at 37 C. Then the sections were washed with DIG-1 buffer and treated with DIG-3 buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$]. After this, the sections were treated with a chromagen solution (337 μg/ml 4-nitroblue tetrazolium chloride, 175 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate in DIG-3 buffer) until a visible signal was detected. The reaction was stopped by adding a reaction stop solution [10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0)]. The sections were observed under light microscope and the number of *KiSS-1*-expressing cells was counted.

Immunohistochemistry

The brains of lactating and nonlactating OVX rats on d 8 were obtained as mentioned above. Every second section through the AVPV (eight sections, from 0.36 mm anterior to 0.48 mm posterior to the bregma) or every fourth section through the ARC (11 sections, from 1.8 to 3.96 mm posterior to the bregma) from each rat was stained with antirat metastin, the cross-reactivity of which was previously described (15). Briefly, the mouse monoclonal antibody (no. 254 raised in Takeda Pharmaceutical Co., Ltd.) used in the present study was raised against C-terminal 17 amino acid sequence (36–52) of rat metastin and recognizes the specific region of rat metastin (43–51) (15). Rat metastin has a C terminal with RY-amide, which is different from human metastin with RF-amide C terminal. This antibody did not cross-react with other RY peptides such as neuropeptide Y and atrial natriuretic peptide (15). In addition, our recent study showed that the immunohistochemical results with this antibody were well consistent with *KiSS-1* mRNA *in situ* hybridization (14). Brain tissue sections from each rat were incubated with the antirat metastin antibody (1:50,000) for 1 d at RT, followed by incubation with Alexa Fluor 488-conjugated donkey antimouse IgG (1:800; Molecular Probes, Eugene, OR). The sections were mounted with an antifade reagent (FloroGuard; Bio-Rad, Hercules, CA). Fluorescence images were obtained on a Apotome microscope (Apotome; Carl Zeiss, Jena, Germany). Eleven digital photomicrographs of each ARC per rat were processed using the National Institutes of Health (NIH) Image analysis program. The ARC area was outlined on the gray-scale image and processed for density measurement. Nonspecific background density points were eliminated using the same threshold for each rat.

Metastin (kisspeptin-54) challenge and blood sampling

On d 2, OVX animals were stereotaxically implanted with a stainless-steel guide cannula (22 gauge; Plastics One, Roanoke, VA) into the 3V with the tip end at −0.8 mm posterior and 7.5 mm ventral to the bregma, according to a rat brain atlas (26). Metastin (kisspeptin-54) injection and blood sampling were performed in freely moving conscious rats on d 8. Human metastin (kisspeptin-54) was dissolved in ultrapure water (UPW) at 0.02, 0.2, and 2 nmol per 2 μl immediately before the injection. Animals received a single infusion of the drug solution into the 3V at the rate of 1 μl/min for 2 min using a microsyringe pump (EICOM, Kyoto, Japan) through an internal cannula (28 gauge; Plastics One), starting immediately after the first blood sampling. Blood samples (100 μl) were collected every 6 min for 3 h through a silicone cannula (Shin-Etsu Polymer, Tokyo, Japan) that had been inserted into the right atrium via the right jugular vein on the day before blood sampling. An equivalent volume of rat red blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the atrial cannula after each blood collection. Control animals were injected with UPW into the 3V in the same manner.

At the end of blood sampling, all animals were injected with 3% brilliant blue into the 3V through the internal cannula to verify the cannula placement. The data obtained from animals with right cannula placement were then analyzed.

LH assay

LH contents in 50-μl plasma samples were measured by a double-antibody RIA using a rat LH RIA kit provided by the National Hormone and Pituitary Program (Baltimore, MD). Plasma LH concentrations were expressed in terms of the National Institute of Diabetes and Digestive and Kidney Diseases rat LH RP-3. The least detectable level was 0.156 ng/ml and the intra- and interassay coefficients of variation were 7.0% at 2.4 ng/ml and 6.9% at 2.6 ng/ml, respectively.

Statistical analysis

The data obtained by real-time RT-PCR study were statistically analyzed by a two-way (nonlactating *vs.* lactating and OVX *vs.* OVX+E2) ANOVA. Statistical differences in the number of *KiSS-1* expressing cells and the intensity of metastin (kisspeptin-54) immunoreactivities in the ARC between nonlactating and lactating rats were determined by Student's *t* test. LH pulses were detected by the PULSAR computer program (28). Mean LH concentration for the 3-h sampling period was calculated for each individual and then for each group. Statistical differences in mean LH concentration between groups were determined by two-way (suckling and metastin treatment) ANOVA followed by the Bonferroni/Dunn test.

Results

KiSS-1 and GPR54 mRNA level

The quantitative RT-PCR analysis showed a significant effect of the suckling stimulus on *KiSS-1* mRNA level in the ARC-ME [F (1, 12) = 41.534, $P < 0.001$] but not the AVPV and POA (two-way ANOVA; lactating *vs.* nonlactating and OVX *vs.* OVX+E2, Fig. 1A). *KiSS-1* mRNA level in the AVPV was slightly higher in the OVX+E2 group than the OVX group, but there was no significant effect of estrogen [F (1, 12) = 3.546, $P = 0.084$]. There were no significant effects of estrogen and interaction between suckling stimulus and estrogen on *KiSS-1* mRNA levels in any of the regions examined.

GPR54 levels in the AVPV of lactating groups were significantly lower than in nonlactating controls [F (1, 12) = 7.828, $P < 0.05$], whereas no significant effect of estrogen treatment or interaction between suckling stimulus and estrogen was found in GPR54 mRNA levels in any of the regions examined (Fig. 1B).

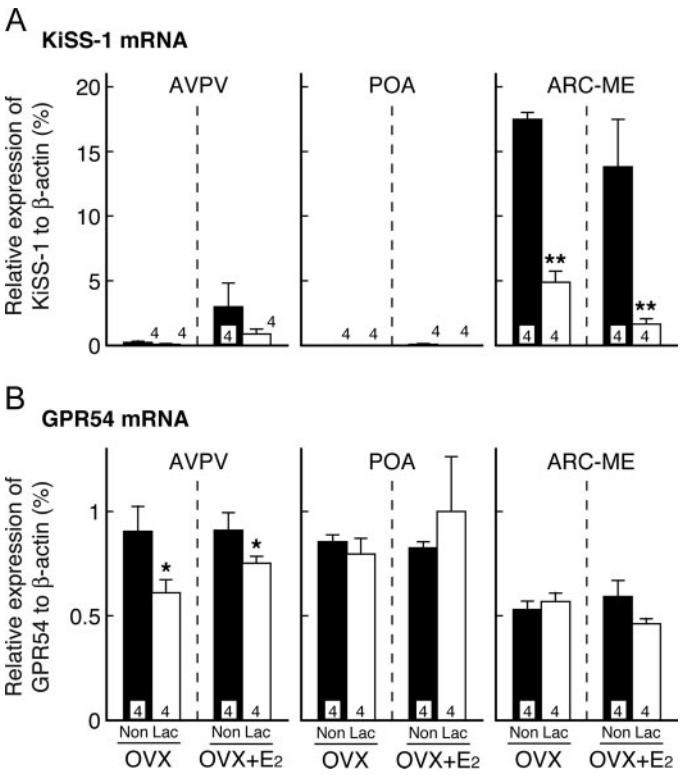

FIG. 1. KiSS-1 (A) and GPR54 (B) mRNA levels in the AVPV, POA, and ARC-ME in nonlactating (Non, *solid column*) and lactating (Lac, *open column*) OVX rats. *Number in or on each column* indicates number of animals used. Values are mean ± SEM. Data were analyzed by two-way ANOVA (lactating *vs.* nonlactating and OVX *vs.* OVX+E2). The analysis revealed statistical effect of suckling stimulus on KiSS-1 mRNA expression in the ARC [F (1, 12) = 41.534, $P < 0.001$] and GPR54 expression in the AVPV [F (1, 12) = 7.828, $P < 0.05$]. No significant effects of estrogen treatment and a suckling stimulus and estrogen interaction were found. *Asterisks* indicate statistical differences from nonlactating OVX rats (*, $P < 0.05$; **, $P < 0.01$).

In situ hybridization of KiSS-1 neurons in ARC

Many *KiSS-1* mRNA-expressing cells were located in the anterior part (Fig. 2A) and posterior part (Fig. 2, B and C) of the ARC in nonlactating OVX rats. On the other hand, few *KiSS-1*-expressing cells were found in both anterior and posterior parts of the ARC in lactating rats (Fig. 2, D and E). No hybridization signals were observed in the brain section incubated with sense probes for *KiSS-1* (Fig. 2F).

The number of *KiSS-1*-expressing cells in the ARC in lactating OVX rats was significantly ($P < 0.05$, Student's *t* test) lower than in the nonlactating OVX controls (Fig. 2G). No hybridization signals were found in the AVPV in both nonlactating and lactating groups (n = 4, data not shown).

Immunohistochemistry of metastin (kisspeptin-54) neurons

Metastin (kisspeptin-54)-immunoreactive cells and fibers were densely located in the ARC of all the nonlactating controls (Fig. 3A), whereas few metastin (kisspeptin-54)-immunoreactive cells were found in lactating OVX rats (Fig. 3B). NIH image analysis showed that areas of metastin (kisspeptin-54) immunoreactivities in the ARC were significantly ($P < 0.05$, Student's *t* test) smaller in the lactating OVX

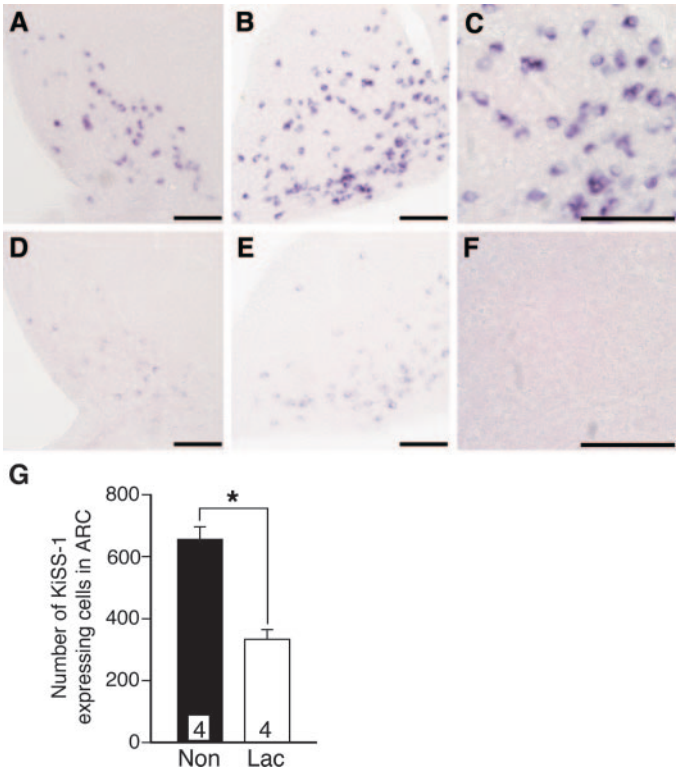

FIG. 2. *In situ* hybridization of KiSS-1 mRNA in ARC in representative nonlactating (A, B, and C) and lactating (D and E) OVX rats. A and D show KiSS-1 mRNA hybridization signal in the anterior part of ARC; B and E show the signal in the posterior part of ARC. C shows high magnification of B. No signals were found in section incubated with sense probe for KiSS-1 (F). Number of KiSS-1-expressing cells in lactating OVX rats (Lac, *open bar*) were significantly (*, $P < 0.05$; Student's *t* test) lower, compared with nonlactating OVX control (Non, *solid bar*) (G). *Number in each column* indicates number of animals used. Values are mean ± SEM. *Scale bars*, 100 μm.

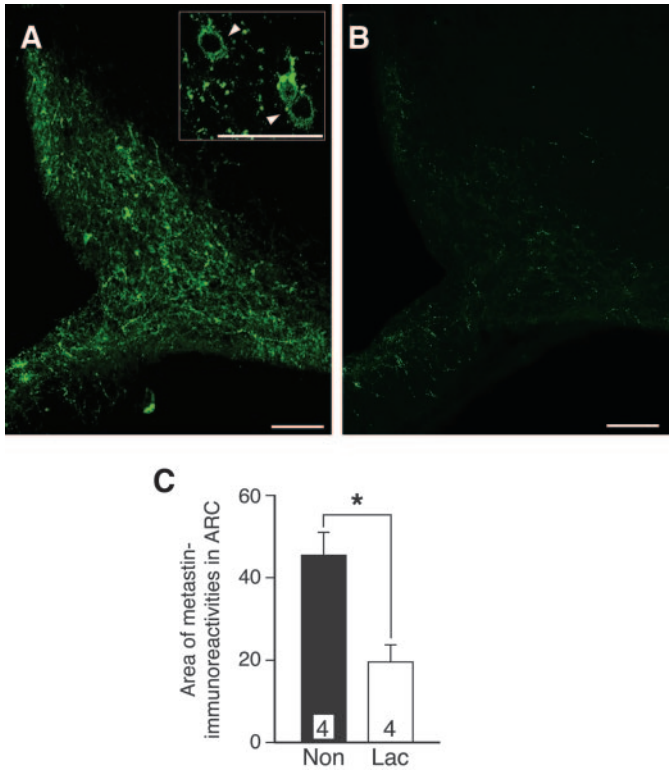

FIG. 3. Immunohistochemistry of metastin (kisspeptin-54) in ARC. Photomicrographs of metastin (kisspeptin-54)-immunoreactive (ir) cells (*green*) in ARC in representative nonlactating (A) and lactating (B) OVX rats on d 8. *Arrowheads* indicate metastin (kisspeptin-54)-ir cell bodies in the ARC in nonlactating OVX rats (*inset* in A). Semiquantitative analysis of metastin (kisspeptin-54)-ir in ARC by NIH image analysis showing that areas of metastin (kisspeptin-54)-ir in lactating OVX rats (Lac, *open bar*) were significantly (*, $P < 0.05$; Student's *t* test) lower than the nonlactating OVX control (Non, *solid bar*) (C). *Number in each column* indicates number of animals used. Values are mean ± SEM. *Scale bars*, 100 μm (A and B); 50 μm (*inset*).

rats, compared with the nonlactating OVX rats (Fig. 3C). No immunoreactive cells and fibers were found in the AVPV in both nonlactating and lactating groups (n = 4, data not shown).

Effect of 3V injection of metastin (kisspeptin-54) on LH release

Frequent LH pulses were found in vehicle-injected nonlactating OVX rats, whereas LH pulses were strongly suppressed throughout the sampling period in vehicle-injected lactating OVX mothers (Fig. 4, A and B). Metastin (kisspeptin-54) injection caused an increase in plasma LH concentrations in nonlactating OVX rats at any of the doses (Fig. 4A). The metastin (kisspeptin-54) injection also increased plasma LH levels in lactating OVX rats, and the plasma LH concentrations peaked at 1–2 h after the injection (Fig. 4B). The LH levels were maintained at a high level for the rest of the sampling period after the level peaked in many of the both nonlactating and lactating rats (Fig. 4, A and B).

The analysis for the mean LH concentrations using a two-way ANOVA revealed the significant effect of the suckling stimulus [F (1, 34) = 6.034, $P < 0.05$] and metastin treatment [F (3, 34) = 22.487, $P < 0.001$] on mean LH concentrations. Mean LH concentrations in metastin (kisspeptin-54)-treated groups at any dose were significantly higher than in vehicle-treated groups ($P < 0.001$, Bonferroni/Dunn test) (Fig. 4C).

Discussion

The present study provides the first evidence of a profound reduction in expression of KiSS-1 gene and its product, metastin (kisspeptin-54), in the ARC in lactating OVX rats, suggesting that the suckling stimulus is a potent inhibiting factor for KiSS-1 mRNA/metastin (kisspeptin-54) expression in the ARC. The lactating OVX rats may still retain the responsiveness to exogenous metastin (kisspeptin-54) because LH secretory responses to central metastin (kisspeptin-54) administration in lactating mothers are similar to those in nonlactating rats, and GPR54 mRNA expression in the POA, in which the majority of the GnRH neurons are located, was not different between groups. It is plausible that the lowered KiSS-1 mRNA/metastin (kisspeptin-54) expression in the ARC could be related, at least in part, to the inhibition of pulsatile LH secretion during lactation.

Metastin (kisspeptin-54) has been reported to have a profound stimulatory effect on GnRH/LH release (18, 19, 22). It has also been proposed that the mediobasal hypothalamus (MBH), including the ARC, contains a GnRH-pulse gener-

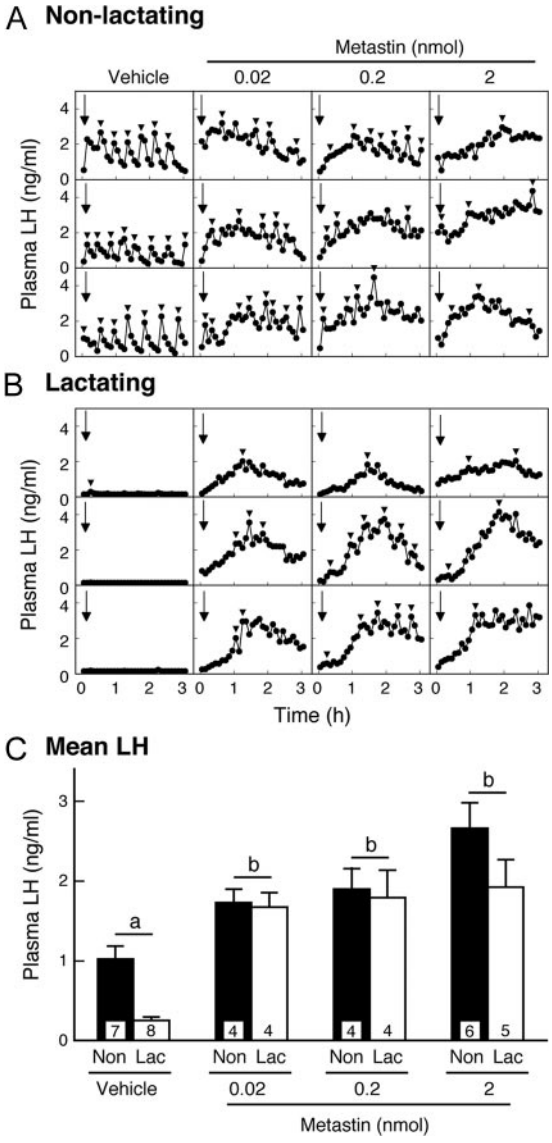

FIG. 4. Plasma LH profiles in representative animals injected with human metastin (kisspeptin-54) (0.02, 0.2, or 2 nmol per 2 μl in UPW) into 3V in nonlactating (A) or lactating (B) OVX rats on d 8. Drugs were injected into 3V immediately after onset of blood sampling (*arrows*). Blood samples were collected for 3 h at 6-min intervals. *Arrowheads* indicate peaks of LH pulses identified with PULSAR computer program. Mean plasma LH concentrations in nonlactating (Non, *solid bars*) or lactating (Lac, *open bars*) OVX rats injected with 0.02, 0.2, or 2 nmol metastin (kisspeptin-54) or vehicle into 3V (C). *Numbers in each column* indicate number of animals used. Values are means ± SEM. *Values with different letters* are significantly ($P < 0.001$) different from each drug injected-group (by two-way ANOVA followed by Bonferroni/Dunn).

ating mechanism (29–32). GnRH neurons express GPR54 mRNA (22) and have a close proximity to metastin (kisspeptin-54) fibers in the POA (15). The ARC metastin (kisspeptin-54) neurons could induce GnRH release via GPR54 located in the GnRH neuronal cell bodies in the POA region. We, however, still could not exclude the possibility that ARC metastin (kisspeptin-54) neurons control GnRH release by acting on GnRH nerve terminals in the ME. The projection of ARC metastin (kisspeptin-54) neurons to control GnRH neurons still remains to be determined.

In the present study, the KiSS-1 mRNA level was suppressed to a low level in the ARC regardless of the steroidal milieu during lactation. To mimic the plasma E2 level during lactation (33), we gave animals a low-level E2 treatment that caused negative feedback effect on pulsatile LH secretion (25) but no induction of afternoon LH surges (34). The result is consistent with our previous reports indicating that a low level of E2 treatment showed a negative feedback effect on plasma LH levels but no significant influence on KiSS-1 mRNA levels (14, 15). On the other hand, an E2 treatment that is high enough to induce LH surges causes a decrease in KiSS-1 mRNA levels in the ARC (14–16, 35, 36). The mechanism underlying the discrepancy of the effect of low and high E2 levels on ARC KiSS-1 mRNA levels remains unknown. Further studies on the production and release of the peptide in the ARC-ME region or POA are required to address this point.

Lactating OVX rats also showed a suppressed level of KiSS-1 mRNA in the AVPV. On the other hand, estrogen treatment slightly increased the AVPV KiSS-1 mRNA level in nonlactating rats, although the effect was not significant. The increase is consistent with our previous study showing that both high and low E2 treatments significantly increases KiSS-1 mRNA levels in the AVPV in female rats (14). Smith *et al.* (16) first showed that a high estrogen level positively regulates KiSS-1 mRNA expression in the AVPV. Thus, the positive regulation of AVPV metastin (kisspeptin-54) neurons by estrogen suggests a role of AVPV metastin (kisspeptin-54) neurons in preovulatory LH surge (14, 16, 35). In this context, the change in AVPV KiSS-1 mRNA level seems to contribute less to the suckling-induced suppression of LH pulses than does the ARC KiSS-1 mRNA level.

Roa *et al.* (37) suggested that sensitivity to metastin (kisspeptin-54) was reduced during lactation, compared with diestrous rats, because intracerebroventricular injection of kisspeptin-10, a metastin agonist, caused LH increase in lactating rats at 1 nmol but not 0.01 nmol, whereas diestrous rats responded to kisspeptin-10 at both doses. In the present study, 0.02 nmol metastin (kisspeptin-54) injection increased plasma LH concentrations in both lactating and nonlactating OVX rats, suggesting that the suckling stimulus may not affect the LH secretory response to exogenous metastin (kisspeptin-54) at 0.02 nmol. Thus, 0.01 nmol might be a critical dose, causing a different sensitivity to metastin (kisspeptin-54) in lactating and nonlactating rats. Because GPR54 mRNA levels in the AVPV, in which some GnRH neurons are located (38), were significantly lower in lactating rats than in nonlactating rats in the present study, decreased GPR54 expression in GnRH neurons may also contribute to reduced sensitivity to exogenous metastin (kisspeptin-54) during lactation. Roa *et al.* (37) also suggested that KiSS-1 mRNA levels in the hypothalamus of lactating rats were comparable with that in diestrous rats, whereas our present data showed that KiSS-1 mRNA levels in the ARC were significantly lower in lactating OVX rats, compared with nonlactating OVX rats. Roa *et al.* (37) measured the KiSS-1 mRNA level in the whole hypothalamus, whereas we measured it in discrete brain tissue such as the ARC region.

Extensive tissue may hamper identification of nucleus-specific change in KiSS-1 mRNA expression, as Roa *et al.* (37) mentioned in their previous report.

The present study showed that the metastin (kisspeptin-54)-induced LH level was maintained at a high level after it slowly reached a peak in both nonlactating and lactating groups. This result is consistent with the previous studies demonstrating that central or peripheral administration of metastin (kisspeptin-54) induces continuous LH release for more than 3 h (15, 18). Messager *et al.* (22) indicated that GnRH secretion in the cerebrospinal fluid persisted, even after the end of the intracerebroventricular metastin (kisspeptin-54) infusion in sheep. A possible explanation of these studies is that centrally injected metastin (kisspeptin-54) lasts even after the end of injection to keep stimulating GnRH/LH release.

Neural inputs to the ARC for suckling-induced suppression of KiSS-1 mRNA/metastin (kisspeptin-54) expression remain to be determined. The suckling stimulus activates neurons projecting to the ARC (39) and originating from various regions including in or outside the MBH (40). Neural inputs to the MBH, which are conveyed through the dorsal part of the hypothalamus, are involved in suckling stimulus-induced suppression of LH secretion (12, 41). Taken together with the present results, neural inputs derived from the suckling stimulus may be directly or indirectly conveyed to the MBH to inhibit KiSS-1 mRNA/metastin (kisspeptin-54) expression in the ARC. γ-Aminobutyric acid and/or catecholamines may be candidates for the inhibitory inputs to the metastin (kisspeptin-54) neurons in the ARC. This is because γ-aminobutyric acid concentrations in the cerebrospinal fluid were increased in lactating rats (42), and the brain stem catecholaminergic neurons in A1 region were activated by suckling stimulus and innervated the ARC (39, 40). Further study is required to address these points.

In conclusion, the present study demonstrated that KiSS-1 mRNA/metastin (kisspeptin-54) expression is inhibited in the ARC by suckling stimulus, suggesting that the inhibition is most probably involved in the strong suppression of LH secretion in lactating rats. It is also possible that the suckling stimulus-induced suppression of KiSS-1 mRNA/metastin (kisspeptin-54) expression in the ARC is associated with changes in function other than GnRH/LH suppression during lactation.

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