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—Original Article—

Augmentation of gonadotropin-releasing hormone receptor expression in the post-lactational mammary tissues of rats

Ryota TERASHIMA[1)], Titaree LAOHARATCHATATHANIN[2)], Shiro KURUSU[1)] and Mitsumori KAWAMINAMI[1)]

[1)]*Laboratory of Veterinary Physiology, School of Veterinary Medicine, Kitasato University, Aomori 034-8628, Japan*
[2)]*Clinic for Small Domestic Animals and Radiology, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok 10530, Thailand*

Abstract. Gonadotropin-releasing hormone (GnRH) is a neurohormone of the hypothalamus controlling pituitary gonadotropin secretion and hence gametogenesis. While it has also been believed that GnRH is synthesized and functions in various peripheral tissues, the expression of GnRH receptor (GnRH-R) in peripheral tissues is not well-described. We previously found that annexin A5, which is increased in the pituitary gonadotropes by GnRH, is dramatically increased in rat mammary epithelial cells after weaning, suggesting that local GnRH is responsible for this increase. Annexin A5 is a member of the annexin family of proteins and is thought to be involved in various regulatory mechanisms, including apoptosis. In the present study, we examined GnRH-R expression in the mammary tissues after weaning. Although GnRH-R mRNA was not detected in the mammary tissues during lactation, it was dramatically increased after weaning. Forced weaning at mid-lactation (day 10) also promoted the expression of GnRH-R transcripts in mammary tissues within 2 days. Furthermore, western blotting analysis with anti-GnRH-R showed that the expression of an immuno-positive 60-kDa protein, whose size was equivalent to that of rat GnRH-R, was confirmed to increase after weaning. These findings clarified the induction of GnRH-R in the mammary tissues after weaning and suggest that GnRH is involved in the involution and tissue remodeling of post-lactating rat mammary tissues.

Key words: Annexin A5, Gonadotropin-releasing hormone (GnRH), GnRH receptor, Involution, Mammary gland

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Gonadotropin-releasing hormone (GnRH) regulates reproduction by controlling gonadotropin secretion. Although the primary action of GnRH is to stimulate gonadotropin secretion in the anterior pituitary gonadotropes, the expression of GnRH has been observed in peripheral tissues, particularly the ovary [1, 2], testis [3, 4], placenta [5, 6], and breast, [4] as well as in tumor cells [7–9], suggesting a role as an autocrine/paracrine regulator of secretory properties or cell growth [10–12]. Additionally, GnRH analogues have been used as therapy in hormone-dependent cancer [13]. GnRH receptor (GnRH-R) signaling directly regulates cell proliferation, apoptosis, or mortality [14–16]. GnRH-R contents and its binding activity are crucial for the therapeutic effect of the GnRH agonist on tumor cells [17, 18]. Although GnRH-R has been extensively detected in variety of normal tissues and tumor cells, the physiological expression of GnRH-R in extra-pituitary tissues remains unclear.

The mammary gland cycles through development, milk production, and involution in each successive pregnancy. Post-lactational involution consists of abundant apoptosis of the mammary epithelial cells and tissue remodeling. It has been shown that the accumulation of milk in the alveoli after cessation of suckling, known as milk stasis, causes local synthesis of the cytokine leukemia inhibitory factor and transforming growth factor beta 3 [19–21]. These cytokines promote involution via the signal transducer and activator of transcription 3 pathway [22]. We previously observed abundant expression of annexin A5 in mammary epithelial cells after lactation [23].

Annexin A5 is a member of the calcium-dependent phospholipid-binding protein family [24–26]. GnRH stimulates the expression of annexin A5 in the pituitary gonadotropes [27, 28], ovarian luteal cells [29], and Leydig cells [30]. GnRH is thought to facilitate regression and apoptosis of the corpus luteum and increase annexin A5 expression in pseudopregnant rats [29]. Because annexin A5 expression is increased in rat mammary epithelial cells two days after weaning [23], it has been hypothesized that GnRH stimulates annexin A5 expression and mammary involution. However, GnRH-R expression in the mammary gland, although GnRH is present [31, 32], remains unclear. In the present study, we investigated whether GnRH-R is expressed in rat mammary tissues. We found that the augmentation of GnRH-R expression occurs just after pup removal.

Materials and Methods

Wistar-Imamichi rats bred in our laboratory were used in this study. Rats were maintained in rooms with controlled temperature and light at 23 ± 3°C and 14 h light/10 h dark cycle (lights on at

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Correspondence: M Kawaminami (e-mail: mitsumor@vmas.kitasato-u.ac.jp

Table 1. Primers used in the current study

Target	Primer (5′-3′)
GnRH-R	Forward: AATCATCTTCGCCCTCACAC
	Reverse: AGCACGGGTTTAGAAAAGCA
GnRH-R exon 1	Forward: CCGTCCTTGGAGAAATATGG
	Reverse: AGCGGCATGACGATTAGAGT
GnRH-R exon 2	Forward: TCTTCAGGATGATCTACCTAGCC
	Reverse: CCTGATGAAGGACTCGTGTG
GnRH-R exon 3	Forward: CCAAGAATAATATCCCAAGAGCA
	Reverse: TCCCGTATATGGGTTTCAGC
RPL19	Forward: GGAAGCCTGTGACTGTCCAT
	Reverse: CCATGAGAATCCGCTTGTTT

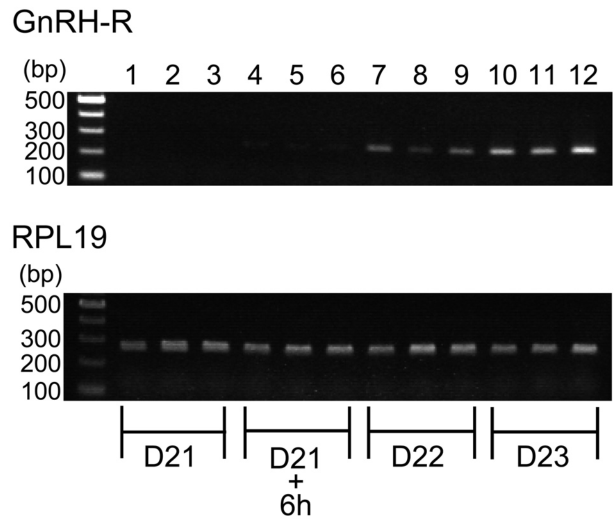

Fig. 1. RT-PCR analysis of GnRH-R mRNA in mammary tissues after lactation. RT-PCR was performed with total RNA isolated from mammary tissues of three rats each on day 21 before weaning and 6 h after weaning on days 21, 22, and 23. GnRH-R mRNA was detected using the primers shown in Table 1. RPL19 was used as an internal control. The lane on the left side is the 100-bp DNA ladder marker. The primer sets for GnRH-R and RPL19 mRNA were designed to yield 251-bp and 264-bp fragments.

0500 h). They were allowed free access to laboratory chow and tap water. Adult female rats showing at least two regular four-day estrous cycles were mated. The day of parturition was defined as day 0 (D0) of lactation and the number of pups was adjusted to eight on D1. Pups were removed from their dam on D21 of lactation and mid-lactational forced weaning was performed on D10. All experiments were performed according to the guidelines for animal experiments of Kitasato University and approved by the Committee for Laboratory Animals, Care and Use at School of Veterinary Medicine, Kitasato University.

Inguinal mammary tissues were collected from rats on D12, 21, 22, 23, or two days after forced weaning on D10. The tissues of D21 (weaning day) rats were harvested at 0 or 6 h after pup removal. The tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription-PCR (RT-PCR) for *GnRH-R* or ribosomal protein L19 (*RPL19*) was performed using Premix Taq^{TM} (Ex Taq^{TM} Version 2.0; Takara Bio, Shiga, Japan) for 35 or 22 cycles, respectively, at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 60 sec, with an initial denaturing step at 94°C for 2 min and a final elongation step at 72°C for 7 min. Primers used in this study are listed in Table 1. Amplified products were separated by 2% agarose gel electrophoresis and detected by ethidium bromide staining.

Inguinal mammary tissues were also collected from rats each day from D20 to 24 for western blot analysis. The tissues of D21 (weaning day) were harvested at 0 or 6 h after pup removal. The anterior pituitary of the rat was collected on D23. The tissues were homogenized and boiled for 5 min in SDS sample buffer. The samples containing 20 µg of protein were electrophoresed on 12% polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked with 5% skim milk (Wako Pure Chemicals, Osaka, Japan) for 1 h at room temperature and then incubated with primary antibodies: anti-GnRH receptor, mouse monoclonal antibody (1:200; Acris Antibodies GmbH, Herford, Germany), or anti-β-actin mouse monoclonal antibody (1:1,000; C4, Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at 4°C. The GnRH-R antibody was raised by immunization of the N-terminal 1–29 amino acid peptide of human GnRH-R, which shows 82.8% homology with rat GnRH-R and less than 40% homology with other rat proteins. After washing, the membranes were incubated with peroxidase-conjugated goat IgG fraction to mouse IgG (1:20,000; ICN Pharmaceuticals, Aurora, OH, USA) for 2 h at room temperature. Immunoreactive protein was detected with ECL Plus Western Blotting Detection Reagents or ECL Prime Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). The signal was detected by exposure of the membrane to an X-ray film for 5, 10, or 15 min with an ImageQuant LAS 4000 digital imaging system (GE Healthcare).

Results and Discussion

RT-PCR examination for GnRH-R mRNA showed no amplification in the mammary tissues of lactation D20, a day before weaning (Fig. 1A). GnRH-R mRNA was dramatically increased over time until two days after weaning on D23 (Fig. 1B). Next, the expression was decreased to trace levels on D26 and 29 (Fig. 1A). This indicates that the expression of GnRH-R is well-regulated and that the cessation of suckling stimuli triggers this expression. Ikeda *et al.* found that GnRH mRNA was expressed in the mouse mammary tissues during the lactating and involution periods, but did not detect GnRH-R mRNA by PCR [31]. This may be because Ikeda *et al.* did not determine the regulation of GnRH-R mRNA expression during the narrow period just after weaning.

We further confirmed the expression of GnRH-R in mammary tissues with different sets of primers in forced weaned rats. The *GnRH-R* gene consists of three exons and encodes 984 base pairs through exons 1–3 in rodents [33, 34]. We examined the expression of exons 1, 2, and 3 simultaneously. Mammary tissues were collected from a forced weaned rat after two days and from a lactating rat. The expression of each exon was equally stimulated after weaning (Fig. 2). This result again revealed that the cessation of suckling induces GnRH-R mRNA expression in the mammary tissues and that full-length mRNA is synthesized.

We previously reported that the expression of annexin A5 is dramatically increased in the epithelial cells of mammary tissues [23]. We predicted that GnRH acts locally in the mammary gland during post-lactational involution. We also published several reports regarding the relationship between GnRH and annexin A5 in various tissues [27–30]. Currently, the physiological function of annexin A5 is unknown, but it is thought to be involved in apoptosis and tissue remodeling. The present data support that GnRH affects degeneration and tissue remodeling in the mammary epithelium after lactation.

To confirm the translation of GnRH-R mRNA, western blotting analysis using a GnRH-R antibody against the N-terminal peptide sequence was performed. The results confirmed two immunoreactive bands, approximately 60 kDa and 30 kDa proteins, in the lactating and post-lactating mammary tissues. Both bands were also observed in the anterior pituitary tissue (Fig. 3). A previous study that also used a GnRH-R monoclonal antibody against the N-terminal 1–29 amino acid residues, which differed from the antibody used in the present study, detected an approximately 60-kDa protein in the rat pituitary gland [35]. GnRH-R in mice and rats is a seven-transmembrane, G-protein-coupled receptor of 327 amino acid residues with two or three N-terminal glycosylation sites and can be detected at 55–70 kDa by SDS-PAGE [36]. Therefore, the 60-kDa band is considered a natural GnRH-R. This band was increased after weaning and reached a peak on D23, coinciding with mRNA results. Interestingly, there was another 30-kDa immunoreactive band, which decreased after weaning. Although the sequence of the 30-kDa protein is not known

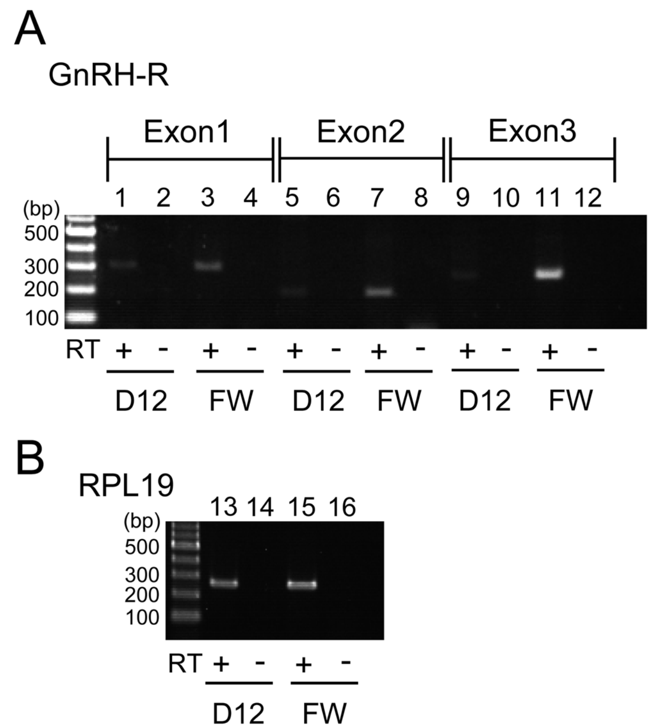

Fig. 2. RT-PCR analysis with exon-specific primers for GnRH-R. (A) Primers for exons 1–3 of the GnRH-R gene were used. RT-PCR was performed on DNase-treated total RNA isolated from the mammary tissues on lactation day 12 (D12) and 2 days after forced weaning on day 10 (FW). Amplification of GnRH-R mRNA (lane 1–4; exon 1, lane 5–8; exon 2, lane 9–12; exon 3) was performed using templates with or without reverse transcription reaction (RT) to prevent the amplification of genomic DNA. Three primer sets specific to exons 1, 2, and 3 were designed to amplify 306-, 201-, and 271-bp amplicons, respectively. (B) RPL19 was used as an internal control for the two rats used (lane 13–16).

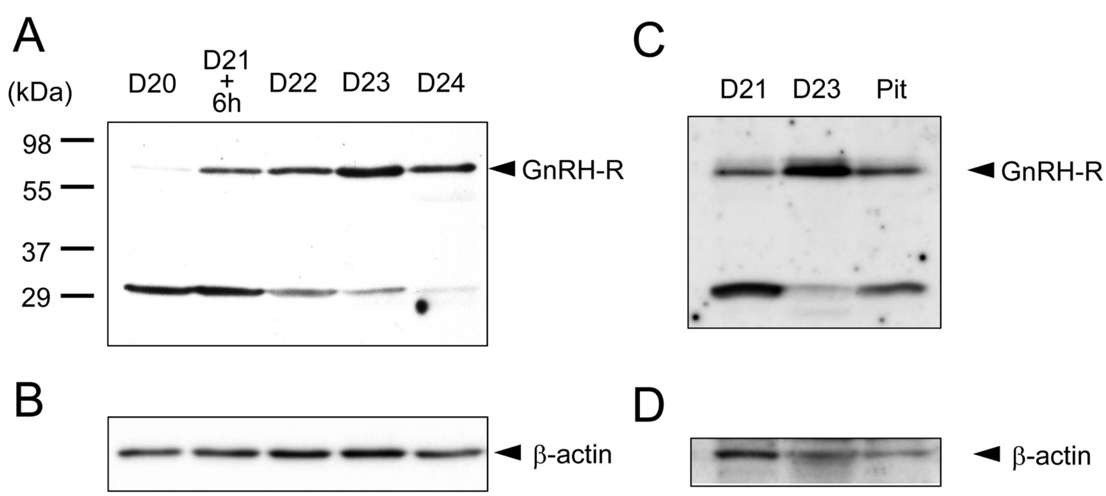

Fig. 3. Western blotting analysis of GnRH-R proteins during and after lactation. (A, B) Western blotting was performed with mammary tissue samples on lactation day 20 (D20) and 6 h after weaning on day 21 (D21+6h), day 22 (D22), day 23 (D23), and day 24 (D24). β-actin was used as an internal control. The experiments were repeated twice using mammary tissues from different rats. (C, D) Western blotting was performed on the mammary tissues from D21 (on weaning) and D23, and the anterior pituitary tissue. β-actin is used as an internal control.

and was observed even during lactation while there was no detectable level of GnRH-R mRNA, the GnRH-R variant may have been present. Theoretically, alternative splicing would produce a shorter GnRH-R. Alternatively, impairment of post-translational glycosylation may produce a GnRH-R with a lower apparent molecular weight on SDS-PAGE [36]. The molecular weight of nascent GnRH-R is 37 kDa. Future studies are needed to investigate the post-transcriptional or post-translational modification of GnRH-R in the mammary gland.

Time-specific expression of GnRH-R in the mammary gland after pup removal strongly suggests that the expression is related to changes in the endocrine milieu after lactation, specifically the cessation of massive prolactin release. Prolactin release is stimulated by suckling during the lactation period. We previously reported that prolactin suppresses annexin A5 expression in the corpus luteum, suggesting negative control of GnRH function by prolactin. Experimental suppression of prolactin release caused luteal regression, and a GnRH antagonist was shown to inhibit the process of apoptosis [29]. Further studies are needed to clarify the role of GnRH in apoptosis and the signaling pathways involved in the post-lactational mammary gland.

In summary, we detected a post-lactational increase in GnRH-R expression in rat mammary tissues. These results suggest that local GnRH is involved in the involution of mammary tissues after weaning to induce epithelial apoptosis and tissue remodeling.

Conflict of Interests: None of the authors have any potential conflicts of interest associated with this research.

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