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Comparative Expression Profiling of Lactogenic Hormone Receptor and It's Signaling Molecules of Bovine Mammary Glands during lactation

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Abstract

Milk synthesis is known to be modulated by peptide hormones such as prolactin (PRL), growth hormone (GH), and insulin-like growth factor I (IGF-I). Previous studies suggested that PRL and IGF-I acted directly on mammary epithelial cells and were involved in lactation. Meanwhile, GH is thought to be indirectly involved in lactation by stimulating the secretion of IGF-I. It is controversial as growth hormone receptors (GHR) is expressed in the mammary epithelial cells. In order to clarify whether GH acted directly on mammary gland tissue, we investigated the prolactin receptors (PRLR), IGF-I receptors (IGF-IR), and GHR as well as the gene expression levels of the downstream signaling molecule for each receptor in the mammary gland tissue of Holstein cows during different stages of lactation. The results revealed that the mRNA expressions of PRLR and IGF-IR were highest during early lactation, and the mRNA expression of the GHR was highest during mid-lactation. We also found that the expression profiling of the signal transducer and activator of transcription 5 (STAT5) genes was similar to that of the GHR gene. On the other hand, the expression profiling of the PRLR gene was similar to that of the SHP2 gene. These results suggest that GH acts on the mammary glands directly, milk synthesis and secretion are chiefly stimulated in mid-lactation, and the timing of the action is different for PRL and IGF-I.

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Keywords

Lactation, Growth Hormone, Prolactin, Signal Transduction, Mammary Gland

1. Introduction

The development of mammary gland begins from the development of the mammary duct system from sexual maturity into mid-pregnancy. The mammary alveolus is formed from mid-pregnancy, the development of the mammary glands is almost completed in late pregnancy, and the mammary epithelial cells of the mammary alveolus begin to produce real milk postpartum. However, an involution of the mammary glands occurs with the decrease in mammary alveolus cell formation in latter lactation, causing milk yield to decrease remarkably. Mammary gland development in the period from pregnancy to lactation is caused by the interaction between the hormones and the growth factors. It is known that milk synthesis is modulated by peptide hormones such as prolactin (PRL), growth hormone (GH), and insulin-like growth factor I (IGF-I) [1].

PRL is widely known to play a key role in the development and differentiation of mammary gland based on the results of research on rodents. PRL acts directly on the differentiation and maintenance of secretory cells through the prolactin receptors (PRLR) in mammary epithelial cells [2]. Previous reports suggested that IGF-I also acted directly in the mammary epithelial cells, and was involved in the proliferation and differentiation of breast epithelial cells [3]. On the other hand, it is thought that GH is indirectly involved in milk lactation by stimulating the secretion of IGF-I [4] [5]. However, it had been reported that hormone receptors (GHR) was expressed in the mammary epithelial cells [6] and that GH could directly modulate casein and leptin gene expression in the mammary epithelial cells [7]. As it has been reported that administration of recombinant bovine GH to lactating dairy cow increases milk yield [8], it is very important to clarify the action mechanism of GH during lactation.

The downstream of PRLR, IGF-I receptors (IGF-IR), and GHR was involved in common signal transduction molecules, such as Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), Src homology 2 domain-containing transforming protein C (SHC), and insulin receptor substrate 1 (IRS-1). Previous study indicated that PRL transmitted through JAK2/STAT5 [9] and, in particular, that PRL was involved in the transcription of β-casein through STAT5 [10]-[12]. Among three receptors, Src homology 2 domain containing protein-tyrosine phosphatase (SHP2) is known as a downstream signaling molecule of PRLR. Previous studies indicated that SHP2 was activated by prolactin and took part in the transcription of β-casein genes [13]. Though STAT1 and STAT3 are reported as downstream signaling molecules of GHR among three receptors [14], it is not clear whether the activation of these signal molecules is involved in milk synthesis.

Therefore, in order to clarify whether GH acted directly on mammary gland tissue, we investigated the mRNA expression of PRLR, IGF-IR, and GHR as well as that of the downstream signaling molecule for each receptor in the mammary gland of Holstein cows during different stages of lactation.

2. Materials and Methods

2.1. Animals, Tissue Sampling and Preparation

The mammary gland from Holstein cow (n = 12) was removed within 20 min after slaughter during defined stages. Small pieces (1 - 2 g) of mammary glands was frozen in liquid nitrogen and stored at −80˚C. The classification of the animals was established as follows: 1) Early lactation stage (day 6 - 18 of lactation, n = 3); 2) Middle lactation stage (day 124 - 158 of lactation, n = 3); 3) Late lactation stage (day 276 - 306 of lactation, n = 3); 4) Dry period (1 month after the beginning of dry period, n = 3). The treatment of the animals was according to "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" (The Physiological Society of Japan).

2.2. RNA Extraction

Total RNA was isolated from mammary glands using the TRIzol reagent (Life Technologies), according to the manufacturer's instructions. The concentration of the isolated total RNA was determined by measuring the optical density at 260 nm, and the purity of the RNA was determined based on the ratio of the absorbance at 260 nm

relative to the absorbance at 280 nm.

2.3. End-Point RT-PCR

Two micrograms of RNA were then processed for cDNA synthesis using a ReverTra Ace RT Master Mix (TOYOBO, Japan). Conditions for the enzymatic amplification were optimised for all the factors studied. For every PCR amplification, the linear range was verified by introducing increasing cDNA amounts as well as cycle numbers. PCR was performed using the EX Taq DNA Polymerase (TAKARA, Japan). Amplification conditions included 30 (PRLR, GHR, STAT3, SHC and IRS-1), 33 (STAT5, STAT1 and SHP2), 35 cycles (IGF-1R and JAK2) of denaturation at 94˚C for 1 min, annealing at 55˚C (60˚C JAK2 and SHC) for 1 min. A single denaturation step at 94˚C for 2 min and a final extension step at 72˚C for 2 min were performed, except for GAPDH: 19 cycles of denaturation at 94˚C for 45 s, annealing at 55˚C for 45 s and extension at 72˚C 45 s. The primer sequences are shown in **Table 1**. GAPDH was used as an endogenous control. The PCR products were separated in a 2% agarose gel, stained with ethidium bromide, and analyzed using Fluor-S MultiImager (Bio-Rad, Hercules, CA). The intensity of leptin abundance was assigned as a ratio to that of GAPDH abundance. The sequences of all PCR products were verified by sequencing.

2.4. Statistical Analysis

In all of the experiments, the values are expressed as the means ± standard error of the mean, with at least 3 replicates in each experimental group. Statistical significance was determined by a one-way ANOVA followed by the Turkey-Kramer analysis. The test was considered significant if $P < 0.05$.

Table 1. Sequences of primers used for RT-PCR amplification.

Gene	Accession number	Primers (5' to 3')
PRLR	NM_174155.3	Forward CCATCCTTTCTGCTGTCAT
		Reverse CTTGCTCCGTGTGTTCTTT
GHR	NM_176608.1	Forward ACCCAGTGGAAAATGGACCCTT
		Reverse CTGTCTGTGTCTGACCCTTCAGTC
IGF-IR	NM_001244612.1	Forward TTAAAATGGCCAGAACCTGAG
		Reverse ATTATAACCAAGCCTCCCAC
JAK2	DT897449	Forward TTGGCAATGACAAACAAGGA
		Reverse ATCTCATCTGGGCATCCATC
STAT5	NM_001012673	Forward TGCATCCGCCATATTCTGTA
		Reverse AGTCGCAGCTCCTCAAATGT
SHC	NM_001164061.1	Forward GTGAGGTCTGGGGAGAAGC
		Reverse GGTTCGGACAAAGGATCACC
IRS-1	XM_003585773.3	Forward CATGCACGAGACAATCCTGG
		Reverse CCTGTTGGTGCTAGGACTC
SHP2	NM_174742.2	Forward CGGTCTGGCAATACCACTTT
		Reverse TCGTGTCCTTTCCTCTTGCT
STAT3	NM_001012671.2	Forward CAACCCCAAGAACGTGAACT
		Reverse GAAGGTACCTGGGGGCTTAG
STAT1	XM_003583326.2	Forward AGCAAGCGTAACCTTCAGGA
		Reverse CATTCTTTGCCACACCATTG
GAPDH	NM_001034034.1	Forward TGACCCCTTCATTGACCTTC
		Reverse GTCTTCTGGGTGGCAGTGAT

3. Results

3.1. The mRNA Expression of Lactogenic Hormone Receptor in Mammary Glands during Lactation

We extracted RNA from the mammary gland of Holstein cows at varying lactation periods (early lactation, middle lactation, and late lactation) and dry periods. Then we analyzed the mRNA expression of PRLR, IGF-IR, and GHR using end-point RT-PCR. The expression of PRLR and IGF-IR were the highest in early lactation and it decreases significantly after mid-lactation (Figure 1). On the other hand, however, GHR gene expression increases from early lactation until mid-lactation, and then significantly decreases in late lactation (Figure 1).

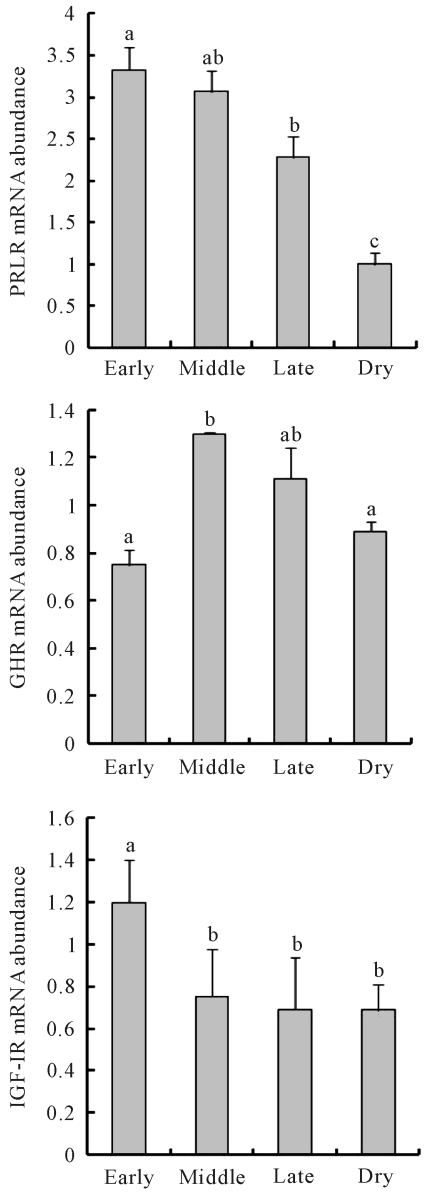

Figure 1. PRLR, GHR and IGF-1R expression in mammary glands during lactation. Mammary glands were collected from early lactation stage (Early), middle lactation stage (Middle), late lactation stage (Late) and dry period Holstein cows. And then total RNA was isolated and subjected to end-point RT-PCR analysis. Each mRNA abundance assigned as a ratio to GAPDH mRNA abundance. The Results are shown as the ±S.E.M (n = 3). Points with a different superscript are significantly different ($P < 0.05$).

3.2. The mRNA Expression of JAK2, STAT5, SHC and IRS-1mRNA in Mammary Glands during Lactation

We then analyzed the mRNA expression of JAK2, STAT5, SHC, and IRS-1 that are shared as PRLR, IGF-IR, and GHR downstream signaling molecules. We found that the expression of JAK2, STAT5, and SHC genes significantly increased from early to mid-lactation, and significantly decreased after late lactation (**Figure 2**). IRS-1 gene exhibited the highest expression in early lactation, and it decreases significantly after late lactation.

3.3. The Expression of SHP2, STAT3, and STAT1 mRNA in Mammary Glands during Lactation

Finally, we analyzed the mRNA expression of SHP2, which is only found downstream of the PRLR among three receptors; and we examined STAT3 and STAT1 mRNA expression, the signal molecules that exist only downstream of the GHR. The expression of STAT3 was increased from early lactation through mid-lactation, and then significantly decreased during non-lactation (**Figure 3**). We did not observe any significant changes in the expression of the STAT1 gene during lactation.

4. Discussion

The expression level of the GHR gene significantly increased from early lactation through mid-lactation and significantly decreased from late lactation onward. The result of the change in GHR gene expression level by lactation period suggests that GH directly acts on the mammary glands. In addition, based on the result that the

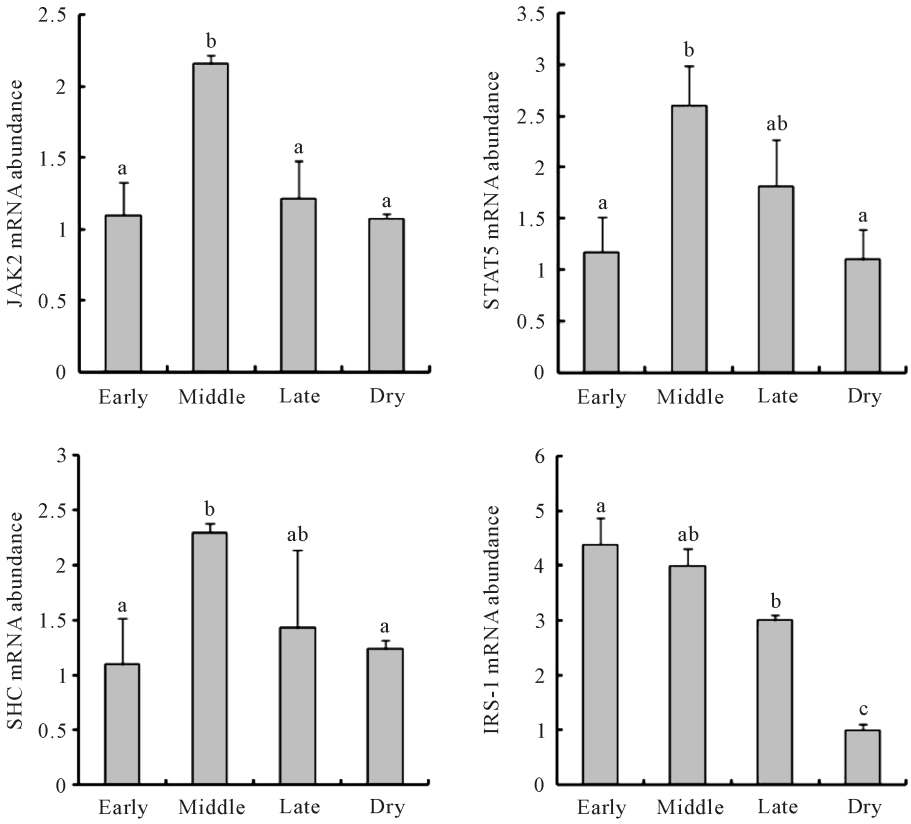

Figure 2. JAK2, STAT5, SHC and IRS-1 expression in mammary glands during lactation. Mammary glands were collected from early lactation stage (Early), middle lactation stage (Middle), late lactation stage (Late) and dry period Holstein cows. And then total RNA was isolated and subjected to end-point RT-PCR analysis. Each mRNA abundance assigned as a ratio to GAPDH mRNA abundance. The Results are shown as the ±S.E.M (n = 3). Points with a different superscript are significantly different ($P < 0.05$).

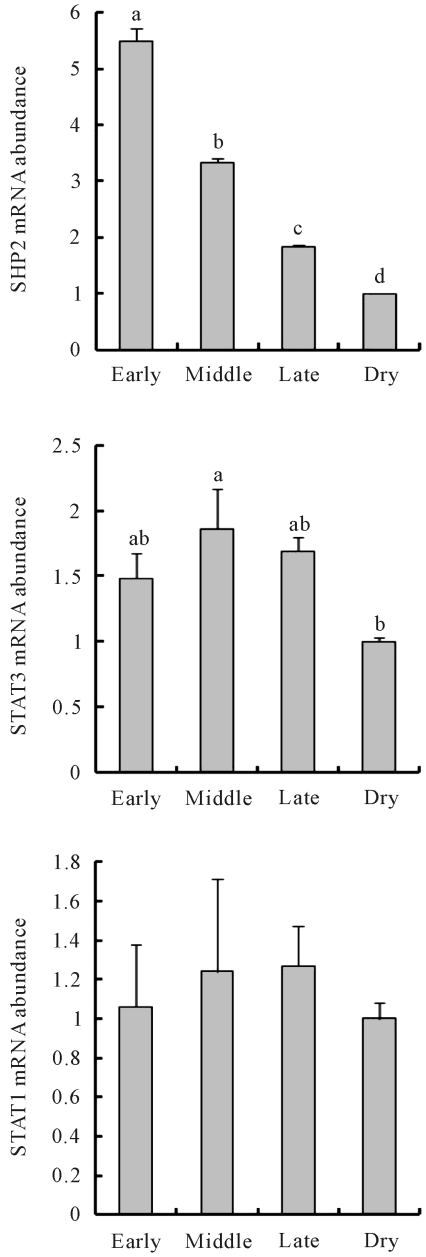

Figure 3. SHP2, STAT3 and STAT1 expression in mammary glands during lactation. Mammary glands were collected from early lactation stage (Early), middle lactation stage (Middle), late lactation stage (Late) and dry period Holstein cows. And then total RNA was isolated and subjected to end-point RT-PCR analysis. Each mRNA abundance assigned as a ratio to GAPDH mRNA abundance. The Results are shown as the ±S.E.M (n = 3). Points with a different superscript are significantly different ($P < 0.05$).

PRLR gene and the IGF-IR gene expression levels were highest in early lactation while the GHR gene expression level was highest in the early lactation, it can be assumed that the action time for GH differs from that of PRL and IGF-I.

The expression level of the JAK2 and STAT5 genes significantly increased from early lactation through mid-lactation and significantly decreased after late lactation. The expression profiling of these genes was similar to the expression profiling of the GHR. This result implies that GH enhances the activity of JAK2/STAT5 signals in mammary gland. It has been reported that PRL acts directly on the mammary glands and promotes the tran-

scription of the β-casein gene through JAK2/STAT5 pathway [10]. However, the expression profiling of the JAK2 and STAT5 were different from those of the PRLR. These results indicate that PRL promotes the β-casein synthesis through JAK2 and STAT5 in early lactation and mid-lactation, and that JAK2/STAT5 signals are increasing due to participation by GH in mid-lactation. The expression profiling of SHC was also similar to that of GHR. This result implies that GH enhances the SHC signal in mammary gland. The role of the SHC signals (including PRL and IGF-I) in milk synthesis, however, has remained unknown, so further research is required.

The mRNA expression level of SPH2 was highest in early lactation and decreased significantly in mid-lactation. SHP2 gene expression profiling was found to be similar to PRLR gene one. This result is consistent with previous reports indicating that SHP2 was activated by PRL and was involved in the synthesis *β*-casein [13]. In addition, as the milk yield was highest during early lactation, we speculate that SHP2 pathway by PRL through in early lactation has greater influence on milk production than the pathway through JAK2/STAT5. STAT3 gene expression profiling resembles GHR and JAK2 gene expression profiling, which suggests that JAK2/STAT3 pathways are activated by GH in the mammary gland. Although previous study reported that the activation of STAT3 by GHs in rat livers [14], there has been no reports of this in mammary tissue. Subsequent research is necessary to investigate whether the activation of JAK2/STAT3 pathways by GH in mammary tissue influences milk synthesis.

5. Conclusion

In conclusion, our data suggest that GH acts on the mammary gland directly, and that milk synthesis and secretion are chiefly stimulated in mid-lactation. It is known that milk production in dairy cows is generally highest in early lactation before decreasing thereafter. Although our results suggest that PRL has a higher lactation effect than GH, but we posit that GH also acts directly on the mammary gland and has lactation effects.

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