Prolactin regulates LAT1 expression via STAT5 (signal transducer and activator of transcription 5) signaling in mammary epithelial cells of dairy cows

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ABSTRACT

The L-type amino acid transporter 1 (LAT1, also known as SLC7A5) is a transporter that allows the uptake of large neutral amino acids into mammalian cells. In dairy cows, LAT1 is highly expressed in lactating mammary tissues and involved in milk protein synthesis. Prolactin (PRL) has a lactogenic role and is capable of inducing milk production in ruminants. However, the relationship between PRL stimulation and LAT1 expression in dairy cow mammary gland has not been well understood. In this study, we showed that PRL stimulation increased expression of LAT1 and β-casein in mammary epithelial cells of dairy cows. The stimulatory effect of PRL on milk protein production was inhibited by LAT1-specific inhibitor or LAT1 knockdown, suggesting that PRL-induced milk protein production is involved in LAT1 expression. To determine whether the PRL signaling pathway participates in regulation of LAT1 expression, PRLR (PRL receptor) or STAT5 (signal transducer and activator of transcription 5) was knocked down by short interfering (si)RNA in mammary epithelial cells of dairy cows. Western blot results showed that knockdown of PRLR or STAT5 with siRNA markedly decreased PRL-stimulated LAT1 expression. In addition, we observed a marked increase in plasma membrane expression of LAT1 in PRL-stimulated cells compared with control cells. These observations indicated that PRL signaling can regulate LAT1 expression and activity in mammary epithelial cells of dairy cows, contributing to increased amino acid availability and milk protein synthesis in mammary gland of dairy cow.

Key words: mammary epithelial cell, prolactin, LAT1 (L-type amino acid transporter 1), milk protein

INTRODUCTION

Amino acids are essential nutrients for protein synthesis and other metabolic functions. The L-type amino acid transporter 1 (LAT1, also known as SLC7A5) is important for the transport of neutral amino acids, including nutritionally essential AA (Christensen, 1990). The L-type amino acid transporter 1 is part of the SLC7 family and forms a heterodimer with the heavy chain of 4F2 (4F2hc, also known as SLC3A2) via a disulfide bond on the cell surface (Mastroberardino et al., 1998; Napolitano et al., 2015). When expressed in mammalian cells together with 4F2hc, LAT1 induces the transport of neutral AA from the circulation into cells (Nakamura et al., 1999).

Prolactin (PRL) is known as a galactopoietic hormone and is capable of inducing milk production (Lacasse et al., 2016). The galactopoietic role of PRL has been well established in rodents (Neville et al., 2002). In dairy cows, PRL content increases in blood plasma around parturition (Edgerton and Hafs, 1973). In cultured bovine mammary epithelial cells, PRL upregulates CSN mRNA expression (Choi et al., 1988). The long-term inhibition of PRL reduces milk yield in dairy cows, indicating its galactopoietic role in ruminants (Lacasse et al., 2011; Lollivier et al., 2015). Although PRL signaling has been extensively studied in milk protein synthesis during lactation, less information is available to describe the relationship between AA...
transport and PRL signaling, especially in mammary glands of dairy cows. In this study, we hypothesized that PRL regulates LAT1 expression and activity in mammary epithelial cells of dairy cows. To test this hypothesis, we evaluated the effects of different concentrations of PRL on LAT1 expression in mammary epithelial cells of dairy cows. Then, using a specific inhibitor and gene knockdown, we determined whether PRL signaling regulates LAT1 expression, thereby controlling milk protein synthesis.

**MATERIALS AND METHODS**

**Cell Culture and Treatment**

Mammary epithelial cells were prepared from mammary parenchymal tissues of 3 lactating Holstein cows as described previously (Lin et al., 2018). All procedures involving animals were in accordance with institutional guidelines and were approved by the Northeast Agricultural University Animal Care and Use Committee (2019–2, Harbin, China). The 3 cows were in their third parity and at 90 DIM. All cows were in good health and had SCC <50,000 cells/mL. Mammary epithelial cells were isolated by collagenase digestion. Briefly, the mammary tissues were minced with surgical scissors and digested for 2 h at 37°C with 1 mg/mL collagenase III (Solarbio, Beijing, China). Then, the digest was filtered through a nylon mesh and the filtrate was centrifuged at 150 × g for 10 min. The cell pellet was resuspended and plated on plastic cell culture dishes coated with rat tail collagen (0.5%; Sigma-Aldrich Inc., St. Louis, MO) and incubated in Dulbecco’s modified Eagle’s medium/ Nutrient Mixture F-12 medium (DMEM/F12, Life Technologies, Carlsbad, CA) containing 10% (vol/vol) fetal bovine serum (Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, and 1 μg/mL hydrocortisone (Sigma-Aldrich). The primary cells were trypsinized at ~80% confluency with trypsin-EDTA (0.25%, Life Technologies) and passaged. Expression of cytokeratin 18 and β-casein was detected by immunofluorescence as described (Lin et al., 2018) before each experiment to ensure that the cells were purified mammary epithelial cells and capable of lactation. All experiments were conducted using cells within 10 passages.

To detect the effects of different concentrations of PRL on LAT1 and 4F2hc expression, mammary epithelial cells were plated at 1 × 10⁶ cells/well in 6-well culture plates. When cells grew to 90% confluence, the medium was changed to DMEM/F12 with 0, 1, 2.5, 5, 10, or 20 μg/mL of PRL. After 24 h of treatment, cells in triplicate wells were collected for total RNA and protein extraction.

To investigate whether LAT1 was involved in PRL-stimulated milk protein synthesis, mammary epithelial cells were grown in 6-well plates (1 × 10⁶ cells/well) to 90% confluence. Then cells were treated for 24 h with 5 μg/mL PRL plus 2.5 mM 2-amino-2-norbornanecarboxylic acid (BCH, an inhibitor of LAT1; Cayman Chemical, Ann Arbor, MI) or 0.02% dimethyl sulfoxide (DMSO) as a control.

To investigate the effect of PRL on plasma membrane expression of LAT1, mammary epithelial cells were grown in 6-well plates (1 × 10⁶ cells/well) to 90% confluence. Then, the cells were treated with 5 μg/mL PRL for 24 h.

**RNA Isolation and Quantitative Real-Time PCR**

Sample preparation and quantification of mRNA abundance was carried out as described (Yang et al., 2017). Briefly, total RNA was extracted from each sample using TRIzol reagent (Life Technologies), and RNA concentrations were measured using a NanoDrop 2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was performed with a PrimeScript Reagent Kit with gDNA Eraser (Takara, Dalian, China). The quantitative real-time PCR (qPCR) was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) in a Roche LightCycler 96 real-time PCR system (Roche Diagnostics GmbH, Mannheim, Germany). Primers for LAT1 and reference genes were designed with Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA) and are shown in Table 1. The qPCR was performed in a 20-μL volume containing 10 μL of 2× ChamQ Universal SYBR qPCR Master Mix, 0.4 μL each of 10 μM primers, 1 μL of cDNA, and 8.2 μL of DNase-/RNase-free water. The amplification program consisted of 1 cycle of 95°C for 30 s and 40 cycles of 95°C for 10 s and 60°C for 30 s, followed by an additional cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s to generate a melting curve. The qPCR reactions on each sample were performed in triplicate. Expression of LAT1 was normalized using the geometric mean of 3 endogenous genes, ACTB, RPS9, and UXT. The relative RNA expression levels were calculated by the 2^ΔΔCT method (Schmittgen and Livak, 2008).

**Transfection**

Short interfering (si)RNAs against the long isoforms of PRLR, LAT1, and STAT5 were purchased from GenePharma (Shanghai, China). Corresponding scrambled siRNAs purchased from GenePharma were used as negative controls. Transient transfection was performed in cultured mammary epithelial cells of dairy
cows using Lipofectamine 3000 reagent (Life Technologies) as described (Lin et al., 2018). For knockdown of the target gene expression, cells in 6-well plates (1 × 10^6 cells/well) were transfected with specific siRNAs in the presence of 5 μg/mL PRL. At 24 h after transfection, cells were harvested for protein extraction and Western blot analysis. All experiments were performed in triplicate.

**Western Blot Analysis**

For Western blot analysis, total proteins were extracted from mammary epithelial cells using RIPA buffer (Beyotime, Shanghai, China) containing 0.5 mM phenylmethylsulfonyl fluoride (Beyotime), 5 μg/mL aprotinin (Sigma-Aldrich), and 5 μg/mL leupeptin (Sigma-Aldrich). Plasma membrane proteins and cytosol proteins were extracted with a membrane and cytosol protein extraction kit (Beyotime). Protein concentrations were determined by the bicinchoninic acid method. Then, ~40 μg of protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with antibodies against LAT1 (1:1,000 dilution; Cell Signaling Technology, Danvers, MA), 4F2hc (1:500 dilution; Proteintech Group Inc., Rosemont, IL), β-casein (1:500 dilution, Bioss Antibodies, Beijing, China), PRLR (1:1,000 dilution; Cell Signaling Technology), STAT5 (1:1,000 dilution; Cell Signaling Technology), phosphorylated (p)-STAT5 (1:1,000 dilution; Cell Signaling Technology), β-actin (1:1,000 dilution; Cell Signaling Technology), or Na^+-K-ATPase (1:100,000 dilution; Abcam, Cambridge, MA). After washing with Tris-buffered saline-Tween, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:500 dilution; ZSGB-BIO, Beijing, China) at room temperature for 1 h. The protein bands were detected using enhanced chemiluminescence detection system (Cell Signaling Technology) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Warrendale, PA). The target protein expression in cell lysate and cytosol was normalized to expression of β-actin. Expression of LAT1 in plasma membrane was normalized to that of Na^+-K-ATPase.

**Statistical Analysis**

Results are presented as means ± standard errors of the means (SEM). Data from 3 independent experiments were subjected to statistical analysis. All statistical tests were performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA). To analyze the effects of different concentrations of PRL on LAT1 and 4F2hc expression, ANOVA was performed to evaluate differences among the groups. Bonferroni’s multiple comparison test was used for post-ANOVA comparisons. To analyze the effects of PRL, BCH, or gene knockdown on STAT5 signaling molecules and LAT1 and CSN2 expression, a 2-tailed unpaired t-test was used to compare means between the PRL and BCH treatment groups or between the gene knockdown group and control group. Differences were considered significant at P < 0.05.

**RESULTS**

PRL Regulates LAT1 Expression and β-Casein Synthesis in Mammary Epithelial Cells

To investigate whether PRL can affect LAT1 expression, mammary epithelial cells were cultured in DMEM/F12 with PRL at concentrations ranging from 0 to 20 μg/mL. The qPCR results showed that the abundance of LAT1 mRNA was upregulated gradually and peaked at 5 μg/mL PRL (Figure 1A). Western blot analyses also showed that levels of LAT1 and 4F2hc were highest in the medium with 5 μg/mL PRL (Figure 1B, C, D).

To determine whether LAT1 expression and milk protein synthesis were directly regulated by PRL

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Table 1. Primer sequences used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Product size (bp)</th>
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<tr>
<td>LAT1</td>
<td>NM_174613.2</td>
<td>F: ACCCTCACTGTCGTTGTCAGG</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTCCCAGTTCTGATGACG</td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>NM_173979.3</td>
<td>F: TGGCCGCACTGACAACTAC</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACAGCAAGCAGTTGCGGTA</td>
<td></td>
</tr>
<tr>
<td>RPS9</td>
<td>NM_001101152.2</td>
<td>F: GGTAGCGGACGATGATGGG</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGGCCACCTTCCGATCTTT</td>
<td></td>
</tr>
<tr>
<td>UXT</td>
<td>NM_001037471.2</td>
<td>F: GATCTATGTCGGCCCTTGGAT</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTTGGTGGGTCTGCGG</td>
<td></td>
</tr>
</tbody>
</table>

1LAT1 = l-type amino acid transporter 1, encoded by SLC7A5, the solute carrier family 7 member 5 gene; ACTB = β-actin; RPS9 = ribosomal protein S9; UXT = ubiquitously expressed prefoldin like chaperone; F = forward; R = reverse.
signaling, mammary epithelial cells were cultured in medium with 5 μg/mL PRL for 24 h. Compared with control, the abundance of LAT1, 4F2hc, and β-casein protein increased significantly (Figure 1E, F; \( P < 0.05, P < 0.01 \)). We then transfected mammary epithelial cells with PRLR siRNA in the presence of 5 μg/mL PRL. Western blot results showed that PRLR siRNA abolished the stimulatory actions of PRL on LAT1, 4F2hc, and β-casein expression (Figure 1G, H; \( P < 0.01 \)), indicating that PRL signaling can regulate LAT1 expression and milk protein synthesis in mammary epithelial cells of dairy cows.

**PRL Regulates Milk Protein Synthesis via LAT1**

Mammary epithelial cells were treated with 5 μg/mL PRL in the presence or absence of BCH, a competitive inhibitor of LAT1. As shown in Figure 2A, the addition of BCH significantly inhibited PRL-induced LAT1 expression (\( P < 0.01 \)), which in turn decreased β-casein synthesis (\( P < 0.01 \)). Next, we transfected mammary epithelial cells with LAT1 siRNA; LAT1 siRNA knockdown significantly decreased LAT1 expression compared with control (scrambled) siRNA (Figure 2C, D; \( P < 0.01 \)). The expression of β-casein was also decreased in the PRL + LAT1 knockdown group compared with the PRL + scrambled siRNA group (Figure 2C, D; \( P < 0.01 \)).

**PRL Induces LAT1 Expression by Activating STAT5**

To investigate whether PRLR–STAT5 signaling is involved in PRL-induced LAT1 expression, mammary epithelial cells were cultured in medium with 5 μg/mL PRL for 24 h. Compared with the control, treatment of mammary epithelial cells with 5 μg/mL PRL significantly increased the expression of PRLR, STAT5, and p-STAT5, as well as the expression of LAT1 and 4F2hc (Figure 3A, B; \( P < 0.01 \)). Next, we transfected mammary epithelial cells with PRLR siRNA. Compared with a scrambled control siRNA, PRLR siRNA knockdown significantly decreased the expression of STAT5 and p-STAT5 (Figure 3C, D; \( P < 0.01 \)). Then, we transfected mammary epithelial cells with STAT5 siRNA in the presence of PRL. Compared with a scrambled control siRNA, PRLR siRNA significantly decreased the expression of STAT5 and p-STAT5 (Figure 3C, D; \( P < 0.01 \)). The expression of LAT1 and 4F2hc also decreased significantly (Figure 3C, D; \( P < 0.01 \)). After transfection, we observed a significant decrease in STAT5 expression in the presence of PRL. At 24 h after transfection, we observed a significant decrease in STAT5 expression in the presence of PRL. Then, we transfected mammary epithelial cells with STAT5 siRNA in the presence of PRL. Compared with the control scrambled siRNA group, the STAT5 siRNA knockdown inhibited the stimulatory effects of PRL on STAT5 activation and LAT1/4F2hc expression (Figure 3E, F; \( P < 0.01 \)).
To investigate changes in cell surface LAT1 expression, we isolated plasma membranes from cultured mammary epithelial cells and determined LAT1 expression in response to 5 μg/mL PRL stimulation. We observed that PRL stimulation caused a marked increase in the expression of LAT1 in the plasma membrane compared with control (Figure 4A, B; P < 0.01), whereas the expression of LAT1 in the cytosol was significantly decreased in the PRL-treated group compared with the control group (Figure 4C, D; P < 0.01).
DISCUSSION

During lactation, the mammary gland requires active uptake of AA to support milk synthesis (Manjarín et al., 2014); LAT1 is an indispensable transporter of EAA (Hyde et al., 2003). In mammary tissues of lactating cows, LAT1 is highly expressed on the plasma membrane of alveolar epithelial cells, suggesting its roles in providing EAA to the mammary gland (Lin et al., 2018).

Prolactin is known to be mammogenic and lactogenetic in both monogastric and ruminant mammals (Lacasse et al., 2016). The actions of PRL on AA uptake by mammary tissues have been described in rodents. In rat mammary gland, PRL has an important role in the regulation of AA uptake (Viña et al., 1981). The sodium-coupled neutral amino acid transporter 2 (SNAT2) is characterized as a classical system A transporter and is important for the uptake of small neutral AA into the mammary gland to promote cell proliferation during gestation and to induce milk protein synthesis during lactation (Velázquez-Villegas et al., 2014). Studies in mammary gland explants from lactating rats have demonstrated that PRL treatment increases SNAT2 mRNA and protein abundance (Velázquez-Villegas et al., 2015). In the current study, we found that mRNA levels of LAT1 in cultured mammary epithelial cells of dairy cows were increased with increasing concentrations of PRL and peaked at 5 μg/mL PRL. The expression profile of LAT1 under different PRL concentrations was similar to that of LAT1 mRNA, suggesting that PRL regulates LAT1 expression at least at the transcriptional level in mammary gland of dairy cow. Prolactin signaling begins with its binding to the long isoform of PRLR (Lesueur et al., 1991; Cassy et al., 1998). Knockdown of PRLR in cultured mammary epithelial cells of dairy cows decreased LAT1 and 4F2hc expression, demonstrating that PRL signaling could directly regulate LAT1 expression in mammary epithelial cells of dairy cows.

Previous studies have demonstrated that PRL can increase milk protein gene expression and secretion in rodents and that suppression of PRL inhibits lactation (Taylor and Peaker, 1975; Guyette et al., 1979; Flint and Vernon, 1998). However, PRL has not always been considered a galactopoietic hormone in ruminants, because the effect of inhibiting PRL function on milk production in dairy cows is less obvious (Karg et al., 1972; Smith et al., 1974). Moreover, Plaut et al. (1987) observed that injecting dairy cows in early lactation with recombinant PRL at 120 mg/d for 14 d did not significantly affect milk yield. Although the involvement of PRL in the control of ruminant lactation has been controversial, more recent studies have provided good evidence that PRL is also galactopoietic in dairy cows (Lacasse et al., 2016). Experiments in vivo showed that inhibition of PRL with quinagolide decreased milk production in dairy cows (Lacasse et al., 2011), whereas increasing the PRL concentration by administering a dopamine antagonist increased milk production (Lacasse and Ollier, 2015). Experiments in vitro have also demonstrated that PRL is galactopoietic in dairy cows. In mammary explants of dairy cows, PRL induces the synthesis and release of α-lactalbumin (Goodman et al., 1983). In cultured bovine mammary epithelial cells, Choi et al. (1988) reported that PRL stimulation, even in the absence of insulin and hydrocortisone, is capable of increasing milk protein mRNA abundance. We also observed upregulated β-casein expression in PRL-treated mammary epithelial cells of dairy cows compared with control cells. Milk protein synthesis requires a steady and sufficient supply of AA (Bequette et al., 1998). Because LAT1 is highly expressed in lactating mammary tissues of dairy cows and is capable of transporting large neutral AA, including the branched-chain AA, the expression and activity of LAT1 likely influence PRL signaling and function. As anticipated, LAT1 inhibitor and gene knockdown in the current study both suppressed PRL-induced β-casein expression, demonstrating that PRL signaling could directly regulate LAT1 expression in mammary epithelial cells of dairy cows.

**Figure 4.** Effect of prolactin (PRL) on plasma membrane expression of LAT1 (l-type amino acid transporter 1) in mammary epithelial cells of dairy cows. (A) Western blot showing the expression of LAT1 in isolated plasma membranes of mammary epithelial cells. Cells were cultured in medium with 5 μg/mL PRL for 24 h. The plasma membrane fraction was then isolated for Western blot analysis; Na+-K-ATPase served as a loading control. (B) Quantification of LAT1 expression from the Western blots in panel A. (C) Western blot showing the expression of LAT1 in the cytosol of mammary epithelial cells. Cells were cultured in medium with 5 μg/mL PRL for 24 h. The cytosol was isolated for Western blot analysis; β-Actin served as a loading control. (D) Quantification of LAT1 expression from the Western blots in panel C. Data are presented as the mean ± SEM of 3 independent experiments. **P < 0.01.
expression in cultured mammary epithelial cells of dairy cows, indicating that PRL signaling-induced milk protein synthesis is dependent on LAT1.

Signal transducer and activator of transcription 5 was originally identified in the lactating mammary gland and is the primary transcription factor responsible for PRL signaling (Kabotyanski et al., 2006). Prolactin signaling induces PRLR dimerization and activation of JAK2 kinase, which in turn activates STAT5 by phosphorylating this molecule (Bole-Feyos et al., 1998). Once activated, STAT5 translocates to the nucleus and binds to specific regulatory sequences in the promoters of target genes such as milk protein genes (Groner and Gouilleux, 1995). In mouse HC11 cells, PRL stimulation of target genes such as milk protein genes (Groner and Gouilleux, 1995). In mouse HC11 cells, PRL stimulation results in a rapid recruitment of p-STAT5 to the CSN2 promoter and enhancer, which in turn regulates CSN2 gene expression (Kabotyanski et al., 2006). However, it had been unclear whether PRL regulates LAT1 expression in bovine mammary epithelial cells via STAT5 activation. In the current study, we showed that the addition of PRL led to a robust increase in STAT5 activity, as shown by the phosphorylation of STAT5 on Tyr694, whereas knockdown of PRLR or STAT5 significantly inhibited PRL-stimulated STAT5 phosphorylation and further decreased LAT1 expression. These results provide good evidence that PRL signaling regulates LAT1 expression by activating PRLR–STAT5 signaling. We also found a putative STAT5 response element in the bovine LAT1 gene promoter (data not shown), which further supports our current hypothesis that PRL regulates LAT1 expression by activating STAT5 signaling.

Here, we showed that PRL upregulates LAT1 expression and activity by facilitating the translocation of LAT1 from the cytosol to the plasma membrane in mammary epithelial cells of dairy cows. The regulation of trafficking of nutrient transporters between intracellular pools and the plasma membrane is a well-established mechanism by which cellular nutrient uptake is modified. With regard to AA transporters, the trafficking of the AA transport system A isoform SNAT2 in insulin-stimulated skeletal muscle cells and 3T3-L1 adipocytes has been studied (Hyde et al., 2002; Hatanaka et al., 2006). In primary human trophoblast cells, mTOR (mammalian target of rapamycin) signaling regulates LAT1 activity by influencing the trafficking of this transporter isoform to the plasma membrane (Rosario et al., 2013). In the present study, we observed that PRL induced an increase in the plasma membrane abundance of LAT1 in mammary epithelial cells of dairy cows. These findings indicate that the expression of LAT1 in cultured bovine mammary epithelial cells may be regulated not just at the transcriptional level; our results provide a possible mechanism for PRL regulation of LAT1 activity at the posttranslational level.

CONCLUSIONS

Prolactin signaling may increase milk protein synthesis by regulating LAT1 expression in mammary epithelial cells of dairy cows. Treatment of bovine mammary epithelial cells with PRL increases movement of LAT1 from an intracellular pool to the membrane, indicating an increase in LAT1 transport activity in lactating mammary tissues of dairy cows.

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