

Effects of Lysine on Milk Protein Synthesis-Related Gene Expression and Protein Phosphorylation in Bovine Mammary Epithelial Cells

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Abstract

This study aimed to investigate the effects of lysine (Lys) on the expression of genes related to milk protein synthesis and protein phosphorylation in bovine mammary epithelial cells (BMECs), and to explore the underlying mechanism of Lys's influence on milk protein synthesis. Third-passage BMECs were randomly divided into 6 groups with Lys concentrations of 0.5 (control), 1.0, 2.0, 4.0, 8.0, and 16.0 mmol/L, with 6 replicates per group. Cells were cultured at 37 °C with 5% CO₂ for 48 h, after which the ATP content in BMECs was measured using a chemiluminescence assay, the expression levels of genes related to milk protein synthesis were determined by real-time quantitative PCR, and the phosphorylation levels of proteins involved in milk protein synthesis were assessed by Western blotting. The results showed that with increasing Lys concentration, ATP content exhibited a marginally significant quadratic increase ($P=0.050$); the expression levels of κ -casein (CSN3) ($P=0.093$), mammalian target of rapamycin (mTOR) ($P=0.005$), eukaryotic initiation factor 4E (eIF4E) ($P=0.076$), and AMP-activated protein kinase α 1 (AMPK α 1) ($P=0.045$) showed significant or marginally significant quadratic changes, all increasing initially and then decreasing; the expression level of α -casein (CSN1S1) ($P=0.081$) and the phosphorylation levels of mTOR ($P=0.038$) and p70 ribosomal protein S6 kinase 1 (S6K1) ($P=0.022$) showed significant or marginally significant linear decreases; the phosphorylation level of AMP-activated protein kinase (AMPK) exhibited a significant linear increase ($P=0.014$). Analysis of variance results showed that Lys supplementation significantly affected ATP content, the expression levels of genes related to milk protein synthesis, and the phosphorylation level of eIF4E ($P<0.05$). Specifically, the following showed better promotional effects: ATP content in the 2.0-16.0 mmol/L groups; CSN1S1, β -casein (CSN2), and signal transducer and activator of transcription 5 (STAT5) in the 1.0-2.0 mmol/L

groups; mTOR gene expression in the 1.0–8.0 mmol/L groups; CSN3 gene expression in the 1.0–4.0 mmol/L groups; Janus kinase 2 (JAK2) gene expression in the 1.0–16.0 mmol/L groups; S6K1 gene expression in the 2.0 mmol/L group; eIF4E gene expression in the 2.0–8.0 mmol/L groups; and eIF4E phosphorylation level in the 2.0–4.0 mmol/L groups. However, the expression levels of CSN1S1, CSN3, STAT5, and mTOR were inhibited in the 16.0 mmol/L group, and the expression level of eukaryotic initiation factor 4E-binding protein 1 (4EBP1) was inhibited in the 1.0–16.0 mmol/L groups. In conclusion, a Lys concentration of 1.0–2.0 mmol/L demonstrated optimal promotion of the expression of genes related to milk protein synthesis in BMECs.

Full Text

Effects of Lysine on Gene Expression and Protein Phosphorylation Related to Milk Protein Synthesis in Bovine Mammary Epithelial Cells

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Abstract

This study investigated the effects of lysine (Lys) on gene expression and protein phosphorylation related to milk protein synthesis in bovine mammary epithelial cells (BMECs) to elucidate the underlying mechanisms. Third-passage BMECs were randomly divided into six groups with six replicates each and cultured at 37 °C with 5% CO₂ for 48 hours in medium containing 0.5 (control), 1.0, 2.0, 4.0, 8.0, or 16.0 mmol/L Lys. ATP content was measured by chemiluminescence, gene expression was determined by real-time quantitative PCR, and protein phosphorylation levels were assessed by Western blotting. The results showed that ATP content exhibited a quadratic increase with rising Lys concentrations ($P=0.050$). Expression levels of α -casein (CSN3) ($P=0.093$), mammalian target of rapamycin (mTOR) ($P=0.005$), eukaryotic initiation factor 4E (eIF4E) ($P=0.076$), and AMP-activated protein kinase α 1 ($AMPK \alpha 1$) ($P=0.045$) showed significant or marginally significant quadratic responses, initially increasing then decreasing. α s1-casein (CSN1S1) gene expression ($P=0.081$) and phosphorylation levels of mTOR ($P=0.038$) and ribosomal protein S6 kinase 1 (S6K1) ($P=0.022$) decreased linearly, while AMPK phosphorylation increased linearly ($P=0.014$) with increasing Lys concentration. ANOVA revealed that Lys supplementation significantly affected ATP content, gene expression levels, and eIF4E phosphorylation ($P<0.05$). Optimal effects were observed at 2.0–16.0 mmol/L for ATP content, 1.0–2.0 mmol/L for CSN1S1, β -casein (CSN2), and signal transducer and activator of transcription 5 (STAT5), 1.0–8.0 mmol/L for mTOR, 1.0–4.0 mmol/L for CSN3, 1.0–16.0 mmol/L for Janus kinase 2 (JAK2), 2.0 mmol/L for S6K1, 2.0–8.0 mmol/L for

eIF4E expression, and 2.0–4.0 mmol/L for eIF4E phosphorylation. However, 16.0 mmol/L Lys inhibited expression of CSN1S1, CSN3, STAT5, and mTOR, while 1.0–16.0 mmol/L Lys suppressed eukaryotic initiation factor 4E binding protein 1 (4EBP1) expression. In conclusion, Lys at 1.0–2.0 mmol/L optimally promotes expression of genes related to milk protein synthesis in BMECs.

Keywords: dairy cow; bovine mammary epithelial cell; lysine; milk protein

Milk protein is a primary component of milk and serves as a crucial indicator of milk quality. Amino acids (AAs) are the main precursors for milk protein synthesis and can influence this process [1]. Lysine (Lys) is an essential amino acid (EAA) that is both critical for milk protein synthesis and limiting in dairy cow nutrition. Therefore, investigating the effects of Lys on milk protein synthesis and its underlying mechanisms is important for regulating milk composition and improving milk quality.

Previous studies have demonstrated that Lys influences milk protein synthesis. Li et al. [2] found that intra-arterial infusion of amino acids promoted milk protein synthesis in dairy cows fed corn straw-based diets. Wang [3] reported that adding EAAs to culture medium increased milk protein synthesis in BMECs. Giallongo et al. [4] observed that rumen-protected Lys supplementation enhanced both milk protein synthesis and milk quality. While these findings indicate that Lys affects milk protein synthesis, most research has focused on in vivo infusion, with limited exploration of in vitro effects and mechanisms. Therefore, this study used BMECs as a model to investigate the effects of varying Lys concentrations on gene expression and protein phosphorylation related to milk protein synthesis, providing a theoretical foundation for further mechanistic studies.

1.1 Reagents and Instruments

Type II collagenase, DMEM/F12 medium, insulin-transferrin-selenium, fetal bovine serum, and 0.25% trypsin/EDTA were purchased from Gibco. Lys (catalog no. L8662), hydrocortisone, epidermal growth factor, prolactin, and agarose were from Sigma. RNAiso PLUS, PrimeScript RT Master Mix, and SYBR Premix Ex Taq™ II were from TaKaRa. Primary antibodies against mammalian target of rapamycin (mTOR) (catalog no. ab2732), phospho-mTOR (catalog no. ab84400), eukaryotic initiation factor 4E (eIF4E) (catalog no. ab72116), phospho-eIF4E (catalog no. ab4774), ribosomal protein S6 kinase 1 (S6K1) (catalog no. ab64804), phospho-S6K1 (catalog no. ab126818), eukaryotic initiation factor 4E binding protein 1 (4EBP1) (catalog no. ab2606), and phospho-4EBP1 (catalog no. ab75767) were from Abcam. Antibodies against AMP-activated protein kinase α 1 (*AMPK* 1) (catalog no. YP0216) and phospho-AMPK α 1 (catalog no. YP0575) were from Immunoway. Primary antibody diluent, secondary antibody diluent, SDS-PAGE electrophoresis buffer, transfer buffer, and ECL chemiluminescent substrate were from Beyotime Biotechnology. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (catalog no.

04-15-06) was from KPL. Major instruments included a Synergy H4 microplate reader (BioTek, USA), ABI 7500 real-time PCR system (ABI, USA), electrophoresis and transfer apparatus, and a protein imaging system (Bio-Rad, USA).

1.2 Primary BMEC Culture and Experimental Design

Mammary tissue was collected from three healthy mid-lactation Holstein cows aged 3–5 years at a slaughterhouse in Hohhot, Inner Mongolia. BMECs were isolated and cultured using the collagenase digestion method described by Sheng et al. [5]. When primary cells reached 80–90% confluence, they were purified and passaged with 0.25% trypsin/EDTA. Third-passage BMECs were seeded onto culture plates at the required density and cultured for 24 hours at 37 °C with 5% CO₂. Upon reaching 80–90% confluence, cells were transferred to starvation medium for 12 hours, then randomly divided into six groups following a single-factor completely randomized design. Lys working solution was added to achieve final concentrations of 0.5 (control), 1.0, 2.0, 4.0, 8.0, and 16.0 mmol/L, with six replicates per group. Cells were cultured for 48 hours. The DMEM/F12 medium contained 0.5 mmol/L Lys, and the concentration range was determined based on previous studies [6, 7] and cell relative growth rate [relative growth rate (%) = (experimental group OD_{490 nm}/control group OD_{490 nm}) × 100].

1.3 Test Indicators and Methods

ATP Content Measurement

BMECs were seeded in 6-well plates at 5×10^5 cells/well. After treatment, supernatant was removed and 200 μ L lysis buffer was added per well. Following complete cell lysis, suspensions were collected and centrifuged at 4 °C, 15,455 \times g for 5 minutes. Supernatants were used immediately for ATP assay according to the kit instructions. ATP standard solutions were prepared at concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μ mol/L. ATP detection reagent was diluted 1:9 to make working solution. In each assay well, 100 μ L working solution was added and incubated at room temperature for 3–5 minutes, followed by 20 μ L sample. After mixing, luminescence was measured using a microplate reader. ATP concentration was calculated from the standard curve and normalized to protein concentration measured by BCA assay, expressed as nmol/mg protein.

Gene Expression Analysis

Total RNA was extracted from BMECs seeded at 5×10^5 cells/well in 6-well plates using the Trizol method. RNA purity and concentration were assessed spectrophotometrically (OD₂₆₀/OD₂₈₀ ratio of 1.8–2.2 indicated good quality). RNA integrity was verified by 2% agarose gel electrophoresis. Total RNA was reverse-transcribed to cDNA using PrimeScript RT Master Mix in a 10 μ L reaction. Gene expression was quantified by real-time PCR using SYBR Premix Ex Taq™ II in a 20 μ L reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the housekeeping gene. Primers for milk protein synthesis-related genes [α s1-casein (CSN1S1), β -casein (CSN2), κ -casein

(CSN3), Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), mTOR, S6K1, 4EBP1, eIF4E, and AMPK [1] are listed in . The PCR program consisted of initial denaturation at 95.0 °C for 30 s, followed by 40 cycles of 95.0 °C for 5 s, 60 °C for 34 s, and 72 °C for 20 s, then a melting curve analysis (95 °C for 5 s, 60 °C for 30 s, 95 °C for 15 s, 51 cycles). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Protein Phosphorylation Analysis

BMECs were seeded in 25 cm² flasks at 5×10^6 cells/flask. After treatment, supernatant was removed and cells were washed twice with phosphate-buffered saline (PBS). Each flask received 250 μ L RIPA lysis buffer containing 0.1% phenylmethylsulfonyl fluoride (PMSF). After 5 minutes of lysis at 4 °C, cells were scraped, collected, and centrifuged at 4 °C, 15,455 \times g for 10 minutes. Supernatants were used for protein phosphorylation analysis. Total protein concentration was determined by BCA assay. For each sample, 60 μ g protein was mixed with 5 \times loading buffer at a 4:1 mass/volume ratio, heated at 100 °C for 5 minutes for denaturation, then subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were incubated overnight at 4 °C with primary antibodies diluted 1:500, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibody diluted 1:1000 for 1 hour at room temperature. After ECL development, images were captured using a protein imaging system and analyzed with Quantity One software. Phosphorylation levels were expressed relative to the control group.

1.4 Statistical Analysis

Data were analyzed using SAS 9.0 software. ANOVA was performed for significance testing, while linear and quadratic regression analyses were conducted using regression procedures. $P < 0.05$ indicated significant differences or regression relationships, $0.05 \leq P < 0.10$ indicated marginally significant trends, and $P \geq 0.10$ indicated non-significant effects.

2.1 Effects of Lys on ATP Content and Milk Protein Synthesis-Related Gene Expression in BMECs

As shown in , relative growth rate (RGR) decreased linearly with increasing Lys concentration ($P < 0.001$), with 4.0-16.0 mmol/L groups significantly lower than control and 1.0-2.0 mmol/L groups ($P < 0.05$). ATP content was significantly higher in 2.0-16.0 mmol/L groups compared to control ($P < 0.05$), peaking at 2.0 mmol/L. Regression analysis revealed a quadratic increase in ATP content with Lys concentration ($P = 0.050$). CSN1S1 expression showed a marginally significant linear decrease ($P = 0.081$), with 1.0-2.0 mmol/L groups significantly higher than other groups ($P < 0.05$), particularly at 2.0 mmol/L, while 4.0-16.0 mmol/L groups were significantly lower than control and 1.0-2.0 mmol/L groups ($P < 0.05$). CSN2 and STAT5 expression were significantly higher in 1.0-2.0 mmol/L groups compared to others ($P < 0.05$), peaking at 2.0 mmol/L. CSN2 expression was lowest at 8.0 mmol/L, while STAT5 expression in 4.0-16.0

mmol/L groups was significantly lower than control ($P < 0.05$). JAK2 expression was significantly higher in 1.0-16.0 mmol/L groups than control ($P < 0.05$). CSN3 ($P = 0.093$), mTOR ($P = 0.005$), eIF4E ($P = 0.076$), and AMPK $\beta 1$ ($P = 0.045$) expression showed significant or marginally significant quadratic responses, initially increasing then decreasing. mTOR expression was significantly higher in control and 1.0-8.0 mmol/L groups than in the 16.0 mmol/L group ($P < 0.05$). eIF4E expression was significantly higher in 2.0-8.0 mmol/L groups ($P < 0.05$). AMPK $\beta 1$ expression was significantly higher in 1.0-8.0 mmol/L groups ($P < 0.05$). S6K1 expression peaked at 2.0 mmol/L, significantly higher than control ($P < 0.05$), and was lowest at 16.0 mmol/L. 4EBP1 expression was significantly reduced in 1.0-16.0 mmol/L groups compared to control ($P < 0.05$).

2.2 Effects of Lys on Phosphorylation of Milk Protein Synthesis-Related Proteins

As shown in and [Figure 1: see original paper], mTOR ($P = 0.038$) and S6K1 ($P = 0.022$) phosphorylation levels decreased linearly with increasing Lys concentration. eIF4E phosphorylation was significantly higher in 2.0-4.0 mmol/L groups than other groups ($P < 0.05$), though the 16.0 mmol/L group did not differ significantly from control ($P > 0.05$). AMPK phosphorylation increased linearly with Lys concentration ($P = 0.014$).

3 Discussion

Casein constitutes approximately 80% of bovine milk protein, comprising primarily CSN1S1, $\alpha 2$ -casein (CSN1S2), CSN2, and CSN3, with CSN1S1 and CSN2 being the most abundant at about 40% and 25%, respectively [9]. CSN1S1, CSN2, and CSN3 are key markers of milk protein synthesis, and their expression directly influences protein production in BMECs. Nan et al. [10] reported that 1.2 mmol/L Lys significantly upregulated CSN1S1, CSN2, and CSN3 expression compared to 0 mmol/L. Our findings confirm that Lys significantly promotes expression of these casein genes, with optimal effects at 1.0-2.0 mmol/L. The dose-dependent response was evident, as high-dose 16.0 mmol/L Lys significantly inhibited CSN1S1 and CSN3 expression.

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and mTOR signaling pathways are two major regulators of protein synthesis [11-12]. In the JAK/STAT pathway, JAK2/STAT5 signaling is particularly important for milk protein synthesis. Liu et al. [13] demonstrated that Lys supplementation significantly increased STAT5 expression in BMECs, and that STAT5 silencing decreased CSN2 expression while overexpression upregulated it, suggesting Lys may influence milk protein synthesis via the JAK2/STAT5 pathway. Our results show that Lys significantly promoted STAT5 and JAK2 expression, with optimal STAT5 activation at 2.0 mmol/L, while high doses (4.0-16.0 mmol/L) significantly inhibited expression. This pattern mirrors the effects on casein gene expression, further supporting the involvement of JAK2/STAT5 signaling in Lys-mediated milk protein synthesis.

The mTOR signaling pathway regulates milk protein synthesis at the translational level [12]. 4EBP1 and S6K1 are two key downstream effectors of mTOR. Upon activation by upstream signals, mTOR modulates protein translation through these pathways. The binding of 4EBP1 to eIF4E inhibits translation, but mTOR-mediated phosphorylation of 4EBP1 releases eIF4E, thereby promoting protein synthesis. Our study found that optimal Lys concentrations enhanced mTOR, S6K1, and eIF4E expression and eIF4E phosphorylation, though this stimulatory effect diminished or became inhibitory at 8.0–16.0 mmol/L. Lys supplementation inhibited 4EBP1 gene expression without significantly affecting its phosphorylation. Bi et al. [14] similarly reported that Lys upregulated mTOR and S6K1 expression while downregulating 4EBP1 in BMECs. These findings suggest Lys may dose-dependently influence milk protein synthesis gene expression through the mTOR signaling pathway.

Amino acids and ATP are crucial factors in protein synthesis, and their availability directly affects milk quality. Mammary protein synthesis and secretion consume approximately 50% of cellular ATP [15]. AMPK is a primary cellular energy sensor that participates in multiple metabolic pathways, regulating metabolic and energy homeostasis [16]. AMPK also functions as an upstream regulator of mTOR, negatively modulating downstream mTOR signaling [17]. Decreased ATP/AMP ratio or nutrient deficiency activates AMPK, promoting ATP catabolism and inhibiting ATP synthesis [18–19]. Thus, AMPK can regulate milk protein synthesis at the translational level through the mTOR pathway. Wang [20] reported that reduced amino acid concentrations decreased cellular nutrient and energy levels, activating AMPK and inhibiting mTOR, S6K1, and 4EBP1 phosphorylation, thereby reducing milk protein synthesis. Our study found that moderate Lys concentrations increased both mTOR gene expression and ATP content while promoting AMPK expression, but high-dose 16.0 mmol/L significantly reduced mTOR and AMPK expression. These discrepancies with previous studies require further investigation.

Our results also demonstrate significant or marginally significant dose-dependent relationships between Lys concentration and ATP content, CSN1S1, CSN3, mTOR, eIF4E, and AMPK expression, and phosphorylation of mTOR, S6K1, and AMPK. Optimal effects were observed at 2.0–16.0 mmol/L for ATP content, 1.0–2.0 mmol/L for CSN1S1, CSN2, and STAT5, 1.0–8.0 mmol/L for mTOR, 1.0–4.0 mmol/L for CSN3, 1.0–16.0 mmol/L for JAK2, 2.0 mmol/L for S6K1, 2.0–8.0 mmol/L for eIF4E expression, and 2.0–4.0 mmol/L for eIF4E phosphorylation. However, 16.0 mmol/L Lys showed diminished or opposite effects. Li [7] found that increasing individual amino acids in BMEC culture medium caused amino acid imbalance that severely affected milk protein synthesis, suggesting that high-dose Lys inhibition may relate to disrupted amino acid ratios. Additionally, Mercier et al. [21] noted that BMEC number may determine milk protein synthesis capacity, and Gao et al. [22] reported that 0.5–2.0 mmol/L Lys promoted BMEC proliferation while higher doses were inhibitory, consistent with our findings. This explains why high-dose Lys suppresses milk protein synthesis-related gene expression. Therefore, Lys

at 1.0-2.0 mmol/L optimally promotes expression of genes involved in milk protein synthesis in BMECs.

Brazil et al. [23] identified the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/mTOR pathway as an important upstream regulator of mTOR signaling, exerting positive control. Akt may also indirectly regulate mTOR through the tuberous sclerosis complex 1 (TSC1)/tuberous sclerosis complex 2 (TSC2) [24]. These findings suggest Lys may regulate mTOR signaling and promote milk protein synthesis gene expression via the PI3K/Akt/mTOR pathway, though this requires further investigation as our study did not examine this pathway.

4 Conclusion

Lysine dose-dependently promotes expression of genes related to milk protein synthesis in BMECs, with optimal effects at 1.0-2.0 mmol/L. High-dose Lys (16.0 mmol/L) inhibits expression of these genes. Lys likely promotes milk protein synthesis through the JAK2/STAT5 and mTOR signaling pathways.

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