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Postprint: Effect of Lysine on Expression of Lactose Synthesis-Related Genes in Dairy Cow Mammary Epithelial Cells

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Abstract

This study aimed to investigate the effects of lysine (Lys) on the expression of lactose synthesis-related genes in bovine mammary epithelial cells (BMECs) and to explore the underlying mechanism of Lys on lactose synthesis. Passage 3 BMECs were randomly divided into 6 groups with 6 replicates each, and the culture medium was supplemented with Lys at concentrations of 0.5 (control), 1.0, 2.0, 4.0, 8.0, and 16.0 mmol/L. Cells were cultured at 37 °C with 5% CO for 48 h. The results showed that appropriate concentrations of Lys exhibited a significant dose-dependent effect on lactose content and the expression of glucose transporter 1 (GLUT1), hexokinase I (HKI), and hexokinase II (HKII) genes (P<0.05). Variance analysis revealed that Lys supplementation significantly or tended to significantly affect lactose content and the expression of lactose synthesis-related genes. Compared with the control group, lactose content was higher in the 2.0-16.0 mmol/L groups (P=0.055), GLUT1 gene expression was significantly increased in the 4.0-16.0 mmol/L groups (P<0.001), -lactalbumin (LALBA) and -1,4-galactosyltransferase-1 (-4GALT1) gene expression were significantly increased in the 2.0-8.0 mmol/L groups (P=0.006 and P<0.001, respectively), and HKII gene expression was significantly increased in the 8.0-16.0 mmol/L groups (P<0.001). However, HKII gene expression was significantly decreased in the 1.0-2.0 mmol/L groups (P<0.001), and HKI gene expression was decreased in the 4.0-16.0 mmol/L groups, especially in the 16.0 mmol/L Lys group, which was significantly lower than the control group (P=0.002). In conclusion, Lys exhibited a significant dose-dependent effect on lactose content and the expression of GLUT1, HKI, and HKII genes, and a Lys concentration of 2.0-8.0 mmol/L demonstrated a better promoting effect on lactose synthesis in BMECs.

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Full Text

Effects of Lysine on Expression of Genes Related to Lactose Synthesis 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 lactose synthesis in bovine mammary epithelial cells (BMECs) and to elucidate the underlying mechanisms. Third-generation BMECs were randomly divided into six groups with six replicates each. The culture medium was supplemented with Lys at concentrations of 0.5 (control), 1.0, 2.0, 4.0, 8.0, and 16.0 mmol/L, and cells were cultured at 37°C with 5% CO for 48 hours. The results demonstrated that appropriate concentrations of Lys exerted a significant dose-dependent effect on lactose content and the expression of glucose transporter 1 (GLUT1), hexokinase I (HKI), and hexokinase II (HKII) genes (P<0.05). Variance analysis revealed that Lys supplementation significantly or tended to significantly affect lactose content and the expression of genes related to lactose synthesis. Compared with the control group, lactose content was higher in the 2.0–16.0 mmol/L groups (P=0.055). GLUT1 gene expression was significantly upregulated in the 4.0–16.0 mmol/L groups (P<0.001), while -lactalbumin (LALBA) and -1,4-galactosyltransferase 1 (-4GALT1) gene expression were significantly increased in the 2.0–8.0 mmol/L groups (P=0.006 and P<0.001, respectively). HKII gene expression was significantly elevated in the 8.0–16.0 mmol/L groups (P<0.001) but was significantly suppressed in the 1.0–2.0 mmol/L groups (P<0.001). HKI gene expression decreased in the 4.0–16.0 mmol/L groups, with the 16.0 mmol/L group being significantly lower than the control (P=0.002). In conclusion, Lys exhibited a significant dose-dependent effect on lactose content and the expression of GLUT1, HKI, and HKII genes. Lys concentrations of 2.0–8.0 mmol/L demonstrated the most favorable promotional effects on lactose synthesis in BMECs.

Keywords: dairy cow; bovine mammary epithelial cells; lysine; lactose; gene expression

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Lactose is the primary component of milk and a key factor limiting milk yield, as production increases with lactose synthesis within a certain range [1-3]. Amino acids (AA) serve as the main precursors for milk protein synthesis and also influence lactose synthesis [4]. Lysine (Lys) is an essential amino acid (EAA) crucial for milk protein synthesis and represents a limiting amino acid in dairy cows [5]. Therefore, investigating the effects and mechanisms of Lys on lactose synthesis is important for regulating milk component synthesis and increasing

milk production. Wang Lina [6] found that supplementing the culture medium with EAAs simultaneously promoted both milk protein and lactose synthesis in a bovine mammary epithelial cell (BMEC) model. Yun Fuyu [7] reported that dietary supplementation with appropriate levels of Lys improved milk yield, milk protein percentage, and lactose percentage in dairy cows. These findings indicate that Lys influences lactose synthesis to some extent. However, previous research has primarily focused on Lys infusion in vivo and its effects on milk protein synthesis in vitro, with limited exploration of how in vitro Lys supplementation affects lactose synthesis and its underlying mechanisms. Thus, further investigation is warranted. Therefore, this study employed BMECs as a model to investigate the effects of different Lys concentrations on the expression of genes related to lactose synthesis, providing a theoretical foundation for further elucidating the mechanisms of Lys action on lactose synthesis in BMECs.

1.1 Reagents and Instruments

Main reagents: Type II collagenase, DMEM/F12 culture medium, insulin-transferrin-selenium sodium, fetal bovine serum (FBS), and 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco; lysine (Lys, L8662), hydrocortisone, epidermal growth factor, prolactin, and agarose were from Sigma; RNAiso PLUS, PrimeScript RT Master Mix, and SYBR Premix ExTaq™ II were from TaKaRa; and the bovine D-lactose enzyme-linked immunosorbent assay kit (EHJ-90716h) was from Xiamen Huijia Biotechnology.

Main instruments: CO incubator (Forma-311, Thermo), inverted microscope (Olympus, Japan), automatic microplate reader (Synergy H4 BioTek, USA), and real-time fluorescence quantitative PCR instrument (ABI-7500, USA).

1.2 Primary BMEC Culture and Experimental Design

Mammary tissue was collected from three healthy mid-lactation Holstein dairy cows aged 3–5 years at the Beiya Halal Slaughterhouse in Hohhot, Inner Mongolia. BMECs were obtained and cultured using the collagenase digestion method described by Sheng et al. [8]. When primary cells reached 80–90% confluence, they were purified and passaged with 0.25% trypsin/EDTA. Third-generation BMECs were seeded in 6-well plates at a density of 5×10 cells/well and cultured in DMEM/F12 medium containing 10% FBS at 37°C with 5% CO for 24 hours. Using a single-factor randomized design, when cells reached 80–90% confluence, the medium was replaced with serum-free DMEM/F12 starvation medium. After 12 hours, cells were divided into six groups receiving different concentrations of Lys working solution to achieve final Lys 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 then cultured for 48 hours at 37°C with 5% CO . The DMEM/F12 medium contained leucine, methionine, glycine, arginine, isoleucine, phenylalanine, threonine, tryptophan, and valine at concentrations of 0.45, 0.13, 0.25, 0.70, 0.42, 0.22, 0.45, 0.04, and 0.45 mmol/L, respectively. Lys concentrations

were determined based on previous studies [9,10] and by measuring cell proliferation rate [RGR(%) = (OD of treatment group / OD of control group) × 100].

1.3 Assay Indicators and Methods

Lactose content in BMECs was measured using a double-antibody sandwich method. Cells were seeded in 6-well plates at 5×10 cells/well. After treatment, cell culture medium was collected and lactose content was determined using the bovine D-lactose ELISA kit according to the manufacturer' s instructions. Standard solutions were prepared by diluting the 1.8 mg/L standard to concentrations of 1,200, 800, 400, 200, and 100 g/L using the standard diluent. Each concentration was added in duplicate (50 L/well) to the enzyme-coated plate. For sample wells, 40 L of sample diluent was added followed by 10 L of the test sample. The plate was sealed and incubated at 37°C for 30 minutes. After incubation, the liquid was discarded and the plate was washed five times with wash buffer. Then, 50 L of enzyme-labeled reagent was added to each well, incubated for 30 minutes, and washed again. Next, 50 L of chromogen A was added to each well, followed by 50 L of chromogen B, and the plate was incubated at 37°C in the dark for 15 minutes. Finally, 50 L of stop solution was added to each well, and OD was measured using an automatic microplate reader with blank wells as the zero reference. Lactose concentration in samples was calculated from the standard curve.

Total RNA extraction from BMECs was performed using the Trizol method. Cells were seeded in 6-well plates at 5×10 cells/well. After treatment, RNA purity and concentration were assessed using a microplate reader. An OD /OD ratio of 1.8–2.2 indicated good RNA purity. RNA integrity was verified by 2% agarose gel electrophoresis. RNA was reverse-transcribed to cDNA using the PrimeScript RT Master Mix kit in a 10 L reaction volume. Gene expression was quantified using the SYBR Premix Ex Taq™ II kit in a 20 L reaction volume. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), -actin, and 18S ribosomal RNA (18S rRNA) were used as housekeeping genes to determine the relative expression of lactose synthesis-related genes: -lactalbumin (LALBA), -1,4-galactosyltransferase 1 (-4GALT1), glucose transporter 1 (GLUT1), hexokinase I (HKI), and hexokinase II (HKII). Primer sequences are shown in . The real-time quantitative PCR program consisted of pre-denaturation at 95.0°C for 30 seconds, followed by 40 cycles of denaturation at 95.0°C for 5 seconds, annealing at 60°C for 34 seconds, and extension at 72°C for 20 seconds. A melting curve was generated by 51 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and 95°C for 15 seconds. Results were analyzed using the geometric mean of the three housekeeping genes (GAPDH, -actin, and 18S rRNA), and relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

1.4 Statistical Analysis

All data were processed using Excel 2010 and analyzed for significance using the ANOVA procedure in SAS 9.0 software. Linear and quadratic regression analyses were performed using the regression procedure. $P<0.05$ indicated significant differences or regression relationships, $0.05<P<0.10$ indicated a tendency toward significance, and $P>0.10$ indicated no significant difference or regression relationship.

2 Results

As shown in , lactose content in BMECs increased significantly in a quadratic manner with increasing Lys concentration (P=0.016), following the regression equation $y = 102.48183 + 15.76510x - 0.88587x^2$ ($R^2 = 0.7770$), where x represents Lys concentration and y represents lactose content. Lactose content was higher in the 2.0-16.0 mmol/L groups (P=0.055), peaking at 4.0 mmol/L, after which the promotional effect diminished with further concentration increases. GLUT1 and HKII gene expression increased significantly in a linear manner with increasing Lys concentration (P=0.002 and P=0.018, respectively). The regression equations were $y = 0.90821 + 0.07748x$ ($R^2 = 0.9360$) for GLUT1 and $y = 0.74129 + 0.05245x$ ($R^2 = 0.7879$) for HKII, where x represents Lys concentration and y represents gene expression level. GLUT1 expression was significantly higher in the 4.0-16.0 mmol/L groups compared with the control ($P<0.001$). HKII expression was significantly elevated in the 8.0-16.0 mmol/L groups ($P<0.001$) but was significantly suppressed in the 1.0-2.0 mmol/L groups, showing a pattern of initial decrease followed by increase. HKI gene expression decreased significantly in a linear manner with increasing Lys concentration (P=0.031), following the regression equation $y = 1.08687 - 0.04321x$ ($R^2 = 0.7272$). HKI expression peaked at 2.0 mmol/L (higher than control) but was lower than control in the 4.0-16.0 mmol/L groups, with 16.0 mmol/L Lys significantly suppressing its expression (P=0.002). LALBA and -4GALT1 gene expression were highest in the 2.0-8.0 mmol/L groups (P=0.006 and $P<0.001$, respectively). Although the 16.0 mmol/L group showed higher expression than the control, the difference was not significant ($P>0.05$).

3 Discussion

In vitro studies have shown that mammary tissue cannot synthesize glucose through gluconeogenesis [15] and must obtain it from the blood. Therefore, the effect of Lys on lactose synthesis in BMECs can only be achieved through regulatory mechanisms. This study found that Lys supplementation promoted lactose content in a significant quadratic manner ($R^2 = 0.7770$ in the regression equation). Variance analysis showed that different Lys concentrations tended to significantly increase lactose content, demonstrating that within an appropriate range, lactose content increased with Lys concentration, with optimal promotion at 4.0 mmol/L. Beyond this concentration, the promotional effect weakened,

indicating a significant dose-dependent relationship between Lys and lactose synthesis.

Glucose uptake by the mammary gland is the rate-limiting step for regulating milk production. GLUT1 is a glucose transporter protein, and hexokinases (HKs) are rate-limiting enzymes for glucose utilization that catalyze the phosphorylation of glucose to glucose-6-phosphate. HKI and HKII are the two HK isoforms expressed in mammary tissue [16], and HKII expression is closely associated with glucose uptake [17]. Fueger et al. [18] found that HKII overexpression promoted glucose absorption. Our results showed that Lys concentration promoted GLUT1 and HKII expression in a significant linear manner, with 8.0-16.0 mmol/L Lys significantly enhancing their expression, while 1.0–2.0 mmol/L Lys suppressed HKII expression. Lys affected HKI expression in a significant linear decreasing manner, with 2.0 mmol/L promoting expression but higher doses showing inhibitory effects, similar to findings by Zhao Yanli [19] that leucine promoted HKI expression but high doses suppressed it. These results suggest that Lys may promote glucose uptake and phosphorylation. The finding that Lys supplementation tended to increase lactose content, albeit with diminished effects at high doses, may be explained by the promotion of glucose uptake and phosphorylation at appropriate Lys concentrations coupled with the inhibition of HKI expression at high doses, further explaining the dose-dependent relationship of Lys on lactose synthesis.

Lactose synthase consists of -4GALT1 and LALBA. LALBA is the regulatory subunit that controls lactose synthesis and secretion, while -4GALT1 is the catalytic subunit that functions only in the presence of LALBA to catalyze the formation of lactose from UDP-galactose and glucose via -1,4-glycosidic bonds, thereby controlling lactose synthesis efficiency and regulating milk yield [20]. This study found that 2.0–8.0 mmol/L Lys significantly promoted LALBA and -4GALT1 expression, while the promotional effect diminished at 16.0 mmol/L Lys. This pattern aligns with the observed effects on lactose content, further suggesting that increased lactose content in BMECs may be associated with Lys-induced promotion of LALBA and -4GALT1 expression.

In summary, Lys exhibits a dose-dependent promotional effect on lactose content and the expression of GLUT1, HKI, and HKII genes. The optimal promotional ranges were 2.0–16.0 mmol/L for lactose content, 4.0–16.0 mmol/L for GLUT1 expression, 8.0–16.0 mmol/L for HKII expression, and 2.0–8.0 mmol/L for LALBA and -4GALT1 expression. However, the promotional effect of 16.0 mmol/L Lys on LALBA and -4GALT1 expression was diminished, 1.0–2.0 mmol/L Lys significantly suppressed HKII expression, and 4.0–16.0 mmol/L Lys inhibited HKI expression. Therefore, Lys concentrations of 2.0–8.0 mmol/L demonstrated the most effective promotion of lactose synthesis in BMECs.

4 Conclusion

Lys exhibited a dose-dependent effect on lactose content and the expression of GLUT1, HKI, and HKII genes, with optimal promotional effects observed at concentrations of 2.0–8.0 mmol/L. At a concentration of 16.0 mmol/L, Lys inhibited HKI gene expression and diminished its promotional effect on lactose synthesis.

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