Effects of Methionine on the Expression of Genes and Proteins Related to Milk Fat Synthesis in Dairy Cow Mammary Epithelial Cells (Postprint)

Authors: Zhao Yanli, Chen Lu, Shi Binlin, Guo Xiaoyu, Yan Sumei

Date: 2017-10-11T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of methionine (Met) on the expression of genes and proteins related to milk fat synthesis in bovine mammary epithelial cells (BMECs), in order to explore the mechanism by which Met influences milk fat synthesis. Third-passage BMECs were randomly divided into 6 treatments (6 replicates per treatment), with Met concentrations in the culture medium of 0.13, 0.26, 0.39, 0.52, 0.65, and 0.78 mmol/L. After culturing for 48 h at 37 °C and 5% CO_2, the content of triglycerides (TG) and the relative expression levels of milk fat synthesis-related genes and proteins, including peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding protein 1 (SREBP1), were determined in BMECs. The results showed that Met concentration had no significant effect on TG content in BMECs ($P>0.05$). The relative expression levels of fatty acid-binding protein 3 (FABP3), acetyl-CoA carboxylase A (ACACA), and PPARγ genes in BMECs treated with 0.52–0.78 mmol/L Met were significantly higher than those in other treatments ($P<0.05$). The relative expression level of lipoprotein lipase (LPL) gene in BMECs was higher with 0.26–0.39 mmol/L Met treatment, significantly higher than that with 0.65–0.78 mmol/L Met treatment ($P<0.05$). The relative expression level of fatty acid synthase (FASN) gene was numerically higher with 0.39–0.52 mmol/L Met treatment, and 0.52 mmol/L Met treatment was significantly higher than 0.13–0.26 mmol/L and 0.65–0.78 mmol/L Met treatments ($P<0.05$). Met concentration significantly affected the expression of SREBP1 and acylglycerol-3-phosphate acyltransferase 6 (AGPAT6) genes and SREBP1 and PPARγ proteins ($P<0.05$), with the highest relative expression observed with 0.26 mmol/L Met treatment. The results indicate that Met concentration influences the expression of genes involved in fatty acid uptake and de novo synthesis, as well as the expression of milk fat synthesis regulatory factors PPARγ and SREBP1 at both gene and protein levels, and that a Met concentration of

0.26–0.52 mmol/L demonstrates better promoting effects on fatty acid de novo synthesis and long-chain fatty acid uptake in BMECs.

Full Text

Effects of Methionine on Expression of Genes and Proteins Related to Milk Fat Synthesis in Bovine Mammary Epithelial Cells

ZHAO Yanli, CHEN Lu, SHI Binlin, GUO Xiaoyu, YAN Sumei*

College of Animal Science, Inner Mongolia Agricultural University, Hohhot 010018, China

Abstract

This study investigated the effects of methionine (Met) on the expression of genes and proteins related to milk fat synthesis in bovine mammary epithelial cells (BMECs) to explore the underlying mechanisms. Third-passage BMECs were randomly divided into six treatments (six replicates each) and cultured in media containing 0.13, 0.26, 0.39, 0.52, 0.65, or 0.78 mmol/L Met. After 48 h of incubation at 37°C and 5% CO_2, intracellular triglyceride (TG) content and the relative expression levels of milk fat synthesis-related genes and proteins (PPARγ and SREBP1) were measured. The results showed that Met concentration had no significant effect on TG content in BMECs ($P>0.05$). The relative expression levels of fatty acid-binding protein 3 (FABP3), acetyl-CoA carboxylase A (ACACA), and PPARγ genes were significantly higher in the 0.52–0.78 mmol/L Met treatments compared to other treatments ($P<0.05$). The relative expression of lipoprotein lipase (LPL) gene was higher in the 0.26–0.39 mmol/L Met treatments, significantly exceeding that in the 0.65–0.78 mmol/L treatments ($P<0.05$). Fatty acid synthase (FASN) gene expression was numerically higher in the 0.39–0.52 mmol/L Met treatments, with the 0.52 mmol/L treatment showing significantly higher expression than the 0.13–0.26 mmol/L and 0.65–0.78 mmol/L treatments ($P<0.05$). Met concentration significantly affected the expression of SREBP1 and AGPAT6 genes as well as SREBP1 and PPARγ proteins ($P<0.05$), with the highest relative expression observed in the 0.26 mmol/L Met treatment. These results demonstrate that Met concentration influences the expression of genes involved in fatty acid uptake and de novo synthesis, as well as the gene and protein expression of the milk fat synthesis regulators PPARγ and SREBP1. Met concentrations of 0.26–0.52 mmol/L showed the best promoting effects on fatty acid de novo synthesis and long-chain fatty acid uptake in BMECs.

Keywords: dairy cow; bovine mammary epithelial cells; methionine; milk fat

Introduction

Milk fat is a crucial component of milk and an important indicator of milk quality. Studies have shown that amino acids (AA), as essential precursors for milk protein synthesis, not only affect milk protein synthesis but also influence milk fat synthesis and composition [1]. Methionine (Met), an essential amino acid for dairy cows, significantly affects both milk protein synthesis and de novo fatty acid synthesis in the mammary gland [2]. Therefore, investigating the effects and mechanisms of Met on milk fat synthesis is important for improving milk quality. In vivo studies have found that abomasal infusion of 40 g/d Met increased medium- and short-chain fatty acid (SMCFA) (C4–C14) and long-chain fatty acid (LCFA) content in dairy cow mammary glands by 11.1% and 9.8%, respectively [2]. In vitro studies reported that 30 g/mL Met upregulated the expression of acetyl-CoA carboxylase A (ACACA), a gene involved in fatty acid de novo synthesis, in BMECs [3]. These findings indicate that Met affects milk fat synthesis and composition, but previous research has focused primarily on the effects of Met on milk secretion and milk fat synthesis, with limited exploration of the underlying mechanisms. Therefore, further experimental investigation is warranted. This study used BMECs as a model to investigate the effects of Met on the expression of genes and proteins related to milk fat synthesis, providing a theoretical basis for further exploration of the mechanisms through which Met influences milk fat synthesis in BMECs.

Materials and Methods

1.1 Reagents and Chemicals

DMEM/F12 basal medium (12400-024), fetal bovine serum (FBS, 10099-141), Type II collagenase (17101-015), insulin-transferrin-selenium (ITS, 51500-056), penicillin-streptomycin solution (15140-122), and 0.05% trypsin-EDTA solution (25300054) were purchased from Gibco. Methionine (Met, M5308), agarose (A9539), hydrocortisone (H0135), prolactin (L6520), epidermal growth factor (EGF, E4127), Oil Red O working solution (O9755), and rabbit anti-peroxisome proliferator-activated receptor gamma (PPARγ) antibody (AV32880) were obtained from Sigma. Mouse anti-sterol regulatory element-binding protein 1 (SREBP1) antibody (ab3259) was purchased from Abcam. RIPA lysis buffer (P0013C), phenylmethylsulfonyl fluoride (PMSF, ST506), BCA protein assay kit (P0012), Western primary antibody dilution buffer (P0023A), Western secondary antibody dilution buffer (P0023D), Western SDS-PAGE electrophoresis buffer (P0014B), Western transfer buffer (P0012B), and ECL chemiluminescent substrate (P0018) were from Beyotime (Beijing). RNAiso Plus (D9109B), PrimeScript™ RT Master Mix (DRR036A), and SYBR® Premix Ex Taq™ II (DRR820A) were purchased from TaKaRa. Tris-HCl buffer (TBST), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (10494-1-AP), HRP-labeled goat anti-rabbit secondary antibody (04-15-06), HRP-labeled goat anti-mouse secondary antibody (LK2003), and protein-free blocking buffer (C520041) were obtained from HyClone, Proteintech, KPL, and Sangon Biotech,

respectively.

1.2 Solution Preparation

Met working solution: Prior to the experiment, 0.1597 g of Met powder was dissolved in 20 mL DMEM/F12 basal medium to prepare a 52 mmol/L Met stock solution, which was filtered through a 0.22 μm filter. The stock solution was used to prepare cell culture media with final Met concentrations of 0.13, 0.26, 0.39, 0.52, 0.65, and 0.78 mmol/L in the BMECs culture system.

Growth medium preparation: The growth medium was prepared by adding reagents to 100 mL DMEM/F12 basal medium to achieve final concentrations of 10% FBS, 1% ITS, 1 μg/mL hydrocortisone, 0.5% ITS, 10 ng/mL EGF, 5 μg/mL prolactin, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.5 μg/mL amphotericin B.

1.3 BMECs Culture

BMECs were cultured using the collagenase digestion method as described by Sheng et al. [4]. Mammary tissue was collected from healthy lactating Holstein cows at a local slaughterhouse in Hohhot, Inner Mongolia, and transported to the laboratory on ice. Approximately 1 cm^3 of tissue was excised from the deep layer and placed in PBS containing 3× antibiotic-antimycotic solution. The tissue was washed three times with 3× antibiotic-antimycotic PBS, rinsed with 75% ethanol for 30 s, and washed three times with 1× PBS. Alveolar-rich regions were minced and transferred to a 5 mL sterile enzyme-free centrifuge tube. The minced tissue was incubated with an equal volume of 0.5% Type II collagenase at 37°C for 1 h, with inversion mixing every 20 min to ensure complete digestion. After filtration through an 80-mesh screen, the cell suspension was centrifuged at 1,300 r/min for 5 min. The pellet was washed twice with PBS (1,300 r/min for 3 min each) and resuspended in growth medium. Cells were seeded into 25 cm^2 ventilated culture flasks and incubated at 37°C and 5% CO_2 until primary cells reached approximately 90% confluence, at which point they were purified and passaged using 0.05% trypsin-EDTA solution.

1.4 Experimental Design

Third-passage BMECs were collected and suspended in growth medium, then seeded onto cell culture plates at the required density and incubated at 37°C and 5% CO_2 for 24 h. A single-factor randomized design was employed, with BMECs randomly divided into six treatments (six replicates each) after 24 h of culture. Based on preliminary results from our research group [5], different Met concentrations were selected: 0.13, 0.26, 0.39, 0.52, 0.65, and 0.78 mmol/L. The lowest Met concentration was more than five times the arterial Met concentration reported in dairy cows by Rius et al. [6] and Wang [7]. When cell confluence reached 80–90%, cells were serum-starved in DMEM/F12 medium for

12 h, followed by addition of culture medium containing different Met concentrations. Cells were then incubated at 37°C and 5% CO_2 for 48 h to investigate the effects of Met on milk fat synthesis in BMECs.

1.5.1 Determination of Triglyceride (TG) Content in BMECs Intracellular TG content was determined using the method of Ramírez-Zacarías et al. [8], with absorbance (OD) values representing TG content. Cell suspension was seeded in 24-well plates at a density of 5$×10^{4}$ cells/mL and cultured for 48 h according to the experimental design. After removing the culture medium and washing twice with PBS, cells were fixed with 0.2 mL of 4% paraformaldehyde for 1 h. Following two PBS washes, cells were stained with 0.5 mL Oil Red O working solution for 2 h in the dark. After three PBS washes, plates were air-dried, and 0.3 mL isopropanol was added to extract lipids for 30 min. OD values were measured at 510 nm using a microplate reader. Each treatment had six replicates.

1.5.2 Determination of Milk Fat Synthesis-Related Gene Expression in BMECs Expression levels of milk fat synthesis-related genes, including fatty acid synthase (FASN), ACACA, stearoyl-CoA desaturase (SCD), fatty acid-binding protein 3 (FABP3), lipoprotein lipase (LPL), PPARγ, SREBP1, 1-acylglycerol-3-phosphate O-acyltransferase 6 (AGPAT6), mitochondrial glycerol-3-phosphate acyltransferase (GPAM), phosphatidic acid phosphatase 1 (LPIN1), butyrophilin subfamily 1 member A1 (BTN1A1), and xanthine dehydrogenase (XDH), were detected by quantitative real-time PCR (Thermo, USA). Primers were designed using Primer 5.0 (Table 1). Cell suspension was seeded in 24-well plates at 2$×10^{5}$ cells/mL and cultured for 48 h before total RNA extraction. Total RNA was extracted using the Trizol method, and RNA purity and concentration were assessed using a microplate reader. RNA samples with A260/A280 ratios between 1.8 and 2.2 were considered pure. RNA integrity was verified by electrophoresis on a 2% gel. Reverse transcription was performed using PrimeScript™ RT Master Mix according to the manufacturer' s instructions in a 10 μL reaction volume. Gene expression was detected using SYBR® Premix Ex Taq™ II kit in a 20 μL reaction volume, with GAPDH as the housekeeping gene. The qPCR program consisted of: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 34 s, and 95°C for 20 s; followed by melting curve analysis (95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 51 cycles). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

1.5.3 Determination of Milk Fat Synthesis-Related Protein Expression in BMECs Cell suspension was seeded in 25 cm^2 culture flasks at 1$×10^{6}$ cells/mL and cultured for 48 h according to the experimental design. After removing the supernatant and washing adherent cells twice with PBS, 250 μL of RIPA lysis buffer containing 0.1% PMSF was added. Cells were lysed at 4°C for 5 min, and the lysate was centrifuged at 12,000 r/min for 10 min at 4°C. The supernatant was collected for detection of PPARγ and SREBP1

protein expression. Protein samples (60 μg) were mixed with 5× loading buffer at a 4:1 ratio, denatured at 100°C for 5 min, and separated by SDS-PAGE (80 V for 40 min on the stacking gel, 120 V for 100 min on the separating gel). Proteins were transferred to PVDF membranes, washed with distilled water for 1 min, blocked at room temperature for 1 h, and washed three times with TBST (2 min each). Membranes were incubated overnight at 4°C with rabbit anti-PPARγ (1:250) and mouse anti-SREBP1 (1:50) primary antibodies. After three TBST washes (5 min each), membranes were incubated with HRP-labeled goat anti-rabbit (1:1,000) and goat anti-mouse (1:500) secondary antibodies at room temperature for 1 h, followed by three TBST washes (8 min each). Protein bands were visualized using ECL chemiluminescent substrate and imaged using a gel imaging system (Tanongis-1000, Shanghai Tianneng Biotechnology). Band intensities were analyzed using Quantity One software. Relative protein expression levels were expressed as ratios to the 0.13 mmol/L Met treatment.

1.6 Data Analysis

All data were calculated and organized using Excel 2007. Statistical significance was analyzed using one-way ANOVA in SAS 9.0 software. Differences were considered significant at $P<0.05$ and tended to be significant at $0.05<P<0.10$.

Results

2.1 Effects of Met on TG Content and Milk Fat Synthesis-Related Gene Expression in BMECs

As shown in Table 2 , different Met concentrations had no significant effect on intracellular TG content in BMECs ($P>0.05$), although numerically higher values were observed in the 0.39–0.65 mmol/L groups. The relative expression of FABP3 gene was significantly higher in the 0.39–0.78 mmol/L Met treatments compared to the 0.13–0.26 mmol/L treatments ($P<0.05$), with the 0.52 and 0.78 mmol/L treatments showing significantly higher expression than the 0.39 mmol/L treatment ($P<0.05$). The 0.52 mmol/L Met treatment significantly increased FASN gene expression compared to the 0.13–0.26 mmol/L and 0.65–0.78 mmol/L treatments ($P<0.05$). LPL gene expression was higher in the 0.26–0.39 mmol/L Met treatments, significantly exceeding that in the 0.65–0.78 mmol/L treatments ($P<0.05$). ACACA and PPARγ gene expression levels were significantly higher in the 0.52–0.78 mmol/L Met treatments compared to all other treatments ($P<0.05$). Both SREBP1 and AGPAT6 gene expression increased initially and then decreased with increasing Met concentration, but all treatments remained higher than the 0.13 mmol/L treatment. Notably, the 0.26 mmol/L Met treatment showed significantly higher SREBP1 expression than other treatments ($P<0.05$), and significantly higher AGPAT6 expression than the 0.13 mmol/L and 0.39–0.52 mmol/L treatments ($P<0.05$). No significant differences were observed among Met treatments for SCD, GPAM, LPIN1, XDH, and BTN1A1 gene expression ($P>0.05$).

2.2 Effects of Met on Milk Fat Synthesis-Related Protein Expression in BMECs

As shown in Table 3 and Figure 1 [Figure 1: see original paper], the relative expression levels of PPARγ and SREBP1 proteins were significantly higher in the 0.26–0.78 mmol/L Met treatments compared to the 0.13 mmol/L treatment ($P<0.05$), with the 0.26 mmol/L treatment showing the highest expression, significantly exceeding all other treatments ($P<0.05$). No significant differences in PPARγ protein expression were observed among the 0.39–0.78 mmol/L Met treatments ($P>0.05$). SREBP1 protein expression was significantly higher in the 0.39 mmol/L treatment compared to the 0.52–0.78 mmol/L treatments ($P<0.05$), while the 0.78 mmol/L treatment showed the lowest expression, significantly lower than all other treatments ($P<0.05$).

Discussion

Methionine, an essential amino acid for dairy cows, has been shown in vivo to affect milk protein synthesis while increasing SMCFA (C4–C16) de novo synthesis and LCFA content in the mammary gland [2]. In vitro studies have reported that 30 μg/mL Met upregulates ACACA expression in BMECs [3]. These findings suggest that Met influences milk fat synthesis, though the underlying mechanisms remain unclear. Building on previous research and our group' s preliminary results [5], this study designed different Met concentration gradients to investigate its effects on milk fat synthesis from the perspective of related gene and protein expression. Since essential amino acids at physiological concentrations have minimal effects on mammary metabolism in dairy cows, concentrations several times higher than normal plasma levels are required to produce significant effects [13]. Therefore, the lowest Met concentration in this study was more than five times the arterial Met concentration reported in dairy cows [6,7].

LPL and FABP3 are important genes involved in LCFA uptake and intracellular transport in various mammalian tissues [14]. LPL determines adipocyte size and fat accumulation by catalyzing the hydrolysis of triglycerides from chylomicrons and very low-density lipoproteins, releasing glycerol and fatty acids for tissue storage and utilization [15]. FABP3 is primarily associated with intracellular LCFA transport. Our results demonstrate that appropriate Met concentrations significantly promote FABP3 and LPL gene expression, indicating that Met enhances LCFA uptake and transport in BMECs.

SREBP1 belongs to the nuclear transcription factor family and is a key transcriptional regulator of lipogenic genes, controlling the expression of fatty acid synthesis-related genes. FASN and ACACA are two critical enzymes in milk fatty acid de novo synthesis, with ACACA being a rate-limiting enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. During lactation, FASN-encoded protein regulates SMCFA (C4–C16) synthesis in the mammary gland [16]. SREBP1 positively regulates FABP3, ACACA, and FASN gene expres-

sion, thereby controlling fatty acid transport and de novo synthesis. SREBP1 function and expression are regulated by PPARγ [11], which can also positively regulate ACACA, FASN, FABP3, AGPAT6, and SREBP1 genes in dairy goat mammary epithelial cells [17] and LPL gene expression in adipocytes [18]. Previous studies have shown that Met supplementation upregulates ACACA expression in BMECs [3], and abomasal Met infusion in cows fed grass silage increased SMCFA (C4–C14) de novo synthesis with increasing Met dose, reaching an 11.1% increase at 40 g/d, though C16 content remained unchanged [2]. Our findings indicate that appropriate Met concentrations promote SREBP1 and PPARγ gene and protein expression, and upregulate FASN and ACACA gene expression. Thus, Met may enhance SMCFA synthesis by upregulating SREBP1 and PPARγ expression, subsequently increasing FASN and ACACA expression.

Triglycerides constitute 98% of milk fat, and their content directly reflects milk fat synthesis. Our results show that appropriate Met concentrations promote SMCFA de novo synthesis and LCFA uptake/transport in BMECs but do not significantly affect TG synthesis, though the mechanism remains unclear. Research indicates that SCFA incorporation into TG synthesis in bovine mammary tissue is regulated by diacylglycerol acyltransferase 1 (DGAT1), which, compared to other TG synthesis-related genes, has a relatively minor but still important role in milk fat synthesis [11]. Fatty acids with chain lengths >C10 are activated by long-chain acyl-CoA synthetase 1 (ACSL1) and bind to FABP3 before entering TG synthesis [11], and ACSL1 overexpression promotes LCFA incorporation into TG [19]. This study found that Met upregulates PPARγ and FABP3 expression but does not significantly affect TG synthesis. Activation of PPARγ in BMECs upregulates DGAT1 expression without affecting ACSL1 expression [20], suggesting that Met' s effect on TG synthesis may be related to ACSL1, though relevant research is limited and requires further investigation.

GPAM, AGPAT6, and LPIN1 are major genes involved in TG synthesis, encoding key enzymes for milk fat synthesis [19]. GPAM catalyzes the esterification of acyl-CoA to the sn-1 position of glycerol-3-phosphate to form lysophosphatidic acid. AGPAT6 catalyzes the esterification of a second acyl-CoA to the sn-2 position to form phosphatidic acid (PA). LPIN1 dephosphorylates PA to form diacylglycerol, after which a third acyl-CoA is esterified to the sn-3 position to synthesize TG. XDH plays an important role in coupling milk fat globules to the apical membrane, while BTN1A1 assists in milk fat droplet formation during secretion. Our results show that Met had no significant effects on GPAM, LPIN1, BTN1A1, and XDH gene expression or TG content, but significantly promoted AGPAT6 expression. A study in lactating mice found that AGPAT6 knockout prevented milk fat synthesis, yet in AGPAT6-rich cell membranes, no increased GPAM or AGPAT6 activity was detected when using sn-1-acylglycerol and acetyl-CoA as substrates [21]. Other studies have identified AGPAT6 as a microsomal glycerol-3-phosphate acyltransferase (GPAT), also known as GPAT4 [22], and GPAT4 overexpression in mice did not affect TG content [23]. These findings suggest that TG synthesis is regulated not only at the transcriptional

level by GPAM and AGPAT6 expression but also by the expression and activity of their encoded proteins. Therefore, our observation that Met significantly promoted AGPAT6 expression without significantly affecting TG synthesis may be related to the expression and activity of GPAM and AGPAT6 proteins, requiring further investigation.

Considering the results on TG synthesis, milk fat-related gene expression, and SREBP1 and PPARγ protein expression, Met concentration affects fatty acid uptake and de novo synthesis. The 0.26–0.78 mmol/L Met treatments showed relatively high SREBP1 and PPARγ protein expression. As Met concentration increased, FABP3 and ACACA gene expression increased, particularly in the 0.52–0.78 mmol/L treatments, though high Met concentrations (0.65–0.78 mmol/L) inhibited LPL and FASN expression. Based on multiple indicators of milk fat synthesis, the optimal Met concentration range appears to be 0.26–0.52 mmol/L. Regression analysis further indicated that ACACA and PPARγ gene expression increased with Met concentration, with optimal expression of FABP3, FASN, AGPAT6, PPARγ protein, and SREBP1 gene and protein achieved at Met concentrations of 0.73, 0.42, 0.55, 0.53, 0.46, and 0.44 mmol/L, respectively. However, since 0.73 mmol/L Met inhibited LPL expression, the optimal Met concentration was determined to be 0.42–0.55 mmol/L. In practice, NRC (2001) recommends that Met supply for lactating dairy cows should account for 2.4% of dietary metabolizable protein. While most studies on Met and milk fat have focused on milk fat percentage and yield, mechanistic studies are scarce. Therefore, the results obtained from this in vitro study require further validation in vivo.

In conclusion, Met concentration influences the expression of genes involved in fatty acid uptake and de novo synthesis, as well as the gene and protein expression of the milk fat synthesis regulators PPARγ and SREBP1. Met concentrations of 0.26–0.52 mmol/L showed the best promoting effects on fatty acid de novo synthesis and long-chain fatty acid uptake in BMECs.

References

[1] MAXIN G, RULQUIN H, GLASSER F. Response of milk fat concentration and yield to nutrient supply in dairy cows[J]. Animal: An International Journal of Animal Bioscience, 2011, 5(8): 1299–1310.

[2] VARIKKO T, VANHATALO A, JALAVA T, et al. Lactation and metabolic responses to graded abomasal doses of methionine and lysine in cows fed grass silage diets[J]. Journal of Dairy Science, 1999, 82(12): 2659–2673.

[3] LI Xiyan. Effects of lysine and methionine ratio on casein synthesis and its mechanism in bovine mammary epithelial cells[D]. Master' s thesis. Beijing: Chinese Academy of Agricultural Sciences, 2011.

[4] SHENG R, YAN S M, QI L Z, et al. Effect of the ratios of acetate and β-hydroxybutyrate on the expression of milk fat- and protein-related genes in

bovine mammary epithelial cells[J]. Czech Journal of Animal Science, 2015, 60(12): 531-541.

[5] CHANG Chencheng. Effects of methionine and methionine-containing dipeptides on expression of genes related to milk protein synthesis in bovine mammary epithelial cells[D]. Master' s thesis. Hohhot: Inner Mongolia Agricultural University, 2015.

[6] RIUS A G, APPUHAMY J A D R N, CYRIAC J, et al. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids[J]. Journal of Dairy Science, 2010, 93(7): 3114-3127.

[7] WANG Xinpeng. Effects of amino acid and fatty acid infusion into the external pudendal artery on milk protein synthesis and amino acid uptake in dairy cows[D]. Master's thesis. Hohhot: Inner Mongolia Agricultural University, 2015.

[8] RAMÍREZ-ZACARÍAS J, CASTRO-MUÑOZLEDO F, KURI-HARCUCH W, et al. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O[J]. Histochemistry, 1992, 97(6): 493-497.

[9] ZHOU Y, AKERS R M, JIANG H. Growth hormone can induce expression of four major milk protein genes in transfected MAC-T Cells[J]. Journal of Dairy Science, 2008, 91(1): 100-108.

[10] QI L Z, YAN S M, SHENG R, et al. Effects of saturated long-chain fatty acid on mRNA expression of genes associated with milk fat and protein biosynthesis in bovine mammary epithelial cells[J]. Asian-Australasian Journal of Animal Sciences, 2014, 27(3): 414-421.

[11] BIONAZ M, LOOR J J. Gene networks driving bovine milk fat synthesis during the lactation cycle[J]. BMC Genomics, 2008, 9: 366.

[12] ZHANG Yangdong. Effects of lipopolysaccharide on milk fat and protein in lactating dairy cows and its mechanism[D]. PhD thesis. Harbin: Northeast Agricultural University, 2011.

[13] HANIGAN M D, CROMPTON L A, METCALF J A, et al. Modelling mammary metabolism in the dairy cow to predict milk constituent yield, with emphasis on amino acid metabolism and milk protein production: model construction[J]. Journal of Theoretical Biology, 2001, 213(2): 223-239.

[14] LEHNER R, KUKSIS A. Biosynthesis of triacylglycerols[J]. Progress in Lipid Research, 1996, 35(2): 169-201.

[15] BONNET M, LEROUX C, CHILLIARD Y, et al. Rapid communication: nucleotide sequence of the ovine lipoprotein lipase cDNA[J]. Journal of Animal Science, 2000, 78(11): 2994-2995.

[16] WAKIL S J. Fatty acid synthase, a proficient multifunctional enzyme[J]. Biochemistry, 1989, 28(11): 4523-4530.

[17] SHI H, LUO J, ZHU J, et al. PPARγ regulates genes involved in triacylglycerol synthesis and secretion in mammary gland epithelial cells of dairy goats[J]. PPAR Research, 2013, 2013: 310948.

[18] DESVERGNE B, MICHALIK L, WAHLI W. Transcriptional regulation of metabolism[J]. Physiological Reviews, 2006, 86(2): 465–514.

[19] BIONAZ M, LOOR J J. ACSL1, AGPAT6, FABP3, LPIN1, and SLC27A6 are the most abundant isoforms in bovine mammary tissue and their expression is affected by stage of lactation[J]. The Journal of Nutrition, 2008, 138(6): 1019–1024.

[20] KADEGOWDA A K G, BIONAZ M, PIPEROVA L S, et al. Peroxisome proliferator-activated receptor-γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents[J]. Journal of Dairy Science, 2009, 92(9): 4276–4289.

[21] BEIGNEUX A P, VERGNES L, QIAO X, et al. Agpat6—a novel lipid biosynthetic gene required for triacylglycerol production in mammary epithelium[J]. The Journal of Lipid Research, 2006, 47(4): 734–744.

[22] CHEN Y Q, KUO M S, LI S, et al. AGPAT6 is a novel microsomal glycerol-3-phosphate acyltransferase[J]. The Journal of Biological Chemistry, 2008, 283(15): 10048–10057.

[23] NAGLE C A, VERGNES L, DEJONG H, et al. Identification of a novel sn-glycerol-3-phosphate acyltransferase isoform, GPAT4, as the enzyme deficient in Agpat6-/- mice[J]. The Journal of Lipid Research, 2008, 49(4): 823–831.

Note: Figure translations are in progress. See original paper for figures.