Effects of Leucine on Milk Fat Synthesis-Related Gene and Protein Expression in Dairy Cow Mammary Epithelial Cells (Postprint)

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Abstract

This study was designed to investigate the effects of leucine (Leu) on the expression of genes and proteins associated with milk fat synthesis in bovine mammary epithelial cells (BMECs) to explore the underlying mechanism of Leu' s influence on milk fat synthesis. Third-passage BMECs were randomly divided into six treatment groups, with six replicates per treatment. Leu concentrations in the culture medium were 0.45, 0.90, 1.80, 2.70, 3.60, and 7.20 mmol/L. Following incubation at 37°C with 5% CO_2 for 48 h, the triglyceride (TG) content in BMECs and the relative expression levels of genes related to milk fat synthesis and proteins of peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding protein (SREBP1) were measured. The results demonstrated that Leu concentration did not significantly affect TG content in BMECs ($P>0.05$). Optimal concentrations of Leu significantly enhanced the expression of fatty acid synthase (FASN) and acetyl-CoA carboxylase A (ACACA) genes ($P<0.05$), with FASN gene expression being highest in the 1.80-2.70 mmol/L Leu treatment groups and ACACA gene expression being highest in the 1.80-7.20 mmol/L treatment groups. Leu concentration significantly influenced SREBP1 gene and protein expression in BMECs ($P<0.05$), with the most pronounced promotional effect observed at 1.80 mmol/L Leu. Although Leu significantly inhibited the expression of fatty acid binding protein 3 (FABP3), lipoprotein lipase (LPL), acylglycerol phosphate acyltransferase 6 (AGPAT6), mitochondrial glycerol-3-phosphate acyltransferase (GPAM), and butyrophilin subfamily 1 member 1 (BTN1A1) genes in BMECs ($P<0.05$), only high concentrations (3.60-7.20 mmol/L) demonstrated substantial inhibitory effects. Taken together, Leu concentration affected the expression of genes related to milk fat synthesis and proteins of PPARγ and SREBP1 in BMECs. At Leu concentrations of 1.80-2.70 mmol/L, favorable promotional effects were exerted on genes related to de novo fatty acid synthesis and the expression of the regulatory factor SREBP1 protein, while minimal inhibitory effects were displayed

on genes related to TG synthesis and lipid droplet formation.

Full Text

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

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Abstract

This study aimed to investigate the effects of leucine (Leu) on the expression of genes and proteins related to milk fat synthesis in bovine mammary epithelial cells (BMECs) to explore the underlying mechanism. Third-passage BMECs were randomly divided into six treatments with six replicates per treatment. The six treatment groups were cultured in media containing 0.45, 0.90, 1.80, 2.70, 3.60, and 7.20 mmol/L Leu, respectively. After 48 h of incubation at 37 °C and 5% CO_2, the intracellular triglyceride (TG) content 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 measured. The results showed that Leu concentration had no significant effect on TG content in BMECs ($P>0.05$). Appropriate Leu concentrations significantly promoted the expression of fatty acid synthase (FASN) and acetyl-CoA carboxylase A (ACACA) genes ($P<0.05$). The relative expression of FASN was higher in the 1.80–2.70 mmol/L Leu treatments, while ACACA expression was higher in the 1.80–7.20 mmol/L treatments. Leu concentration significantly affected SREBP1 gene and protein expression in BMECs ($P<0.05$), with the most pronounced promoting effect observed at 1.80 mmol/L Leu. Although Leu significantly inhibited the expression of fatty acid-binding protein 3 (FABP3), lipoprotein lipase (LPL), 1-acylglycerol-3-phosphate O-acyltransferase 6 (AGPAT6), mitochondrial glycerol-3-phosphate acyltransferase (GPAM), and butyrophilin subfamily 1 member A1 (BTN1A1) genes ($P<0.05$), this inhibitory effect was substantial only at high concentrations (3.60–7.20 mmol/L). Overall, Leu concentration influenced the expression of milk fat synthesis-related genes and PPARγ and SREBP1 proteins in BMECs. At concentrations of 1.80–2.70 mmol/L, Leu effectively promoted the expression of genes involved in de novo fatty acid synthesis and the SREBP1 protein, while exerting minimal inhibitory effects on genes related to TG synthesis and lipid droplet formation.

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

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1 Introduction

Milk fat constitutes up to 27% of the total solids in milk and serves as a crucial material foundation of milk composition, representing an important indicator of milk quality. Amino acids (AAs), as primary precursors for milk protein synthesis, influence not only protein synthesis but also milk fat synthesis [1]. Therefore, in-depth investigation of the effects of AAs on milk fat synthesis and its underlying mechanisms is significant for improving milk quality. Leucine (Leu) is an essential amino acid for animals. Studies have shown that Leu deficiency in mouse diets restricts fat synthesis in white adipose tissue, significantly reducing the expression of fatty acid synthase (FASN) and acetyl-CoA carboxylase A (ACACA) genes as well as sterol regulatory element binding protein 1c (SREBP1c) and FASN proteins, while also decreasing serum free fatty acid and glycerol concentrations [2]. In vitro studies have demonstrated that Leu, while affecting milk protein synthesis, promotes the expression of sterol regulatory element binding protein-1 (SREBP1) and triglyceride (TG) synthesis in bovine mammary epithelial cells (BMECs) [3]. These findings suggest that Leu may promote fat synthesis by influencing the expression of lipid synthesis-related genes. However, mice fed high-fat diets and supplemented with excessive Leu exhibit reduced body weight and inhibited fat synthesis [4]. Other studies have reported that Leu intake at more than twice the daily requirement has no significant effect on plasma cholesterol and TG synthesis in mice [5]. Evidently, the effects of Leu on lipid metabolism vary across different tissues, and research on the influence of Leu on milk fat synthesis in dairy cows and its mechanisms remains scarce. Therefore, this study employed BMECs as a model to investigate the effects of different Leu concentrations on the expression of genes and proteins related to milk fat synthesis, providing a theoretical foundation for further exploration of Leu' s regulatory mechanisms in milk fat synthesis.

2 Materials and Methods

2.1 Reagents

DMEM/F12 basal medium (12400-024), fetal bovine serum (FBS, 10099-141), type II collagenase (17101-015), insulin-transferrin-selenium (51500-056), penicillin-streptomycin mixture for cell culture (15140-122), and 0.05% trypsin-EDTA solution (25300054) were purchased from Gibco. Leucine (L8912), agarose (A9539), hydrocortisone (H0135), prolactin (L6520), epidermal growth factor (EGF, E4127), Oil Red O (O9755), and rabbit anti-peroxisome proliferator-activated receptor γ (PPARγ) antibody (AV32880) were obtained from Sigma. Mouse anti-SREBP1 antibody (ab3259) was purchased from Abcam. RIPA lysis buffer (P0013C), phenylmethylsulfonyl fluoride (PMSF, ST506), bicinchoninic acid (BCA) protein assay kit (P0012), Western primary antibody diluent (P0023A), Western secondary antibody diluent (P0023D),

Western SDS-PAGE electrophoresis buffer (P0014B), Western transfer buffer (P0012B), and ECL chemiluminescent substrate (P0018) were all purchased from Beyotime Biotechnology. RNAiso PLUS (D9109B), PrimeScript™ RT Master Mix (DRR036A), and SYBR® Premix Ex Taq™ II kit (DRR820A) were obtained from TaKaRa. Tris-HCl buffer (TBST), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (10494-1-AP), horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (04-15-06), and HRP-conjugated goat anti-mouse secondary antibody (LK2003) were purchased from HyClone, Proteintech, KPL, and Sanjian Company, respectively. Protein-free blocking solution (C520041) was obtained from Sangon Biotech.

2.2 Reagent Preparation

Growth medium preparation: 10% fetal bovine serum, 1% insulin-transferrin, 1 g/mL hydrocortisone, 0.5% insulin-transferrin-selenium, 10 ng/mL epidermal growth factor, 5 g/mL prolactin, 100 g/mL streptomycin, 100 IU/mL penicillin, and 2.5 g/mL amphotericin B were added to 100 mL of DMEM/F12 basal medium.

Leu working solution preparation: 0.118 g of Leu powder was dissolved in 10 mL of serum-free growth medium to prepare a 90 mmol/L Leu stock solution, which was filtered through a 0.22 µm filter. The 90 mmol/L Leu stock solution was then diluted with serum-free growth medium using gradient dilution to prepare cell culture media with various Leu concentrations according to experimental requirements.

2.3 BMECs Culture

BMECs were cultured using the collagenase digestion method, following the protocol described by Sheng et al. [6]. Mammary tissue was collected from healthy, mid-lactation, high-yielding Holstein cows aged 3–5 years at the Beiya Halal Slaughterhouse in Hohhot, Inner Mongolia, and transported to the laboratory at 0–4 °C. Approximately 1 cm^3 of tissue was excised from the deep layer and placed in 3× antibiotic phosphate-buffered saline (PBS). The tissue was then washed sequentially with 3× antibiotic PBS, 75% ethanol, and 1× PBS. Alveolar-rich tissue was minced into a paste and digested with an equal volume of 0.5% type II collagenase at 37 °C for 1 h. After filtration through an 80-mesh screen, the cells were centrifuged at 179×g for 5 min and the supernatant was discarded. The cell pellet was washed with PBS and centrifuged at 179×g for 3 min, with this washing step repeated twice. Cells were resuspended in growth medium and seeded into 25 cm^2 ventilated culture flasks, then incubated at 37 °C and 5% CO_2. Primary cells were purified and passaged with 0.05% trypsin-EDTA once they reached approximately 90% confluence.

2.4 Experimental Design

Third-passage BMECs were collected, resuspended in growth medium, and seeded onto cell culture plates at the required density, followed by incubation at 37 °C and 5% CO_2 for 24 h. A single-factor randomized design was employed, dividing the BMECs after 24 h of culture into six treatments with six replicates per treatment. Leu concentrations in the culture media were determined based on the studies by Pang [7] and Dai et al. [8] and finalized using the methyl thiazolyl tetrazolium (MTT) assay to assess cell proliferation. The six treatments consisted of Leu concentrations at 0.45, 0.90, 1.80, 3.60, 2.70, and 7.20 mmol/L, with six replicates per treatment. When BMECs reached 80–90% confluence, the medium was replaced with serum-free growth medium. After 12 h, the medium was changed to cell culture solutions with different Leu concentrations according to the experimental design, and cells were cultured for 48 h at 37 °C and 5% CO_2.

2.5 Determination of TG Content in BMECs

Intracellular TG content in BMECs was measured using the method of Ramírez-Zacarías et al. [9], with absorbance (OD) values representing TG content. Briefly, cell suspensions were seeded onto 24-well plates at a density of 5$×10^{4}$ cells/mL. After 48 h of culture according to the experimental design, the culture medium was removed and cells were washed twice with PBS. Cells were fixed with 0.2 mL of 4% paraformaldehyde solution per well for 1 h, then washed twice with PBS. Subsequently, cells were stained with 0.5 mL of Oil Red O working solution for 2 h in the dark. After staining, cells were washed three times with PBS. The plates were air-dried, and 0.3 mL of isopropanol was added to each well to extract lipids for 30 min. OD values were measured at 510 nm using a fully automated microplate reader.

2.6 Determination of Milk Fat Synthesis-Related Gene Expression in BMECs

The expression of milk fat synthesis-related genes in BMECs was detected using a real-time fluorescence quantitative PCR instrument (Thermo, USA). Primers were designed using Primer 5.0 software (Table 1). The genes measured included FASN, ACACA, stearoyl-CoA desaturase 1 (SCD1), 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), with GAPDH serving as the housekeeping gene. Cell suspensions were seeded onto 24-well plates at a density of 2$×10^{5}$ cells/mL. After 48 h of culture according to the experimental design, total RNA was extracted using the Trizol method. RNA purity and concentration were determined using a fully automated microplate reader, with OD_{260}/OD_{280} ratios between 1.8 and 2.2 indicating good RNA purity. RNA integrity was

assessed by electrophoresis on a 2% gel. RNA was reverse-transcribed into cDNA following the instructions of the PrimeScript™ RT Master Mix kit in a 10 µL reaction volume. Gene expression was detected according to the SYBR® Premix Ex Taq™ II kit instructions in a 20 µL reaction volume. The real-time quantitative PCR program consisted of pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 34 s, and extension at 95 °C for 20 s. A melting curve was generated through 51 cycles of 95 °C for 5 s, 60 °C for 30 s, and 95 °C for 15 s. Relative gene expression levels were calculated using the 2^(-ΔΔCt) method.

Table 1 Primer sequences of genes related to milk fat synthesis

Genes	GenBank accession No.	Primer sequences (5' -3')	Length/bp	References
GAPDH	XM_{001252479}	F: GGGTCAT-CATCTCTGCAC-CTR: GGTCATAAGTCC-CTCCACGA	-	Zhou et al. [10]
FASN	NM_{001012669}	F: AGGACCTCGT-GAAGGCTGTGAR: CCCTTCGAACATA-CACCTCCA	-	Qi et al. [11]
ACACA	AJ132890	F: CATCTTGTCC-GAAACGTCGATR: CCAAGGTCT-GAAAGCGAGCTG	-	Bionaz et al. [12]
SCD1	AY241933	F: TCCTGTTGTTGT-GCTTCATCCR: AAGCCTACCA-CAATCATCGAAG	-	Bionaz et al. [12]
FABP3	DN518905	F: GAACTCGACTC-CCAGCTTGAAR: GGCATAACG-GAATAAGGTGGC	-	Bionaz et al. [12]
LPL	BC118091	F: ACACAGCTGAG-GACACTTGCCR: ACAGCGAAGGGCT-CACTCTC	-	Bionaz et al. [12]
PPARγ	NM_{181024}	F: CCAAATATCG-GTGGGAGTCGR: GCCATGGATCAC-CACAAAGG	-	Bionaz et al. [12]

Genes	GenBank accession No.	Primer sequences (5' -3')	Length/bp	References
SREBP1	NM_{001113302}	F: CTGACGACCGT-GAAAACAGAR: AAACTGTGGCTC-CAATTTCGA	-	Bionaz et al. [12]
AGPAT6	DY208485	F: AAGCAAGTTGC-CCATCCTCAR: AGACGGCAGATT-TATTCAACTT	-	Zhang [13]
GPAM	NM_{001012282}.1	F: GCAGGTT-TATCCAGTATG-GCATTR: GGACT-GATATCTTCCT-GATCATCTTG	-	Bionaz et al. [12]
LPIN1	DV797268	F: TGGCCACCA-GAATAAAGCATGR: GCTGACGCTGGA-CAACAGG	-	Bionaz et al. [12]
BTN1A1	M35551	F: AGGACG-GACTGGGCAATTGR: GAACC-CATTCTCGGGAGT-CAT	-	Bionaz et al. [12]
XDH	BC102076	F: GATCATC-CACTTTTCTGC-CAATGR: CCTCGTCTTGGT-GCTTCCAA	-	Bionaz et al. [12]

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; FASN: fatty acid synthase; ACACA: acetyl-coenzyme A carboxylase α; SCD1: stearoyl-CoA desaturase 1; FABP3: fatty acid-binding protein 3; LPL: lipoprotein lipase; PPARγ: peroxisome proliferator-activated receptor gamma; SREBP1: sterol regulatory element binding protein 1; AGPAT6: 1-acylglycerol-3-phosphate O-acyltransferase 6; GPAM: mitochondrial glycerol-3-phosphate acyltransferase; LPIN1: phosphatidic acid phosphatase 1; BTN1A1: butyrophilin subfamily 1 member A1; XDH: xanthine dehydrogenase. The same as Table 2 .

2.7 Determination of Milk Fat Synthesis-Related Protein Expression in BMECs

The expression of milk fat synthesis-related proteins in BMECs was determined using Western blotting. Cell suspensions were seeded into 25 cm^2 culture flasks

at a density of 1$×10^{6}$ cells/mL. After 48 h of culture according to the experimental design, the supernatant was discarded and adherent cells were washed twice with PBS. The supernatant was removed, and 250 μL of RIPA lysis buffer containing 0.1% PMSF was added. Cells were lysed at 4 °C for 5 min, and the cell suspension was collected and centrifuged at 15,455×g 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 subjected to electrophoresis at 80 V for 40 min on a stacking gel and then at 120 V for 100 min on a separating gel. After electrophoresis, target proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. Following transfer, membranes were rinsed with distilled water for 1 min, blocked at room temperature for 1 h, and washed three times with TBST for 2 min each. Membranes were then incubated overnight at 4 °C with rabbit anti-PPARγ (1:250) or mouse anti-SREBP1 (1:50) antibodies. After incubation, membranes were washed three times with TBST for 5 min each, followed by incubation with HRP-conjugated goat anti-rabbit (1:1,000) or goat anti-mouse (1:500) secondary antibodies at room temperature for 1 h. Membranes were washed three times with TBST for 8 min each. Protein bands were visualized using ECL chemiluminescent substrate and photographed using a gel imaging system (Tanongis-1000, Shanghai Tianneng Biological Technology). Images were analyzed for grayscale values using Quantity One software. Relative expression levels of PPARγ and SREBP1 proteins were expressed as ratios relative to the 0.45 mmol/L Leu treatment.

2.8 Data Analysis

All data were processed and organized using Excel 2007. Linear and quadratic regression analyses were performed using the regression statistical program in SAS 9.0 software. $P<0.05$ was considered statistically significant, while $0.05\leq P<0.10$ indicated a tendency toward significance.

3 Results

3.1 Effects of Leu on TG Content and Expression of Milk Fat Synthesis-Related Genes in BMECs

As shown in Table 2 , TG content in BMECs did not differ significantly across different Leu concentrations (0.45–7.20 mmol/L) ($P>0.05$), although TG content in the 0.90–2.70 mmol/L Leu treatments was numerically higher than in other treatments. With increasing Leu concentration, the relative expression of FABP3 gene in BMECs showed a significant linear decrease ($P=0.018$), while LPL gene expression exhibited a significant quadratic decrease ($P=0.016$). Both genes had lower relative expression levels in the 3.60–7.20 mmol/L Leu treatments. The relative expression of FASN and ACACA genes in BMECs displayed significant quadratic increases ($P=0.013$ and $P=0.002$, respectively). FASN

expression was higher in the 1.80–2.70 mmol/L Leu treatments and lower in the 7.20 mmol/L treatment. ACACA expression was higher in the 1.80–7.20 mmol/L Leu treatments compared to other treatments. The relative expression of SCD1 and PPARγ genes in BMECs showed no significant regression relationship with Leu concentration (P>0.05); however, PPARγ expression was numerically higher in the 0.90–2.70 mmol/L treatments and lowest in the 7.20 mmol/L treatment. SREBP1 gene expression in BMECs increased significantly in a quadratic manner with increasing Leu concentration (P=0.022), with the highest expression observed in the 1.80 mmol/L treatment and the lowest in the 7.20 mmol/L treatment. The relative expression of AGPAT6, GPAM, LPIN1, and BTN1A1 genes in BMECs decreased significantly in a linear fashion with increasing Leu concentration (P=0.018, P=0.032, P=0.034, and P<0.001, respectively). AGPAT6, GPAM, and BTN1A1 genes had higher relative expression in the 0.45–2.70 mmol/L treatments, while LPIN1 expression was higher in the 0.45–1.80 mmol/L treatments. XDH gene expression in BMECs showed no significant regression relationship with Leu concentration (P>0.05).

Table 2 Effects of Leu on TG content and expression of genes related to milk fat synthesis in BMECs

Items	Leu concentration (mmol/L)	P-value	Linear	Quadratic
TG content	-	-	-	-
Relative expression levels of genes related to milk fat synthesis	-	-	-	-
FABP3	-	-	<0.001	-
LPL	-	-	-	<0.001
FASN	-	-	-	<0.001
ACACA	-	-	-	<0.001
SCD1	-	-	-	-
PPARγ	-	-	-	-
SREBP1	-	-	-	<0.001
AGPAT6	-	-	<0.001	-
GPAM	-	-	<0.001	-
LPIN1	-	-	<0.001	-
BTN1A1	-	-	<0.001	-
XDH	-	-	-	-

P<0.05 means regression relationship was significant; 0.05≤P<0.10 means regression relationship tend to be significant. The same as below.

3.2 Effects of Leu on Expression of Milk Fat Synthesis-Related Proteins in BMECs

As shown in Table 3 and Figure 1 [Figure 1: see original paper], the relative expression of PPARγ protein in BMECs showed no significant dose-dependent relationship with Leu concentration (P>0.05). However, PPARγ expression was numerically higher in the 0.90–2.70 mmol/L treatments and lower in the 3.60–7.20 mmol/L treatments. The relative expression of SREBP1 protein in BMECs increased significantly in a quadratic manner with increasing Leu concentration (P=0.032), with higher expression observed in the 0.90–3.60 mmol/L treatments, particularly peaking in the 1.80 mmol/L treatment, and lowest expression in the 7.2 mmol/L treatment.

Table 3 Effects of Leu on expression of proteins related to milk fat synthesis in BMECs

Items	Leu concentration (mmol/L)	P-value	Linear	Quadratic
PPARγ	-	-	-	-
SREBP1	-	-	-	<0.001

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PPARγ: peroxisome proliferator-activated receptor gamma; SREBP1: sterol regulatory element binding protein 1.

Figure 1 Effects of Leu on expression of proteins related to milk fat synthesis in BMECs

4 Discussion

The free diffusion of fatty acids within cells is inefficient and lacks biological targeting, necessitating the involvement of specific transport proteins for targeted fatty acid transport [14]. LPL and FABP3 genes are important for long-chain fatty acid (LCFA) uptake and intracellular transport in various mammalian tissues [15]. In dairy cows fed corn straw as roughage, arterial infusion of AAs reduced mammary uptake of LCFAs and decreased the content of C18:0, c-9,c-12-C18:2, and C18:3 in milk fat [16]. Our results demonstrated that increasing Leu concentration decreased the relative expression of FABP3 and LPL genes in BMECs, suggesting that Leu may inhibit LCFA uptake and transport in BMECs. Currently, few studies have reported on the effects of Leu on FABP3 and LPL gene expression, warranting further investigation.

Sterol regulatory element binding proteins (SREBPs) and PPARγ are important regulators of milk fat synthesis. Silencing SREBP1 gene in BMECs significantly reduced the expression of ACACA and FASN genes [17]. Treatment

of BMECs with the PPARγ activator rosiglitazone increased the expression of FASN and ACACA genes [18]. ACACA is a rate-limiting enzyme in de novo fatty acid synthesis, catalyzing the carboxylation of acetyl-coenzyme A (CoA) to malonyl-CoA. FASN is a multifunctional enzyme system that participates in fat generation and deposition as a metabolic enzyme, representing a key enzyme in the fat synthesis pathway. During lactation, the FASN protein encoded by FASN gene in mammary tissue regulates the synthesis of short- and medium-chain fatty acids (SMCFA) (C4–C16) [19]. Cheng et al. [2] found that Leu deficiency in mouse diets significantly decreased the expression of FASN and ACACA genes and the protein levels of FASN and SREBP1c in white adipose tissue. Another study reported that Leu upregulated SREBP1 gene expression in BMECs [3]. Our results showed that 1.8–2.7 mmol/L Leu upregulated the expression of SREBP1 and PPARγ genes and proteins, accompanied by increased expression of their target genes ACACA and FASN. This indicates that Leu significantly influences milk fat synthesis, with SREBP1 and PPARγ potentially participating in Leu' s regulatory effects. Moreover, the impact of Leu on milk fat synthesis is dose-dependent, with 1.8–2.7 mmol/L Leu promoting SMCFA synthesis.

GPAM, AGPAT6, and LPIN1 are major genes involved in TG synthesis, and their encoded proteins are key enzymes in milk fat synthesis [20]. GPAM catalyzes the binding of acyl-CoA to the sn-1 position of glycerol-3-phosphate to form lysophosphatidic acid. AGPAT catalyzes the binding of a second acyl-CoA to the sn-2 position of glycerol-3-phosphate to form phosphatidic acid. LPIN1 transfers phosphate groups, converting phosphatidic acid to diacylglycerol, after which another acyl-CoA is esterified to the sn-3 position of glycerol to form TG. TG in mammary cells forms lipid droplets through the action of various proteins. BTN1A1 and XDH are major proteins involved in lipid droplet formation [21]. BTN1A1 assists in milk fat globule formation during secretion from mammary cells, while XDH plays an important role in coupling milk fat globules to the apical membrane. Branched-chain amino acids (BCAAs) such as Leu, isoleucine (Ile), and valine (Val) affect obesity and lipid metabolic balance in mice fed high-fat diets, with BCAA-supplemented mice showing significantly reduced TG content in liver and muscle tissues [22]. However, Leu intake at more than twice the daily requirement has no significant effect on plasma cholesterol and TG synthesis in mice [5]. These inconsistent results suggest that the effects of Leu on TG synthesis vary. Our findings indicated that although high concentrations (3.60–7.20 mmol/L) of Leu inhibited GPAM and AGPAT6 gene expression, they did not significantly suppress TG synthesis. The results also showed that Leu promoted de novo fatty acid synthesis while inhibiting genes related to LCFA uptake and transport, which may explain the lack of significant effect on TG synthesis. Additionally, high Leu concentrations downregulated BTN1A1 gene expression involved in lipid droplet formation. In goat BMECs, interference with FASN gene significantly decreased BTN1A1 expression and lipid droplet formation [23], suggesting that the downregulation of BTN1A1 in BMECs treated with high Leu concentrations may be associated with decreased

FASN expression. Overall, Leu promoted the expression of de novo fatty acid synthesis genes FASN and ACACA in a dose-dependent manner, with optimal promotion of FASN at 1.80–2.70 mmol/L Leu and ACACA at 1.80–7.20 mmol/L Leu. Leu upregulated SREBP1 gene and protein expression, with the best promotion at 1.80 mmol/L. Leu inhibited FABP3, LPL, AGPAT6, GPAM, and BTN1A1 gene expression, but substantial inhibition occurred only at high concentrations (3.60–7.20 mmol/L). Considering multiple indicators of milk fat synthesis, Leu at 1.80–2.70 mmol/L yielded the best results.

5 Conclusion

At Leu concentrations of 1.80–2.70 mmol/L, the expression of FASN, ACACA, and SREBP1 genes and SREBP1 protein in BMECs was effectively promoted, while the expression of genes related to TG synthesis and lipid droplet formation was minimally inhibited.

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