Effect of Methionine Tripeptide on Casein and Oligopeptide Transporter Gene Expression in Bovine Mammary Epithelial Cells (Postprint)

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Abstract

This study aimed to investigate the effects of methionine tripeptide (Met-Met-Met) on the expression levels of casein and peptide transporter genes in bovine mammary epithelial cells (BMECs). Passage 3 bovine mammary epithelial cells cultured by enzymatic digestion were used as the experimental model. Treatments consisted of 0 (control), 40, 50, 60, 70, and 80 g/mL methionine tripeptide supplemented into the culture medium, with 5 replicates per treatment and 1 culture well per replicate; cells were cultured for 24, 48, and 72 h, and the relative proliferation rate of bovine mammary epithelial cells was measured; the complete experiment was repeated twice to determine the optimal culture time. Treatments consisted of 0 (control), 40, 50, 60, 70, and 80 g/mL methionine tripeptide supplemented into the culture medium, with 3 replicates per treatment and 1 culture well per replicate; cells were cultured for the optimal duration, and casein gene expression levels were detected by real-time quantitative PCR to determine the appropriate methionine tripeptide concentration; the complete experiment was repeated three times. With the optimal culture time and methionine tripeptide concentration, peptide transporter gene expression levels were measured, with methionine tripeptide-free medium serving as the control, 3 replicates per treatment, and 1 culture well per replicate; the complete experiment was repeated three times. Results demonstrated that the relative proliferation rate was highest following 24 h culture of BMECs with methionine tripeptide supplementation; following 24 h culture with 60 g/mL methionine tripeptide, the expression levels of αs1-casein and β-casein genes were highest, and the expression levels of peptide transporter 1 and peptide transporter 2 genes in bovine mammary epithelial cells were significantly higher than those in the control treatment ($P<0.05$). In summary, supplementation of the culture medium with 60 g/mL methionine tripeptide enhances casein and peptide transporter gene expression levels in bovine mammary epithelial cells.

Full Text

Effects of Methionine Tripeptide on Expression Levels of Casein and Small Peptide Transporter Genes in Bovine Mammary Epithelial Cells

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Abstract: This experiment investigated the effects of methionine tripeptide (Met-Met-Met) on expression levels of casein and small peptide transporter genes in bovine mammary epithelial cells (BMECs). Using third-passage BMECs cultured via enzymatic digestion as a model, different concentrations of Met-Met-Met [0 (control), 40, 50, 60, 70, and 80 μg/mL] were added to the culture medium. Each treatment comprised 5 replicates with one culture well per replicate, and cells were cultured for 24, 48, and 72 h to measure relative growth rate (RGR). The entire experiment was repeated twice to determine the optimal culture duration. Subsequently, different concentrations of Met-Met-Met [0 (control), 40, 50, 60, 70, and 80 μg/mL] were added to the culture medium, with each treatment having 3 replicates and one culture well per replicate. Cells were cultured for the optimal duration determined previously, and casein gene expression levels were measured by real-time quantitative PCR to identify the appropriate Met-Met-Met concentration. This portion of the experiment was repeated three times. Finally, using the optimal culture time and appropriate Met-Met-Met concentration, cells were cultured with Met-Met-Met-supplemented medium (treatment) and without Met-Met-Met (control). Each treatment had 3 replicates with one culture well per replicate, and small peptide transporter gene expression levels were measured, with the entire experiment repeated three times. The results showed that the relative proliferation rate was highest when BMECs were cultured with Met-Met-Met for 24 h. When cells were cultured for 24 h with 60 μg/mL Met-Met-Met, the expression levels of αs1-casein and β-casein genes were highest, while the expression levels of small peptide transporter 1 and small peptide transporter 2 genes were significantly higher than those in the control ($P<0.05$). In conclusion, adding 60 μg/mL Met-Met-Met to the culture medium can enhance the expression of casein and peptide transporter genes in BMECs.

Keywords: methionine tripeptide; bovine mammary epithelial cells; casein; small peptide transporters

Classification Number: S823

Milk protein is a crucial indicator of milk quality, with casein accounting for 80% of total milk protein content. Casein comprises four types: αs1-casein (CSN1S1), αs2-casein (CSN1S2), β-casein (CSN2), and -casein (CSN3), which constitute approximately 36.5%–40.7%, 9.7%–10.2%, 26.8%–30.5%, and 6.8%–

9.7% of total casein, respectively [1]. Traditionally, free amino acids were considered sufficient to meet the synthetic metabolic requirements of various animal tissues [2]. However, recent research has revealed that small peptide absorption occurs in ruminants, and small peptides in blood circulation can participate in milk protein synthesis in bovine mammary epithelial cells (BMECs), partially compensating for insufficient free amino acid uptake during mammary protein synthesis [3]. Backwell et al. [4] provided lactating dairy cows with histidine in dipeptide form versus free histidine in equal amounts, demonstrating that the dipeptide form promoted greater milk protein synthesis. Wang [5] found that adding amino acid dipeptides during BMECs culture increased milk protein synthesis. Gao et al. [6] also added different concentrations of methionine-methionine dipeptide, methionine-lysine dipeptide, lysine-lysine dipeptide, and lysine-methionine dipeptide during BMECs culture, confirming that appropriate concentrations of dipeptides could promote CSN2 gene and protein expression.

Two small peptide transporters have been identified in bovine mammary tissue: peptide transporter 1 (PepT1) and peptide transporter 2 (PepT2) [7-8]. These transporters function based on directed proton gradients and negative membrane potentials within their systems [9-10], transporting most dipeptides and tripeptides but generally not peptides with more than three amino acid residues [1,4].

Previous studies on milk protein synthesis in BMECs have preliminarily investigated dipeptides composed of methionine, the first limiting amino acid. However, no reports exist on methionine tripeptide (methionyl-methionyl-methionine, Met-Met-Met). Therefore, this experiment added different concentrations of Met-Met-Met during BMECs culture to investigate its effects on relative proliferation rate and the expression levels of casein and small peptide transporter genes, providing a theoretical basis for improving milk protein yield and milk quality.

1.1 Experimental Materials

DMEM/F12 medium, type II collagenase, penicillin-streptomycin solution, 0.25% trypsin/EDTA, and insulin-transferrin solution were purchased from Gibco. Fetal bovine serum (FBS) was obtained from BI. The tissue/cell total RNA extraction kit (DP431) was from TIANGE. PrimeScript RT Master Mix, SYBR Premix Ex TaqTM II, ABI PrismTM (KR0390-v8.13), 6× loading buffer, and DL2000 DNA marker were purchased from TAKARA (Shanghai). Hydrocortisone was from Sigma. MTT, DMSO, and amphotericin B were from Amresco. DPBS was from HyClone. Met-Met-Met was synthesized by Sangon Biotech (Shanghai) with a purity of 98.10% and relative molecular mass of 411.61 according to the synthesis report.

Major instruments included: CO2 incubator (HF-240, Heal Force Bio-Meditech Holdings), inverted microscope (Olympus), automatic microplate reader (Synergy H4, BioTek), gradient PCR instrument (Veriti Thermal Cycler, Thermo),

real-time quantitative PCR instrument, and electrophoresis system (Bio-Rad).

1.2 Experimental Design

This experiment employed a single-factor completely randomized design. For determining the optimal culture duration, different concentrations of Met-Met-Met [0 (control), 40, 50, 60, 70, and 80 μg/mL] were added to the culture medium, with each treatment having 5 replicates (one culture well per replicate). Cells were cultured for 24, 48, and 72 h, and BMECs relative growth rate (RGR) was measured. The entire experiment was repeated twice. To determine the appropriate Met-Met-Met concentration, different concentrations [0 (control), 40, 50, 60, 70, and 80 μg/mL] were added to the culture medium, with each treatment having 3 replicates (one culture well per replicate). Cells were cultured for the optimal duration determined previously, and casein gene expression levels were measured by real-time quantitative PCR. This portion was repeated three times. Finally, using the optimal culture time and appropriate Met-Met-Met concentration, cells were cultured with Met-Met-Met-supplemented medium (treatment) and without Met-Met-Met (control). Each treatment had 3 replicates (one culture well per replicate), and small peptide transporter gene expression levels were measured, with the entire experiment repeated three times.

1.3 BMECs Culture Method

BMECs were obtained via enzymatic digestion. Healthy Holstein cow mammary tissue was obtained (from Beiya Halal Cold Storage, Hohhot, Inner Mongolia). Surface tissue was removed, and approximately 1 cm^3 tissue blocks were excised from deep tissue and placed in pre-cooled DPBS. In a biosafety cabinet, tissue blocks were washed with DPBS, surface layers were trimmed, and the trimmed blocks were minced into a paste in centrifuge tubes. An equal volume of 0.5% collagenase II solution was added, and the mixture was digested for 1 h at 37 °C in 5% CO2, with shaking every 20 min. The digest was filtered through an 80-mesh cell strainer, and the filtrate was centrifuged at 1,300 r/min for 5 min. The supernatant was discarded, and BMECs induction medium [DMEM/F12 containing 10% FBS, 1 mL penicillin-streptomycin, 0.5 mL insulin-transferrin, 100 μL amphotericin B, and 100 μL hydrocortisone per 100 mL] was added. Cells were resuspended, transferred to 25 cm^2 culture flasks, and cultured at 37 °C in 5% CO2. Cell growth was observed daily. When cells reached 85%–95% confluence, BMECs were purified based on their differential sensitivity to trypsin digestion compared to bovine mammary fibroblasts and passaged. Third-passage cells were used in this study.

1.4.1 MTT Assay for Relative Proliferation Rate

Cell relative proliferation rate was measured using the MTT method [12] to assess cell viability. Third-passage BMECs were collected and suspended in BMECs induction medium, then seeded in 96-well plates (Corning, 3599) at 1$×10^{4}$ cells/well. After 48 h incubation at 37 °C in 5% CO2, medium

was removed and replaced with serum-free BMECs induction medium for 24 h starvation. Serum-free induction medium containing different Met-Met-Met concentrations was then added, and cells were cultured for 24, 48, or 72 h. Four hours before the end of culture, 20 µL MTT (5 mg/mL) was added to each well. After 4 h, supernatant was discarded, 150 µL DMSO was added to each well, and plates were shaken for 10 min. Absorbance at 490 nm (OD_{490}) was measured using an automatic microplate reader. Each treatment had 5 replicates (one culture well per replicate), and the entire experiment was repeated twice. Relative proliferation rate was calculated as: (OD_{490} of treatment / OD_{490} of control) × 100.

1.4.2 Real-Time Quantitative PCR for Casein Gene Expression

Third-passage BMECs were seeded in 6-well plates (Corning, 3599) at 2$×10^{5}$ cells/well and cultured for 48 h at 37 °C in 5% CO2. Medium was then removed and replaced with serum-free BMECs induction medium for 24 h starvation. Serum-free induction medium containing different Met-Met-Met concentrations was added, with each treatment having 3 replicates (one culture well per replicate). Cells were cultured for the optimal duration determined from proliferation assays. Total RNA was extracted using the tissue/cell total RNA extraction kit. RNA integrity and purity were assessed by 1% agarose gel electrophoresis and microplate reader. Reverse transcription was performed using PrimeScript RT Master Mix. Real-time quantitative PCR was conducted using SYBR Premix Ex TaqTM II in a 20 µL reaction volume, with each replicate analyzed three times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene to measure expression of CSN1S1, CSN1S2, CSN2, and CSN3 genes. Primer sequences and parameters are shown in Table 1 . The PCR program was: 95.0 °C for 30 s; 40 cycles of 95.0 °C for 30 s, annealing temperature for 30 s, and 72.0 °C for 20 s. Melting curve analysis was performed from 70–95 °C, increasing 0.5 °C every 6 s for 51 cycles. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

1.4.3 Real-Time Quantitative PCR for Small Peptide Transporter Gene Expression

Third-passage BMECs seeded in 6-well plates were cultured using the optimal culture time determined from proliferation assays and the appropriate Met-Met-Met concentration determined from casein expression analysis. Each treatment had 3 replicates (one culture well per replicate). After culture, total RNA was extracted and reverse-transcribed. Using GAPDH as the reference gene, expression levels of small peptide transporters (PepT1 and PepT2) were measured with primers shown in Table 1. The PCR program and melting curve analysis were identical to section 1.4.2, and results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Table 1 Primer sequences and parameters

Gene	Primer sequences (5' –3')	GenBank No.	Length/bp
αs1-casein (CSN1S1)	F: AATCCATGCCCAACA-GAAAGR: TCAGAGC-CAATGGGATTAGG	BC109618	56.0
αs2-casein (CSN1S2)	F: AGCTCTCCACCAGTGAG-GAAR: GCAAGGCGAATTTCTG-GTAA	NM-174528.2	59.0
β-casein (CSN2)	F: GTGAG-GAACAGCAGCAAACAR: CCAGGAGCAAAACCAA-GAAC	NM-181008	56.0
-casein (CSN3)	F: CCAGGAGCAAAACCAA-GAACR: TGCAACTGGTTTCT-GTTGGT	NM-174294	56.0
PepT1	F: TGGTCAATGAGTTCTGC-GAAAGR: CGAGGATGGGCGTTAG-GTAG	HC-402477	59.0
PepT2	F: ATGGCAATGCCCAATGAAR: CACCAACACAGCAACAAA-CAAA	NM-001079582	59.0
GAPDH	F: GGGTCATCATCTCTG-CACCTR: GGTCATAAGTCCCTC-CACGA	XM-001252479	56.0

F: forward primer; R: reverse primer.

1.5 Statistical Analysis

Data were analyzed using SAS 9.0 software. Variance analysis was performed on relative proliferation rate and casein gene expression data, with linear and quadratic regression analysis. Small peptide transporter gene expression data were analyzed by t-test. $P<0.05$ was considered statistically significant.

2.1 Effects of Met-Met-Met Concentration and Culture Time on BMECs Relative Proliferation Rate

As shown in Table 2 , when cells were cultured with different Met-Met-Met concentrations for 24 h, relative proliferation rate showed a linear trend with increasing concentration ($P=0.1012$), peaking at 50 μg/mL. The regression equation

was Y = -0.00347X + 1.22374, R^2 = 0.6461, where X represents Met-Met-Met concentration and Y represents relative proliferation rate, though the curve fit was poor. When cultured for 48 h, relative proliferation rate showed a quadratic trend (P=0.2762) with increasing concentration, described by Y = 0.0001X^2 - 0.0141X + 1.3758, R^2 = 0.7238, indicating initial inhibition followed by promotion of cell growth, but with low regression fit. When cultured for 72 h, cells showed a similar quadratic trend (P=0.0060) with the equation Y = 0.0001X^2 - 0.0062X + 1.0524, R^2 = 0.9940, demonstrating clear initial inhibition followed by promotion of growth with increasing Met-Met-Met concentration. The highest relative proliferation rate occurred at 50 μg/mL Met-Met-Met for 24 h, establishing 24 h as the optimal culture time.

Table 2 Effects of Met-Met-Met concentration and culture time on RGR of BMECs (%)

Culture time (h)	Met-Met-Met concentration (μg/mL)	P-value					Linear	Quadratic
	0	40	50	60	70	80		
24	100.00c	104.53b	109.65a	104.73b	98.80b	95.51d	1012	<0.0001
48	100.00ab	102.22ab	99.05a	97.15b	105.16a	106.01a	762	<0.0001
72	100.00ab	99.36b	93.81b	97.35ab	104.46a	104.61a	0060	<0.0001

Values in the same row with the same or no letter superscripts indicate no significant difference (P>0.05), while different letters indicate significant difference (P<0.05). The same applies below.

2.2 Effects of Met-Met-Met Concentration on Casein Gene Expression in BMECs

As shown in Table 3 , after 24 h culture, CSN1S1, CSN1S2, and CSN2 gene expression levels in BMECs showed quadratic trends with increasing Met-Met-Met concentration. The regression equations were: Y = -0.0009X^2 + 0.1194X - 2.8894, R^2 = 0.7290, P = 0.2710 for CSN1S1; Y = -0.0005X^2 + 0.0740X - 1.5911, R^2 = 0.9165, P = 0.0835 for CSN1S2; and Y = -0.0013X^2 + 0.1566X - 3.7503, R^2 = 0.8872, P = 0.1128 for CSN2, where X represents Met-Met-Met concentration and Y represents gene expression level. This indicated dose-dependent relationships, though not statistically significant (P>0.05). CSN3 expression showed an opposite quadratic trend (Y = 0.0007X^2 - 0.0845X + 3.2983, R^2 = 0.9211, P = 0.0789). Overall, 60 μg/mL Met-Met-Met showed the best promoting effect on casein gene expression.

Table 3 Effects of Met-Met-Met concentration on expression levels of casein genes in BMECs

Gene	Met-Met-Met concentration (μg/mL)	P-value					Linear	Quadratic
	0	40	50	60	70	80		
αs1-casein (CSN1S1)	1.00a	0.44e	0.58d	1.08a	0.81b	0.68c	0.03	<0.0001
αs2-casein (CSN1S2)	1.00a	0.56d	0.72c	0.94b	1.08a	0.91b	0.03	<0.0001
β-casein (CSN2)	1.00a	0.43c	0.75b	1.06a	0.70b	0.44c	0.03	<0.0001
-casein (CSN3)	1.00a	1.00a	0.66c	0.60d	0.66c	0.72b	0.02	<0.0001

2.3 Effects of Optimal Met-Met-Met Concentration on PepT1 and PepT2 Gene Expression in BMECs

As shown in Table 4 , compared with the control, 60 μg/mL Met-Met-Met significantly upregulated PepT1 and PepT2 gene expression (P<0.05).

Table 4 Effects of Met-Met-Met at proper concentration on expression levels of PepT1 and PepT2 genes in BMECs

Gene	Met-Met-Met concentration (μg/mL)	P-value
PepT1	0: 1.00a60: 1.38b	<0.05
PepT2	0: 1.00a60: 1.45b	<0.05

Approximately 90% of milk protein in dairy cows is synthesized from free amino acids in blood, while 10% is derived from amino acids in small peptide form for milk protein synthesis [1,4]. Small peptide absorption and utilization in animal tissues primarily depends on an independent transporter system that operates against concentration gradients of hydrogen (H^+) and calcium (Ca^{2+}) ions. Small peptide transporters offer advantages including fast transport rates, low energy consumption, and non-saturable transport [16], whereas amino acid transporters are substrate-specific with high energy consumption and limited capacity [17], theoretically making small peptide utilization more efficient than free amino acids. Small peptide transporters belong mainly to the solute carrier 15 (SLC15) family, with PepT1 and PepT2 being crucial for small peptide transport in lactating animals. These transporters utilize electronic gradients to move most dipeptides, tripeptides, and many peptidomimetics from extracellular to intracellular spaces [18-19]. PepT1 is a broad-spectrum transporter with low affinity and high capacity, while PepT2 exhibits the opposite

characteristics—high affinity and low capacity [20]. Cui [8] added threonine-phenylalanine-phenylalanine (Thr-Phe-Phe) tripeptide to BMECs culture and found significantly enhanced PepT1 gene expression. Zhou et al. [21-22] inhibited PepT2 transport function in lactating cow mammary explants and observed significantly reduced milk protein synthesis. Additionally, in vitro cultured BMECs could uptake phenylalanine-phenylalanine dipeptide (Phe-Phe) to promote PepT2 expression for milk protein synthesis, indicating that both PepT1 and PepT2 play important roles in mammary small peptide uptake.

Relative proliferation rate is a key indicator of cell viability and proliferation. This study found that Met-Met-Met, as a precursor for casein synthesis, regulated BMECs viability in a concentration-dependent manner. Different proliferation trends emerged with extended culture time; the highest relative proliferation rate (viability) occurred at 24 h, differing from the optimal time for dipeptides [23]. This may be because tripeptides contain more amino acid residues than dipeptides, accelerating cellular metabolic processes upon entry. Prolonged culture may lead to nutrient deficiency or accumulation of metabolites that inhibit cell growth.

When cultured for the optimal duration, 60 μg/mL Met-Met-Met yielded the highest CSN1S1 and CSN2 gene expression, while 70 μg/mL produced the highest CSN1S2 expression. CSN3 showed a decreasing-then-increasing trend, possibly because its primary function is preventing casein aggregation and precipitation [24], leading to some suppression in vitro. These results demonstrate that after 24 h culture with different Met-Met-Met concentrations, 60 μg/mL produced the highest CSN1S1 and CSN2 expression while suppressing CSN3, similar to findings by Sun [23] who observed CSN3 suppression when substituting free methionine with methionine dipeptide in BMECs culture.

Small peptide transport primarily depends on PepT1 and PepT2, both exhibiting significant substrate specificity and multiple transmembrane domains [25-26]. This study demonstrates that optimal Met-Met-Met concentration can promote PepT1 and PepT2 expression, proving BMECs can uptake and utilize longer peptide chains than dipeptides for milk protein synthesis and cell proliferation. However, the specific utilization mechanisms of small peptides in BMECs and the optimal ratio of small peptides to free amino acids for casein synthesis require further investigation, as do the underlying mechanisms.

In conclusion, Met-Met-Met can regulate BMECs proliferation, with 60 μg/mL Met-Met-Met for 24 h producing the highest relative proliferation rate while promoting casein and both small peptide transporter gene expression. This confirms that Met-Met-Met can be transported into cells via these two peptide carriers to participate in milk protein synthesis in mammary cells.

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