Effects of Methionine-Containing Dipeptides on Expression of Genes Related to Milk Protein Synthesis in Dairy Cow Mammary Epithelial Cells: Postprint

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Abstract

This study aimed to investigate the effects of methionine (Met)-containing dipeptides on the expression of milk protein synthesis-related genes in bovine mammary epithelial cells (BMECs). The experiment consisted of three parts, all employing a single-factor completely randomized design, with Met supplementation concentration and culture time set at 60 g/mL (0.402 mmol/L) and 48 h, respectively. In Part 1, eight Met-containing dipeptides [methionine-methionine (P-Met-Met), methionine-lysine (P-Met-Lys), methionine-tryptophan (P-Met-Trp), methionine-phenylalanine (P-Met-Phe), methionine-threonine (P-Met-Thr), methionine-isoleucine (P-Met-Ile), methionine-leucine (P-Met-Leu), methionine-valine (P-Met-Val)] were added to the culture medium, with no dipeptide addition as the control, to determine the expression levels of milk protein synthesis-related genes (αs1-casein, β-casein, -casein, β-lactoglobulin, peptide transporter 2, and aminopeptidase N) in BMECs. In Part 2, eight free amino acids corresponding to the above dipeptides (F-Met-Met, F-Met-Lys, F-Met-Trp, F-Met-Phe, F-Met-Thr, F-Met-Ile, F-Met-Leu, F-Met-Val) were added to the culture medium, with no free amino acid addition as the control, to determine the expression levels of milk protein synthesis-related genes in BMECs. In Part 3, isomolar replacement of the corresponding free amino acids with dipeptides was performed to determine the expression levels of milk protein synthesis-related genes in BMECs as well as intracellular and extracellular aminopeptidase content. The results showed that the P-Met-Met and P-Met-Lys groups upregulated the expression levels of αs1-casein and β-casein genes compared with the control and other dipeptide groups, with the P-Met-Met group showing superior effects to the P-Met-Lys group. The F-Met-Met and F-Met-Lys groups significantly increased the

expression level of the αs1-casein gene compared with the control and other free amino acid groups (P<0.05). Except for the P-Met-Val and P-Met-Leu groups, replacement of free amino acids with other dipeptides increased the expression levels of milk protein and peptide transporter 2 genes to varying degrees, with P-Met-Met demonstrating the best promoting effect. In conclusion, isomolar replacement of free amino acids with Met-containing dipeptides can promote the expression of milk protein genes, with P-Met-Met showing the best effect.

Full Text

Dipeptides Containing Methionine Affect Gene Expressions Related to Milk Protein Synthesis in Bovine Mammary Epithelial Cells

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Abstract

This study investigated the effects of methionine-containing dipeptides on the expression of genes involved in milk protein synthesis in bovine mammary epithelial cells (BMECs). The experiment comprised three parts, all employing a completely randomized single-factor design. Methionine concentration and incubation time were set at 60 μg/mL (0.402 mmol/L) and 48 h, respectively. In Part 1, eight methionine-containing dipeptides [methionine-methionine (P-Met-Met), methionine-lysine (P-Met-Lys), methionine-tryptophan (P-Met-Trp), methionine-phenylalanine (P-Met-Phe), methionine-threonine (P-Met-Thr), methionine-isoleucine (P-Met-Ile), methionine-leucine (P-Met-Leu), and methionine-valine (P-Met-Val)] were added to culture medium, with a no-dipeptide control group. Expression levels of milk protein synthesis-related genes (αs1-casein, β-casein, -casein, β-lactoglobulin, peptide transporter 2, and aminopeptidase N) were measured. In Part 2, eight free amino acid mixtures corresponding to the dipeptides (F-Met-Met, F-Met-Lys, F-Met-Trp, F-Met-Phe, F-Met-Thr, F-Met-Ile, F-Met-Leu, F-Met-Val) were tested against a no-amino-acid control, with gene expression similarly assessed. In Part 3, dipeptides were used to equimolarly replace their corresponding free amino acids, and both gene expression and aminopeptidase A (APA) content inside and outside BMECs were determined.

The results showed that P-Met-Met and P-Met-Lys upregulated αs1-casein and β-casein gene expression compared to the control and other dipeptide groups,

with P-Met-Met showing superior effects. F-Met-Met and F-Met-Lys significantly increased αs1-casein gene expression relative to the control and other free amino acid groups ($P<0.05$). Except for P-Met-Val and P-Met-Leu, dipeptide substitution for free amino acids enhanced the expression of milk protein and peptide transporter genes to varying degrees, with P-Met-Met demonstrating the most pronounced promotional effect. In conclusion, equimolar substitution of free amino acids with methionine-containing dipeptides can promote milk protein gene expression, with P-Met-Met being the most effective.

Keywords: dairy cow; mammary epithelial cells; milk protein; methionine; dipeptide; gene expression

Introduction

Milk protein is a crucial indicator of milk quality, and the composition and content of milk component precursors directly affect protein synthesis in the mammary gland, thereby influencing milk quality. Methionine (Met), as a precursor for milk protein synthesis, is the primary essential amino acid and the first limiting amino acid in dairy cows. It serves as the first amino acid in translation initiation for protein synthesis and is the most important direct methyl donor in the body. A critical requirement for activating the protein translation process is methylation of the ribose connected to nucleotides at the 5' cap structure. Therefore, investigating the effects and mechanisms of methionine on milk protein synthesis is significant for regulating mammary component synthesis and improving milk quality. Wang et al. found that dietary supplementation with methionine hydroxy analog significantly increased milk yield and protein content in dairy cows. However, amino acids utilized for milk protein synthesis are not exclusively supplied as free amino acids from blood; mammary tissue can also uptake non-free amino acids such as small peptides and proteins to meet the demands of milk protein synthesis. Small peptides can compensate for insufficient amino acid uptake by mammary tissue and play functional roles in protein metabolism. Bi et al. reported that methionine-methionine (P-Met-Met) and methionine-lysine (P-Met-Lys) dipeptides promoted BMEC proliferation and β-casein (CSN2) gene expression. Using BMECs as a model, equimolar substitution of free amino acids with P-Met-Met and P-Met-Lys resulted in significantly higher αs1-casein (CSN1S1) gene expression and casein concentration in culture medium compared to free amino acid groups. These findings demonstrate that both amino acids and small peptides influence milk protein synthesis in dairy cows. Consequently, studying the effects of dipeptide substitution for free amino acids on milk protein synthesis provides theoretical and practical significance for understanding the mechanisms of precursor effects and continuously improving milk quality. However, relevant research remains limited. Therefore, this study employed BMECs as an in vitro model to investigate the effects of various methionine-containing dipeptides on the expression of milk protein synthesis-related genes and aminopeptidase N (APN), aiming to identify effective dipeptide combinations and provide a theoretical foundation

for establishing dipeptide nutritional models suitable for dairy cow lactation.

Materials and Methods

1.1 Reagents and Instruments

Collagenase II, DMEM/F12 medium, insulin-transferrin solution, fetal bovine serum (FBS), trypsin/EDTA, and antibiotic-antimycotic were purchased from Gibco. Hydrocortisone, epidermal growth factor, prolactin, methionine, lysine, tryptophan, phenylalanine, threonine, isoleucine, leucine, and valine were obtained from Sigma. Eight dipeptides (P-Met-Met, P-Met-Lys, P-Met-Trp, P-Met-Phe, P-Met-Thr, P-Met-Ile, P-Met-Leu, P-Met-Val) were purchased from Shanghai Science Peptide Biological Co., Ltd. (product numbers PO14103105 through PO14103112). MTT, DMSO, and amphotericin B were from Amresco, PBS from HyClone, SYBR Premix Ex Taq II and PrimeScript RT Master Mix from TaKaRa, and RNAprep pure Cell/Bacteria Kit from TIANGEN. Major instruments included an inverted microscope (Olympus), automatic microplate reader (Synergy H4, BioTek), cell counter (Cytorecon, ECI), CO_2 incubator (HF-240, Likon Biomedical Technology Holdings), real-time PCR system (ABI-7500, ABI), and electrophoresis apparatus (Bio-Rad).

1.2 Experimental Design

The study consisted of three parts using completely randomized single-factor designs. Based on preliminary results, methionine concentration and incubation time were set at 60 μg/mL (0.402 mmol/L) and 48 h, respectively, using DMEM/F12 medium containing 17.24 μg/mL Met. Part 1 examined eight methionine-containing dipeptides (P-Met-Met, P-Met-Lys, P-Met-Trp, P-Met-Phe, P-Met-Thr, P-Met-Ile, P-Met-Leu, P-Met-Val) against a no-dipeptide control, with each dipeptide at 0.402 mmol/L. Part 2 tested eight free amino acid mixtures (F-Met-Met, F-Met-Lys, F-Met-Trp, F-Met-Phe, F-Met-Thr, F-Met-Ile, F-Met-Leu, F-Met-Val) corresponding to the dipeptides, with each amino acid concentration matching its respective dipeptide group. Part 3 investigated equimolar dipeptide substitution for free amino acids, with eight independent comparisons between each dipeptide and its corresponding amino acid mixture. All three experiments included six replicates per group, and FBS was excluded from culture media.

1.3 Primary BMEC Culture

BMECs were isolated using collagenase digestion. Healthy Holstein cow mammary tissue was obtained, surface layers removed, and approximately 1 cm^3 samples collected in ice-cold PBS. After washing in PBS, tissues were minced and digested with 0.5% collagenase II for 1 h at 37°C and 5% CO_2, with gentle agitation every 20 min. Digested material was filtered through 80-mesh cell strainers, centrifuged at 179×g for 5 min, and resuspended in complete medium.

Cells were cultured in 25 cm^2 flasks at 37°C and 5% CO_2. Upon reaching 80-90% confluence, cells were purified and passaged based on differential trypsin sensitivity between BMECs and fibroblasts. Third-passage BMECs were used for experiments.

1.4.1 Milk Protein Synthesis-Related Gene Expression in BMECs

Third-passage BMECs were seeded at 2$×10^{5}$ cells/well in 6-well plates with six replicates per group. Total RNA was extracted using the RNAprep pure kit, with integrity and purity assessed by 1.5% agarose gel electrophoresis and microplate reader. Reverse transcription used PrimeScript RT Master Mix, and quantitative PCR was performed with SYBR Premix Ex Taq II in 20 µL reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene for quantifying expression of milk protein synthesis-related genes [αs1-casein (CSN1S1), β-casein (CSN2), -casein (CSN3), β-lactoglobulin (LGB), peptide transporter 2 (PEPT2), and aminopeptidase N (APN)] using primers listed in . The PCR program consisted of pre-denaturation at 95.0°C for 30 s, followed by 40 cycles of denaturation at 95.0°C for 30 s, annealing for 30 s, and extension at 72.0°C for 20 s, with a final extension at 72°C for 7 min. Melting curve analysis ramped from 70°C to 95°C at 0.5°C increments every 6 s for 51 cycles. Results were analyzed using the $2^{(-\Delta\Delta Ct)}$ method.

1.4.2 Aminopeptidase A (APA) Content in BMECs and Culture Medium

After 48 h incubation, culture medium was centrifuged at 15,000 g/min for 10 min at 4°C to collect supernatant. Cells were washed three times with PBS, lysed with 70 µL lysis buffer per well for 30 min at 4°C, scraped, and centrifuged at 15,000 g/min for 10 min at 4°C. APA content in both fractions was measured using ELISA kits according to manufacturer protocols.

1.5 Data Processing and Analysis

Data were compiled in Excel. Statistical analysis used SAS 9.0 software: Parts 1 and 2 employed one-way ANOVA with Duncan' s multiple comparison test, while Part 3 used t-tests to compare dipeptide and free amino acid groups. Significance was declared at $P<0.05$, with trends noted at $0.05\leq P<0.10$.

Results

2.1 Effects of Methionine-Containing Dipeptides on Milk Protein Synthesis-Related Gene Expression in BMECs

As shown in , CSN1S1 expression was significantly higher in P-Met-Met and P-Met-Lys groups compared to the control and other dipeptide groups ($P<0.05$), with P-Met-Met showing superior effects. CSN2 expression was significantly elevated in P-Met-Met and P-Met-Lys groups versus the control, P-Met-Thr,

P-Met-Trp, P-Met-Leu, P-Met-Ile, and P-Met-Val groups (P<0.05), while P-Met-Phe was significantly higher than P-Met-Thr, P-Met-Leu, P-Met-Ile, and P-Met-Val groups (P<0.05). CSN3 expression was significantly increased in P-Met-Met, P-Met-Lys, P-Met-Phe, and P-Met-Ile groups compared to P-Met-Thr, P-Met-Trp, P-Met-Leu, and P-Met-Val groups (P<0.05). No significant effects were observed on LGB, PEPT2, or APN expression among the eight dipeptides (P>0.05).

2.2 Effects of Free Amino Acids on Milk Protein Synthesis-Related Gene Expression in BMECs

demonstrates that CSN1S1 expression was significantly higher in F-Met-Met and F-Met-Lys groups than in the control and other free amino acid groups (P<0.05). CSN2 expression was significantly elevated in F-Met-Met, F-Met-Lys, F-Met-Phe, and F-Met-Leu groups compared to F-Met-Thr (P<0.05). For CSN3, F-Met-Met, F-Met-Phe, F-Met-Trp, and F-Met-Leu groups showed significantly higher expression than F-Met-Thr (P<0.05), while F-Met-Lys was significantly higher than F-Met-Phe, F-Met-Thr, F-Met-Trp, F-Met-Leu, F-Met-Ile, and F-Met-Val groups (P<0.05). PEPT2 expression was significantly increased in F-Met-Met versus F-Met-Trp, F-Met-Leu, F-Met-Ile, and F-Met-Val groups (P<0.05), and in F-Met-Lys versus F-Met-Ile (P<0.05). No significant differences were observed for LGB or APN expression among groups (P>0.05).

2.3 Effects of Equimolar Dipeptide Substitution for Free Amino Acids on Gene Expression and APA Content in BMECs

reveals that compared to F-Met-Met, P-Met-Met significantly upregulated CSN1S1, CSN2, and CSN3 expression (P<0.05). P-Met-Lys versus F-Met-Lys significantly increased CSN2 expression (P<0.05) and tended to elevate PEPT2 expression (P=0.09). P-Met-Phe significantly enhanced CSN1S1 and APN expression compared to F-Met-Phe (P<0.05). P-Met-Trp showed significantly higher CSN1S1 and PEPT2 expression than F-Met-Trp (P<0.05) with a trend for increased APN (P=0.06). P-Met-Leu versus F-Met-Leu significantly decreased CSN2 expression (P<0.05) while increasing PEPT2 expression (P<0.05) and tending to elevate APN (P=0.06). P-Met-Ile significantly upregulated CSN1S1, CSN3, and PEPT2 compared to F-Met-Ile (P<0.05). P-Met-Val significantly increased PEPT2 and APN expression versus F-Met-Val (P<0.05).

Extracellular APA content tended to be lower in P-Met-Met and P-Met-Lys groups compared to their corresponding free amino acid groups (P=0.06 and P=0.07, respectively), with no significant changes observed for other dipeptide substitutions (P>0.05). Intracellular APA content was not significantly affected by any dipeptide substitution (P>0.05).

Discussion

Approximately 90% of milk protein is synthesized from amino acids in mammary tissue, with over 90% of these amino acids absorbed from blood. However, research indicates that mammary tissue can also utilize peptide-bound essential amino acids for milk protein synthesis, though the mechanism remains unclear. Zhou et al. found that lysine-containing dipeptides promote milk protein synthesis in BMECs. Small peptide absorption by animal tissues depends on independent transport systems, primarily H^+- and Ca^{2+}-dependent carriers operating against concentration gradients. Peptide transporters exhibit rapid transport rates, low energy consumption, and low saturation characteristics, contrasting with free amino acid transporters and theoretically enabling higher utilization efficiency. PEPT2 is a low-capacity, high-affinity peptide carrier that primarily transports dipeptides, tripeptides, and peptidomimetic drugs. Studies in lactating cow mammary explants demonstrated that inhibiting PEPT2 function significantly reduces milk protein synthesis. Zhou et al. showed that BMECs can uptake phenylalanine-phenylalanine (P-Phe-Phe) dipeptide to promote PEPT2 expression for milk protein synthesis, suggesting PEPT2 plays an important role in mammary peptide uptake. Aminopeptidase A (APA) on BMEC surfaces hydrolyzes small peptides into free amino acids, representing one mechanism for providing amino acids for milk protein synthesis. When amino acid levels cannot meet lactation demands, metabolic signals regulate APA expression and activity. Thus, small peptides can be utilized by mammary tissue through both peptide transporters and APA hydrolysis pathways.

Liu et al. reported that small peptide infusion in goats promoted APN gene expression and significantly enhanced milk protein synthesis. Our results showed that P-Met-Met and P-Met-Lys increased CSN1S1 and CSN2 expression compared to controls, promoting milk protein synthesis with P-Met-Met showing superior effects. Except for P-Met-Met, P-Met-Phe, and P-Met-Thr groups, other dipeptide groups exhibited significantly or tendentially higher PEPT2 expression than free amino acid groups. APA detection revealed its presence both intracellularly and extracellularly, with most dipeptide groups showing significantly or tendentially higher APN expression than corresponding amino acid groups, indicating BMECs can produce APA to hydrolyze dipeptides for milk protein synthesis while also utilizing intact dipeptides.

Research on methionine-containing peptides demonstrated differential utilization efficiency among dipeptide types, with peptide-bound methionine utilization ranging from 35% to 122% of free methionine efficiency. P-Met-Val, P-Met-Leu, and leucine-methionine (P-Leu-Met) showed higher utilization than corresponding free amino acids. Our study found that equimolar substitution of free amino acids with eight methionine-containing dipeptides enhanced CSN1S1, CSN2, and CSN3 expression for most dipeptides except P-Met-Val and P-Met-Leu, with P-Met-Met showing the strongest promotion. Compared to F-Met-Met, P-Met-Met increased expression by 23.1%, 15.3%, and 18.5% for CSN1S1, CSN2, and CSN3, respectively. P-Met-Phe and P-Met-Lys also showed notable

increases of 12.5%, 6.1%, 11.8% and 8.0%, 15.8%, 3.7%, respectively. These results indicate higher utilization efficiency of dipeptides versus free amino acids in BMECs. The elevated PEPT2 and APN expression in dipeptide groups further supports that BMECs can synthesize milk protein through both dipeptide and amino acid uptake pathways, with small peptides being more effective promoters. However, the specific mechanisms of peptide cellular uptake and the optimal dipeptide-to-free-amino-acid ratio for milk protein synthesis require further investigation.

In conclusion, equimolar substitution of free amino acids with methionine-containing dipeptides promotes milk protein gene expression, with P-Met-Met, P-Met-Lys, P-Met-Phe, and P-Met-Ile showing particularly beneficial effects, especially P-Met-Met.

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