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Methionyl-Methionine Promotes α-s1 Casein Synthesis in Bovine Mammary Gland Explants by Enhancing Intracellular Substrate Availability and Activating JAK2-STAT5 and mTOR-Mediated Signaling Pathways[1–3]

Jian-Xiang Yang,[4,6] Cai-Hong Wang,[4,6] Qing-Biao Xu,[4] Feng-Qi Zhao,[4,5] Jian-Xin Liu,[4] and Hong-Yun Liu[4]*

[4]Institute of Dairy Science, College of Animal Sciences, Zhejiang University, Hangzhou, China; and [5]Laboratory of Lactation and Metabolic Physiology, Department of Animal Science, University of Vermont, Burlington, VT

Abstract

Background: Interest is increasing in the role of peptide-bound amino acids (AAs) in milk protein synthesis because studies have found that the uptake of some essential AAs by the mammary gland cannot meet the requirements for milk protein synthesis. Although the role of dipeptides in milk protein synthesis is clearly established, little is known about the underlying mechanisms.

Objective: The objective of this study was to determine whether small peptides can be taken up intact by the peptide transporters in mammary tissue explants and the underlying mechanisms of the effects of methionyl-methionine (Met-Met) supplementation on milk protein synthesis.

Methods: Mammary tissue explants were cultured in conditional medium and then treated with different concentrations of Met-Met that replaced 0%, 5%, 10%, 15%, 20%, and 25% of free Met for another 24 h. In some experiments, explants were cultured with an optimal dose of Met-Met with or without the inhibitors of peptide transporter 2 [PepT2; diethylpyrocarbonate (DEPC), 0.1 mmol/L] and aminopeptidase N (APN; bestatin, 20 μmol/L) for 24 h.

Results: The substitutions of 15% free Met with Met-Met significantly promoted α-s1 casein (α_{s1}-CN) expression in the mammary explants ($P < 0.05$). The inhibition of the PepT2 by DEPC or APN by bestatin significantly decreased the Met-Met–stimulated increase of α_{s1}-CN expression ($P < 0.05$). Compared with the control group (0% Met-Met), absorption of Val, Met, Leu, Phe, Lys, and His was improved, and mRNA abundance of the neutral and basic AA transporter was increased in the 15% Met-Met group ($P < 0.05$). In addition, the mRNA abundance of the mammalian target of rapamycin (*mTOR*), p70 ribosomal S6 kinase 1 gene, eukaryotic initiation factor 4E binding protein 1 gene , Janus kinase 2 (*JAK2*), and signal transducer and activator of transcription 5 (*STAT5*) was increased in the 15% Met-Met–treated group ($P < 0.05$).

Conclusion: Met-Met promoted α_{s1}-CN synthesis in cultured bovine mammary gland explants, and this stimulation may be mediated by enhanced intracellular substrate availability and by activating JAK2-STAT5 and mTOR signaling pathways. *J Nutr* 2015;145:1748–53.

Keywords: amino acid uptake, bovine mammary gland explants, methionyl-methionine, milk protein synthesis, peptide transporter 2

Introduction

Studies have found that the uptake of some essential amino acids (AAs)[7] by the mammary gland cannot meet the requirements for milk protein synthesis (1, 2). Thus, it was proposed that the peptide-bound AAs play a role in milk protein synthesis. A large portion of AAs circulating in the blood is in the form of peptides

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[3] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[6] These authors contributed equally to this work.
[7] Abbreviations used: AA, amino acid; APN, aminopeptidase N; DEPC, diethylpyrocarbonate; JAK2, Janus kinase 2; MEC, mammary epithelial cell; Met-Met, methionyl-methionine; mTOR, mammalian target of rapamycin; PepT2, peptide transporter 2; STAT5, signal transducer and activator of transcription 5; S6K1, p70 ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; α_{s1}-CN, α_{s1} casein.

*To whom correspondence should be addressed: E-mail: hyliu@zju.edu.cn.

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(3, 4). The utilization of these circulating small peptides for milk protein synthesis appears to be a general phenomenon (2). Backwell et al. (5) reported that intravenously infused peptide-bound AA promoted milk protein synthesis in goat mammary glands. Research that used lactating mouse mammary gland explants has provided evidence that small peptides containing Met could serve as a source of Met for the synthesis of secreted protein, and some Met dipeptides have a higher efficiency in milk protein synthesis than free Met (6). Recently, our studies demonstrated that small peptides containing Met, Lys, or Phe improved αs1 casein (α_{s1}-CN) synthesis in cultured bovine mammary gland explants (7, 8).

Although the role of dipeptides in milk protein synthesis is clearly established, little is known about the underlying mechanisms. It was hypothesized that small peptides can be taken up intact by the peptide transporters in mammary epithelial cells (MECs), as in the intestinal cells, and used for milk protein synthesis (9–11). It was found that the peptide transporter 2 (PepT2) serves as an integral membrane protein for the cellular uptake of dipeptides and tripeptides and peptide-like drugs in rat mammary gland extracts and human milk epithelial cells (11). PepT2 was also detected in bovine mammary glands (12). However, the enterocytes contain high levels of intracellular peptidases that can hydrolyze peptides into their constituent AAs. Kim et al. (13) detected peptidase activity in the brush border and soluble fractions of rat intestinal mucosa for 13 dipeptides and 5 tripeptides. These peptidases may play important roles in peptide absorption and utilization. It is important to know whether these peptidases are also expressed in MECs. To our knowledge, the expression of aminopeptidase N (APN) was found in caprine mammary gland and regulated by circulating plasma peptides (14). In addition to being used as the substrates of protein synthesis, small peptides may function as signaling molecules to promote AA absorption and milk protein synthesis in the mammary gland (8).

In this study, we investigated the effects of methionyl-methionine (Met-Met) supplementation on α_{s1}-CN synthesis, AA uptake, and the expression of PepT2, AA transporters, and peptidases in lactating bovine mammary explants. In addition, we investigated the mechanisms underlying the effects of Met-Met by inhibition of PepT2 and peptidase activities and by its effects on the expression of mammalian target of rapamycin (*mTOR*) and Janus kinase 2-signal transducer and activator of transcription 5 (*JAK2-STAT5*) signaling molecules.

Methods

Mammary tissue preparation and explants culture. The use of all animals in this study was approved by the Institutional Animal Care and Use Committee of Zhejiang University of China. Mammary tissues were obtained from 3 slaughtered mid-lactation Holstein dairy cows in a local abattoir and diced into pieces (1 mm^3) under sterile conditions. The tissue explants were seeded in 6-well cell culture plates with 2 mL of growth medium [DMEM-F12 supplemented with 10% FBS, 1% L-glutamine, 5 μg/mL insulin, 0.5 μg/mL prolactin, 5 μg/mL transferrin, 1 μg/mL hydrocortisone, 100 IU/mL penicillin, and 100 IU/mL streptomycin (Sigma)] and incubated at 37°C in a humidified incubator with 5% CO_2.

Treatments of cultured mammary gland explants. Mammary tissue explants were cultured in the above-mentioned growth medium that had no FBS and was supplemented with the following amounts of essential AAs to meet the optimal growth requirements of MECs: 210 μg Lys/mL, 60 μg Met/mL, 123 μg Thr/mL, 117 μg Phe/mL, 214 μg Leu/mL, 120 μg Ile/mL, 154 μg Val/mL, and 45 μg His/mL (7). All AAs are L-isomers. After 3 h, the conditional medium was replaced with the treatment medium in which 0%, 5%, 10%, 15%, 20%, and 25% of total free Met was substituted by Met-Met, respectively, and the explants were further cultured for another 24 h. In some experiments, explants were cultured with an optimal dose of Met-Met with or without the inhibitors of PepT2 [diethylpyrocarbonate (DEPC), 0.1 mmol/L] and peptidase APN (bestatin, 20 μmol/L) for 24 h.

qRT-PCR. The procedures of qRT-PCR were described previously (15). Briefly, total RNA was isolated from the explants, and reverse transcription was performed with an RT Kit (Takara). The mRNA abundance for genes was quantified with SYBR PrimeScript reagent kit (Takara) and primers listed in **Supplemental Table 1**. The relative expression of target genes was normalized to the expression of β-actin and calculated with the $2^{-\Delta\Delta CT}$ method.

Western blot analysis. Protein levels were determined by Western blot analysis according to previously described procedures with minor modifications (16). Briefly, mammary explants were lysed and centrifuged. The protein concentration of the supernatant fluids was measured with BCA protein assay (Beyotime). Protein (40 μg) was separated on polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Membranes were first incubated with primary antibodies for α_{s1}-CN (1:5000; Abcam), PepT2 (1:1500; Abcam), and β-actin (1:1000; Boster), and then with a HRP-conjugated secondary antibody (Boster). The protein bands were detected with a chemiluminescence system (CLiNX Science) and analyzed with ImageJ (version 1.32j; NIH). Band intensities were normalized against corresponding bands of β-actin.

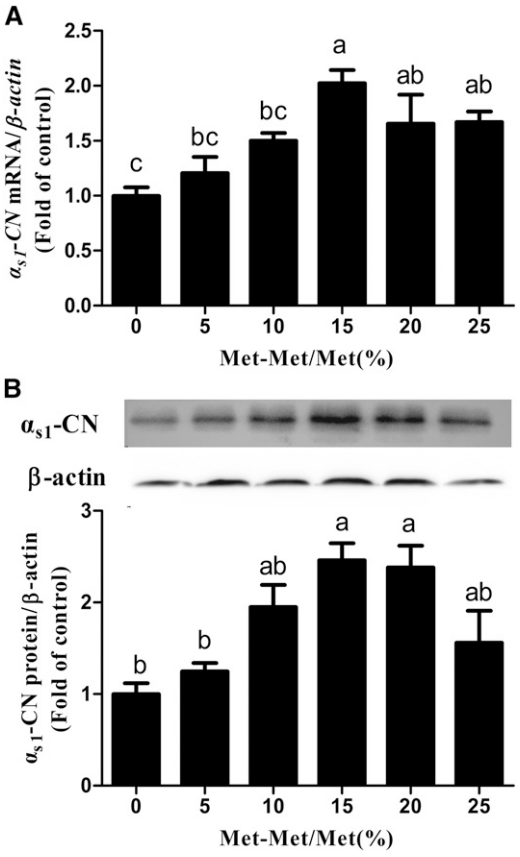

FIGURE 1 Relative mRNA (A) and protein (B) expression of α_{s1}-CN in bovine mammary explants cultured with 0%, 5%, 10%, 15%, 20%, or 25% of total Met as Met-Met. Values are means ± SEMs ($n = 3$ per group). Means without a common letter differ, $P < 0.05$. Met-Met, methionyl-methionine; α_{s1}-CN, α_{s1} casein.

AA absorption. The amount of AAs absorbed by the mammary explants was determined by measuring the difference of AA concentrations in culture media before and after the incubation of the explants. AA contents in the media were measured with the Amino Acid Auto-Analyzer Model L-8900 (Hitachi).

Statistical analysis. All experiments were performed with 3 replicates, and each experiment was independently repeated 3 times by using mammary explants from 3 cows. Data were analyzed by using the GLM procedure of SAS software (version 9.0; SAS Institute). Two-factor ANOVA was used to determine the effects of Met-Met and DEPC or bestatin on α_{s1}-CN synthesis. Other data were analyzed with 1-factor ANOVA. Statistical significance for all analyses was set at $P < 0.05$.

Results

Met-Met enhances α_{s1}-CN expression. The effects of Met-Met as a partial substitution of free Met (5–25%) on the α_{s1}-CN expression in mammary gland explants are shown in **Figure 1**. The mRNA abundance of *α_{s1}-CN* increased when 15%, 20%, and 25% of free Met was substituted by Met-Met ($P < 0.05$; Figure 1A). In addition, 15% and 20% substitutions significantly enhanced α_{s1}-CN protein expression ($P < 0.05$; Figure 1B).

Met-Met stimulates PepT2 expression. The PepT2 expression in mammary explants under different Met-Met concentrations is shown in **Figure 2**. The mRNA abundance of *PepT2* significantly increased when 15% free Met was replaced by Met-Met ($P < 0.05$; Figure 2A). A similar result was observed for PepT2 protein expression ($P < 0.05$; Figure 2B).

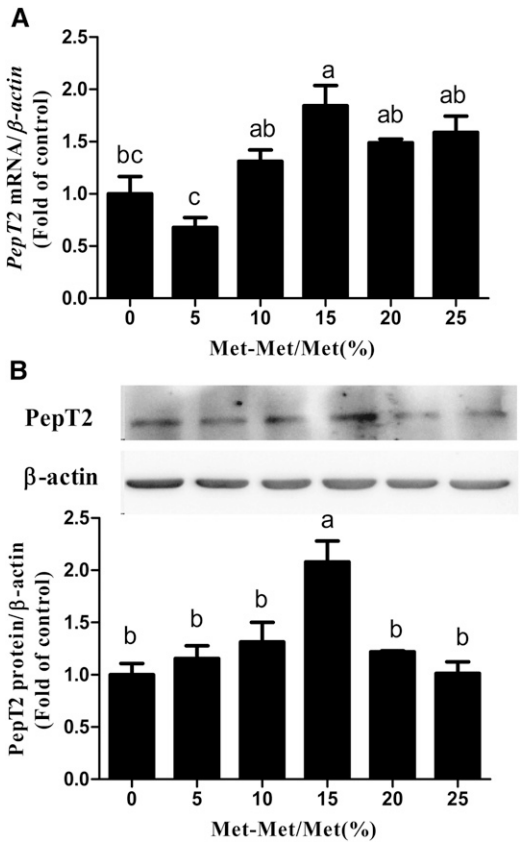

FIGURE 2 Relative mRNA (A) and protein (B) expression of PepT2 in bovine mammary explants cultured with 0%, 5%, 10%, 15%, 20%, or 25% of total Met as Met-Met. Values are means ± SEMs ($n = 3$ per group). Means without a common letter differ, $P < 0.05$. Met-Met, methionyl-methionine; PepT2, peptide transporter 2.

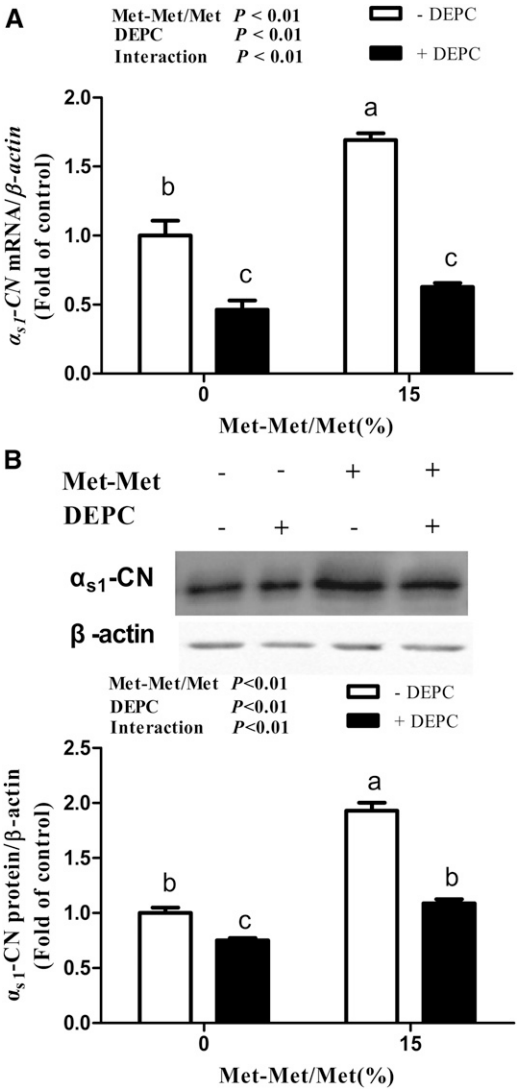

FIGURE 3 Effects of DEPC on Met-Met–stimulated expression of *α_{s1}-CN* mRNA (A) and protein (B) in bovine mammary explants cultured with or without 15% of total Met as Met-Met. Values are means ± SEMs ($n = 3$ per group). Means without a common letter differ, $P < 0.05$. DEPC, diethylpyrocarbonate; Met-Met, methionyl-methionine; α_{s1}-CN, α_{s1} casein.

PepT2 inhibitor DEPC reduces Met-Met–stimulated α_{s1}-CN expression. When DEPC was added, the increase of *α_{s1}-CN* mRNA abundance stimulated by Met-Met (15% of total Met) was totally abolished (**Figure 3**A), and the increase in the protein level of α_{s1}-CN stimulated by Met-Met was reduced from ~100% to 40% (Figure 3B). DEPC itself significantly inhibited α_{s1}-CN expression regardless of the presence of Met-Met (Figure 3A, B).

Peptidase affects Met-Met–stimulated α_{s1}-CN expression. Substitution of 15% free Met by Met-Met significantly stimulated the mRNA abundance of *APN* ($P < 0.05$; **Figure 4**A). Adding 20 μmol/L bestatin to the medium markedly decreased the Met-Met–induced increase of *α_{s1}-CN* mRNA abundance ($P < 0.05$; Figure 4B) and α_{s1}-CN protein synthesis ($P < 0.05$; Figure 4C).

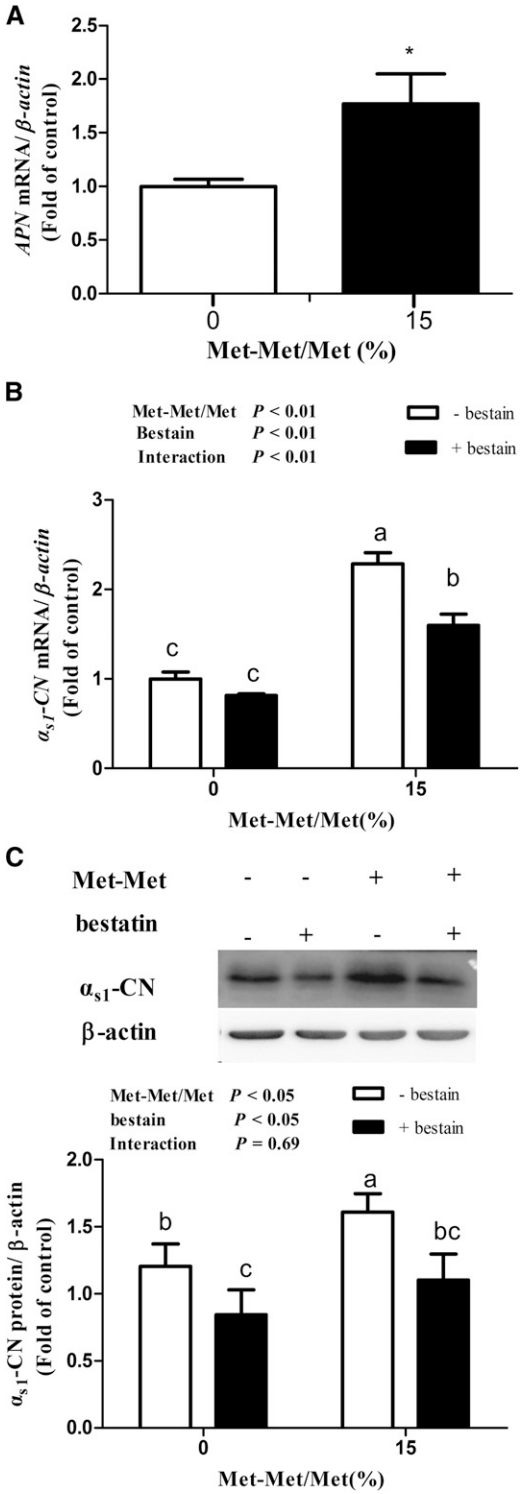

FIGURE 4 Relative mRNA abundance of *APN* (A) and α_{s1}-*CN* (B) and protein expression of α_{s1}-CN (C) in bovine mammary explants cultured with or without 15% Met-Met of total Met and bestatin. Values are means ± SEMs (n = 3 per group). *Different from 0%, $P < 0.05$. Means without a common letter differ, $P < 0.05$. APN, aminopeptidase N; Met-Met, methionyl-methionine; α_{s1}-CN, α_{s1} casein.

Met-Met stimulates AA absorption and mRNA expression of AA transporters. When 15% free Met in the medium was replaced by Met-Met, the absorption of Val, Met, Leu, Phe, Lys, and His by mammary explants and the mRNA abundance of sodium- and chloride-dependent neutral and basic AA transporter genes increased significantly ($P < 0.05$; **Figure 5**A, B). There were no significant changes in mRNA abundance of the neutral AA transporter gene, the L-type AA transporter 1 gene, the aromatic AA transporter gene, and the cationic AA transporter 1 gene (Figure 5B).

Met-Met increases the expression of JAK2-STAT5 *and mTOR signaling molecules.* Treatment of mammary explants with Met-Met (15% of total Met) significantly increased the mRNA abundance of *STAT5*, *JAK2*, *mTOR*, the p70 ribosomal S6 kinase 1 (*S6K1*), and the eukaryotic initiation factor 4E binding protein 1 (*4E-BP1*) compared with the control group ($P < 0.05$; **Figure 6**).

Discussion

Studies have indicated that free AAs are not the only substrates for milk protein synthesis; small peptides that contain 2 or 3 AA residues can also be used as precursors for milk protein synthesis in the mammary gland (5, 17). As one of the most limiting AAs for the synthesis of milk proteins by dairy cows, Met and its peptide form have been investigated for decades for their utilization in mammary tissue explants or epithelial cells (6–8). In this study, we showed that substitution of 15% free Met with Met-Met significantly promoted α_{s1}-CN expression in cultured mammary gland explants of lactating dairy cows. The result is

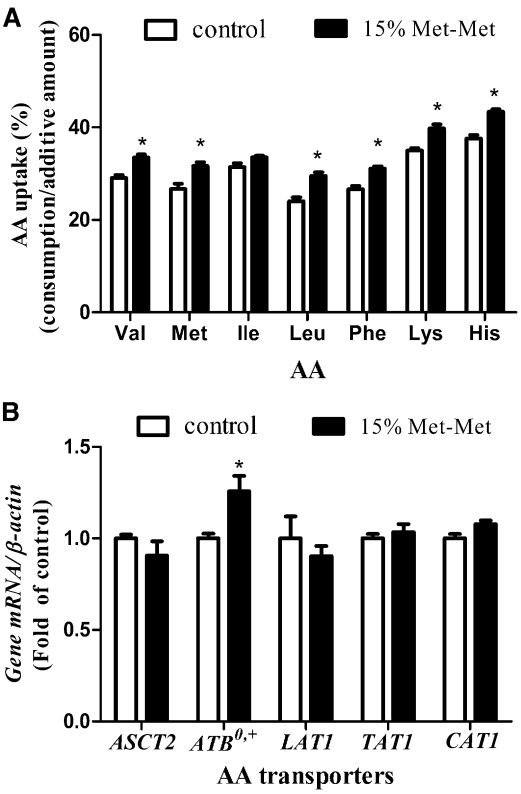

FIGURE 5 Absorption of AAs (A) and relative mRNA abundance of AA transporters (B) in bovine mammary explants cultured with or without 15% Met-Met of total Met. Values are means ± SEMs (n = 3 per group). *Different from 0%, $P < 0.05$. AA, amino acid; *ASCT2*, neutral amino acid transporter 2 gene; $ATB^{0,+}$, sodium- and chloride-dependent neutral and basic amino acid transporter gene; *CAT1*, cationic amino acid transporter 1 gene; *LAT1*, L-type amino acid transporter 1 gene; Met-Met, methionyl-methionine; *TAT1*, aromatic amino acid transporter gene.

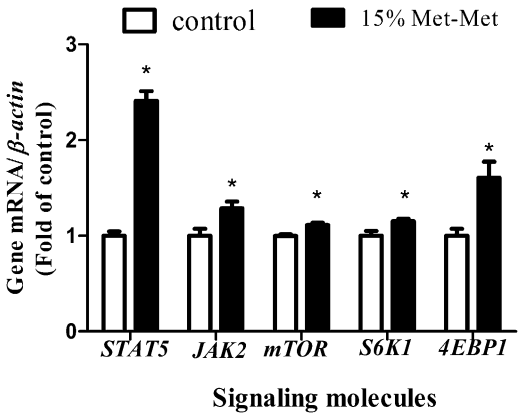

FIGURE 6 Relative mRNA abundance of *STAT5*, *JAK2*, *mTOR*, *S6K1*, and *4E-BP1* in bovine mammary explants cultured with or without 15% Met-Met of total Met. Values are means ± SEMs ($n = 3$ per group). *Different from 0%, $P < 0.05$. *JAK2*, Janus kinase 2 gene; Met-Met, methionyl-methionine; *mTOR*, mammalian target of rapamycin gene; *STAT5*, signal transducer and activator of transcription 5 gene; *S6K1*, p70 ribosomal protein S6 kinase 1 gene; *4E-BP1*, eukaryotic initiation factor 4E binding protein 1 gene.

consistent with previous findings that small peptides containing Met can be taken up and used for milk protein synthesis in lactating mouse mammary gland explants (6). In addition, our previous study demonstrated that Met-Lys significantly increased α_{s1}-*CN* mRNA abundance compared with free Met and Lys in cultured bovine MECs (7). Pan et al. (17) also found that Met-Met, Met-Val, and Leu-Met have higher efficiencies in milk protein synthesis than free Met. Thus, results from all of these studies support the hypothesis that Met dipeptides can be used with a higher efficiency than equivalent free Met in milk protein synthesis. Our present study also indicated that the best substitution ratio of Met-Met to free Met is 15%.

Small peptides can be taken up intact by mammary tissue via carrier-mediated transport systems (18). PepT1 and PepT2 are the 2 main transporters for peptides, but only PepT2 was detected in mammary tissue (13). PepT2 belongs to the family of H^+-coupled peptide transporters, and its activity is affected by H^+ (19). The histidyl residues of PepT2 are the most likely AA residues involved in H^+ binding and translocation in H^+-coupled transport systems (20). This study found that replacement of 15% free Met by Met-Met significantly promoted the expression of PepT2 in the mammary explants. DEPC, a chemical known to modify the histidyl residues of PepT2 and thus block the function of PepT2 (21, 22), significantly decreased the Met-Met–stimulated increase of α_{s1}-CN expression. However, because DEPC is not a specific inhibitor of PepT2 and also has some inhibitory effect on RNAase (23), further studies are required to identify the action of PepT2 in uptake of small peptides in MECs. On the basis of these results, we propose that the stimulating effect of Met-Met on milk protein synthesis might be at least partially via its uptake by PepT2 into MECs.

The role of APN on peptide hydrolysis was extensively studied (24). APN can split off the N-terminal Met from oligopeptides (14, 20). It was shown that knockout or overexpression of APN delays or accelerates the development of rat mammary gland, respectively (24). In addition, abomasal infusion of casein hydrolysate increases the expression of *APN* mRNA and protein by 51% and 58%, respectively, in the mammary gland (24). In this study, we showed that the mRNA abundance of *APN* in the mammary explants can also be increased by supplementation with Met-Met. In the presence of bestatin, an inhibitor of APN, the synthesis of α_{s1}-CN protein was markedly decreased by supplementation with Met-Met. However, because of the nonspecific inhibition of bestatin, further studies that use knockout or RNA interference are needed to confirm the effects of APN (25). Nevertheless, our data also support that Met-Met may be hydrolyzed into free Met by APN in mammary tissue and then used for milk protein synthesis.

Surprisingly, our study showed that Met-Met in culture medium can significantly enhance the absorption of free Val, Met, Leu, Phe, Lys, and His. The enhanced AA absorption by Met-Met may be partially explained by the reduced competition of AA transporters because of the use of peptide transporters. However, to get more direct results of absorption, isotope-labeled AAs should be used in future studies. Small peptides and AAs are transported via 2 independent and different systems (26, 27). When AAs are taken up in the form of peptides, the competition among AAs for transporter-bound sites of carriers can be partly avoided (28). However, the mRNA abundance of the sodium- and chloride-dependent neutral and basic AA transporter gene, a specific AA transporter that transports a broad spectrum of AAs, including neutral and cationic AAs (29), in mammary explants was also increased, consistent with AA absorption. The specific mechanism underlying the stimulation of the AA transporter expression by Met-Met is not known, but the AA transporter is known to function as a nutrition sensor (30). Taken together, our results indicate that the increase in α_{s1}-CN synthesis by Met-Met is partly due to the stimulation of AA absorption in cultured mammary explants.

Because Met-Met stimulated α_{s1}-*CN* mRNA expression, which cannot be explained by enhanced AA absorption, we investigated the effects of Met-Met on signal transduction pathways. Milk protein synthesis is regulated by the JAK2-STAT5 and mTOR pathways (31, 32). JAK2-STAT5 mediates prolactin- and growth factor-stimulated milk protein synthesis at the transcription level (33). Binding of prolactin and growth factors to their corresponding receptors on MECs leads to JAK2 phosphorylation, which, in turn, phosphorylates STAT5. Phosphorylated STAT5 forms dimers, enters the nucleus, and binds to the promoters and enhancers of milk protein genes, inducing their expression. In this study, the mRNA abundance of *JAK2* and *STAT5* increased significantly in mammary explants by supplementation with 15% Met-Met in the medium, which may play a role in Met-Met–stimulated α_{s1}-*CN* mRNA expression. The mTOR signaling pathway is another important pathway that couples nutrient supply to protein synthesis and cell growth (31). It was shown that dietary AAs can regulate protein synthesis in animals by regulating the translation initiation of mTOR (34). In addition, the phosphorylation of mTOR and S6K1 is also increased by AAs (35). In this study, we showed that Met-Met can increase the mRNA abundance of *mTOR*, *S6K1*, and *4E-BP1*, which may lead to increased α_{s1}-CN synthesis. On the basis of these observations, we propose that the effects of Met-Met on *JAK2-STAT5* and *mTOR* pathways might be partly due to increased AA absorption and utilization. However, the precise mechanism awaits further investigation.

In conclusion, this study revealed that Met-Met promoted α_{s1}-CN expression in cultured mammary gland explants, most likely by enhancing intracellular substrate availability and by activating JAK2-STAT5 and mTOR signaling pathways. The utilization of Met-Met by mammary tissues involves peptide transport by PepT2 and hydrolyzation by APN.

Acknowledgments

J-XY and H-YL designed the research; J-XY, C-HW, and Q-BX conducted the experiments and analyzed the data; J-XY, F-QZ, J-XL, and H-YL wrote the paper; and H-YL had primary responsibility for the final content. All authors read and approved the final manuscript.

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