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Combination of histidine, lysine, methionine, and leucine promotes β-casein synthesis via the mechanistic target of rapamycin signaling pathway in bovine mammary epithelial cells

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ABSTRACT

The ratio of different AA in the diets of cows is vital to improve milk protein yield. β-Casein is one of the important milk proteins with high nutritive value. However, the suitable ratio of essential amino acids (EAA) for the expression of β-casein in the immortalized bovine mammary epithelial cell line is not fully characterized. This study employed response surface methodology to determine the optimal ratio of His, Lys, Met, and Leu on β-casein expression level in vitro and clarified the effect of the 4 EAA on β-casein via the mechanistic target of rapamycin (mTOR) signaling pathway. A central composite design containing 5 axial points per EAA and 28 combinations of the 4 EAA was used in our study. The results of response surface methodology and the changes of the mTOR-related signaling proteins were determined by western blot. The results showed that β-casein level was significantly affected by all 4 EAA ($R^2 = 0.71$). The optimum conditions for β-casein expression are found to be 5.47 mM of His, 7.48 mM of Lys, 1.17 mM of Met, and 8.21 mM of Leu (His:Lys:Met:Leu = 5:6:1:7) in the designed scope of concentration. The interaction of Leu and Met significantly affected β-casein expression ($P < 0.01$). The phosphorylation of mTOR (Ser^{2481}), regulatory associated protein of target of rapamycin (Ser^{792}), ribosomal protein S6 kinase 1 (Thr^{389}), ribosomal protein S6 ($Ser^{235/236}$), and eukaryotic elongation factor 2 (Thr^{56}) was increased with the supplementation of either single EAA or an optimal combination of EAA. However, the phosphorylation of eukaryotic initiation factor 4E binding protein 1 (Thr^{37}) was decreased with the addition of Lys, Met, or Leu alone. Furthermore, the phosphorylation (P) of eIF2α (Ser^{51}) was decreased when Met was supplemented alone. Under the optimal mixture of 4 EAA, the phosphorylation of mechanistic target of rapamycin complex 1 signaling proteins was significantly greater than the single EAA supplementations and the expression of β-casein was 98% as high as the positive control (i.e., medium with all AA). A similar trend was found with P-ribosomal protein S6 kinase 1 and P-ribosomal protein S6. In conclusion, the extracellular concentrations of His, Lys, Met, and Leu at a ratio of 5:6:1:7 maximized β-casein expression in the immortalized bovine mammary epithelial cell line may occur via activation of the mechanistic target of rapamycin complex 1 signaling pathway.

Key words: bovine mammary epithelial cells, mechanistic target of rapamycin, β-casein, essential amino acid

INTRODUCTION

The lactating bovine mammary gland is a potent milk protein synthesizing factory (Patton, 1969). Milk protein is composed of casein (80%) and whey protein (McLean et al., 1984). Casein is a protein of high human nutritional value and is used as an important index to measure the quality of milk (Fox and Sweeney, 2003). β-Casein is one of the 4 casein proteins and constitutes about 30% of the total casein (Bionaz et al., 2012).

The amount of the essential amino acids (**EAA**) in the diet is important for milk protein synthesis, but the ratio of EAA in the diet is more important. (Park et al., 1976; Haque et al., 2012). Nan et al. (2014) revealed

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that the ratio of Met to Lys had a significant influence on the expression of casein in bovine mammary epithelial cells (**CMEC**). It was also reported that milk protein synthesis efficiency was 24.7% under a grass-based diet with 17.8% CP (Hristov et al., 2004), but this efficiency increased to 30.0% under a grass-based diet with a well-balanced addition of His, Lys, Met, and Leu even when the dietary CP was 15.0% (Haque et al., 2012). Similarly, supplying the 4 EAA (Lys, Met, His, and Leu) also increases the milk protein yield (Haque et al., 2012).

According to previous studies, His, Lys, Met, and Leu were not only the main limiting EAA in grass-based diets (Kim et al., 1999, 2000, 2001; Korhonen et al., 2002; Weekes et al., 2006; Lapierre et al., 2008), but they also were key intracellular factors presumably regulating milk protein synthesis via the mechanistic target of rapamycin complex 1 (**mTORC1**) pathway in CMEC (Prizant and Barash, 2008; Appuhamy et al., 2012; Wang et al., 2014). The mTORC1 consists of the mechanistic target of rapamycin (**mTOR**), the regulatory associated protein of target of rapamycin (**Raptor**), and the G protein β subunit-like protein (**GβL**; Kim, 2009). When mTORC1 is activated by AA, it triggers the phosphorylation of the ribosomal protein S6 kinase 1 (**S6K1**), eukaryotic initiation factor 4E binding protein 1 (**4EBP1**), and ribosomal protein S6 (**RPS6**) to stimulate mRNA translation (Anthony et al., 2000; Prizant and Barash, 2008; Appuhamy et al., 2012). In addition, eukaryotic elongation factor 2 (**eEF2**) is phosphorylated on Thr56 and may be inhibited by mTORC1 or enhanced by adenosine monophosphate-activated protein kinase (**AMPK**), which may be a limiting factor in milk protein synthesis (Christophersen et al., 2002; Arriola Apelo et al., 2014a).

The objectives of this study were to identify the optimal ratio of His, Lys, Met, and Leu for β-CN protein synthesis and to examine the role of mTORC1 pathway in regulating β-CN synthesis with respect to the optimum supplementations of the EAA in the immortalized bovine mammary epithelial cell line (**CMEC-H**).

MATERIALS AND METHODS

Materials

The individual AA (L-His, L-Lys, L-Met, and L-Leu, catalog no. H5659, L8662, M5308, and L8912, respectively) were purchased from Sigma-Aldrich (Shanghai Trading Co. Ltd., Shanghai, China). The total and site-specific phosphorylated antibodies against mTOR (Ser^{2481}, catalog no. 2913/YP1134), 4EBP1 (Thr^{37}, catalog no. 0018/YP0001), RPS6 ($Ser^{235/236}$, catalog no. 4139/YP0832), and eIF2α (Ser^{51}, catalog no. 1507/YP0093) were purchased from Immuno Way (Immuno Way Biotechnology Company, Barksdale Professional Center, Newark, DE). The total and site-specific phosphorylated antibodies against Raptor (Ser792, catalog no. 2280/2083), S6K1 (Thr389, catalog no. 9202/9205), eEF2 (Thr56, catalog no. 2332/2331), and G protein β subunit-like (GβL, catalog no. 3274) were purchased from Cell Signaling Technology Inc., (Danvers, MA). The β-CN antibody (catalog no. orb18512) was purchased from Biorbyt Ltd. (Cambridge, UK) and the antibody against β-actin (catalog no. ab8226) used as a loading control was purchased from Abcam (Shanghai Trading Co. Ltd.).

Cell Culture

The CMEC-H cells were established as described in our previous work (Hu et al., 2016). The CMEC-H cells exhibit the typical cobblestone morphology characteristic and propagation in long-term culture. These cells can express stem cell markers and can be induced to differentiate and synthesize milk proteins. The CMEC-H cells were cultured in 10-cm dishes (catalog no. 172958, Thermo Scientific, Rockford, IL) at 38°C with 5% CO_2. Dulbecco's modified Eagle's medium (**DMEM**)/Ham's F-12 (diluted 1:1, catalog no. 1439945/1491066, Invitrogen Trading Co. Ltd., Shanghai, China) containing 10% fetal bovine serum (Invitrogen Trading Co. Ltd.) and 100 μg/mL of Penicillin-Streptomycin Solution (catalog no. C0222, Beyotime Institute of Biotechnology, Jiangsu, China) was used as the basal growth medium until they reached 80% confluence and then passaged with 0.25% trpysin-0.02% EDTA (catalog no. C0203, Beyotime Institute of Biotechnology).

Experiment 1. Individual Effects of the EAA on Cell Proliferation. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (**MTT**) method was used to evaluate the effect of varying concentrations of His, Lys, Met, and Leu on the ratio of relative growth rate of the CMEC-H cells (Nan et al., 2014; Gao et al., 2015). Cells were seeded in 96-well microplates by adding a 100 μL/well of a suspension of 5,000 cells in DMEM/F-12 containing 10% fetal bovine serum and were cultured for 24 h. Afterward, the cells were serum-starved overnight and then were cultured in 200 μL of medium devoid of all AA (**−AA**; catalog no. ME14034L1, Invitrogen Trading Co. Ltd.) or in –AA medium supplemented with all AA (**+AA**) or with varying concentrations of His (at 0, 0.15, 0.6, 1.2, 4.8, 9.6, 19.2, 28.8, and 43.2 m*M*), Lys (at 0, 0.5, 2, 4, 8, 16, 24, and 36 m*M*), Met (at 0, 0.12, 0.72, 1.44, 4.32, 8.64, 17.28, 25.92, and 38.88 m*M*), and Leu (at 0, 0.45, 1.35, 5.4, 10.8, 21.6, 32.4, and 48.6 m*M*) for 12 h. Each concentration was tested in 6 independent wells and the