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D-Glucose and amino acid deficiency inhibits casein synthesis through JAK2/STAT5 and AMPK/mTOR signaling pathways in mammary epithelial cells of dairy cows

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ABSTRACT

Amino acids and energy deficiency lead to lower milk protein content in dairy cows. However, the known mechanisms involved in this process do not adequately explain the variability of milk protein concentration in the mammary gland. We hypothesized that a deficiency in D-glucose (D-Glc) or AA would inhibit casein synthesis by regulating signaling pathways in mammary epithelial cells. Cow mammary epithelial cells (CMEC) were subjected to combinations of 1 of 3 concentrations of D-Glc (0, 2.50, or 17.5 m*M*) and 1 of 3 concentrations of AA (0, 1.03, or 7.20 m*M*). The effect of each mixture on cell cycle stage was assessed by flow cytometry. The expression levels of β-casein and κ-casein (encoded by *CSN2* and *CSN3*) were measured by quantitative real-time PCR and Western blotting. Phosphorylation of Janus kinase 2 (Jak2), signal transducer and activator of transcription 5a (Stat5a), AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase 1 (S6K1), and eukaryotic factor 4E-binding protein 1 (4EBP1) were analyzed by Western blotting. The percentages of cells in the DNA postsynthetic (G2) and DNA synthesis (S) phases would decrease, with the level of D-Glc or AA declining individually, but no interaction was observed between the D-Glc and AA effects. The CSN2 and CSN3 mRNA and protein were downregulated when D-Glc or AA decreased individually from 17.5 to 2.50 m*M* or from 7.20 to 1.03 m*M*, but D-Glc deficiency had a greater effect according to the regression analysis. The phosphorylation ratio of Jak2 ($Tyr^{1007/1008}$), Stat5a (Tyr^{694}), mTOR (Ser^{2448}), S6K1 (Thr^{389}), and 4EBP1 (Thr^{37}) was downregulated with the level of D-Glc or AA decline, whereas the phosphorylation ratio of AMPK ($Thr^{183/172}$) was upregulated. And the change of D-Glc level had a more marked effect than AA in regulating the activity of these signaling protein above according to the regression analysis. Thus, D-Glc or AA deficiency likely reduced casein transcription via inhibition of the Jak2/Stat5 pathway, and reduced translation via suppression of the mTOR pathway by activation of AMPK, but D-Glc deficiency had a more marked effect. These indicated that deficiency of AA, and especially Glc, suppressed proliferation of CMEC and casein gene and protein expression, associated with inhibition of JAK2/STAT5 and AMPK/mTOR signaling pathways.

Key words: glucose, amino acid, signaling pathway, milk protein

INTRODUCTION

It is estimated that more than 100 billion kg of corn stover (**CS**; maize leaves and stalks) is produced annually in China (Pang et al., 2008), and it is commonly used in the diet of dairy cows on small farms (Zhao and Li, 2009). However, CS contains low concentrations of CP and readily fermentable carbohydrates compared with high-quality forages such as alfalfa hay (Zhu et al., 2013). Milk yield and milk protein content were lower in cows fed with CS than those fed with alfalfa hay, because of lower rumen microbial protein supply and fermentable carbohydrates (Zhu et al., 2013). In our previous study, we found that milk protein content was lower in cows fed CS rather than alfalfa hay, and that this was accompanied by significantly lower blood AA and glucose (**Glc**) concentrations, which might be partly due to rates of feed intake. Toerien et al. (2010)

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showed that jugular infusion of Glc alone or in combination with EAA increased milk protein yield relative to saline infusion, in feed-deprived dairy cows, and Safayi and Nielsen (2013) found similar results in lactating dairy goats. Deficiency of AA and Glc may therefore lead to reduced synthesis of milk protein.

In the mammary glands, AA and Glc play a role in the synthesis of milk protein, not only as precursors (Lobley, 1990; Hanigan and Baldwin, 1994), but also as regulators of signaling (Kimball and Jefferson, 2006; Burgos et al., 2013). Previous studies showed that the Janus kinase (**Jak**)-signal transducer and activator of transcription (**Stat**) signaling pathway plays an important role in casein gene transcription and the mammalian target of rapamycin (**mTOR**) signaling pathway plays an important role in casein translation in the mammary gland (Buser et al., 2007; Nan et al., 2014; Villarino et al., 2015; Yang et al., 2015).

Stimulation of cytokines, growth factors, or nutrients phosphorylates and activates Jak2 (at $Tyr^{1007/1008}$), which then phosphorylates latent STAT5 monomers on a conserved tyrosine (Tyr^{694} or Tyr^{699}, depending on the species). Phosphorylated STAT5 undergoes dimerization and translocation to the nucleus, where it binds to specific elements (gamma activation sequence element) of casein promoter and induces the transcription of casein genes (Yamashita et al., 2001). The mTOR pathway integrates nutrient- and growth-factor-derived signals to regulate growth, the process whereby cells accumulate mass and increase in size (Sarbassov et al., 2004). Phosphorylation of mTOR at Ser^{2448} is indicative of mTOR pathway activity in bovine mammary epithelial cells (Appuhamy et al., 2011). Phosphorylated, and thus activated, mTOR in turn regulates phosphorylation of eukaryotic initiation factor 4E binding protein (**4EBP1**) and ribosomal protein S6 kinase 1 (**S6K1**), which are rate limiting to the initiation steps of milk protein synthesis (Appuhamy et al., 2011). Cellular energy stress activates AMP-activated protein kinase (**AMPK**) by phosphorylating it. Once activated, AMPK inhibits ATP-consuming processes, such as protein synthesis. One of the targets inhibited by activated AMPK is mTOR (Kudchodkar et al., 2007). Burgos et al. (2013) demonstrated that AMPK could suppresses global protein synthesis by inhibiting mTOR signaling in bovine mammary epithelial cells.

Nutrients, for example, AA and small oligopeptides, in appropriate supplemental amounts, regulated the proliferation of cells and the expression of casein through regulation of Jak-Stat and mTOR signaling pathways in dairy cow mammary epithelial cells (**CMEC**; Nan et al., 2014; Yang et al., 2015). Although it has been shown previously that AMPK/mTOR pathway regulates protein translation in CMEC, we know little about regulation of casein transcription by nutrients. Because transcription regulation happened earlier than translation, it is important to improve current knowledge on casein transcription regulation in dairy cow mammary glands. We hypothesized that deficiency of D-Glc or AA would lead to a decrease in milk casein transcription and translation through regulation of the JAK2/STAT5 and AMPK/mTOR signaling pathways. The purpose of this study was therefore to explore the effect of D-Glc and AA deficiency on the phosphorylation of the signaling proteins involved in casein synthesis in CMEC.

MATERIALS AND METHODS

Cell Preparation and Treatments

The CMEC were obtained as previously reported (Wang et al., 2014). Briefly, mammary gland tissue was dissected from the udder of mid-lactation healthy Holstein dairy cow and cut into 1-mm^3 pieces using sterile techniques. The explants were planted into a 25-cm^2 cell culture bottle (Corning, Oneonta, NY) coated with 5 mg/mL sterile rat tail collagen type II (diluted with 0.006 *M* acetic acid; Shengyou, Hangzhou, China) and cultured with complete Dulbecco's modified Eagle's medium/Ham's F-12 (**DMEM/F12**; diluted 1:1, Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 μg/mL of penicillin-streptomycin solution (Beyotime Institute of Biotechnology, Shanghai, China), and 25 mg/mL of amphotericin B in an incubator at 37°C with 5% CO_2. At 80% confluence, the tissue explants were removed from the culture. Epithelial and fibroblast cells were segregated using 0.25% trypsin and 0.15% trypsin plus 0.02% EDTA (Beyotime Institute of Biotechnology, Shanghai, China) as previously described (Wang et al., 2014), yielding primary CMEC. Prior to experimental treatments, the purified CMEC were plated into 6-well plates at a density of 1.0×10^5 cells per well. Upon reaching 80% confluence, CMEC were starved in specific medium without D-Glc and all AA including EAA and NEAA (Leagene Biotechnology, Beijing, China) overnight and subsequently incubated in media containing 1 of 3 concentrations of D-Glc (0, 2.5, or 17.5 m*M*) and 1 of 3 concentrations of AA (0, 1.03, or 7.20 m*M*). The highest concentrations of D-Glc (17.5 m*M*) and AA (7.20 m*M*) were those of complete DMEM/F12, and were more than 3-fold greater than the normal blood concentrations of dairy cows (Rius et al., 2010). All the treatment media were free of serum and adjusted to pH 7.40. The cells were harvested for RNA