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 mM) and 1 of 3 concentrations of AA (0, 1.03, or 7.20 mM). 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 mM or from 7.20 to 1.03 mM, but d-Glc deficiency had a greater effect according to the regression analysis. The phosphorylation ratio of Jak2 (Tyr1007/1008), Stat5a (Tyr694), mTOR (Ser2448), S6K1 (Thr389), and 4EBP1 (Thr37) was downregulated with the level of d-Glc or AA decline, whereas the phosphorylation ratio of AMPK (Thr183/172) 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)
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^{984} or Tyr^{986}, 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% CO₂. 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 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 × 10⁷ 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 mM) and 1 of 3 concentrations of AA (0, 1.03, or 7.20 mM). The highest concentrations of d-Glc (17.5 mM) and AA (7.20 mM) 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
incubated with 1 mL of propidium iodide (50 µg/mL, Sigma-Aldrich, Shanghai, China) for 0.5 h at 37°C, and then rinsed 3 times with PBS, followed by incubation with 1 mL of RNase A (0.1 mg/mL, diluted with PBS, Sigma-Aldrich) for 0.5 h at 37°C, and 3 additional washes with PBS. Finally, the cells were fixed overnight with ice-cold 70% ethanol at 4°C, and then rinsed 3 times with PBS, washed 3 times with PBS, and resuspended with PBS. The cell cycle stage of the cells was analyzed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) and Modfit software (Verity Software House, Topsham, ME; Kampa-Schittenhelm et al., 2013).

**Analysis of Cell Cycle Progression by Flow Cytometry**

The treated cells were collected and washed 3 times with PBS (135 mM NaCl, 4.7 mM KCl, 10 mM Na2HPO4, and 2 mM NaH2PO4; pH 7.4). Each sample was fixed overnight with ice-cold 70% ethanol at 4°C, and then rinsed 3 times with PBS, followed by incubation with 1 mM of RNase A (0.1 mg/mL, diluted with PBS, Sigma-Aldrich) for 0.5 h at 37°C, and 3 additional washes with PBS. Finally, the cells were incubated with 1 mM of propidium iodide (50 µg/mL, diluted with PBS, Sigma-Aldrich) for 0.5 h at 37°C in a darkened environment, washed 3 times with PBS, and resuspended with PBS. The cell cycle stage of the cells was analyzed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) and Modfit software (Verity Software House, Topsham, ME; Kampa-Schittenhelm et al., 2013).

**RNA Extraction and Quantification of CSN2 and CSN3 mRNA**

The RNA was extracted and purified using a cellular RNA extraction kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s protocol. The RNA quantity and purity were confirmed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA samples were reverse-transcribed using Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China), according to the manufacturer’s instructions. The quantitative real-time PCR was performed in a Bio-Rad IQ5 Real-Time PCR cycler (Bio-Rad Laboratories, Hercules, CA), using SYBR Premix Ex TaqII kit (TaKaRa). Each 20-µL PCR reaction system contained 0.8 µL of forward primer, 0.8 µL of reverse primer, 2 µL of template, 10 µL of SYBR Premix Ex TaqII, and 6.4 µL of sterile distilled water. All reactions were performed using the following protocol: 30 s at 95°C for 1 cycle, 5 s at 95°C, 34 s at 60°C, and 30 s at 72°C for 41 cycles. The same reaction was performed using an equal amount of RNAase-free water as a negative control. Primer sequences (5’-3’) for the internal reference gene RPS9 (ribosomal protein S9) and target genes were as follows: **RPS9** (NM_001101152.2; forward: ATGAGGGGCAAGATGGAAGCTG, reverse: ATGAAAGGACGGGATGTTCAC), **CSN2**: (XM_015463786.1; forward: AGT-GAGAACGCAGCAAACAG, reverse: AGCAGAGGCAGAGAGGAGGTTG), **CSN3**: (NM_174294.2; forward: TTCAACTGCGGTCTAAATACTCTAAG, reverse: TCAAAAAACTAATCTGGCATAAAAAG), **Jak2**: (XM_015464499.1; forward: ACAGGGGCTGGCGTTCA, reverse: TATTTGTAACACAGCCTAAGG), and **Stat5**: (NM_001012673.1; forward: AAGACCCGAGCAAGTTCGC, reverse: AGCAGCGTGCGAGTTAGCAT; Nan et al., 2014). The quantitative real-time PCR data were analyzed using the 2−∆∆CT method (Livak and Schmittgen, 2001).

**Immunoblot Analysis of Casein and Signaling Proteins**

Cells were lysed using a proprietary buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, and leupeptin; Beyotime, Shanghai, China), and the concentration of protein in each lysate was measured using a BCA Protein Assay Kit (Beyotime). Approximately 30 µg of total protein was separated on a 10% SDS-PAGE gel, after being denatured by boiling in SDS sample buffer for 5 min. Electrophoretically separated proteins were transferred to polyvinylidene fluoride membranes (0.45 or 0.2 µM Immobilon-P, Merck Millipore, Darmstadt, Germany) at a constant 200 mA. The membranes were blocked with Tris-buffered saline, with Tween-20 (TBST; 1.21 g of Tris, 5.84 g of NaCl, and 1 mL of Tween-20 dissolved in 1 L of double-distilled H2O, pH 7.5) containing 5% (wt/vol) skim milk for 1.5 h at room temperature (∼25°C), and then incubated with primary antibody (diluted in TBST containing 5% skim milk) for 1.5 h at room temperature, or overnight at 4°C. Membranes were then washed 3 times (5 min each) with TBST, and incubated with horseradish peroxidase-conjugated secondary antibody (diluted in TBST containing 5% skim milk, 1:2,000, Bioss, Beijing, China) for 1 h at room temperature. The membranes were then washed 3 times (10 min each) with TBST, and then the chemiluminescence of horseradish peroxidase was visualized using Super ECL Plus (Thermo Scientific, Rockford, IL). Protein band intensity was quantified using ImageJ2X software (National Institutes of Health, Bethesda, MD). Primary antibodies against β-actin, Jak2, phosphorylated (p-) Jak2 (Tyr1007/1008), Stat5α, p-Stat5α (Tyr694), raptor, p-raptor (Ser792), S6K1, and p-S6K1 (Thr389) were purchased from Cell Signaling Technology (Danvers, MA). The p-AMPK (Thr183/172), p-mTOR (Ser2448), and p4EBP1 (Thr37) antibodies were purchased from ImmunoWay (Plano, TX) and CSN2 and CSN3 antibodies were purchased from Biorbyt (Cambridge, UK).
level of phosphorylation of each molecule is indicated by a ratio of phosphorylated/total target protein.

**Statistical Analysis**

The data are reported as mean ± standard error and are representative of at least 3 independent experiments. The main effects of d-Glc and AA and their interaction on each response [percentages of cells in the DNA postsynthetic (G2) and DNA synthesis (S) phases, relative mRNA and protein expression ratio of CSN2 and CSN3, phosphorylation ratio of signaling protein] were analyzed using the following statistical model with GLM procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC):

\[
Y_{ijk} = \mu + EAA_i + EG_j + (EAA \times EG)_{ij} + e_{ijk},
\]

where \(Y_{ijk}\) = percentages of cells in the DNA postsynthetic (G2) and DNA synthesis (S) phases, relative mRNA and protein expression ratio of CSN2 and CSN3, phosphorylation ratio of signaling protein; \(\mu\) = mean percentages of cells in the DNA postsynthetic (G2) and DNA synthesis (S) phases, relative mRNA and protein expression ratio of CSN2 and CSN3, phosphorylation ratio of signaling protein; \(EAA_i\) = fixed effect of AA in medium; \(EG_j\) = fixed effect of d-Glc in medium; \((EAA \times EG)_{ij}\) = fixed interactive effect between AA and d-Glc; and \(e_{ijk}\) = random error. Where appropriate, Duncan's test was used to evaluate post hoc effects, and differences between groups were considered significant if \(P < 0.05\). In addition, a regression analysis of each response against AA and d-Glc levels using the REG procedure of SAS (version 9.2, SAS Institute Inc.) was conducted to see which variable would have stronger effect based on the parameter estimates of the slopes.

**RESULTS**

**Effects of d-Glc and AA Deficiency on the Cell Cycle**

The percentage of CMEC in the G2 plus S stages was downregulated by decline in level of d-Glc \((P < 0.001)\) or AA \((P < 0.001)\) individually, but no interaction was observed between the d-Glc and AA effects \((P = 0.19; \text{Figure } 1)\). When AA was 1.03 mmol/L and d-Glc was 17.5 mmol/L or AA was 7.20 mmol/L and d-Glc was 2.50 mmol/L, the percentages of cells in the G2 plus S stages were 6.8% \((P = 0.11)\) or 12.8% \((P = 0.05)\) lower, respectively, than those of cells incubated in high concentrations of AA (17.5 mmol/L) and d-Glc (2.50 mmol/L). Furthermore, when one of AA or d-Glc was absent individually (0 mmol/L), the percentages were 12.9% \((P < 0.05)\) and 25.3% \((P < 0.01)\) lower, respectively. Thus, the absence of d-Glc reduced the number of cells in G2 plus S stages by 12.4% more than the absence of AA, showing that d-Glc had a more significant effect on cell cycle progression. In addition, we conducted a regression analysis of the percentages of cells in the G2 plus S stages against AA and d-Glc levels and we also see that d-Glc would have stronger effect based on the parameter estimates of the slopes (-4.52) than AA (-2.47).

**Effects of d-Glc and AA Deficiency on the Synthesis of CSN2 and CSN3**

As shown in Figure 2, d-Glc \((P < 0.01)\) and AA \((P = 0.03)\) had individual but no interaction effects on CSN2 mRNA \((P = 0.26)\) and protein \((P = 0.49)\) expression and so did CSN3. Decline in level of d-Glc or AA reduced the expression of both CSN2 and CSN3 mRNA and protein in cells (Figure 2). Compared with high level of d-Glc (17.5 mmol/L) and AA (7.20 mmol/L) treatment, the relative mRNA and protein levels of CSN2 or CSN3 decreased respectively by 12.1% \((P = 0.04)\) and 8.6% \((P = 0.02)\) or 7.7% \((P = 0.06)\) and 4.9% \((P = 0.19)\) when d-Glc was 17.5 mmol/L and AA was 1.03 mmol/L. However, the relative mRNA and protein...
levels of CSN2 or CSN3 were 20.8% (P < 0.01) and 20.5% (P < 0.01), or 20.6% (P = 0.02) and 17.1% (P = 0.04) lower, when AA was 7.20 mmol/L and D-Glc was 2.50 mmol/L. Therefore, the relative mRNA and protein levels of CSN2 or CSN3 were an additional 8.7 and 11.9%, or 12.9 and 12.2% lower, respectively, when D-Glc was insufficient than when AA were insufficient, implying that D-Glc is more important for the synthesis of CSN2 and CSN3. Regression analysis of the relative mRNA and protein levels of CSN2 against AA and D-Glc levels showed that parameter estimates of the slopes of D-Glc effect (0.64 and 0.72) was higher than AA (0.42 and 0.56). Regression analysis of the relative mRNA and protein levels of CSN3 against AA and D-Glc levels showed that parameter estimates of the slopes of D-Glc effect (0.59 and 0.76) was also higher than AA (0.42 and 0.47).

Effects of D-Glc and AA Deficiency on Jak2-Stat5 Pathway

The ratios of p-Jak2/Jak2 (P < 0.01) and p-Stat5a/Stat5a (P < 0.01) were declining with the decrease in concentration of D-Glc or AA individually, but no interaction (P = 0.18) was observed between D-Glc and AA effects (Figure 3). Compared with the high level group (D-Glc 17.5 mmol/L and AA 7.20 mmol/L), the p-Jak2/Jak2 and p-Stat5a/Stat5a ratios were 10.1% (P < 0.05) and 19.6% (P = 0.06) lower when D-Glc was 17.5 mmol/L and AA was 1.03 mmol/L, and 9.6% (P < 0.05) and 20.9% (P < 0.01) lower when AA were 7.2 mmol/L and D-Glc was 2.50 mmol/L. Besides, these ratios were 11.5% (P < 0.05) and 31.7% (P < 0.01) lower, respectively, when D-Glc was 17.5 mmol/L and AA were absent, and 41.5% (P < 0.05) and 67.5% (P < 0.01) lower when AA was 7.20 mmol/L and D-Glc was absent. Thus, the absence of D-Glc induced additional respective 30.0 and 35.8% reductions compared with the absence of AA alone. In addition, the parameter estimates of the slopes of D-Glc levels (0.22 and 0.56) against the ratios of p-Jak2/Jak2 and p-Stat5a/Stat5a is higher than AA (0.06 and 0.26).

Effects of D-Glc and AA Deficiency on the AMPK/mTOR Pathway

We also found a dose-dependent relationship between the activation of the AMPK/mTOR pathway and D-Glc.
or AA concentration, although again there were no interactions ($P = 0.59$) between their lower concentrations on this pathway (Figure 4). Compared with the high level group (d-Glc 17.5 mmol/L and AA 7.20 mmol/L), the decline in level of AA (1.03 mmol/L) alone was associated with a higher ($P = 0.21$) p-AMPK/AMPK ratio (2.2%), but lower ($P < 0.05$) ratios of p-mTOR/mTOR, p-S6K1/S6K1, and p-4EBP1/4EBP1 (by 30.5, 8.3, and 1.8%, respectively). However, when d-Glc concentration decreased to 2.50 mmol/L individually, there was a 206% higher ($P < 0.05$) ratio of p-AMPK/AMPK, but the ratios of p-mTOR/mTOR, p-S6K1/S6K1, and p-4EBP1/4EBP1 were lower (by 45.7, 20.3, and 24.5%, respectively; $P < 0.01$). Thus, d-Glc deficiency had a 204% greater effect on p-AMPK/AMPK than AA deficiency, and respective 15.2, 12, and 22.7% greater effects on p-mTOR/mTOR, p-S6K1/S6K1, and p-4EBP1/4EBP1. In addition, the parameter estimates of the slopes of d-Glc levels (−0.21, 1.78, 1.31, and 0.67) against the ratios of p-AMPK/AMPK, p-mTOR/mTOR, p-S6K1/S6K1, and p-4EBP1/4EBP1 was all higher than AA (−0.15, 0.31, 0.98, and 0.19). Thus, the decline in d-Glc level was more effective at activating the AMPK/mTOR pathway.

**DISCUSSION**

Milk production by dairy cows is dependent on mammary epithelial cell proliferation and activity, both of which are influenced by nutrient availability (Singh et al., 2010). The process of cell growth depends on a high rate of protein synthesis and requires high levels of cellular energy. Cell proliferation and activity is also positively related to the percentage of cells in the DNA synthesis (S) and DNA postsynthetic (G2) phases of the cell cycle (Han et al., 2015). In this study, the CMEC cells were starved overnight and treated with 3 levels of AA and 3 levels of d-Glc in a 3 × 3 factorial arrangement of treatments, among which the highest level of d-Glc (17.5 mM) and AA (7.20 mM) were those of normal DMEM/F12, and were more than 3-fold greater than the normal blood concentrations of dairy cows (Rius et al., 2010). We considered the highest level of d-Glc (17.5 mM) and AA (7.20 mM) as sufficient for cell proliferation.

Our results showed that reduce of d-Glc or AA levels resulted in a reduction in the percentage of CMEC cells in the G2 plus S stages. This is in accordance with the results of Han et al. (2015), who showed that low levels of Glc induced mitochondrial apoptosis and prevented the progression of cells into the S phase from G1, causing cell cycle arrest. In a separate study, Nan et al. (2014) observed that supplemental Lys, Met, and Lys and Met mixtures enhanced the proliferation

![Figure 3](https://example.com/figure3.png)
of bovine mammary epithelial cells: a low dose of Lys (0.05 to 0.4 mM) or Met (0.025 to 0.2 mM) had no significant effect on relative growth rate of cells, but when cultured concentration was 0.8 to 1.6 mM of Lys or 0.4 to 0.8 mM Met, the relative growth rate was increased significantly. In this study, we also showed that d-Glc has a bigger influence on CMEC proliferation, and similar findings were reported by Lunt and Vander Heiden (2011), who demonstrated that increased Glc metabolism is selected for in proliferating cells throughout nature. Because Glc can provide the precursors for the chemical constituents (e.g., nucleotides, AA, and lipids) that are used to build macromolecules essential for cell division. A more recent study demonstrated that cells could grow in size using the AA glutamine as the only major carbon source but were unable to proliferate unless a metabolite capable of entering glycolysis was available (Wellen et al., 2010), presumably to support nucleic acid synthesis. These findings support the importance of Glc in providing nucleotide precursors for animal cells in which Glc is the major nutrient available.

The availability of AA is one of the key factors regulating milk protein synthesis (Backwell et al., 1996;
Bobe et al., 1999), but milk protein synthesis is also an energy-consuming process (Lobley, 1990; Hanigan and Baldwin, 1994). We showed that d-Glc or AA deficiency results in lower expression of CSN2 and CSN3 at both the mRNA and protein levels. Consistent with this, Nichols et al. (2016) demonstrated stimulation of milk protein synthesis in response to 5-d EAA and Glc supplementation in lactating dairy cows. They found that infusion of EAA increased milk yield by 4.1 kg/d and milk protein by 256 g/d compared with saline, and the addition of Glc to EAA infusate did not stimulate milk protein yield or concentration, but did decrease milk urea nitrogen, while stimulating skeletal muscle accretion via increased insulin signaling (Nichols et al., 2016). Toerien et al. (2010) showed that when lactating cows were feed-deprived for 22 h and then infused i.v. for 9 h with EAA+ (Glc), Glc only, milk protein yield was increased 33 and 27% by EAA + Glc and Glc infusions, respectively.

In contrast to our results, Appuhamy et al. (2014) reported that the supplementation of media with Glc ± EAA did not affect casein synthesis rates in mammary tissue slices. This disparity might be due to the fact that greater nutrient concentrations in the mammary tissue slices may have partly masked the effects of altered concentrations in the media on cellular energy status. In previous studies, greater milk and milk protein production in lactating cows fed high-starch diets compared with those fed low-starch diets has been reported, and this response in production was observed even though dietary protein was lower than recommended (15.2% CP, DM basis; Rius et al., 2010). The present knowledge (e.g., stronger stimulatory effects of Glc on casein synthesis) may indicate that provision of glucogenic substrate such as postrumen starch during dietary restriction can stimulate the mammary glands to synthesize more milk protein.

AMPK acts as a master regulator of metabolic homeostasis by sensing cellular energy status. It is activated by a low cellular ATP/AMP ratio (Burgos et al., 2013). Activated AMPK phosphorylates downstream targets, activating catabolic pathways, while deactivating synthetic pathways that consume ATP, such as protein synthesis (Xu et al., 2012). We found that a low Glc or AA concentration in the medium increased p-AMPK (Thr172). Consistent with this, Appuhamy et al. (2014) found that addition of Glc or EAA to the medium reduced AMPK phosphorylation in bovine mammary epithelial cells by a similar amount (~23%), whereas addition of both Glc and EAA reduced it by 46%. Hanigan et al. (2001) reported that the contribution of AA to oxidative metabolism and ATP synthesis in the bovine mammary glands is relatively minor at the prevailing in vivo concentrations, but concentrations of AA in our treatment medium (7.20 mmol/L) and EAA in the medium of Appuhamy et al. (2014; 3.50 mmol/L) were several-fold greater than the normal plasma AA and EAA concentrations (Rius et al., 2010) in dairy cows, and thus the contribution of AA to oxidative metabolism have been significant. However, although AA can serve as an important energy substrate to mammary cells, as Glc is the main provider of ATP, Glc deprivation can increase the AMP:ATP ratio directly, so it is no surprise to find that AMPK activation is more sensitive to Glc deficiency than AA in the present study.

Amino acids posttranscriptionally regulate mTOR in the bovine mammary gland (Appuhamy et al., 2014). In this study, we found that p-mTOR (Ser2448), p-S6K1 (Thr389), and p-4EBP1 (Thr47) were downregulated in the presence of low Glc and AA concentrations. In the previously reported study, infusion of EAA and Glc in feed-deprived cows increased phosphorylation of mTOR and S6K1, and promoted protein synthesis in mammary tissue (Toerien et al., 2010). In addition, Gao et al. (2015) demonstrated that addition of leucine and histidine could activate the mTOR pathway by phosphorylating mTOR (Ser2448), S6K1 (Thr389), and 4EBP1 (Thr47) in CMEC, thus promoting casein synthesis. Consistent with this, Appuhamy et al. (2014) demonstrated that, irrespective of the presence of Glc in the medium, supplementation of EAA significantly increased phosphorylation of mTOR at Ser2448 and 4EBP1 at Thr37/46 in bovine mammary epithelial cells and mammary tissue slices. mTOR is another downstream target that is inhibited by AMPK (Kudchodkar et al., 2007). Burgos et al. (2013) showed that activation of AMPK suppressed global protein synthesis via inhibition of mTOR signaling in CMEC. The present study showed that a deficiency of Glc or AA activates the AMPK signaling pathway, but suppresses the
mTOR signaling pathway, and that deficiency of d-Glc had a greater effect on the AMPK-mTOR pathway. Therefore, the suppression of mTOR was potentially contributed to more by energy deficiency-mediated activation of AMPK than it was by AA deficiency, which may help explain the observation that d-Glc deficiency had a greater effect on casein expression. And stronger stimulatory effects of Glc on casein synthesis may indicate that increasing energy supply to the mammary glands could be suggested as a nutritional intervention to stimulate mammary protein production. However, more research is required in this aspect in dairy cows.

CONCLUSIONS

Deficiency of AA, and especially Glc, suppressed proliferation of CMEC and casein gene and protein expression, associated with inhibition of JAK2/STAT5, mTOR, and AMPK signaling pathways. This indicates that not only AA availability, but also Glc availability, is important for the synthesis of milk protein.

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