Effects of AMP-activated protein kinase (AMPK) signaling and essential amino acids on mammalian target of rapamycin (mTOR) signaling and protein synthesis rates in mammary cells

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ABSTRACT

Regulation of mammary protein synthesis potentially changes the relationships between AA supply and milk protein output represented in current nutrient requirement models. Glucose and AA regulate muscle protein synthesis via cellular signaling pathways involving mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK). The objective of this study was to investigate the effects of essential AA (EAA) and acetate or glucose on mTOR and AMPK signaling pathways and milk protein synthesis rates. A bovine mammary epithelial cell line, MAC-T, was subjected to different media containing 0 or 3.5 mmol/L EAA concentrations with 0 or 5 mmol/L acetate or 0 or 17.5 mmol/L glucose in 2 separate 2 × 2 factorial studies. In a separate set of experiments, lactogenic bovine mammary tissue slices were subjected to the same treatments except that the low EAA treatment contained a low level of EAA (0.18 mmol/L). Supplementation of EAA enhanced phosphorylation of mTOR (Ser2448) and eukaryotic initiation factor 4E binding protein 1 (4EBP1, Thr37/46), and reduced phosphorylation of eukaryotic elongation factor 2 (eEF2, Thr56) in MAC-T cells. Concentration of ATP and phosphorylation of AMPK increased and decreased, respectively, in the presence of EAA in MAC-T cells. Acetate, EAA, or glucose numerically reduced AMPK phosphorylation by about 16% in mammary tissue slices. Provision of EAA increased phosphorylation of mTOR and 4EBP1, intracellular total EAA concentration, and casein synthesis rates in mammary tissue slices, irrespective of the presence of acetate or glucose in the medium. Phosphorylation of mTOR had a marginally negative association with AMPK phosphorylation, which was

positively related to eEF2 phosphorylation. Casein synthesis rates were positively and more strongly linked to mTOR phosphorylation than the negative link between eEF2 phosphorylation and casein synthesis rates. A 100% increase in mTOR phosphorylation was associated with an increase in the casein synthesis rate of 0.74%·h⁻¹, whereas a 100% increase in eEF2 phosphorylation was related to a decline in the casein synthesis rate of 0.33%·h⁻¹. Although AMPK phosphorylation was responsive to cellular energy status and had a negative effect on mTOR-mediated signals in bovine mammary epithelial cells, its effect on milk protein synthesis rates appeared to be marginal compared with the mTOR-mediated regulation of milk protein synthesis by EAA.

Key words: AMP-activated protein kinase (AMPK), essential amino acid, casein synthesis, energy substrate

INTRODUCTION

Poor N utilization efficiency of dairy cows leads to significant N losses in urine and feces (Huhtanen and Hristov, 2009). This loss of N represents both an economic and environmental cost. Increasing urinary N excretion is indicative of greater catabolism of AA in the body, with splanchnic tissues being a major contributor. Splanchnic catabolism of AA is proportional to arterial AA concentrations, which are influenced by AA uptake by nonsplanchnic tissues, such as the lactating mammary gland. In contrast, AA uptake by mammary glands appears to be a function of arterial concentration, blood flow, and metabolic demand. Therefore, if AA supply to the animal remains constant while use for milk protein synthesis is increased, the increased demand for AA by mammary tissue will draw down arterial AA concentrations, leading to reduced AA catabolism by the splanchnic tissues (Hanigan et al., 1998). Moreover, AA are not only precursors for protein synthesis but they also play a significant regulatory role (Kimball and Jefferson, 2006). Protein synthesis regulation potentially violates the assumption of fixed

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efficiencies of absorbed AA conversion to milk protein in current nutrient requirement models. Understanding milk protein synthesis regulation is therefore important for improving protein and AA requirement models.

We previously (Appuhamy et al., 2011, 2012) demonstrated that essential AA (**EAA**) enhanced milk protein synthesis rates by signaling via the mammalian target of rapamycin (**mTOR**) pathway. Phosphorylation of mTOR at Ser2448 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 eukaryotic elongation factor 2 (**eEF2**), which are rate limiting to the initiation and elongation steps of milk protein synthesis, respectively (Appuhamy et al., 2011).

Increased availability of glucose, and thereby improved cellular energy status, has also been shown to stimulate protein synthesis in muscle cells (Bolster et al., 2002; Jeyapalan et al., 2007; Deshmukh et al., 2008). These signals involve AMP-activated protein kinase (**AMPK**), which is able to sense cellular energy status. Cellular energy stress activates AMPK by phosphorylating it. Once activated, AMPK inhibits ATP-consuming processes, such as protein synthesis, while enhancing ATP-producing processes, such as fatty acid oxidation and glucose uptake (Mukherjee et al., 2008). One of the targets inhibited by activated AMPK is mTOR (Kudchodkar et al., 2007).

Consistent with the fact that milk protein synthesis is an energy-consuming process (Lobley, 1990; Hanigan and Baldwin, 1994), Burgos et al. (2013) showed that activation of AMPK repressed global protein synthesis via inhibition of mTOR signals in bovine mammary epithelial cells. These authors activated AMPK with 2-deoxyglucose. However, examining the effect of the energy substrates acetate and glucose on AMPK and, specifically, milk protein synthesis would be more beneficial and relevant from a nutritional standpoint. Moreover, Safayi and Nielsen (2013) recently suggested that N balance differences can lead to differential glucose and acetate effects on milk protein synthesis, indicating the importance of testing the acetate and glucose effects compared with AA availability. Therefore, the objectives of the present study were to investigate the effects of glucose, acetate, and EAA concentrations on AMPK, mTOR, 4EBP1, eEF2, and casein synthesis rates, and to explore relationships among the signaling proteins and casein synthesis rates in bovine mammary epithelial cells.

MATERIALS AND METHODS

The Animal Care and Use Committee of Virginia Tech approved all procedures. This study was con-

ducted according to the Federation of Animal Science Societies' *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010).

Cells and Tissues

Two separate sets of experiments were conducted using an immortalized bovine mammary epithelial cell line, MAC-T (Huynh et al., 1991), and mammary tissue slices from lactating cows. Cells were seeded on 6-well plates at a cell density of 5,625 cells/cm² and cultured at 37°C in 5% CO₂ to about 90% confluency. Cells were grown in Dulbecco's modified Eagle medium/F12 (**DMEM/F12**; Invitrogen, Carlsbad, CA) supplemented with 8% (vol/vol) fetal bovine serum and containing 3.51 mg of D-glucose, 100 U of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B (HyClone Laboratories Inc., Logan, UT) per milliliter. Four multiparous lactating Holstein cows were screened for mastitis, deprived of food for 12 h, and slaughtered in accordance with the US Department of Agriculture Food Safety and Inspection Services regulations. Mammary tissue slices (130 ± 30 mg) were prepared within 30 min of slaughter from a clean rear quarter using a Stadie-Riggs handheld microtome and placed in 25-mL Erlenmeyer flasks containing 4 mL of treatment medium, as described previously (Appuhamy et al., 2011).

Treatments and Experimental Design

MAC-T Cell Experiments. The MAC-T cells were serum starved overnight and treated with 2 levels of EAA and 2 levels of sodium acetate in a 2 × 2 factorial arrangement of treatments. Essential AA levels were those of complete DMEM/F12 (3.5 mmol/L) or DMEM/F12 devoid of EAA (0 mmol/L). Sodium acetate levels were 0 or 5 mM (in the absence of glucose). In a separate experiment, the cells were treated with the same EAA levels and 2 D-glucose levels: 0 and 17.5 mmol/L (in the absence of acetate) again in a 2 × 2 factorial arrangement of treatments. The 17.5 mmol/L level is the normal glucose level in complete DMEM/F12. The high levels of acetate, glucose, and EAA were more than 3-fold greater than normal blood concentrations in dairy cows (Purdie et al., 2008; Rius et al., 2010). The basal media to which EAA, glucose, or acetate was added were completely deprived of EAA, glucose and acetate, and contained 3.7 mmol of non-EAA (standard DMEM/F12 concentrations), 0.1 mg of insulin, 0.02 mmol of phenol red, 0.50 mmol of sodium pyruvate, and 14.0 mmol of sodium bicarbonate per L. All the treatment media were free of serum and adjusted to a pH of 7.4.

After a 1-h incubation in treatment media, culture plates were placed on ice and 2 mL/well of stop buffer (sterile PBS containing 1 mM NaF and 10 μ M Na₃VO₄) was added. Cells were lysed by scraping cells into ice-cold lysis buffer containing 1% (vol/vol) protease and phosphatase inhibitor cocktail (ThermoScientific, Rockford, IL). Cell lysates from 6 wells were combined and each treatment was repeated 4 times on 4 different days. Using a separate experimental replicate ($n = 3$), ATP content in MAC-T cells was measured as described by Manfredi et al. (2002). Briefly, after treatment medium had been removed, 250 μ L of 0.5 mol/L perchloric acid was added into each well and the cells were scraped. Cell lysates were centrifuged ($23,000 \times g$ for 10 min at 4°C) and the supernatant was neutralized by adding 2 mol/L potassium hydroxide (4:1, vol/vol). After another round of centrifugation ($23,000 \times g$ for 10 min at 4°C), the supernatant was injected into an HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) and separated with 2 mobile phases using a Phenomenex Kinetex 2.6- μ m C18 column (4.6 $\times$ 50 mm; Phenomenex, Torrance, CA). Mobile phase A consisted of 5 mM tetrabutylammonium hydrogen sulfate, 100 mM KH₂PO₄ (pH 6.0), and 2.5% acetonitrile. Mobile phase B was the same except it contained 25% acetonitrile (pH 5.5; Bernocchi et al., 1994; Williams et al., 2008). Nucleotide concentrations were detected with a UV/visible spectrophotometer at 260 nm and quantified using a standard curve of commercially available ATP.

Mammary Tissue Slice Experiments. The mammary tissue slice experimental design and treatments were the same as for MAC-T cells, except that an altered level of EAA (5% of regular DMEM/F12 concentrations) for the -EAA treatments was chosen to measure fractional protein synthesis rates (**FSR**) using ²H₅-Phe as previously described (Appuhamy et al., 2011). Duplicate mammary tissue slices from each cow were randomly assigned to treatment media in the 25-mL Erlenmeyer flasks, purged with a 95:5 mix of O₂:CO₂ for 20 s, sealed with rubber stoppers, and placed in a shaking (60 oscillation/h) water bath at 37°C (time = 0). Flasks were opened at 75 min to add ²H₅-Phe, purged with the gas mix again, and incubated in the shaking water bath for another 45 min. The medium maintained pH throughout the incubation, as indicated by the phenol red. Tissue remained viable during the incubation as evidenced by ²H₅-Phe incorporation into mammary protein over the last 45 min of the incubation. Label incorporation rates were found to be linear for 8 h (unpublished data), consistent with the findings of others (Bauman et al., 1973; Dimenna and Emery, 1980; O'Quinn et al., 2002), where various metabolic

and synthetic processes remained linear for at least 2 h when mammary tissue was incubated in this manner.

Following incubation, tissue slices were retrieved and homogenized as previously described (Appuhamy et al., 2011). Aliquots of cell homogenate supernatant were mixed with equal volumes of 2 $\times$ Laemmli buffer, incubated at 80°C for 8 min, and stored at -80°C until immunoblot analyses. Casein-enriched protein precipitates were obtained by adjustment of pH to 4.6. The effectiveness of this enrichment procedure was determined from 6 random mammary tissue slices by Coomassie blue staining after electrophoretic separation on 12% SDS-PAGE gel. Casein bands were determined by visual comparison with purified bovine casein (Sigma-Aldrich, St. Louis, MO). Protein band intensities were measured with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Casein was found to represent $49 \pm 10\%$ of the total stained protein in a lane (results not shown). This indicates that the FSR results in the present study can reasonably be related to milk protein synthesis rates, although other proteins were present in the precipitate. Protein-bound [*E_{PB}* (%)] and intracellular [*E_{IC}* (%)] ²H₅-Phe enrichments, and intracellular EAA concentrations were determined as previously described (Appuhamy et al., 2011).

Random mammary tissue samples from each cow were obtained simultaneously with the tissues slices and used to prepare microscopy slides, which were stained with hematoxylin and eosin (Sigma) as described previously (Daniels et al., 2009). Stained images of mammary tissue were captured with a top-mounted digital microscope camera (Olympus DP10, Opelco, Dulles, VA) under 40 $\times$ magnification. Four images, each representing mammary tissues harvested from each of 4 cows, are presented in Figure 1. As evidenced by Figure 1, the harvested mammary tissue slices contained a high proportion of epithelial cells. Therefore, the observed cellular signaling effects can be reasonably related to milk protein synthesis.

Protein Immunoblot Analysis

Cell lysate aliquots (20 μ g of protein) were subjected to protein immunoblot analyses as previously described (Escobar et al., 2006). Electrophoretically separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) and incubated with antibodies (Cell Signaling Technology, Danvers, MA) recognizing phosphorylated forms of AMPK (Thr172), mTOR (Ser2448), 4EBP1 (Thr37/46), and eEF2 (Thr56). Visualized immunoblots were then stripped and reprobed with the corresponding total antibodies (Cell Signaling Technology).

Figure 1. Representative images of mammary tissues (stained with hematoxylin and eosin) from 4 lactating cows at 20× magnification. Color version available in the online PDF.

Because we were unable to detect total 4EBP1, the 4EBP1 phosphorylation was calculated relative to the α -tubulin intensities (Figure 2). Blots were visualized using enhanced chemiluminescence (ECL Plus, GE Healthcare, Piscataway, NJ) and digitized and integrated using Un-Scan-It (Silk Scientific Inc., Orem, UT). Phosphorylation state was calculated as the ratio of phosphorylated form to the total.

Fractional Rate of Protein Synthesis

Casein-rich precipitates from mammary tissue slices were hydrolyzed with 6 N HCl in 0.1% phenol at 110°C for 20 h. The hydrolysate and the supernatant collected from the precipitation were desalted by ion exchange chromatography, and converted to *N*-(*tert*-butyldimethyl) AA derivatives as described previously (Bequette et al., 1999). The E_{PB} and E_{IC} were quantified by GC-MS (GC-MS Trace GC Ultra-DSQII, Thermo Electron Corp., Waltham, MA) using selected ion monitoring to determine the ratio of the 239 and 234 m/z ions. Calibration curves were generated to determine E_{PB} and E_{IC} as previously described (Bequette et al., 2002). The FSR were calculated as

$$FSR = \frac{E_{PB} \times 100}{E_{IC} \times t},$$

where t is the incubation time of mammary tissue slices in media containing 2H_5 -Phe (0.75 h) and the units of FSR are %·h⁻¹.

Statistical Analysis

The main effects of acetate or glucose and EAA and their interaction on phosphorylation state of signaling proteins (in both MAC-T cells and mammary tissue slices), ATP content (only in MAC-T cells), and FSR and intracellular EAA concentrations (only in mammary tissue slices) were analyzed using the following statistical model with the MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC):

$$Y_{ijk} = \mu + EAA_i + ES_j + (EAA \times ES)_{ij} + e_{ijk},$$

where Y_{ijk} = phosphorylation state, ATP content, FSR, or intracellular EAA concentration; μ = mean phosphorylation state, ATP content, FSR, or intracellular

Figure 2. Representative Western immunoblot images for phosphorylated (Phos_) AMPK (Thr172), mTOR (Ser2448), 4EBP1 (Thr37/46), and eEF2 (Thr56) and their total forms in the presence (+) and absence (–) of essential amino acids (EAA) and glucose (GL) in MAC-T cell medium. AMPK = AMP-activated protein kinase; mTOR = mammalian target of rapamycin; 4EBP1 = eukaryotic initiation factor 4E binding protein; eEF2 = eukaryotic elongation factor 2.

EAA concentration; EAA_i = fixed effect of EAA in medium; ES_j = fixed effect of energy substrate (acetate or glucose) in medium; $(EAA \times ES)_{ij}$ = fixed interactive effect between EAA and energy substrate; and e_{ijk} = random error. Phosphorylation state was normalized to

the average value (across replicates) of negative control treatments. Meaningful relationships among phosphorylation state of the signaling proteins, intracellular total EAA concentration, and FSR in mammary tissue slices were quantified separately with simple linear regression models and multiple linear regression models using the REG procedure of SAS. Mean response variable values (within treatment within each cow) across both acetate-EAA and glucose-EAA experiments were used in the regression analyses. The normalized phosphorylation ratios were used for corresponding regression analyses. Hence, a 1-fold (or 100%) increase in phosphorylation of a signaling protein is equivalent to the amount of phosphorylation measured in the absence of EAA and the energy substrates.

RESULTS

MAC-T Cells

Acetate-EAA Experiment. Addition of essential amino acids (+EAA) reduced ($P = 0.03$) AMPK phosphorylation by 30%, with or without acetate in the medium (Table 1). Consistently, EAA supplementation increased ($P = 0.03$) ATP content in MAC-T cells by 38% (60.6 vs. 83.8 $\mu\text{mol}/\text{mg}$ of protein) irrespective of the presence of acetate. Only EAA affected mTOR and eEF2 phosphorylation. Addition of EAA increased mTOR phosphorylation ($P < 0.01$) and decreased eEF2 phosphorylation ($P = 0.02$), regardless of acetate in the medium. Acetate did not have an individual or an interactive effect with EAA on the phosphorylation state of the signaling proteins or ATP content in MAC-T cells.

Table 1. Effects (least squares means) of essential AA (EAA) and acetate (AC; top half of table) or EAA and glucose (GL; bottom half of table) on signaling protein phosphorylation and cellular ATP concentrations in MAC-T cells

Variable ¹	Treatment				SEM ²	P-value		
	−EAA−AC	−EAA+AC	+EAA−AC	+EAA+AC		EAA	AC	EAA × AC
Acetate and EAA experiment								
AMPK (Thr172), AU ³	1.00 ^a	0.83 ^{ab}	0.63 ^b	0.65 ^b	0.18	0.03	0.49	0.35
mTOR (Ser2448), AU	1.00 ^a	2.94 ^{ab}	4.72 ^{ab}	5.56 ^b	1.01	0.01	0.18	0.59
eEF2 (Thr56), AU	1.00 ^a	0.89 ^{ab}	0.76 ^b	0.78 ^{ab}	0.07	0.02	0.48	0.37
ATP, μmol/mg of protein	57.1 ^a	64.0 ^a	88.2 ^b	79.3 ^b	8.10	0.02	0.93	0.33
Glucose and EAA experiment								
	−EAA−GL	−EAA+GL	+EAA−GL	+EAA+GL	SEM	EAA	GL	EAA × GL
AMPK (Thr172), AU	1.00 ^a	0.63 ^{ab}	0.64 ^{ab}	0.54 ^b	0.10	0.06	0.05	0.24
mTOR (Ser2448), AU	1.00 ^a	0.99 ^b	1.30 ^a	1.45 ^b	0.16	0.04	0.71	0.65
4EBP1 (Thr37/46), AU	1.00 ^a	1.18 ^a	1.75 ^b	1.70 ^b	0.08	<0.001	0.36	0.16
eEF2 (Thr56), AU	1.00 ^a	1.01 ^b	0.90 ^{ab}	0.88 ^b	0.04	0.03	0.89	0.79

^{a,b}Means in a row with superscripts without a common letter differ, $P < 0.10$.

¹AMPK = AMP-activated protein kinase; mTOR = mammalian target of rapamycin; eEF2 = eukaryotic elongation factor 2; 4EBP1 = eukaryotic initiation factor 4E binding protein.

²Pooled standard error of the means.

³Normalized phosphorylation ratios (phosphorylated protein:total protein) expressed in arbitrary units (AU).

Table 2. Effects (least squares means) of essential AA (EAA) and acetate (AC; top half of table) or EAA and glucose (GL; bottom half of table) on signaling protein phosphorylation, total intracellular EAA concentrations (ICEAA), and fractional synthesis rates of casein (FSR) in bovine mammary tissue slices

Variable ¹	Treatment				SEM ²	P-value		
	–EAA–AC	–EAA+AC	+EAA–AC	+EAA+AC		EAA	AC	EAA × AC
Acetate and EAA experiment								
AMPK (Thr172), AU ³	1.00	0.88	0.89	0.71	0.13	0.25	0.22	0.81
mTOR (Ser2448), AU	1.00 ^{ab}	0.79 ^a	1.64 ^b	1.72 ^b	0.30	0.01	0.81	0.58
4EBP1 (Thr37/46), AU	1.00 ^a	1.05 ^a	2.1 ^b	2.01 ^b	0.23	<0.001	0.87	0.86
eEF2 (Thr56), AU	1.00	1.57	1.05	0.82	0.37	0.22	0.54	0.16
ICEAA, mol/L ⁴	0.32 ^a	0.41 ^a	1.45 ^b	1.57 ^b	0.09	<0.001	0.26	0.85
FSR, %·h ^{–1}	0.97	0.94	1.50	1.04	0.23	0.14	0.25	0.31
Glucose and EAA experiment								
	–EAA–GL	–EAA+GL	+EAA–GL	+EAA+GL	SEM	EAA	GL	EAA × GL
AMPK (Thr172), AU	1.00	0.77	0.85	0.76	0.09	0.44	0.10	0.48
mTOR (Ser2448), AU	1.00 ^a	1.21 ^{ab}	1.88 ^b	1.53 ^{ab}	0.24	0.04	0.79	0.27
4EBP1 (Thr37/46), AU	1.00 ^a	1.25 ^{ab}	2.57 ^b	2.14 ^b	0.76	0.01	0.82	0.47
eEF2 (Thr56), AU	1.00	1.33	1.02	0.87	0.28	0.21	0.57	0.16
ICEAA, mol/L	0.30 ^a	0.31 ^a	1.43 ^b	1.13 ^b	0.15	<0.001	0.34	0.32
FSR, %·h ^{–1}	0.98 ^a	0.77 ^{ab}	1.57 ^b	1.31 ^{ab}	0.22	0.02	0.30	0.92

^{a,b}Means in a row with superscripts without a common letter differ, $P < 0.10$.

¹AMPK = AMP-activated protein kinase; mTOR = mammalian target of rapamycin; 4EBP1 = eukaryotic initiation factor 4E binding protein; eEF2 = eukaryotic elongation factor 2.

²Pooled standard error of the means.

³Normalized phosphorylation ratios (phosphorylated protein:total protein) expressed in arbitrary units (AU).

⁴Sum of the intracellular concentrations of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine.

Glucose-EAA Experiment. Addition of glucose ($P < 0.05$) or EAA ($P = 0.06$) equally reduced AMPK phosphorylation by 23% (Table 1). Addition of both glucose and EAA reduced ($P < 0.05$) AMPK phosphorylation by 46%. We observed no interaction between the glucose and EAA effects ($P = 0.24$). Addition of EAA increased ($P = 0.04$) and decreased ($P = 0.03$) mTOR (38%) and eEF2 (12%) phosphorylation, respectively. Moreover, EAA supplementation increased 4EBP1 phosphorylation ($P < 0.01$) by 58%, regardless of glucose concentration. Glucose did not have an individual or an interactive effect with EAA on phosphorylation of mTOR, 4EBP1, or eEF2.

Mammary Tissue Slices

Acetate-EAA Experiment. Supplementation of EAA ($P = 0.25$) or acetate ($P = 0.22$) to EAA-deficient and energy substrate-deprived (without both acetate and glucose) media did not significantly affect AMPK phosphorylation but reduced it numerically by 15% (Table 2). As observed in MAC-T cells, EAA supplementation increased ($P < 0.01$) mTOR phosphorylation by 54%, regardless of acetate in medium. Moreover, 4EBP1 phosphorylation ($P < 0.01$) increased 2-fold with the addition of EAA. Unlike in MAC-T cells, EAA supplementation did not affect eEF2 phosphorylation in mammary tissue slices ($P = 0.22$). Intracellular EAA concentration increased more than 3-fold with addition

of EAA to medium, which marginally enhanced ($P = 0.14$) fractional casein synthesis rates.

Glucose-EAA Experiment. Supplementation of EAA or glucose did not affect AMPK phosphorylation (Table 2). Provision of glucose numerically reduced ($P = 0.10$) AMPK phosphorylation by 17%, regardless of the presence or the absence of EAA in media. As in other experiments, EAA supplementation increased both mTOR ($P = 0.04$) and 4EBP1 ($P = 0.01$) phosphorylation. Supplementation of EAA or glucose did not affect eEF2 ($P = 0.21$) in mammary tissue slices. Intracellular EAA concentration in mammary tissue slices increased ($P < 0.01$) by more than 3-fold when EAA was added to medium. Supplementation of EAA increased FSR ($P = 0.02$) by 65% (88 vs. 144%·h^{–1}) irrespective of the presence or absence of glucose in media. Supplementation with glucose did not affect intracellular EAA concentrations or casein synthesis rates.

Relationships Among Signaling Proteins, FSR, and EAA. When analyzed with simple linear regression models, phosphorylation of mTOR was positively and negatively associated with 4EBP1 ($P = 0.02$) and eEF2 ($P = 0.01$) phosphorylation, respectively (Table 3). A 1-fold increase in mTOR phosphorylation was related to 0.81- or –0.83-fold changes in 4EBP1 and eEF2 phosphorylation, respectively. Moreover, mTOR phosphorylation explained about a quarter of the variation in phosphorylation of each ($R^2 = 0.26$ and 0.25,

Table 3. Regression analyses among phosphorylation states of signaling proteins (arbitrary units), total intracellular concentrations of essential AA (ICEAA, mol/L), and fractional synthesis rates of casein (FSR, %·h⁻¹) in bovine mammary tissue slices¹

Independent variable ²	Dependent variable	Regression coefficient	<i>P</i> -value	Adjusted-R ²
Simple linear regressions				
mTOR	4EBP1	0.81	0.02	0.26
	eEF2	-0.83	0.01	0.25
AMPK	mTOR	-0.52	0.03	0.16
	4EBP1	-0.59	0.13	0.06
	eEF2	0.92	<0.01	0.40
ICEAA	mTOR	0.42	0.01	0.35
	4EBP1	0.85	<0.001	0.51
	eEF2	-0.43	0.01	0.31
	AMPK	-0.16	0.13	0.08
AMPK	FSR	-0.45	0.15	0.06
mTOR	FSR	0.93	<0.01	0.42
4EBP1	FSR	0.28	0.03	0.20
eEF2	FSR	-0.50	0.01	0.23
ICEAA	FSR	0.24	0.10	0.11
Multiple linear regressions				
ICEAA	mTOR	0.33	0.04	0.42
AMPK		-0.39	0.13	
AMPK	eEF2	1.02	0.01	0.59
mTOR		-0.11	0.74	
ICEAA		-0.21	0.31	
mTOR	4EBP1	0.58	0.11	0.38
ICEAA		0.42	0.09	
mTOR	FSR	0.74	0.01	0.51
eEF2		-0.33	0.10	
mTOR	FSR	0.80	0.04	0.34
ICEAA		0.04	0.84	

¹When analyzed across acetate-EAA and glucose-EAA experiments.²mTOR = mammalian target of rapamycin; AMPK = AMP-activated protein kinase; 4EBP1 = eukaryotic initiation factor 4E binding protein; eEF2 = eukaryotic elongation factor 2.

respectively). Phosphorylation of AMPK individually had a negative relationship with mTOR phosphorylation; a 1-fold increase in AMPK phosphorylation was associated with a 0.52-fold decline ($P = 0.03$) in mTOR phosphorylation. A notable positive relationship ($R^2 = 0.40$; $P < 0.01$) was found between AMPK phosphorylation and eEF2 phosphorylation. Even when analyzed with mTOR phosphorylation in a multivariate regression model (Table 3), AMPK still had a strong positive ($P < 0.01$; $R^2 = 0.59$) association with eEF2. A 1-fold increase in AMPK phosphorylation was associated with an almost 1-fold increase in eEF2 phosphorylation (Table 3).

Increasing intracellular EAA concentrations were positively related with increasing mTOR ($P = 0.01$) or 4EBP1 ($P < 0.01$) phosphorylation but negatively associated ($P < 0.01$) with eEF2 phosphorylation. The link between intracellular EAA and mTOR phosphorylation occurred independent ($P = 0.04$) of the link between AMPK phosphorylation and mTOR phosphorylation. When analyzed together with mTOR in a multiple linear regression model, intracellular EAA tended ($P = 0.09$) to positively affect 4EBP1 phosphorylation independent of mTOR phosphorylation.

Phosphorylation of mTOR ($P < 0.01$) and 4EBP1 ($P = 0.03$) and phosphorylation of eEF2 ($P = 0.01$) were positively and negatively associated with FSR, respectively. Phosphorylation of mTOR individually explained 42% of casein synthesis rate variation. When analyzed together, both eEF2 ($P = 0.10$) and mTOR ($P = 0.01$) phosphorylation, respectively, had positive and negative relationships independently with casein synthesis rates. Intracellular EAA concentrations alone tended to be positively associated with casein synthesis rates ($P = 0.10$). However, when analyzed together with mTOR phosphorylation, the intracellular EAA effect was nullified ($P = 0.84$), whereas the mTOR effect remained significant ($P = 0.04$).

DISCUSSION

We previously demonstrated that phosphorylation of AMPK on Thr172 was negatively associated with mTOR-mediated anabolic signals in bovine mammary epithelial cells (Appuhamy et al., 2009). Burgos et al. (2013) observed a similar link between 2-deoxyglucose-induced AMPK phosphorylation and mTOR signals that ultimately repressed global protein synthesis in

bovine mammary epithelial cells. In the current work, addition of glucose or EAA to medium reduced ($P < 0.06$) AMPK phosphorylation in MAC-T cells, whereas acetate did not affect it. Moreover, the decline in AMPK phosphorylation for essential AA supplementation was associated with increased ATP content in MAC-T cells. The insignificant effect of acetate supplementation on energy status of the bovine mammary epithelial cells was unexpected because acetate is known to be the most important substrate contributing to oxidative metabolism in the dairy cow mammary glands (Bickerstaffe et al., 1974). Given that the MAC-T cells had been grown in medium with high concentrations of glucose and EAA and devoid of acetate over many passages before the experiments, MAC-T cells might prefer glucose and EAA over acetate. Although Hanigan et al. (2001) reported that the contribution of EAA to oxidative metabolism and ATP synthesis in the bovine mammary glands is relatively minor at the prevailing in vivo concentrations, concentrations of EAA in media were several-fold greater (3.5 mmol/L) than the normal plasma EAA concentrations (~ 0.80 mmol/L; Rius et al., 2010) in dairy cows, and thus the contribution of EAA to oxidative metabolism may have been significant. However, neither acetate, nor glucose, nor EAA had statistically significant effects on AMPK phosphorylation, and thereby cellular energy status, but supplementation of each marginally reduced ($0.16 < P\text{-value} < 0.25$) AMPK phosphorylation by 15 to 17% in mammary tissue slices. It is possible that potentially greater nutrient concentrations in mammary tissue slices and the presence of some EAA (0.18 mmol/L) in the negative control medium may have partly masked the nutrient effects on cellular energy status in mammary tissue slices as compared with MAC-T cells.

Irrespective of the presence of acetate or glucose in the medium, supplementation of EAA significantly increased phosphorylation of mTOR at Ser2448 in MAC-T cells and mammary tissue slices. Phosphorylation of mTOR at Ser2448 is a good biomarker of mTOR pathway activation (Chiang and Abraham, 2005). Although both glucose and EAA significantly reduced AMPK phosphorylation, only EAA significantly activated mTOR, suggesting a stronger EAA effect on mTOR that does not involve much AMPK in MAC-T cells. Consistently, intracellular concentrations of EAA (sum of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine concentrations) were positively related to mTOR phosphorylation ($P = 0.04$) independent of AMPK phosphorylation in mammary tissue slices. Intracellular EAA concentrations explained 35% ($P = 0.01$) of the variability in mTOR phosphorylation, whereas AMPK phosphorylation alone was negatively

associated with mTOR phosphorylation ($P = 0.03$) and explained 16% of the variability.

Eukaryotic factor 4E binding protein 1 is a major downstream target of mTOR (Li et al., 2005). The observed positive relationship ($P = 0.02$) between mTOR phosphorylation and 4EBP1 phosphorylation agrees with this connection. Moreover, hyperphosphorylation of 4EBP1 enhances the initiation step of mRNA translation. Hence, the substantially increased 4EBP1 phosphorylation for EAA supplementation to MAC-T cell and tissue slice media signifies EAA-mediated positive signals on mRNA translation initiation in mammary cells. In our previous experiments, a 1-fold increase in mTOR phosphorylation was associated with a 0.89-fold increase in 4EBP1 phosphorylation (Appuhamy et al., 2011). In the current work, a 1-fold increase in mTOR phosphorylation was associated with a 0.81-fold increase in 4EBP1 phosphorylation. Phosphorylation of 4EBP1 was positively associated with intracellular EAA concentration, as was its upstream kinase mTOR. However, when analyzed with mTOR phosphorylation in the same regression model, EAA still had a notable ($P = 0.09$) association with 4EBP1 phosphorylation, indicating an mTOR-independent mediation of 4EBP1 phosphorylation by EAA. Zhang and Zheng (2012) recently discovered an mTOR-independent control of 4EBP1 phosphorylation. Our finding suggests an involvement of EAA in this control. Neither acetate nor glucose had a significant effect on 4EBP1 phosphorylation in either MAC-T cells or mammary tissue slices. Burgos and Cant (2010) observed a significant increase in 4EBP1 phosphorylation in bovine mammary acini with the addition of a mixture of acetate and glucose to medium deprived of acetate, glucose, and all AA, including nonessential AA. However, addition of acetate and glucose did not change 4EBP1 phosphorylation in the presence of AA in their experiments. In partial agreement with these observations, addition of glucose to medium deprived of EAA numerically ($P = 0.38$) increased 4EBP1 phosphorylation by 18% (phosphorylation status = 1.00 vs. 1.18 units) in MAC-T cells but the differences were not that notable when medium contained EAA (phosphorylation status = 1.75 vs. 1.70 units). The nonessential AA present in the negative control treatments of the present experiments could presumably substitute for energy substrate by contributing to oxidative metabolism and ATP synthesis.

Eukaryotic elongation factor 2 mediates the elongation step of mRNA translation. Phosphorylation of eEF2 at Thr56 by eEF2 kinase inhibits its function. The effect of nutrient supply on phosphorylation of eEF2 at Thr56 in mammary cells has rarely been examined. Nevertheless, Christophersen et al. (2002)

suggested that eEF2 could be a limiting factor specifically in milk protein synthesis. In the present study, as well as our previous experiments, we showed a negative association between EAA supply and phosphorylation state of eEF2, verifying that EAA not only control the initiation step of mRNA translation but also the elongation step in mammary epithelial cells. Moreover, the inverse relationship between mTOR phosphorylation and eEF2 phosphorylation is also consistent with our previous findings (Appuhamy et al., 2011, 2012). The substantially positive relationship between AMPK phosphorylation and eEF2 phosphorylation in bovine mammary cells is a distinctive finding from the present experiments. This association was independent of the relationship of eEF2 with mTOR. Horman et al. (2002) and Chan et al. (2004) reported a positive link between phosphorylation of AMPK and phosphorylation of eEF2 in heart muscle cells and liver cells, respectively. The link between AMPK and eEF2 likely occurs via eEF2 kinase because phosphorylated AMPK has been shown to directly phosphorylate, and thus activate, eEF2 kinase (Browne et al., 2004).

Provision of extracellular EAA increased casein synthesis rates of mammary tissue slices. When analyzed across both experiments, intracellular EAA concentrations were positively ($P = 0.10$) related to casein synthesis rates in a simple linear regression model. A greater proportion of this link appeared to occur via mTOR as the relationship was negated ($P = 0.84$) when mTOR phosphorylation was included in the model. Although eEF2 phosphorylation was associated with mTOR phosphorylation, it tended ($P = 0.10$) to have a negative effect on casein synthesis rates, independent of mTOR phosphorylation. Nonetheless, mTOR phosphorylation was more strongly connected to casein synthesis rates than to eEF2 phosphorylation. A 1-fold increase in mTOR phosphorylation was independently associated with a $0.74\% \cdot \text{h}^{-1}$ ($P = 0.01$) fractional casein synthesis rate increase, whereas a 1-fold increase in eEF2 phosphorylation was related to a $0.33\% \cdot \text{h}^{-1}$ ($P = 0.10$) decline in casein synthesis rates.

In parallel with cell signaling, the effects of extracellular acetate or glucose provisions on casein synthesis rates were minimal. Effects of acetate supply on milk protein synthesis are quite inconsistent across in vivo studies in the literature. Continuous intraruminal infusion of acetate in dairy cows (Rook and Balch, 1961) and dairy goats (Lough et al., 1983) have been reported to increase milk fat yields, without any effect on protein yield, whereas in Safayi and Nielsen (2013), provision of acetate increased milk protein yield particularly in early-lactating cows but did not affect it in cows in late lactation. Increasing milk protein responses for

acetate infusions in Safayi and Nielsen (2013) were associated with increasing negative N balance in dairy cows. We did not see such an effect of cellular N balance on acetate responses. Supplementation of acetate to medium deprived of EAA, acetate, and glucose did not change casein synthesis rates, but addition of EAA alone increased it by 55%. Addition of both acetate and EAA did not affect casein synthesis rates. Purdie et al. (2008) reported a 12% increase in milk protein yield in response to AA infusion and no change in milk protein yield when AA was infused with acetate. Provision of glucose with or without EAA in the medium did not affect casein synthesis rates in mammary tissue slices. In contrast to our results, Toerien et al. (2010) demonstrated that jugular infusion of glucose alone or with EAA increased milk protein yield in dairy cows. Moreover, abomasal infusion of starch increased protein yield in dairy cows regardless of the infusion of casein (Rius et al., 2010). Nonetheless, in vivo glucose effects can occur through many mechanisms that cannot be perfectly tested with in vitro systems. For example, additional glucose can spare AA from utilization in liver gluconeogenesis and thereby more AA can be delivered to the mammary glands. Moreover, elevated plasma glucose concentrations stimulate secretion of insulin, which in turn enhances AA uptake and protein synthesis by the mammary gland (Al-Trad et al., 2009; Burgos and Cant, 2010; Appuhamy et al., 2011).

CONCLUSIONS

In summary, AMPK phosphorylation is positively responsive to decreasing ATP content in bovine mammary epithelial cells. Besides glucose, EAA can serve as an important energy substrate to mammary cells when supplied in high concentrations. Although phosphorylation of AMPK inversely affected mTOR signals, the stimulatory effects of EAA on mTOR signals were significantly stronger than the inverse effects of AMPK. Phosphorylated, and thus activated, mTOR considerably enhanced casein synthesis rates by phosphorylating 4EBP1, which is rate limiting to the initiation of protein synthesis. On the other hand, phosphorylated, and thus activated, AMPK had a positive association with phosphorylation of eEF2, which is detrimental to protein synthesis. However, the positive effect of mTOR phosphorylation on protein synthesis rates was significantly stronger than the negative effect of eEF2 phosphorylation. Overall, EAA strongly enhanced milk protein synthesis by signaling via mTOR pathway independent of AMPK, which was responsive to cellular energy status but had a marginal effect on milk protein synthesis rates.

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