Regulatory Roles of Essential Amino Acids, Energy, and Insulin in Mammary Cell Protein Synthesis

J. A. D. Ranga Niroshan Appuhamy

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Mark D. Hanigan, Chair

R. Michael Akers,

Ronald E. Pearson

Katharine F. Knowlton

Jeffery E. Escobar

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ABSTRACT

Dairy cows inefficiently convert dietary protein to milk protein causing economic and environmental costs. Amino acids (AA), insulin, and glucose significantly enhance muscle protein synthesis efficiencies. The objectives of this research project were 1) to investigate the regulatory effects of essential AA (EAA) and their interactions with insulin, glucose and acetate on mammary protein synthesis rates, 2) to investigate whether branched chain amino acids (BCAA): leucine , isoleucine , and valine , become limiting for milk protein synthesis when Met and Lys supply were not limiting, and 3) to develop a mathematical representation for the EAA and insulin effects on cellular signals for protein synthesis. MAC-T cells were treated with EAA, insulin, glucose, and acetate to observe their individual and interactive effects on phosphorylation of mTOR, rpS6, S6K1, 4EBP1, eEF2, eIF2α, Akt, and AMPK. These signaling effects on protein synthesis rates were examined with mammary tissue slices. A mathematical representation of the insulin and EAA effects was developed. The effects of supplementing BCAA on milk protein synthesis were investigated using nine Holstein cows, assigned to 7 d continuous jugular infusions of saline, Met and Lys, and Met and Lys plus BCAA.

Multiple essential amino acids, Leu, Ile, Met, and Thr were able to substantially regulate protein synthesis rates in bovine mammary cells by increasing ($P < 0.05$)

phosphorylation of mTOR, S6k1, 4EBP1, and decreasing ($P < 0.10$) eEF2 phosphorylation. Insulin considerably ($P < 0.10$) exerted similar signaling effects in MAC-T cells, independent of EAA. Supplementation of only acetate increased ($P = 0.09$) mammary cell energy status as indicated by reduced AMPK phosphorylation in MAC-T cells. Neither acetate nor glucose had substantial regulatory effects on mammary protein synthesis rates. Although Met and Lys supplementation increased ($P < 0.01$) milk protein yields and protein efficiencies, there were no apparent benefits of BCAA supplementation under the feeding circumstances of our study. The developed mathematical model adequately represented the regulatory effects of EAA and insulin. Such mathematical representations of regulatory effects of EAA and their interaction with other nutrients may improve our current AA requirement models to predict AA requirements of dairy cows with increased accuracy.

DEDICATION

I dedicate this dissertation to my beloved father Ranjan, mother Neeta, brothers Sanjeewa, Sanjaya, and Sameera, and my grandmother Beeta

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CHAPTER 1:

Introduction

When compared to swine and poultry, dairy cows are fairly inefficient at converting dietary N to productive protein; milk and muscle. This leads to greater N excretion in urine and feces causing an economic loss to dairy produces and serious environmental pollution problems. Surplus protein feeding to dairy cows is the major reason for excessive nitrogen excretion and thereby reduced N efficiencies. Usage of a wide variety of feedstuffs, nutrient remodeling in the rumen, and lack of knowledge about post absorptive metabolism of amino acids (AA) contribute to inaccurate predictions of dairy cows' amino acid (AA) requirements (Lapierre et al., 2006). Amino acid requirements of dairy cows are therefore tipically expressed as an aggregate AA requirement, namely metabolizable protein (MP). This definition necessitates that MP at or above requirements includes a significant safety margin to ensure an adequate supply of AA for maximum production.

On the other hand, emerging evidence suggests that there are inherent limitations for protein translation in the bovine mammary glands (Moshel et al., 2006). Since protein synthesis rates depend on translation rates (Conner et al. 2006) and AA uptake by the mammary gland appears to be positively correlated with translation efficiencies (Bequette et al., 2003) these limitations likely reduce mammary AA uptake allowing AA to be catabolized by other tissues. Splanchnic tissues can clear 60% of the daily supply of postabsorptive AA (Hanigan et al., 2005). Splanchnic tissue clearance of AA is directly proportional to plasma AA concentrations**.** Since AA concentrations in blood reflect the balance of absorption, catabolism, and anabolism, splanchnic catabolism can be reduced

by decreasing the absorbed supply or by increasing anabolic use of AA (Hanigan et al., 2004). Feeding low protein diets reduces influx of AA into blood and increases anabolic use for milk protein synthesis which increases AA removal from blood. Therefore a feeding strategy combining these concepts should decrease AA catabolism and improved N efficiency in dairy cows. Feeding cows with low CP diets supplemented with AA critical for milk protein synthesis has been recognized as a promising strategy for improving N use efficiencies without compromising milk production of dairy cows (Lapierre et al., 2006). Therefore, identification of AA potentially critical for milk protein synthesis is important.

In addition to being precursors for protein synthesis, AA also regulate translation rates (Kimball 2001). This mechanism is not consistent with the current limiting AA paradigm used for balancing rations. For example, lysine and methionine co-limit milk protein synthesis in cows fed corn-based diets (Nichols et al., 1998). Conventionally we think lysine and methionine are limiting based on substrate availability. However the developing understanding of the regulatory role of AA in protein synthesis directs us to think in a new way. We now suggest that methionine and lysine deficiencies themselves may represent lack of stimulation on milk protein synthesis or corn-based diets may be deficient in some other AA that limit milk protein synthesis, besides the substrate limiting effects of lysine and methionine.

Most AA-induced signals are transmitted via the protein kinase, mammalian target of rapamycin (mTOR, Proud, 2004). mTOR-mediated AA signals for protein synthesis have been thoroughly studied in muscle cells (Moshel et al., 2006). Amino acids, in particular leucine, activate mTOR by phosphorylation. Once activated, mTOR

directly phosphorylates ribosomal protein S6 (rpS6) kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1, Kimball, 2002), and mediates dephosphorylation of eukaryotic elongation factor 2 (eEF2, Proud, 2004). Increasing phosphorylation of rpS6, S6K1 and 4EBP1, and dephosphorylation of eEF2 are associated with increasing muscle protein synthesis rates. Besides AA, hormones, in particular insulin, and energy substrates such as glucose regulate muscle protein synthesis. These signals are respectively mediated by protein kinase B (PKB/Akt) and the cellular energy sensor, AMP-activated protein kinase (AMPK).

Several studies have demonstrated the positive impacts of AA and energy supplementation, and increased plasma insulin concentrations on milk protein synthesis in cows. For example, Rulquin and Pisulewski (2006) observed significant increases in milk protein yield in response to leucine supplementation. Rius et al. (2010) observed increased milk protein yields in cows fed protein deficient but high energy diets. Similarly, abomasal infusion of casein increased milk protein yields of dairy cows by 10% whereas a combined infusion of insulin and casein increased protein yield by 28% (Griinari et al., 1997). These observations therefore lead to a hypothesis that AA, energy, and insulin mediated cellular signaling mechanisms found in muscles cells also exist in bovine mammary epithelial cells. If true, one can devise a better feeding strategy that would lead to improved N utilization efficiencies in dairy cows. Moreover, knowledge of the prospective regulatory effects of AA and their interactions with energy substrates such as glucose and acetate, and insulin will allow for improvements in our mathematical representation of AA requirements of dairy cows.

The objectives of this research project were therefore to 1) investigate the effects of total essential amino acids (EAA) and their interactions with energy substrate such as glucose and acetate, and insulin on mTOR signals and protein synthesis rates in bovine mammary epithelial cells, 2) identify the individual EAA having significant effects on mTOR signals and protein synthesis rates in bovine mammary epithelial cells, 3) investigate whether branched chain amino acids, leucine , isoleucine , and valine , become limiting for milk protein synthesis when Met and Lys supply were not limiting, and 4) develop a mathematical representation for the EAA and insulin effects on mTOR signals.

CHAPTER 2:

Literature Review

Nitrogen utilization efficiency of dairy cows

Dairy cows have been found to be fairly inefficient at converting dietary nitrogen (N) to muscle and milk proteins. Moorby and Theobald. (1999), Spears et al. (2003), and Nadeau et al. (2007) reported that N utilization of dairy cows was approximately 25%. Hence, dairy cows utilize only one fourth of dietary N for protein synthesis while the balance is lost in urine and feces. This N loss is a significant economic loss for dairy producers since protein is the most expensive nutrient fed to dairy cows (Hanigan et al., 1998). Moreover, urine and feces have been recognized as critical sources of environmental pollution. For example, ammonia emitted from dairy manure is a major air and water pollutant contributing to eutrophication, aerosol formation, acid rain, and impaired visibility (USEPA, 2004). An average dairy cow has been found to excrete about 150 g of N in urine per day (Spanghero and Kowalski, 1997, Kebreab et al., 2001). Assuming all urinary N is converted to amonnia and there are nine million dairy cows in the United States (USDA, 2006), the dairy industry can therefore emit approximately 6.0 x 10^5 metric tons of ammonia to atmosphere annually. This is significantly large contribution to US air pollution compared to the annual ammonia emissions from permitted industrial sources of 50 metric tons (Chitikela and Ritter, 2004). Therefore an improvement in nitrogen utilization efficiency will significantly reduce dairy industry contributions to environmental pollution.

Why this much low nitrogen utilization efficiency in dairy cows?

Considering the significant correlation between N intake and N excretion, in particular urinary N (Kebreab et al., 2001), one may speculate that dairy cows may be fed surplus proteins beyond the requirement. This speculation is quite reasonable as there are no clearly defined requirements of amino acids (AA) for dairy cows. Defining AA requirements for ruminants is challenging task due to the usage of large variety of feedstuff and remodeling of dietary nutrients by rumen microorganisms (Lapierre et al., 2006). Therefore, AA requirements of dairy cows are expressed as an aggregate AA requirement, namely metabolizable protein (MP) requirement. Such a definition demands dairy cows to be fed with high safety margins of the defined MP requirement to ensure adequate supply of AA for maximum production. Moreover, N utilization efficiency of dairy cows is relatively less compared to that of monogastric livestock species. In poultry or swine, this efficiency may average 30 to 35% and even approach 40% (Rotz, 2004). Unlike ruminants, protein needs of monogastric animals can be more closely met. Their individual amino acid (AA) requirements have been well defined. This allows for greater N use efficiencies because lower protein diet supplemented with individual AA can maintain similar production compared to what observed with higher protein diet (Lapierre et al., 2006). Implementation of such an approach in dairy nutrition and feeding requires understanding role of individual essential AA in tissue protein synthesis.

Essential amino acids for dairy cows

Essential AA (EAA) have to be supplied through the diet as animals are not able to synthesis them at a rate that is adequate to fulfill requirement of normal growth and function. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine,

threonine, tryptophan, and valine have been recognized as EAA for dairy cows (Schwab et al., 1976). Hanigan et al. (2000) pointed out the need of improving the representation of postabsorptive metabolism of EAA in current protein requirement models.

Post absorptive metabolism of amino acids and nitrogen utilization efficiency

Amino acid metabolism in liver

After exiting the gut tissues, amino acids first flow to the liver where they may be subjected to substantial metabolism (Lapierre et al., 2006). When presented to the liver, AA may 1) pass directly through and become available for peripheral tissues or 2) may be removed from blood and incorporated into liver proteins, subjected to oxidation, or utilized (primarily alanine and glutamine) for gluconeogenesis (Bequette et al. 2003). Liver AA affinity is very low with only 0 to 10% of AA in blood being removed in a single pass (Hanigan, 2005). Thus most absorbed AA reach arterial blood. However, approximately 50% of the cardiac output, and thus systemic AA, recirculate to the liver. Therefore AA not used for productive purposes or catabolized by peripheral tissues will be recycled to the splanchnic tissues (Hanigan, 2005). Branched-chain AA (BCAA), leucine, isoleucine, and valine exhibit minimum hepatic clearance (approximately 1% of absorbed BCAA, (Hanigan et al., 2004). In contrast, substantial amount of histidine, methionine and phenylalanine are removed by the liver and subjected to both oxidation and liver protein synthesis (Bequette et al., 2003).

In mammalian cells, BCAA catabolism involves two major steps. The first step is reversible transamination of BCAA catalyzed by the branched-chain aminotransferase (BCAT). In the second step, branched-chain α-keto acids are oxidatively decarboxylated to produce the corresponding branched-chain acyl-CoA derivatives. This irreversible reaction is catalyzed by the mitochondrial branched-chain α-keto acid dehydrogenase

enzyme complex (BCKD, (DeSantiago et al., 1998)). Since liver lacks the first of these 2 enzyme complexes, it is unable to catabolize BCAA and thus almost all of the dietary supply of BCAA is passed through the liver and available for peripheral tissues such as muscle and the mammary gland. Since the supply of BCAA in blood is reflective of that in the diet, they are a reasonable signal of AA availability for protein synthesis in non-hepatic tissues (Bequette et al., 2003). Hepatic clearance of other AA depends on plasma AA concentrations. The liver removes more AA when their plasma concentrations are high. Since, plasma AA concentrations indicate the balance between absorption and tissue metabolism and the liver removes most by mass action, enhancements of non-hepatic tissue metabolism would reduce AA removal by the liver (and gut tissues as well). Increased use of AA to synthesize milk protein would therefore reduce AA removal by the liver. This would ultimately reduce N excretion via urine and improve N utilization efficiencies of dairy cows.

Amino acid uptake and utilization in the mammary gland

Amino acid uptake by the mammary gland

Mepham (1982) stated that AA uptake by mammary gland could depend on 3 factors: 1) arterial concentration of AA, 2) mammary blood flow, and 3) functionality of transporters or carrier systems in the basal membrane of the secretory cells. Madsen et al. (2005) observed that dietary supplements of rumen protected lysine and methionine increased arterial lysine and methionine concentrations, but mammary uptake of these AA did not change in dairy goats. Bequette et al. (2000) concluded that mammary gland cells can adjust AA uptake, irrespective to their plasma concentrations, by controlling mammary blood flow. These authors observed increased (33%) mammary blood flow in

response to low levels of plasma histidine in dairy goats. Moreover, mammary gland's capacity to remove plasma His increased 43-fold, whereas the gland's capacity to remove other plentifully available AA declined by two- to threefold. These observations suggest of mammary uptake of AA is a function of internal metabolism and not simply plasma concentrations. Shotwell et al. (1982) concluded that the activity of some transport systems in Chinese hamster ovary cells is regulated by a mechanism which acts at the level of translation. Christensen (1990) reported that AA transporter activity depends on intracellular AA concentrations which are determined mainly by translation or protein synthesis rates. Guan et al. (2004) also reported such a positive correlation between essential AA uptake and their demand for milk protein synthesis in the porcine mammary gland.

Metabolism of amino acids in the mammary gland

Once AA enter the cells, they can participate in 1) milk protein synthesis, 2) synthesis of other proteins (i. e., structural proteins and enzymes), or 3) catabolic reactions (i. e., oxidation). Additionally, AA can pass unchanged into milk, blood or lymph (Mepham, 1982). Mammary glands tend to extract BCAA in excess compared to their requirements for milk protein synthesis. The excess BCAA presumably undergo oxidation in lactating mammary gland (Hanigan et al., 2004). Expression of BCAT and BCKD activity have been reported to significantly increase in the mammary gland during lactation (DeSantiago et al., 1998).

Besides BCAA, mammary glands also tend to extract Arginine and Lysine in excess. Although Lysine is considered a limiting for milk protein synthesis in dairy cows when fed corn ingredient-based diets (Nichols et al., 1998), it appeared to be almost

always taken up in excess by the udder (Bequette et al. 2003). Annison and Linzell (1964) showed that 24 to 44% of CO_2 produced by goat mammary gland could not be accounted for glucose or acetate oxidation. This indicates that EAA are also energy sources.

Excess arginine undergoes hydrolysis into ornithine and urea. Ornithine 1) acts as a carbon precursor for proline, 2) acts as a N precursor for several non-essential amino acids, and 3) give rise to the polyamine spermidine which is able to substitute for lactogenic cortisol (Mepham, 1982). Moreover, Lacasse et al (1996) found nitric oxide synthase in the vascular endothelium and secretory epithelium of the bovine mammary gland. Nitric oxide synthase (NOS) catalyzes the reaction converting arginine to nitric oxide involved in blood flow regulation. Lacasse et al (1996) also observed that co-infusion of arginine with N omega-nitro-arginine (NONA), inhibitor of NOS, markedly reduced NONA's ability to decrease mammary blood flow. Therefore the excess arginine can also act as a precursor for nitric oxide in the bovine mammary gland.

Extraction of Phe, Met, Thr, and His by mammary glands appears to more closely match the amounts required for milk protein output (Bequette et al., 2003). Clark et al. (1978) observed that Met and Thr were limiting for milk protein synthesis in bovine mammary cell culture. A number of studies have shown that supplementation of Met, either alone or together with Lys, significantly improved milk protein synthesis in dairy cows fed corn based diets (Weekes et al., 2006).

Clark (1975) mentioned that high producing cows fail to produce to their genetic capacity due to limited supply of key nutrients. Considering EAA as the "key nutrients", Mepham (1982) indicated that although the "key nutrients" are present, their transfer to

protein could be limited by inadequate stimulation (e. g. a key hormone) for protein synthesis. Moshel et al. (2006) indicated limitations in bovine mammary gland at mRNA translation level based on the studies by Persuy et al. (1992) and Rijnkels et al. (1995). Christophersen et al (2002) observed that some protein factors involved in mRNA translation were more critical to milk protein synthesis than to muscle and liver protein syntheses in lactating dairy cows. Moreover, Toerien and Cant (2007) concluded that some protein factors critical for mRNA translation are not present in a maximally active form in bovine mammary glands. All these observations suggest that besides excessive protein feeding, some physiological limitations at milk protein translation may also be responsible for poor N utilization efficiencies in dairy cows. Therefore it is worth taking a look at mRNA translation mechanisms in mammalian cells.

mRNA translation and critical control points

The mechanisms involved in protein synthesis or messenger RNA (mRNA) translation in mammary epithelial cells is similar to that in other mammalian cells (Mepham, 1982). The process of translating mRNA into protein is generally divided into three major steps: initiation, elongation, and termination. Initiation involves the binding of ribosome to the start codon on the messenger RNA sequence. Elongation is characterized by translocation of ribosome along the mRNA while translating the copied genetic code into a chain of amino acids that are supplied by transfer RNA (tRNA). Termination occurs when the ribosome and the newly formed polypeptide chain both detach from the mRNA after reaching the end of the protein-coding region on the mRNA (Voorma et al., 1994). Rates of protein synthesis are determined by the amount of mRNA transcribed, the number of ribosomes present in a cell, and the translational efficiency per

ribosome (Anthony et al., 2001a). Translation efficiency per ribosome depends on translation initiation and elongation rates respectively controlled by eukaryotic initiation (eIF) and elongation (eEF) factors (Connors et al., 2008).

Translation initiation and its control

Translation initiation has been recognized as a key site of protein synthesis control (Goldstein et al., 1974). Protein synthesis is initiated universally with the amino acid methionine. The codon used for initiation is almost always AUG (Drabkin and RajBhandary, 1998). During the initiation process, the 80S pre-initiation complex consisting of initiator methionyl-tRNA (met-tRNAi), and 43S and 60S ribosomal subunits binds mRNA at the initiation codon of mRNA (Pestova et al., 1996). As Figure 2-1 shows, this process basically involves two steps: 1) binding of met-tRNAi to the 40S ribosomal subunit to form 43S preinitiation complex, and 2) binding of mRNA to the 43S preinitiation complex. Both steps are subjected to regulation (Anthony et al., 2001a). Formation of the 43S preinitiation complex is mediated by the heterotrimeric protein eIF2 (three subunits, α, β, and γ). Phosphorylation of eIF2 on Ser51 of the α subunit impairs its facilitation of the 43S complex formation (Suragani et al., 2005).

mRNA binding to the 40S ribosomal subunit is mediated by another heterotrimeric protein referred to as eIF4F, which consists of eIF4A, an RNA helicase, eIF4E, the mRNA cap binding protein, and eIF4G, an adaptor protein containing binding sites for eIF4A, and eIF4E (Haghighat et al., 1996). Since eIF4G also has a binding site for eIF3, the eIF4F complex interacts with 43S preinitiation complex through eIF3 (Figure 2-1) and binds to 5’ cap of mRNA with the help of cap-binding protein eIF4E. Once it binds to the 5’-cap, it starts scanning along non-coding regions of mRNA until it

locates the start codon (Pestova et al., 1996). After arrival at the start codon, the eIF4F complex directs the met-tRNAi-carrying 43S ribosomal subunit to the start codon and protein synthesis is initiated.

Mechanisms controlling translation initiation

In eukaryotic cells, eIF4E-mediated cap-binding of the 43S subunit is rate limiting for translation initiation (Rau et al., 1996). Two mechanisms for regulating translation initiation of mammalian cells are recognized with respect to eIF4E. The first mechanism involves phosphorylation of eIF4E itself at Ser11. This phosphorylation reaction enhances the affinity of eIF4E for the 5'-cap structure of mRNA (Minich et al., 1994). However, several *in-vivo* studies revealed increased protein synthesis rates despite low levels of eIF4E suggesting the phosphorylation state of eIF4E is not critical for protein synthesis (Kimball, 2002).

The second mechanism involves the binding of eIF4E to a family of eIF4E binding proteins referred to as 4E-BP. Pause et al., (1994) reported three forms of 4E-BP; 4E-BP1, 2, and 3 and found that the functionality of these proteins can be modulated through alteration of their phosphorylation status. Unphosphorylated 4E-BP1 competitively binds eIF4E and prevents its binding to eIF4G to form cap-binding complex, eIF4F. Hyperphosphorylation of 4E-BP1 results in dissociation of eIF4E making more eIF4E available to support faster translation initiation. Proud and Denton (1997) reported that virtually all eIF4E could be bound to 4E-BP1 in rat adipocytes because binding of eIF4E to 4E-BP1 occurred at a stoichiometry of 1:1 and considerable excess of 4E-BP1 has been found relative to eIF4E in these cells. As eIF4E-mediated

translation initiation is important for translation of most of the mRNAs**,** rate of phosphorylation of 4E-BP1 limits global protein synthesis.

Eukaryotic initiation factor 4E binding protein 1 is phosphorylated at multiple sites. Gingras et al. (2001) reported that phosphorylation of 4E-BP1 is hierachial because phosphorylation of some sites were required for subsequent phosphorylations. For example, phosphorylation at Thr37/46 was a prerequisite for phosphorylations at Thr70 and Ser65. Therefore, phosphorylation of Thr37/46 itself is a reasonable marker of hyperphosphorylation of 4EBP1.

Translation initiation of 5'-TOP mRNA

Besides the initiation and elongation rates that determine translation rate per ribosome, the number of ribosomes available for translation is also important in determining overall efficiency of protein synthesis. Messenger RNAs encoding ribosomal proteins and some of the protein factors involved in translation such as eEFs and eIF4G (Jefferson and Kimball, 2003) are characterized by 5' terminal oligopyrimidine tracts (5'-TOP). This element is comprised of a cytidine residue at the 5' cap site followed by an uninterrupted stretch of up to 13 pyrimidines (Avni et al., 1997). Phosphorylation of ribosomal protein S6 (rpS6) by its kinase (S6K1) has been found to up-regulate 5'-TOP-mRNA (Kimball, 2002). Studies delineating the mechanism of action of rpS6 in enhancing 5'-TOP-mRNA translation revealed that rpS6 was present near the eIFs and mRNAs suggesting that rpS6 might support translation initiation of this specific and important group of mRNA (Anthony et al., 2001a).

Regulation of translation elongation

In the elongation step of translation, the ribosome moves one codon ahead while peptidyl t-RNA moves from the A-site to the P-site, following formation of a new peptide bond (Wang and Proud, 2006). This translocation of ribosome is mediated mainly by eEF2. Phosphorylation of eEF2 at Thr56 impairs its function as it is unable to bind to the ribosome. This phosphorylation is catalyzed by eEF2 kinase. Christophersen et al. (2002) found 20x and 50x greater eEF2 levels in the mammary gland than the levels found respectively in liver and skeletal muscle of lactating dairy cows. This indicates that eEF2 is critical for milk protein synthesis.

Overall eIF2, 4EBP1, eEF2, and S6K1 and its substrate rpS6 play significant roles in regulating protein synthesis. Activity of these proteins is modulated through alterations of their phosphorylation state which has been found to respond to hormones, in particular insulin (Proud, 2006), AA (Kimball, 2002), and energy substrates such as glucose (Jeyapalan et al., 2007). Many studies have demonstrated that insulin, AA, and glucose enhance protein synthesis via signaling through a major protein kinase, mammalian target of rapamycin (mTOR, Figure 2-2).

Mammalian target of rapamycin (mTOR)

Mammalian Target of Rapamycin (mTOR) was discovered in studies into the mechanism of action of rapamycin, an immunosuppressant used clinically to prevent kidney graft rejection. Sabatini et al. (1994) discovered a 249 kDa protein (2549 amino acids) as the target of rapamycin and named it first as RAFT. They continued to work on cDNA libraries of this protein and discovered that RAFT was the mammalian homolog of target of rapamycin (TOR) in yeast. RAFT was later named mTOR. Mammalian target of

rapamycin is a member of a lipid kinase family, phosphoinositide-3-kinase related kinases (PI3KK), although it is able to phosphorylate only proteins at Threonine or Serine residues (Wang and Proud, 2006). Several studies have shown that mTOR exists in two functionally and structurally distinct protein complexes i. e., mTOR complex 1 (TORC1) and complex 2 (TORC2) (Yang et al., 2006). Both complexes contained mTOR and two other protein factors called LST8 and GβL. However the regulatory associated protein of mTOR (Raptor) and the rapamycin insensitive component of mTOR (rictor) are the two distinct proteins present in TORC1 and TORC2 respectively. Rapamycin inhibits not all (Yang et al., 2006) but several functions of mTOR by its action on TORC1. Importantly, TORC2 is rapamycin insensitive and its functions are poorly characterized (Yang et al., 2006). Rapamycin-sensitive mTOR complex 1 (mTORC1) have been well studied and found to promote protein synthesis, cell growth, and cell proliferation in response to hormones, growth factors, and nutritional cues (Foster et al., 2009).

Ribosomal protein S6 kinase 1 (S6K1) and 4EBP1 have been well characterized as direct substrates of mTOR (Avruch et al., 2009). Both S6K1 and 4EBP1 have been shown to possess a similar regulatory motif with an AA sequence of Arg-Ala-Ile-Pro in the N-terminus. The interactions of 4EBP1 and S6K1 with mTOR are believed to occur using this motif and therefore it has been named the TOS (mTOR-signaling) motif (Proud, 2004). As its name (regulatory associated protein of mTOR) implies, Raptor facilitates interaction between mTOR and its substrates 4EBP1 and S6K1(Foster et al., 2009). The catalytic domain of mTOR is located near the C-terminus most likely between AA 2430 – 2450 (Cheng et al., 2004). Phosphorylation in this region activates mTOR. Reynolds et al. (2002) concluded that changes in phosphorylation of mTOR at Ser2448

was strongly associated with protein synthesis. Moreover, Cheng et al. (2004) identified Thr2446 as a novel phosphorylation site on mTOR. These authors showed that phosphorylation of mTOR at Thr2446 inhibited mTOR activity through impairment of phosphorylation at other AA residues (e. g. Ser2448) that enhance mTOR activity. Besides the changes in the phosphorylation state of mTOR itself, phosphorylation of raptor can also modulate mTOR function (e. g. phosphorylation state of 4EBP1 and S6K1). Phosphorylation of raptor at Set792 was negatively associated with mTOR (Gwin et al., (2008).

Mammalian target of rapamycin appears to control phosphorylation of eEF2 on Thr56 by modulating the activity of eEF2 kinase. Redpath et al. (1996) observed an inverse association between mTOR activity and eEF2 kinase activity. Proud (2004) reported that this link between mTOR and eEF2 kinase is mediated via S6K1 since they found that S6K1 can phosphorylate eEF2 kinase at Ser366. mTOR-induced phosphorylation of 4EBP1 and S6K1, and dephosphorylation of eEF2 ultimately led to improved protein synthesis efficiencies. However, phosphorylation and thus inactivation of eIF2 by general control non-repressible kinases (GCNs) has been shown to be mTOR-independent (Anthony et al., 2004).

Regulation of mTOR pathway by amino acids

Amino acid availability regulates mTOR signaling. This is not surprising because AA are the building blocks of proteins. In most studies that tested the effects of individual AA through addition to an AA deprived media, only Leu had significant stimulatory effects on mTOR signals (Kimball, 2001). However, Proud (2004) reported that the other AA could also have considerable control over mTOR signals when

removed from AA rich media. Hara et al. (1998) observed that withdrawal of leucine or arginine is each nearly as effective in downregulating mTOR signals in Chinese hamster ovary cells as withdrawal of all amino acids. However leucine was found to uniquely stimulate mTOR signals in muscle cells (Avruch et al., 2009, Escobar et al., 2006).

It is unclear whether mTOR signals responds to extracellular or intracellular levels of AA. The results from experiments conducted by Anne Beugnet and her group (Beugnet et al., 2003) supported the idea that intracellular AA regulate mTOR signaling. However, the upstream protein factors that link AA stimuli to mTOR still remain to be identified. Several candidate protein factors such as Ras homologue enriched in brain (Rheb), tuberous sclerosis complex 2 (TSC2), Vps34, and Rag-GTPase have been proposed.

A small GTPase named Rheb (Figure 2-2) was proposed to mediate the stimulatory effects of AA on mTOR. When loaded with GTP. Rheb tends to activate mTOR through an unknown mechanism (Roccio et al., 2005). The level of GTP loading on Rheb appears to be controlled by GTPase-activating protein TSC2 (Figure 2-2). Therefore it was speculated that AA acted through TSC2 to regulate mTOR activity via Rheb. However, Smith et al. (2005) demonstrated that TSC2 is not required for regulation of mTOR by AA. On the other hand, Nobukuni et al. (2005) pointed out the role of class 3 phosphatidylinositol-3OH kinase (PI3K), Vps34 in the control of mTOR by AA. However, Kim et al. (2008) reported that *Drosophila* with Vps34-null mutations had normal TOR activity suggesting that Vps34 is not critical for mTOR activity. These authors identified new protein factors, Rag GTPases as activators of TORC1 in response to AA signals in *Drosophila*. Despite the lack of a clear mechanism for mediating mTOR

phosphorylation, the stimulatory effects of AA on mTOR signaling in muscle cells have been extensively studied. Amino acid supplementation increased phosphorylation of mTOR (Ser2448), 4EBP1 (Thr70), S6K1 (Ser389), and rpS6 (Ser235/236 and Ser240/244) in skeletal muscle tissues while enhancing muscle protein synthesis in-vivo (Escobar et al., 2006, Orellana et al., 2007). Therefore increased phosphorylation of these signaling proteins at these AA residues could be reasonable indicators of improved protein synthesis.

Regulation of mTOR signals by insulin

Upon binding to its tyrosine kinase receptor, insulin promotes autophosphorylation of its receptor which lead to activation. Activated insulin receptor in turn activates the phosphatidylinositol-3-kinase (PI3K) through phosphorylation of adaptor proteins such as insulin receptor substrate 1 (IRS1) (Fujita et al., 2007). Phosphorylation of PI3K leads to phosphorylation of Akt/protein kinase B (PKB, Figure 2-2) at Thr308 or Thr473. Akt-mediated phosphorylation (Thr1462, Ser924, or Thr1518) of TSC2 impairs its GTPase activity (Figure 2-2) while leaving Rheb in GTP-loaded form. GTP-loaded Rheb activates mTOR. Therefore, the mechanism through which insulin activates mTOR is independent of the mechanism through which AA affect mTOR. Suryawan et al. (2004) reported that insulin and amino acid supplementations differentially activate mTOR pathways in muscle tissues.

Insulin increases phosphorylation of 4EBP1 at both Ser65 and Thr70. AA mainly increase its phosphorylation at Thr37/46 whereas insulin can also increase this phosphorylation to a certain extent (Proud, 2004). Chang et al. (2004) identified Ser2448 as the Akt mediated phosphorylation site of mTOR. Browne and Proud (2004) identified

new phosphorylation site in eEF2 kinase (Ser78) which was responsive to insulin in an mTOR-dependent but S6K1-independent manner.

Cellular energy state controlling mTOR

Protein synthesis is an energetically expensive process. For example, Hanigan et al. (1998) reported that protein synthesis in bovine mammary gland require 37% of the available ATP. When cells undergo an energy stress, they tend to shut down protein synthesis as an energy conservation stratergy. Since mTOR signals strongly stimulate cellular protein synthesis, deteriorating cellular energy state appears to inhibit mTOR signals. This inhibition has been found to occur via cellular energy sensing protein, AMP-activated protein kinase (AMPK). AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric kinase complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits (Gwinn et al., 2008). AMPK is activated under conditions of energy stress, when intracellular ATP levels decline and intracellular AMP levels increase, as occur during nutrient deprivation or hypoxia. AMP directly binds to AMPK and activates it by phosphorylating the α subunit at Thr172 (Hardie, 2008). Once activated, AMPK phosphorylates TSC2 at Ser1387 and thereby activates it (Huang and Manning, 2008). Activated TSC2 in turn impairs mTOR activity through its GTPase activity on Rheb (Figure 2-2). Importantly, AMPK is able to directly control phosphorylation of S6K1 (Inoki et al., 2003) and eEF2 kinase (Browne and Proud, 2004) independent of mTOR. Moreover, Hardie et al. (2008) reported that AMPK can negatively affect the activity of raptor by directly phosphorylating it.

The signaling effects of AA, insulin and glucose have been extensively studied in muscle and liver cells (Moshel et al., 2006) but little work has been done to explore these

effects in other tissues such as mammary tissues that produce substantial amounts of protein. The literature provides a fair amount of evidence for a hypothesis that similar signaling mechanisms may occur even in the mammary gland.

Effects of insulin, and energy and amino acid supply on milk protein yields

Abomasal infusion of casein increased milk protein yields of dairy cows by 10% whereas infusion of insulin and casein increased protein yield by 28% (Griinari et al., 1997). On the other hand, feeding a high protein diet supplemented with BCAA: leucine, isoleucine and valine increased milk protein synthesis in lactating sows but infusion of insulin did not have apperant effects. Again, combined effects of insulin and dietary protein were associated with the highest milk protein yields (Dunshea et al., 2005). Supplementation of protein, in particular BCAA, has resulted in mixed observation in dairy cows. For example, Mackle et al. (1999) observed insignificant effects of BCAA supplementations on milk protein yields in dairy cows but Rulquin and Pisulewski (2006a) observed significant increase in milk protein yield for Leucine supplementation. Moreover, Broderick et al. (2003) reported a linear increase in milk protein yields in response to increasing dietary protein and energy (represented by increasing NDF) levels. However, these results do not help to distinguish whether increased milk protein yields are due to enhanced microbial protein synthesis or due to the effects of post-absorptive metabolism of these nutrients. However, Toerien et al. (2009) observed increased milk protein synthesis for jugular infusion of glucose which was further elevated by infusion of some EAA.

Potential regulatory effects of amino acids in the mammary gland and their impact on protein requirement models

These observations all indicate potential interplay among metabolites arising from different combinations of nutrient supply, and this is inconsistent with current nutrient requirement models where a single nutrient is assumed to limit production. As discussed previously, the mammary gland is able to adjust transporter activity and mammary blood flow to maintain optimal milk protein synthesis despite the deficiencies in some AA. These adjustments are associated with milk protein synthesis rates as increased AA uptake or enhanced mammary blood flow ultimately aim to cater the demand of AA for protein synthesis. Since AA are able to regulate protein synthesis rates, AA should consequently influence AA entry into the mammary gland. Therefore uptake and utilization efficiency of an AA may have to depend on potential regulatory effects of the other AA or metabolites on protein synthesis. This does not agree with the fixed transfer efficiencies of AA in current requirement models. Moreover, under these circumstances, more than one AA can be limiting or critical for milk protein synthesis in disagreement with the single limiting AA concept of animal nutrition.

Bequette et al. (2003) mentioned that few experiments tested the aggregate effect of AA (i. e. casein infusion) on milk protein synthesis rather than testing the individual effects which is more important. However, an *in vivo* study testing this many treatments (e. g. ten treatments for ten essential amino acids) would demand a large number of animals and thus require a large investment of time, labor, and money because cows' milk protein responses to individual AA are highly variable (50% coefficient of variation, (Clark et al., 1978)). Under such circumstances, mammary tissue or cell culture

approaches are helpful in testing the effects of individual AA on milk protein synthesis. Moreover, as the balance of AA use between mammary tissue and other tissues in the body is influenced by hormones and other metabolites such as ATP (Hanigan et al., 2004), investigations into interactions of AA with insulin and energy substrates such as glucose and acetate could be warranted.

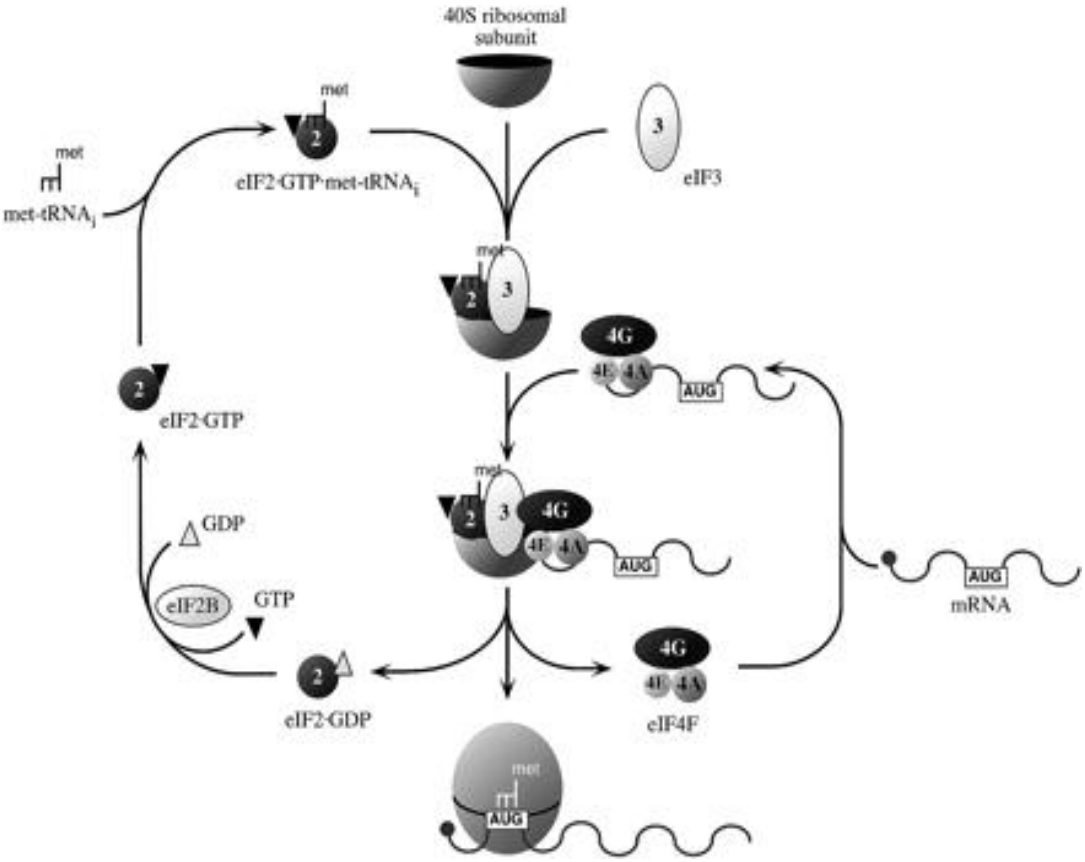

Figure 2-1. Initiation of mRNA translation in eukaryotic cells. The diagram highlights the two key regulatory steps in translation initiation: the binding of initiator methionyl-tRNA to the 40S ribosomal subunit and the binding of mRNA to the 40S ribosomal subunit (Kimball, 2002).

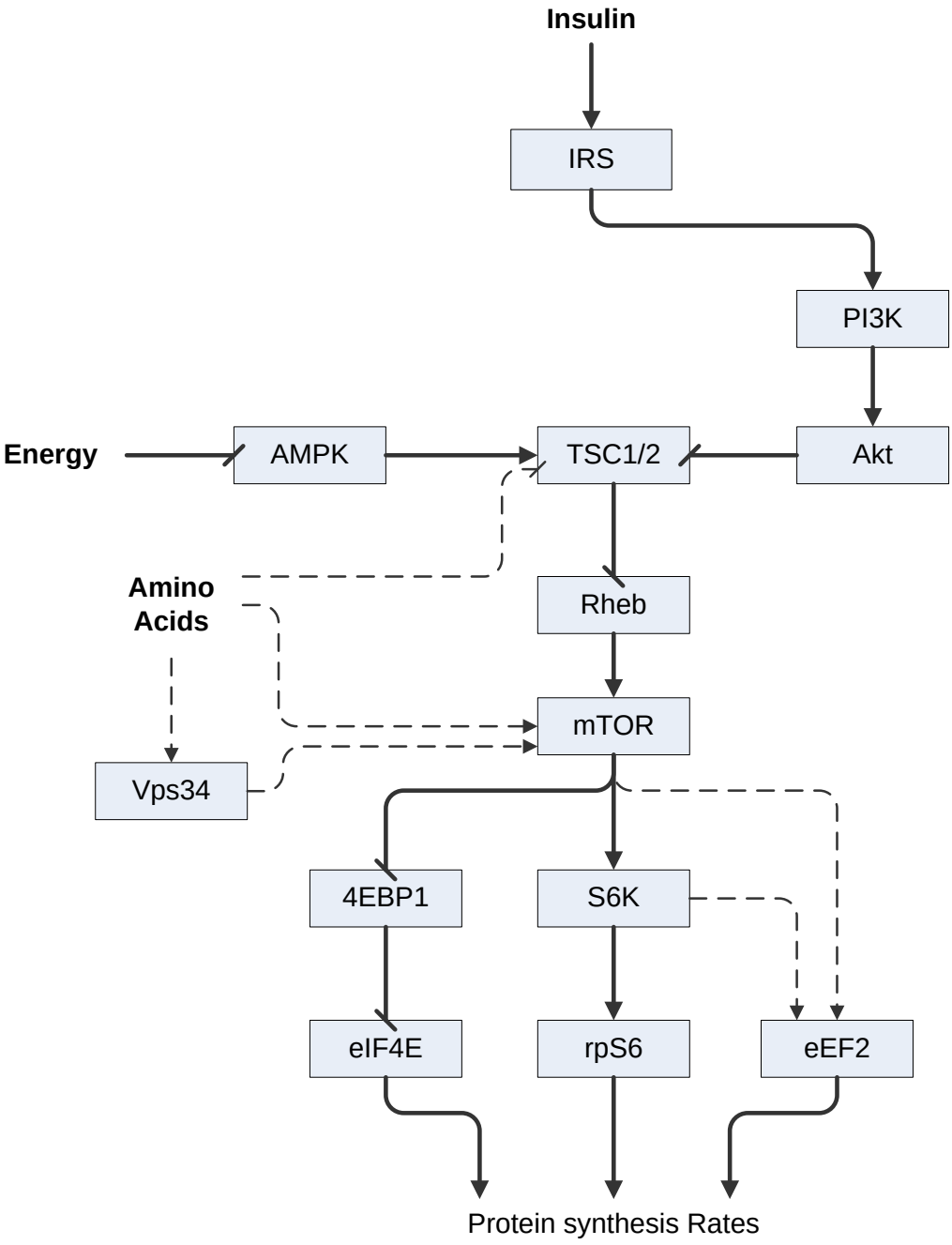

Figure 2-2. mTOR signaling pathway influenced by amino acids, insulin and cellular energy status as demonstrated in muscle cells.

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CHAPTER 3:

Essential Amino Acids and Insulin Mediated Cellular Signals for Protein Synthesis in Bovine Mammary Cells

ABSTRACT

Supplementation of amino acids (AA) and increased plasma insulin concentrations have been found to enhance mil protein synthesis in dairy cows. Little work has explored cellular mechanisms associated with these stimulatory effects. Objective of this study was to investigate the effects of essential AA (EAA) and insulin on cellular signaling for protein synthesis in bovine mammary cells. Bovine mammary epithelila cell line, MAC-T, was grown in 0 or 3.5 mM EAA with 0 or 100 ng/mL insulin in a 2×2 factorial arrangement of treatments. In separate factorial experiments, lactogenic bovine mammary tissue slices were incubated in $^{2}H_5$-Phe enriched media with same levels of insulin with 0.18 or 3.5 mM EAA. Phoshorylation state of protein kinase B (Akt), mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase 1 (S6K1), eukaryotic initiation factor (eIF) 4E binding protein 1 (4E-BP1), eukaryotic elongation factor 2 (eEF2), and eIF2α were analyzed. $^{2}H_5$-Phe enrichment in protein ($^{2}H_5$-Phe_PRT) was measured in mammary tissue slices. In MAC-T cells, deprivation of EAA and insulin respectively reduced phosphorylation of mTOR, S6K1, 4E-BP1, and eEF2 by 46 to 60% and 30 to 40%, respectively. In mammary tissue explants, EAA deprivation also reduced phosphorylation of these signaling proteins and $^{2}H_5$-Phe-PRT content by 30 to 40% and 60%, respectively. Extracellular insulin availability increased phosphorylation of Akt by 83% but reduced phosphorylation of eIF2α by 48% in an mTOR-independent manner.

Interactive effects of EAA and insulin on mTOR were non-significant supporting the hypothesis that protein synthesis in bovine mammary glands is a variable function of at least EAA availability and insulin signaling.

INTRODUCTION

When dairy cattle are fed to meet NRC requirements, about 25% of dietary nitrogen (**N**) is converted to productive N (milk or muscle) and 75% is lost in feces and urine (Bequette et al., 2003). This loss of N represents both an economic and environmental cost. Approximately one-third of the N loss occurs through catabolism of amino acids (**AA**) by ruminal microbes, with the remainder catabolized by postabsorptive tissues. A large proportion of the postabsorptive loss occurs in the splanchnic tissues (Hanigan et al., 2004). Since splanchnic tissues remove AA in a concentration dependent manner, it has been hypothesized that N utilization efficiency could be significantly improved if blood AA concentrations were reduced which would lead to a reduction in splanchnic AA removal from blood (Hanigan, 2005). As AA concentrations in blood reflect the balance of absorption, catabolism, and anabolism, splanchnic catabolism can be reduced by decreasing the absorbed supply or by increasing anabolic use, namely, milk protein synthesis.

Protein synthesis rates depend primarily on translation initiation and elongation rates that are regulated by a number of protein factors such as eukaryotic initiation factors (**eIF**) and eukaryotic elongation factors (**eEF**) (Connors et al., 2008). Activity of these protein factors is determined by the degree of phosphorylation that responds to various signals such as hormones (particularly insulin) and metabolites (particularly AA). Understanding the impact of these signals on protein synthesis in mammary epithelial

cells is critical to achieving a quantitative understanding of AA requirements for tissue maintenance and milk production. It is also critical to improving the efficiency of nitrogen utilization in the animal because the balance of AA use between mammary tissue and other tissues in the body is influenced by hormones and metabolite supply (Hanigan et al., 1998). The interplay among metabolites arising from variations in nutrient supply is demonstrated at the animal level by the work of Rius et al. (2010), where animals in a protein deficient state responded to dietary energy by increasing milk and milk protein yields. Similarly, abomasal infusion of casein increased milk protein yield of dairy cows by 10% whereas infusion of insulin and casein increased protein yield by 28% (Griinari et al., 1997). These responses are inconsistent with current nutrient requirement models where a single nutrient is assumed to limit production. They also demonstrate the plasticity of AA metabolism resulting in varying postabsorptive N efficiencies which is also inconsistent with current requirement models.

Large amount of work has demonstrated the positive effects of AA and insulin on protein synthesis in skeletal muscle and liver (Moshel et al., 2006). Insulin and AA induced cellular signals regulating protein synthesis and these signals have been shown to converge on mammalian target of rapamycin (**mTOR**) (Proud, 2004). Additionally, AA and insulin appeared to regulate protein synthesis via altering the phosphorylation of eukaryotic initiation factor 2 (**eIF2**) (Zhang et al., 2002, Orellana et al., 2007). Little work has explored these cellular signals and the potential interactions in bovine mammary epithelial cells. Knowledge of these mechanisms will allow improvements in the mathematical representation of our AA requirement models leading to more accurate predictions of their requirements.

We hypothesized that insulin and AA independently and positively affect protein synthesis in mammary epithelial cells through similar signaling mechanisms that have been described in skeletal muscle cells. The objective of this study was to investigate the effects of essential AA (**EAA**) and insulin on cellular signaling and protein synthesis in bovine mammary epithelial cells (**BME**) as well as in lactogenic bovine mammary tissue slices (**MTS**).

MATERIALS & METHODS

Cells and tissues

Two separate experiments were conducted using immortalized BME cell line, MAC-T, established by Huynh et al. (1991) and MTS. The MAC-T cells were seeded into 6-well plates at a cell density of 5×10^4 and grown to about 90% confluency in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; Invitrogen, Carlsberg, CA) containing 3.51 g/L of D-glucose and supplemented with 8% (v/v) of fetal bovine serum, 100 U/mL penicilin, 100 ug/mL streptomycin, and 0.25 ug/mL amphotericin B (HyClone Laboratories Inc., Logan, UT). Cells were kept in a humidified atmosphere of 5% CO_2 at 37°C. The Mammary tissues slices were prepared from the rear quarter of 4 multiparous lactating Holstein cows immediately after slaughter. These cows were screened for mastitis pathogens prior to slaughter to insure collection of healthy tissues. Cows were removed from feed for 12 h prior to slaughter. A series of tissue slices of 130 ± 30 mg were prepared using a Stadie-Riggs hand-held microtome and placed in 25-ml Erlenmeyer flasks containing 4 ml of treatment media. All slices were prepared within 30 min of animal slaughter.

Treatments and experimental design

The MAC-T cells were serum starved overnight and then treated with 2 levels of EAA and 2 levels of insulin in a 2×2 factorial arrangement of treatments (Table 1). Amino acid levels were the EAA concentrations in regular DMEM/F12 (+**EAA**, 0.5 Lys, 0.12 Met, 0.70 Arg, 0.45 Leu, 0.42 Ile, 0.45 Val, 0.15 His, 0.22 Phe, 0.45 Thr, and 0.04 Trp; all in mM) or DMEME/F12 devoid of EAA (-**EAA**). Bovine insulin (Sigma, St. Louis, MO) levels were 0 (-**I**) or 100 ng/mL (+**I**). MAC-T cells were incubated with treatment media for 1 h. Cessation of the treatments was ensured by placing the plates on ice followed by addition of ice-cold stop buffer containing 1 mM NaF and 10 μM Na_3VO_4 in sterile PBS. Cells were lysed by scraping ice-cold buffer containing 20 mM HEPES (pH 7.4), 50 mM NaF, 100 mM KCl, 0.2 mM, 50 mM β-glycerophosphate, 1% (v/v) Triton X-100, 1 mM benzamadine, 1 mM DTT, 0.5 mM Na_3VO_4, and 1% (v/v) of protease and phosphatase inhibitor cocktail (ThermoScientific, Rockford, IL). Cell lysates from 6 wells were combined, centrifuged at 12,000×g for 10 min at 4°C. The resulting supernatants were subjected to Western immunoblotting analyses. The cell culture experiments were repeated four times in four different days.

The experimental design and treatments for the MTS were the same except that the EAA deprived treatments (-EAA) consisted of 0.175 mM EAA (5% of the regular concentrations in DMEM/F12, Table 1). The cell culture experiments were first conducted with complete deprivation of EAA and insulin from greater concentrations (3.50 and 100 ng/mL, respectively) in order to have a high chance of seeing significant signaling effects. The tissue culture experiments were conducted later to test the

relationships of these signaling effects to mammary protein synthesis rates. The protein synthesis rates were determined by measuring $^{2}H_5$-Phe enrichments in mammry tissue proteins as described below. With the aim of maintaining a constant $^{2}H_5$-Phe to Phe ratio of 0.20 in all treatment media, some amounts of Phe and the other EAA were left in media. As preliminary dose response experiments revealed that at least 0.70 mM (20% of regular EAA concentration in DMEM/F12) should be present in media to induce significant mTOR-dependent signals (i.e., phosphorylation of ribosomal protein S6) for protein synthesis, the 0.175 mM EAA (5% of regular EAA concentration in DMEM/F12) concentration was chosen as -EAA treatments (Table 1) for MTS.

Two flasks containing individual mammary tissue slices of each cow were randomly assigned to each of 4 treatment media (Table 1), purged with 95:5 mix of O_2:CO_2 for 20 s, sealed with rubber stoppers, and placed in a shaking water bath at 37°C. Flasks were opened at 90 min for the addition of $^{2}H_5$-Phe, purged as described above and placed back in the shaking water bath. Incubations were stopped by placing the flasks on ice and immediately adding ice-cold stop buffer at 120 min. Tissue slices were retrieved, washed twice in the ice-cold stop buffer, mixed 1:7 (w:v) with lysis buffer, and homogenized (Power Gen 1000; Fisher Scientific, Waltham, MA). Cell lysates from both cell culture and MTS were centrifuged at 12,000×g for 15 min at 4°C. Aliquots of supernatant were combined with equal volumes of 2x Laemmli-sodium dodecyl sulfate (SDS) sample buffer (20% glycerol, 5% β-mercaptoethanol, 4% SDS, 0.004% bromophenol blue, 0.125 M Tris-HCl pH =6.8), incubated at 80°C for 8 min, and stored at -80°C for subsequent Western immunoblotting analyses to determine signaling protein phosphorylation. Mammary tissue proteins, predominantly casein, were precipitated

from 300 μl aliquots of the cell lysate supernatants from tissue culture experiments by adjusting the pH to 4.6 via the addition of 12 μl of 5% (v/v) phosphoric acid followed by centrifugation at 4,000×g for 20 min at 4°C. The precipitate was washed twice with PBS and analyzed for $^{2}H_5$-Phe incorporation (**$^{2}H_5$-Phe-PRT**) as described below. The supernatant was analyzed for intracellular $^{2}H_5$-Phe (**$^{2}H_5$-Phe-INT**) after precipitating rest of the proteins using 10% (w/v) 5-sulfosalicylic acid.

Western Immunoblotting analysis

Aliquotes of cell lysates containing 20 μg protein were separated in 7-14% polyacrylamide gels, electrophoretically transferred to PVDF membranes (Millipore; Billerica, MA), and probed with polyclonal antibodies for the phosphorylated forms of insulin receptor substrate 1 (IRS-1, Ser1101), protein kinase B (Akt, Ser473), mTOR (Ser2448), ribosomal protein S6 kinase 1 (S6K1, Thr389), eukaryotic elongation factor 2 (eEF2, Thr56), eukaryotic initiation factor 4E binding protein 1 (4EBP1, Thr37/46), and eukaryotic initiation factor 2-α (eIF2α, Ser51). Cell lysates from MAC-T cells were tested for phosphorylation state (PS) of all these proteins. However, eIF2α and IRS1 were not examined in tissue explants. Blots were developed using an enhanced chemiluminescense kit (ECL Plus, Amersham; Piscataway, NJ), visualized using ECL film and a medical film processor (SRX 101A Konica Minolta; Wayne, NJ) and digitized and integrated using Un-Scan-It (Silk Scientific, Inc., Orem, UT). The blots were then stripped (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl buffer at pH 6.8) for 10 min at 75°C while shaking, blocked and re-probed with polyclonal antibodies for the total form of each signaling protein. All the antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Phosphorylation state of each signaling protein

was calculated as the ratio of the phosphorylated form to the total form and presented as arbitrary units.

$^{2}H_5$-Phe enrichment analysis

Casein precipitates were hydrolyzed with 6N HCl in 0.1% phenol at 110°C for 20 h. The casein hydrolysate and the supernatant collected after casein precipitation were desalted by ion exchange chromatography, and converted to N-(tert-butyldimethyl) AA derivatives as described previously (Bequette et al., 1999). The $^{2}H_5$-Phe_PRT and $^{2}H_5$-Phe_INT were quantified by gas chromatograph-mass spectrometer (GC-MS: Trace GC Ultra- DSQII , Thermo Electron Corporation; Waltham, MA) using selected ion monitoring to determine the ratio of the 239 and 234 m/z ions. Calibration curves were generated from gravimetric mixtures of these two ions as previously described by Bequette *et al*. (2002).

Statistical Analysis

The individual and interactive effects of insulin and EAA on phosphorylation state of the signaling proteins and $^{2}H_5$-Phe_PRT were analyzed using following statistical model:

$$Y_{ijk} = \mu + EAA_i + I_j + (AA\times I)_{ij} + e_{ijk},$$

where Y_{ijk} = phosphorylation state of signaling proteins or $^{2}H_5$-Phe-PRT, μ= mean phosphorylation state or $^{2}H_5$-Phe-PRT, EAA_i = fixed effect of the EAA in media, I_j = fixed effect of insulin in media, $(AA\times I)_{ij}$ = fixed interactive effect between EAA and insulin, e_{ijkl} = random error. The phosphorylation state and $^{2}H_5$-Phe_PRT were normalized to that of media containing both EAA and insulin (+EAA+I) before analysis. The effects of EAA and insulin on $^{2}H_5$-Phe_PRT were analyzed including $^{2}H_5$-Phe-INT

into the statistical model as a covariate. Statistical significance for the treatment effects and Pearson's correlation coefficients for associations among the phosphorylation state of signaling proteins and $^{2}H_5$-Phe-PRT were obtained by MIXED and CORR procedures in SAS 9.1 (Cary, NC), respectively.

In MAC-T cell experiments, four separate multiple regression analyses: 1) phosphorylation state of mTOR against phosphorylation state of Akt with class effect of EAA, 2) phosphorylation state of Akt against phosphorylation state of IRS1 with class effect of insulin, 3) phosphorylation state of eIF2α against phosphorylation state of mTOR with class effect of EAA, and phosphorylation state of eEF2 against phosphorylation state of mTOR and S6K1 were conducted using REG procedure in the SAS 9.1.

RESULTS

Effects of EAA and insulin on PS of some signaling proteins in MAC-T cells

Table 2 presents the least square means (LSM) for phosphorylation state of some signaling proteins in MAC-T cells, tested pertained to four EAA and insulin combinations in a 2 x 2 factorial design as shown in Table 1. Figure shows representative Western immunoblot images of the phosphorylated forms of these signaling proteins. Insulin deprivation reduced ($P < 0.001$) phosphorylation of Akt whereas EAA deprivation had no observable effect on phosphorylation of Akt. Omission of both Insulin and EAA reduced ($P < 0.028$) phosphorylation of mTOR. The highest phosphorylation of mTOR was observed when both EAA and insulin were present in the media (+EAA+I). Deprivation of insulin, in the presence of EAA (+EAA-I), did not substantially reduce ($P = 0.205$) phosphorylation of mTOR but EAA withdrawal, in the

presence of insulin (-EAA+I), resulted in significantly decreased ($P < 0.022$) phosphorylation of mTOR. Compared to +EAA+I, and +EAA-I, deprivation of both EAA and insulin (-EAA-I) reduced ($P < 0.022$) phosphorylation of mTOR by 65% and 40%, respectively (Table 2). There was no interaction ($P = 0.736$) between EAA and insulin on phosphorylation of mTOR. Pearson's correlation coefficients for relationships among phosphorylations of the signaling proteins in MAC-T cells are given in Table 3-3 . Phosphorylation of mTOR tended to be positively correlated (r = 0.49, $P = 0.095$) with phosphorylation of Akt in MAC-T cells.

Essential AA deprivation reduced ($P < 0.014$) phosphorylation of 4EBP1 and S6K1 in MAC-T cells (Table 2). On the other hand, phosphorylation of 4EBP1 and phosphorylation of S6K1 declined ($P < 0.030$) when media was devoid of insulin. In line with phosphorylation of mTOR, the highest levels of phosphorylation of 4EBP1 and S6K1 were observed when both EAA and insulin were present in the media (Table 2**).** Removal of insulin did not change ($P > 0.290$) phosphorylation of 4EBP1 and S6K1, in the presence of EAA. However, phosphorylation of 4EBP1 declined ($P = 0.038$) in response to EAA deprivation even if insulin was present in the media (-EAA+I). In the absence of EAA, insulin withdrawal (-EAA-I) further reduced ($P < 0.017$) phosphorylation of 4EBP1 and S6K1. Both phosphorylation of 4EBP1 and S6K1 had considerably positive correlations ($P < 0.050$) respectively of 0.77 and 0.61 with phosphorylation of mTOR in MAC-T cells (Table 3-3**).** Phosphorylation of eEF2 increased ($P = 0.049$) when EAA was removed from the media whereas insulin in media did not have significant impact ($P = 0.156$) on phosphorylation of eEF2 (Table 2). Essential AA deprivation also increased ($P = 0.008$) eIF2α phosphorylation but insulin

removal from media did not change ($P = 0.668$) phosphorylation of eIF2α. Essential AA rich but insulin deprived media (+EAA-I) resulted in the least phosphorylation of eIF2α which was less than phosphorylation of eIF2α of –EAA-I (P = 0.056, Table 2). Phosphorylation of eEF2 had negative (-0.92 and -0.83 respectively) correlations ($P <$ 0.042) with phosphorylation of mTOR and phosphorylation of S6K1 (Table 3-3).

Deprivation of EAA and insulin reduced ($P < 0.025$) phosphorylation of IRS1 at Ser1101 (phosphorylation of IRS1) in MAC-T cells (Table 2). Removal of either EAA or insulin alone did not reduce (P > 0.254) phosphorylation of IRS1 compared to that of media containing both EAA and insulin. However, removal of both EAA and insulin from media tended to reduce ($P = 0.055$) phosphorylation of IRS1. Phosphorylation of mTOR, S6K1, and 4EBP1 had positive correlations (P < 0.020) of 0.77, 0.84, and 0.72 respectively with phosphorylation of IRS1 (Table 3-3).

Regression coefficients and their statistical significance (*P* –values) for effects of phosphorylation of Akt on phosphorylation of mTOR, phosphorylation of mTOR on phosphorylation of eIF2, phosphorylation of IRS1 on phosphorylation of Akt , and both phosphorylation of mTOR and S6K1 on phosphorylation of eEF2 are given in Table 4. Regardless of the positive ($P = 0.002$) effect of EAA on phosphorylation of mTOR, increasing phosphorylation of Akt was associated ($P = 0.018$) with increasing phosphorylation of mTOR. Phosphorylation of IRS1 had no association ($P = 0.690$) with phosphorylation of Akt even when the effects of insulin on phosphorylation of Akt was considered. Phosphorylation of mTOR was not associated with phosphorylation of eIF2α ($P = 0.426$) even when the negative effect of EAA ($P = 0.054$) on phosphorylation of eIF2α was included into the regression model. Multiple regression analysis results in

Table 4 further revealed that increasing phosphorylation of mTOR was associated with decreasing phosphorylation of eEF2 ($P = 0.003$) while phosphorylation of S6K1 had no relationship with phosphorylation of eEF2 ($P = 0.747$) in MAC-T cells.

Effects of EAA and insulin on phosphorylation state of some signaling proteins and $^{2}H_5$-Phe_PRT in MTS

Least square means (LSM) for phosphorylation state of some signaling proteins and $^{2}H_5$-Phe_PRT for the EAA and insulin treatments in MTS experiments are presented in Table 5. Figure 2 presents representative Western immunoblot images for phosphorylation state of these signaling proteins across EAA and insulin treatments. In contrary to the effect of insulin in MAC-T cells, insulin in media did not change ($P = 0.486$) phosphorylation of Akt in MTS (Table 5). Similar to MAC-T cells, EAA in media did not have effects ($P = 0.893$) on phosphorylation of Akt. EAA had an impact ($P = 0.009$) on phosphorylation of mTOR. As results in Table 5 show, greater phosphorylation of mTOR was observed when mammary tissue slices were exposed to EAA. When both EAA and insulin were removed from the media, phosphorylation of mTOR declined ($P = 0.049$) by 50% compared to that of EAA and insulin rich media.

EAA in media significantly affected ($P < 0.090$) phosphorylation of S6K1 and 4EBP1 but insulin did not change ($P > 0.280$) either protein (Table 5) in mammary tissue slices. The presence of both EAA and insulin in media resulted in the highest phosphorylation of S6K1 and phosphorylation of 4EBP1. Deprivation of both EAA and insulin tended to reduce phosphorylation of S6K1 ($P = 0.053$) and phosphorylation of 4EBP1 ($P = 0.056$) compared to that of media containing both EAA and insulin. As

Table shows, phosphorylation of mTOR had positive ($P < 0.035$) correlations of 0.65 and 0.63 respectively with phosphorylation of S6K1 and phosphorylation of 4EBP1. Phosphorylation of S6K1 and 4EBP1 were also positively correlated (r = 0.60, P = 0.067). The results in Table 5 revealed that EAA removal from media increased (P = 0.011) phosphorylation of eEF2 in MTS. The least phosphorylation of eEF2 resulted from media containing both EAA and insulin and was significantly less than that of EAA deprived media containing insulin. Phosphorylation of eEF2 had a significant negative correlation with phosphorylation of S6K1 (r = -0.88, P = 0.004), phosphorylation of mTOR (r = -0.54, P = 0.083), and phosphorylation of 4EBP1 (r = -0.88, P = 0.088, Table 6). $^{2}H_5$-Phenylalanine incorporation into mammary tissue proteins ($^{2}H_5$-Phe-PRT) was considerably reduced ($P < 0.001$) in response to EAA deprivation from media. Insulin withdrawal from media did not have an impact on $^{2}H_5$-Phe-PRT (P = 0.358, Table 5). Phosphorylation of mTOR, S6K1, 4EBP1, and eEF2 were strongly correlated (0.80, 0.87, 0.87, and -0.80 respectively, $P < 0.006$) with $^{2}H_5$-Phe-PRT in mammary tissue slices (Table 6).

DISCUSSION

Insulin induces phosphorylation of Akt on Ser473 (Alessi et al., 1996) through the sequential phosphorylation of its receptor, insulin receptor substrates (e.g., IRS1), and phosphatidylinositol 3-kinase (PI3K) (Gosmanov et al., 2004). Reduced phosphorylation of Akt has been associated with reduced fractional rates of muscle protein synthesis (Prod'homme et al., 2005). In this study, insulin deprivation reduced phosphorylation of Akt in MAC-T cells by more than 80% (Table 2). However in mammary tissue explants, phosphorylation of Akt was not affected by insulin (Table 5). This could presumably be

due to poor insulin diffusion into mammary tissue slices that resulted in low insulin binding to membrane receptors in the internal portions of the slice. Concentrations of EAA in media did not affect phosphorylation of Akt in MAC-T cells or mammary tissue explants as observed in muscle tissue and adipocytes (Liu et al., 2002, Hinault et al., 2004, Suryawan et al., 2009). The lack of interactive effects between insulin and EAA in MAC-T cells indicates that insulin-mediated activation of Akt is independent of EAA availability in BME cells.

Mammalian target of rapamycine (mTOR) is well recognized for its ability to integrate nutrient and hormonal signals controlling protein synthesis (Sadiq et al., 2007). The effect of EAA and insulin on phosphorylation of mTOR in MAC-T cells (Table 2) and the strong positive correlation between phosphorylation of mTOR and protein synthesis rates (indicated by $^{2}H_{5}$-Phe_PRT in Table 5) in MTS reflect a potential role for mTOR in integrating these signals with respect to protein synthesis in bovine mammary gland. EAA availability increased phosphorylation of mTOR in both culture systems; however, insulin increased phosphorylation of mTOR in MAC-T cells, but failed to elicit a response in mammary tissue explants. The positive correlation between phosphorylation of Akt and phosphorylation of mTOR in MAC-T cells (Table 3) is indicative of insulin-induced stimulatory signals on mTOR in BME cells. Moreover, a multivariate analysis that regressed phosphorylation of Akt on phosphorylation of mTOR (Table 4) further revealed a significantly positive relationship between phosphorylation of Akt and phosphorylation of mTOR in MAC-T cells. There were no interactions between EAA and insulin on phosphorylation of mTOR in MAC-T cells (Table 2) supporting the hypothesis that the effects of EAA and insulin on phosphorylation of

mTOR were additive in the BME cells. Reductions in phosphorylation of mTOR in response to removal of either insulin, EAA, or both from the media of MAC-T cells were 25%, 36% and 65%, respectively (Table 2). These additive effects suggest that EAA and insulin mediate phosphorylation of mTOR through two independent pathways in BME cells as previously described in muscle cells (Hinault et al., 2004, Suryawan et al., 2007).

Deprivation of EAA or insulin lowered phosphorylation levels of 4EBP1 and S6K1 in MAC-T cells, which is consistent with the phosphorylation of mTOR results (Table 2); however, only EAA affected these signaling proteins in mammary tissue explants (Table 5). Phosphorylation of 4EBP1 results in release of initiation factor eIF4E facilitating recruitment of 40S ribosomal subunit to the 5'cap of mRNA, a rate limiting step of translation initiation (Richter and Sonenberg. 2005, Lynch et al., 2004). The positive correlation between phosphorylation of 4EBP1 and $^{2}H_{5}$-Phe-PRT evidences the critical role of phosphorylation of 4EBP1 in regulating protein synthesis in BME cells. The 4EBP1 is sequentially phosphorylated at multiple sites (e.g., Thr37/46, Ser70, and Ser65) that appear to have direct effects on releasing eIF4E to support translation initiation (Lasko, 2003). Given the high correlation between Thr37/46 phosphorylation and the rates of protein synthesis in mammary tissue slices (Table 6), it would appear that phosphorylation at the other sites is highly correlated with phosphorylation at Thr37/46 in BME cells. In MAC-T cells, phosphorylation of 4EBP1 decreased in response to the removal of EAA in the presence of insulin (-EAA+I in Table 2). Removal of both EAA and insulin (-EAA-I in Table 2) further reduced phosphorylation of 4EBP1. In connection to these observations, Proud. (2004) reported that AA mainly enhanced phosphorylation of 4EBP1 and insulin can further increase it in mTOR-dependent manner. In both MAC-

T cells and MTS, phosphorylation of 4EBP1 had positive correlations with phosphorylation of mTOR suggesting that 4EBP1 is strongly mediated by phosphorylation of mTOR. This finding is not surprising as 4EBP1 is a direct substrate of mTOR (Avruch et al., 2009).

The other direct substrate of mTOR is S6K1. The positive correlations between phosphorylation of S6K1 and mTOR in both MAC-T cells (Table 3-3) and MTS (Table 6) are consistent with such a link in mammary cells. As observed for mTOR, phosphorylation of S6K1 was markedly reduced by omission of either EAA or insulin from media of MAC-T cells (Table 2) but only EAA deprivation decreased phosphorylation of S6K1 in MTS (Table 5). Although S6K1 phosphorylation also occurs in response to signals arising from PI3K and Ras/MAPK pathways (Martin and Blenis, 2002), the proportion of total variation explained by mTOR phosphorylation was greater than for 4EBP1 suggesting that mTOR likely acted alone under these conditions. This conclusion is consistent with the observations that blocking mTOR action with rapamycin leads to rapid dephosphorylation of S6K1 at Thr389 even when PI3K and Ras/MAPK pathways are stimulated (Tee and Blenis, 2005).

Eukaryotic elongation factor 2 enhances the GTP-dependent translocation of the ribosome along sequential mRNA codons, following recruitments of aminoacyl-tRNA to the mRNA. Phosphorylation of eEF2 at Thr56 by its cognate kinase, eEF2 kinase negates its physiological function (Wang and Proud, 2006). Removal of EAA reduced phosphorylation of eEF2 in both MAC-T cells (Table 2) and MTS (Table 5) irrespective of insulin treatment. The negative correlation between phosphorylation of eEF2 and $^{2}H_{5}$-Phe-PRT in MTS (Table 6) revealed that decreased phosphorylation of eEF2 was

associated with enhanced protein synthesis rates in mammary cells. Moreover, the negative correlation between phosphorylation of eEF2 and mTOR suggests that the reduction in phosphorylation of eEF2 was mediated through enhanced phosphorylation of mTOR in both MAC-T cells and mammary tissue explants. Insulin and AA have been shown to activate eEF2 by phosphorylating, and thus, inactivating eEF2 kinase in an mTOR-dependent manner (Redpath et al., 1996, Proud, 2004). The negative correlation between phosphorylation of S6K1 and phosphorylation of eEF2 (Table 3-3 and Table 6) suggest that mTOR mediated regulation of phosphorylation of eEF2 could occur via phosphorylation of eEF2 kinase by S6K1 in consistent with what reported by Proud (2004). However, regression of phosphorylation of eEF2 against both phosphorylation of mTOR and S6K1 revealed stronger negative relationships of phosphorylation of eEF2 with phosphorylation of mTOR than with phosphorylation of S6K1 (Table 6). Hence, it would appear that phosphorylation of mTOR might mediate phosphorylation of eEF2 kinase and consequently phosphorylation of eEF2 directly or via a different kinase in bovine mammary cells.

Hyper-phosphorylation of S6K1 in response to high intracellular AA concentrations can inhibit insulin mediated phosphorylation of Akt by direct phosphorylation of IRS-1 at Ser1101 (phosphorylation of IRS1) in muscle cells (Tremblay et al., 2007). In partial agreement with this observation, EAA supplementation increased both phosphorylation of S6K1 and IRS1 in MAC-T cells (Table 2). However, the weak correlation between phosphorylation of IRS1 and phosphorylation of Akt (Table 3-3) and insignificant difference in phosphorylation of Akt between +EAA+I and –EAA+I (Table 2) indicate that surplus EAA-induced phosphorylation of S6K1 was

unable to create significantly negative-feedback on insulin signals for protein synthesis in MAC-T cells. Moreover, a regression analysis considering class effect of insulin on phosphorylation of Akt revealed a weak association between phosphorylation of IRS1 and phosphorylation of Akt in BME cells (Table 4). Eukaryotic initiation factor 2- α (eIF2α) plays a critical role in regulating global translation rates by facilitating recruitment of initiator methionyl-tRNA to 40S ribosomal subunit. Cellular stresses such as AA deprivation have been associated with enhanced phosphorylation of eIF2α at Ser51 which inactivates it (Anthony et al., 2004). Among known eIF2 kinases, general control non-repressible kinase (GCN2) has been shown to be responsible for AA mediated phosphorylation of eIF2α in mouse embryonic stem cells (Harding et al., 2000). Hence, the association between EAA deprivation and increase phosphorylation of eIF2α (Table 2) in MAC-T cells could involve GCN2. The non-significant correlation between phosphorylation of mTOR and phosphorylation of eIF2α in Table 3 suggests that activity of eIF2α might not be regulated by mTOR in MAC-T cells. Moreover, a regression analysis accounting for class effect of EAA on phosphorylation of eIF2α further testifies an insignificant association between phosphorylation of mTOR and phosphorylation of eIF2α in bovine mammary epithelial cells (Table 4).

In summary, both insulin and EAA exhibited significant regulatory effects on cellular signals for protein synthesis in BME cells. These signals appeared to be additive, and independently converged on mTOR as observed in well-studied muscle cells. Protein synthesis rates in bovine mammary cells were highly correlated with the phosphorylation states of mTOR, 4EBP1, S6K1, and eEF2 providing evidence that AA not only act as substrates but also as stimulatory signals for protein synthesis in bovine mammary cells.

The interactions of EAA and insulin at mTOR support the hypothesis that milk protein synthesis is a variable function of at least EAA and insulin. This is in disagreement with single limiting nutrient concept of current nutrient requirement models. Amino acid having significant control on translation would affect the sequestration of other amino acid by the mammary glands.

Ruminant mammary glands are able to modulate AA uptake by adjusting mammary blood flow and AA transporter activities in response to internal metabolism namely protein synthesis. Therefore utilization of one AA may depend on availability of another AA. Moreover, the observed effects of insulin on cellular signals for translation in BME cells infers that AA incorporation into milk protein could also vary with extracellular cues such as plasma insulin concentrations. These findings therefore disagree with fixed AA transfer efficiencies in current protein requirement models. Experiments that investigate the regulatory effects of individual EAA may help one identify the limiting AA with enough precision to determine an AA supplementation strategy leading to maximal nitrogen utilization efficiency in dairy cows. Moreover, the knowledge about the regulatory effects of AA and their interaction with other stimuli such as insulin and energy substrate could improve our mathematical representations of AA requirements.

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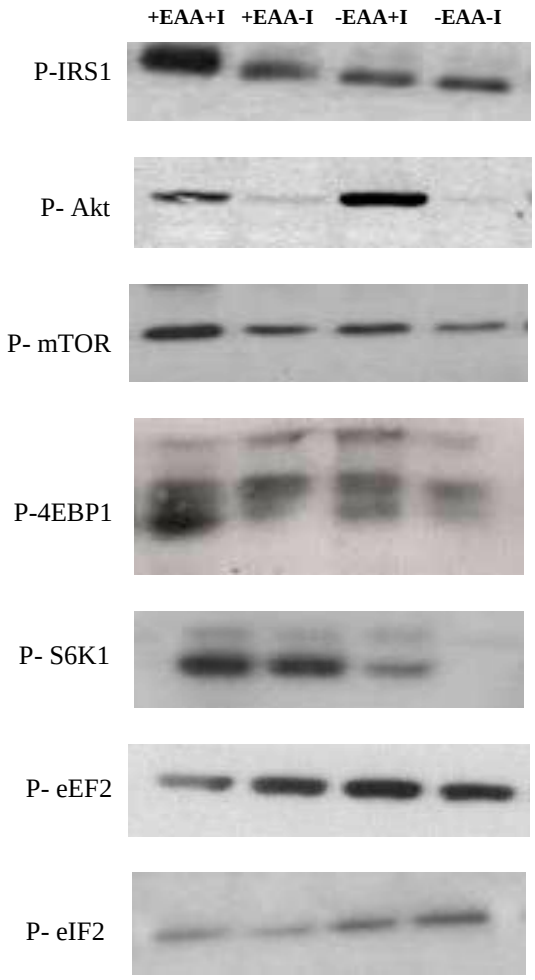

Figure 3-1. Representative Western immunoblotting images of phosphorylated forms (P) of some signaling proteins when MAC-T cells were grown in media containing EAA and insulin (+EAA+I), only EAA (+EAA-I) and only insulin (-EAA+I), and media deprived of both EAA and insulin (-EAA-I) for 1 h.

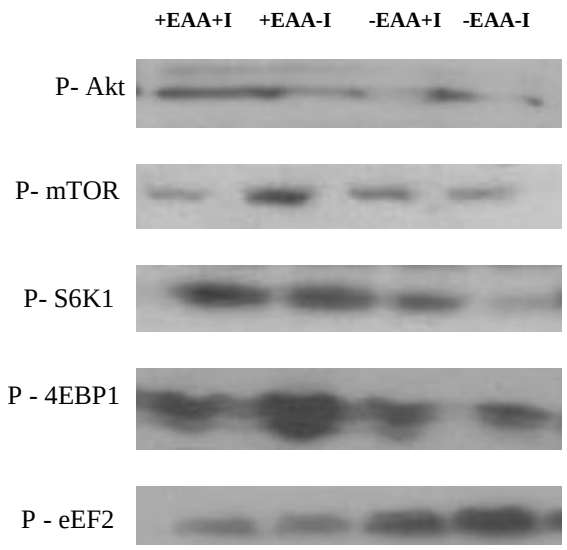

Figure 3-2. Representative Western immunoblotting images of phosphorylated forms of some signaling proteins when mammary tissue slices were grown in both EAA and insulin rich media (+EAA+I), EAA rich media without insulin (+EAA-I), insulin rich but EAA deficient media (-EAA+I), and EAA deficient media without insulin (-EAA-I) for 2 h.

Table 3-1. Combinations of total essential amino acids (EAA) and insulin levels in a 2×2 factorial arrangement of treatments.

	Insulin	
EAA	100 ng/ml (+I)	0 ng/ml (-I)
3.5 mM[1] (+EAA)	+EAA+I	+EAA-I
0[2] or 0.18[3] mM (-EAA)	-EAA+I	-EAA-I

[1]concentrations of EAA in regular DMEM/F12. [2] EAA deprived treatment in MAC-T cell culture experiments. [3]EAA deficient media in cultured mammary tissue slices

Table 3-2. Individual and interactive effects of essential amino acids (EAA) and insulin (I) on phosphorylation state (PHS) of some signaling proteins in MAC-T cells.

Signaling protein	PHS[1]				SEM	*P* - value		
	+EAA+I[2]	+EAA-I[3]	-EAA+I[4]	-EAA-I[5]		EAA	I	EAA×I
Akt	1.00[a6]	0.18[b]	1.14[a]	0.17[b]	0.16	0.777	<0.001	0.723
mTOR	1.00[a]	0.75[ab]	0.54[bc]	0.35[c]	0.13	<0.001	0.028	0.736
S6K1	1.00[a]	0.68[ab]	0.62[bc]	0.29[c]	0.18	0.014	0.029	0.967
4EBP1	1.00[a]	0.84[ab]	0.73[b]	0.29[c]	0.09	<0.001	0.001	0.141
eEF2	1.00[a]	1.55[ab]	1.79[ab]	2.53[b]	0.39	0.050	0.156	0.475
eIF2α	1.00[ba]	0.85[a]	1.35[ba]	1.39[b]	0.11	0.008	0.668	0.512
IRS1	1.00[a]	0.72[ab]	0.64[ab]	0.44[c]	0.12	0.014	0.023	0.475

[1]Least square means (LSM) of PHS, [2]Both EAA and insulin rich media, [3]EAA rich midea without insulin, [4]EAA deprived media with insulin, [5]Both EAA and insulin deprived media, [6]different letters indicate significantly ($P < 0.05$) different LSMs.

Table 3-3. Pearson's correlation coefficients (above diagonal) for phosphorylation state of the signaling proteins, and statistical significance (*P* – values, below diagonal) of the correlations in MAC-T cells.

	Akt	mTOR	S6K1	4EBP1	eEF2	eIF2α	IRS1
Akt		0.49	0.59	0.58	-0.05	-0.11	0.37
mTOR	0.095		0.77	0.61	-0.92	-0.37	0.77
S6K1	0.081	0.047		0.87	-0.83	-0.69	0.84
4EBP1	0.898	0.001	0.010		-0.43	-0.54	0.72
eEF2	0.243	0.042	0.017	0.057		0.38	-0.58
eIF2α	0.659	0.259	0.012	0.068	0.275		-0.51
IRS1	0.365	0.008	0.005	0.019	0.038	0.090	

Table 3-4. Regression analysis results for relationships of phosphorylated (P) mtor with P-Akt, P-Akt with P-IRS1, P-mTOR with P-eIF2α, and P-eEF2 with P-mTOR and P-S6K when class effects (presence = 1 and absence = 0) of essential amino acids (EAA) and insulin in MAC-T cell media were considered.

Dependent variable	Independent variables	parameter estimates	*P* - value
P-mTOR	P-Akt	0.25	0.018
	EAA	0.43	0.002
P-Akt	P-IRSI	-0.29	0.690
	Insulin	1.44	0.001
P-eIF2α	P-Mtor	0.52	0.426
	EAA	-0.68	0.054
P-eEF2	P-S6K1	-0.26	0.747
	P-mTOR	-4.84	0.003

Table 3-5. Individual and interactive effects of essential amino acids (EAA) and insulin (I) on phosphorylation state (PHS) of some signaling proteins in mammary tissue slices.

Signaling protein	PHS				SEM	*P* – value		
	+EAA+I	+EAA-I	-EAA+I	-EAA-I		EAA	I	EAA×I
Akt	1.00[a1]	0.84[a]	1.44[a]	0.89[a]	0.18	0.893	0.486	0.288
mTOR	1.00[a]	0.96[ab]	0.71[ab]	0.50[b]	0.15	0.009	0.341	0.638
S6K1	1.00[a]	0.94[ab]	0.57[ab]	0.36[b]	0.08	0.011	0.282	0.864
4EBP1	1.00[a]	0.80[a]	0.67[a]	0.60[a]	0.14	0.085	0.358	0.661
eIF2α	1.00[a]	1.21[ab]	1.51[ab]	1.50[b]	0.25	0.011	0.443	0.456
$^2H_5$-Phe_PRT	1.00[a]	0.96[a]	0.47[b]	0.31[b]	0.11	<0.0001	0.358	0.648

[1]different letters; a, b and c, indicate significantly ($P < 0.05$) different LSM.

Table 3-6. Pearson's correlation coefficients (above diagonal) for the phosphorylation state of some signaling proteins and $^2H_5$-Phe enrichments in casein ($^2H_5$-Phe_PRT), and statistical significance (*P* – values, below diagonal) of the correlation coefficients in mammary tissue slices.

	Akt	mTOR	S6K1	4EBP1	eEF2	$^2H_5$-Phe-PRT
Akt		0.23	0.51	0.14	0.33	0.23
mTOR	0.413		0.65	0.63	-0.54	0.79
S6K1	0.090	0.029		0.60	-0.88	0.85
4E-BP1	0.649	0.030	0.067		-0.54	0.90
eEF2	0.319	0.083	0.004	0.088		-0.76
$^2H_5$-Phe_PRT	0.401	0.001	0.001	<0.001	0.006	

CHAPTER 4:

Effects of Energy Substrates: Acetate and Glucose and Essential Amino Acids on cellular signals for protein synthesis in bovine mammary epithelial cells

ABSTRACT

Understanding the interactions between amino acids (AA) and energy substrates in determining milk protein synthesis rates will allow improvements in AA requirement models for dairy cows. The objective of this study was to investigate the effects of glucose and acetate interacting with essential AA (EAA) on cellular signals for protein synthesis in bovine mammary cells. MAC-T cells were treated with DMEM/F12 media with or without EAA, glucose (GL) or acetate (AC). Phosphorylation of signaling proteins; AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), ribosomal protein S6 (rpS6), eukaryotic initiation factor 4E binding protein 1 (4EBP1), and eukaryotic elongation factor 2 (eEF2) was assessed by Western analysis. Mammary tissue slices (MTS) obtained from lactating cows were subjected to the same treatments and assessed for the signaling protein phosphorylation and $^{2}H_{5}$-Phenylalanine enrichment in protein precipitated at pH=4.6 as an indication of protein synthesis rates. In MAC-T cells, the absence of acetate tended to increase ($P = 0.094$) phosphorylation of AMPK. In both MAC-T cells and MTS, removal of EAA reduced ($P < 0.075$) phosphorylation of mTOR , 4EBP1 and rpS6. Omission of EAA was associated with increased ($P < 0.035$) phosphorylation of eEF2 whereas omission of glucose caused phosphorylation of rpS6 to decrease ($P = 0.011$) in MAC-T cells. Protein synthesis rates

were unaffected by acetate and glucose in the media but decreased ($P = 0.009$) for EAA withdrawal. Phosphorylation of 4EBP1, rpS6, and eEF2 were strongly ($P < 0.050$) correlated with mTOR in MAC-T cells. Phosphorylation of eEF2 strongly ($P = 0.011$) linked to phosphorylation of AMPK in MTS. Phosphorylation of mTOR and 4EBP1 were positively ($P < 0.020$) correlated with protein synthesis rates while phosphorylation of eEF2 was negatively ($P = 0.015$) associated with protein synthesis rates. Protein synthesis rates tended ($P < 0.090$) to be inversely correlated with phosphorylation of AMPK. Acetate appeared to exert greater influence on AMPK phosphorylation than glucose in MAC-T cells. Essential amino acids were the key regulators of protein synthesis rates in bovine mammary cells.

INTRODUCTION

Poor nitrogen (**N**) utilization efficiency of dairy cows leads to significant N losses in urine and feces. Besides the associated economic cost, N excreted in the urine and feces have been recognized as critical sources of environmental pollution. For example, ammonia emitted, in particular from urine, is one of the major air and water pollutants contributing to eutrophication, aerosol formation, acid rain, and impaired visibility (USEPA, 2004). Increasing urinary N excretion is indicative of greater catabolism of amino acids (**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 non-splanchnic 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 resulting reductions in arterial concentrations of AA should lead to reduced AA catabolism by the splanchnic tissues (Hanigan et al., 1998).

Protein synthesis rates are determined by translation initiation and elongation rates which are regulated by the activity of eukaryotic initiation and elongation factors. Activity of these factors is determined by their phosphorylation state which responds to the availability of nutrients such as AA and glucose. Since proteins are polymers of AA and protein synthesis is an energetically expensive process (Wang and Zhang, 2009), it makes perfect sense that AA and energy substrate availability both play a role in regulating protein synthesis. These nutrient signals have been shown to be associated with the phosphorylation of a major protein kinase, mammalian target of (**mTOR**, Tokunaga et al., (2004)). Phosphorylated and thus activated mTOR in turn enhances protein synthesis rates, in part by phosphorylating ribosomal protein S6 (**rpS6**) and eukaryotic initiation factor 4E binding protein 1 (**4EPB1**, Kimball, 2002).

When cells undergo an energy stress, increases in the AMP : ATP ratio activates AMP-activated protein kinase (**AMPK**) by phosphorylation. Once activated, AMPK shuts down 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). Moreover, AMPK has been shown to inhibit translation via mTOR-independent pathways. For example, AMPK directly activates eukaryotic elongation factor 2 (**eEF2)** kinase (**eEF2K**). Once activated, eEF2K phosphorylates and inactivates eEF2 which plays a critical role in the elongation step of mRNA translation (Chan et al., 2004). The

stimulatory effects of AA and glucose on muscle protein synthesis have been extensively studied (Bolster et al.,2002, Jeyapalan et al., 2007, Deshmukh et al., 2008). Little work has explored signaling effects of EAA and energy in bovine mammary epithelial cells.

Broderick et al. 2003 reported linear increase in milk protein yields in lactating dairy cows in response to increasing dietary protein and energy (represented by increasing NDF) levels. Similarly, duodenally infused casein and ruminally infused propionate significantly increased milk protein yields (Raggio et al., 2006). Rius et al. (2010) abomasally infused starch and casein into lactating dairy cows and observed that energy supply was associated with positive cellular signals for protein synthesis. Similar signaling effects of energy supply were observed by Toerien et al. (2009), in response to jugular infusion of glucose and some essential amino acids (**EAA**). In contrast, only AA supplementation raised milk protein yield when dairy cows were infused with sodium caseinate and energy substrates (**EN**) such as glucose (Clark et al., 1977), acetate or propionate (Lough et al., 1983). Purdie et al. (2008) observed increased milk protein yields for infusion of AA alone while infusion of AA with acetate caused a decrease in milk protein yields suggesting a potential interaction between AA and EN.

We hypothesized that AA and energy substrate may independently affect the signaling pathways regulating protein synthesis in mammary epithelial cells via similar mechanisms demonstrated in muscle cells. The objective of this study was to investigate the individual and interactive effects of essential AA (**EAA**), glucose, and acetate on signaling protein phosphorylation and protein synthesis rates in bovine mammary cells.

MATERIALS & METHODS

Three experiments were conducted. The first experiment (**Expt 1**) tested the effects of EAA and acetate on cellular signaling pathways in MAC-T cells. The second experiment (**Expt 2**) examined the affects of glucose and EAA on cellular signaling pathways in MAC-T cells. The third experiment (**Expt 3**) examined the relationships between the phosphorylation state of the cellular signaling proteins and protein synthesis rates in mammary tissue slices (**MTS**) obtained from lactating cattle.

Expt 1: effects of EAA and acetate on cellular signals for protein synthesis in MAC-T cells

Cell Culture Conditions: An immortalized bovine mammary epithelial cell line, MAC-T, established by Huynh et al. (1991) was used. Cells were seeded into 6-well plates at a density of 5×10^4 and grown to about 90% confluency in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Invitrogen, Carlsberg, CA) containing 3.51 g D-glucose /L and supplemented with 8% fetal bovine serum, and 100 U/mL Penicilin, 100 ug/mL Streptomycin and 0.25 ug/mL Amphotericin B (HyClone Laboratories Inc., Logan, UT). Cells were serum starved overnight before applying treatments.

Treatments and experimental design: Serum starved cells were treated with 2 levels of EAA and 2 levels of acetate in a 2×2 factorial arrangement of treatments (Table 1). Essential Amino acids were included at normal DMEM/F12 concentrations (**+EAA**, 0.5 Lys, 0.12 Met, 0.70 Arg, 0.45 Leu, 0.42 Ile, 0.45 Val, 0.15 His, 0.22 Phe, 0.45 Thr, and 0.04 Trp; all in mM) or completely removed (**-EAA**). Acetate was provided at concentrations of 5 mM (**+AC**) or 0 mM (**-AC**). Glucose was excluded from all treatment media. Glucose free DMEM/F12 with and without EAA was purchased from Invitrogen

(Carlsberg, CA). Sodium acetate and individual EAA were purchased from Sigma (St. Louis, MO).

MAC-T cells were incubated with treatment media for 1 h. Cells were harvested and cell lysates were obtained as described previously (McFadden and Corl, 2009). Each treatment was applied to each well in one 6-well plate of the MAC-T cells. Cell lysates from all 6 wells were combined into a pooled sample which was then centrifuged at 12,000×g for 10 min at 4°C. The supernates were subjected to Western immunoblotting analyses. The experiment was repeated four times on four different days.

Expt 2: effects EAA and glucose on cellular signals for protein synthesis in MAC-T cells

The MAC-T cells were grown up to 90% confluency as described in Expt1. Overnight serum starved cells were treated with +EAA and -EAA and 2 levels of D-glucose ; 3.51 g/L (**+GL**) and 0 g/L (**-GL**) in a 2×2 factorial arrangement of treatments (Table1). D-glucose was purchased from Sigma (St. Louis, MO). All other procedures were carried out as described for Expt1. This experiment was also repeated four times on four different days.

Expt 3: effects of acetate, glucose, and EAA on cellular signals and protein synthesis rates in mammary tissue slices

Tissue Slice Preparation: Mammary tissues slices were prepared from the rear quarter of 4 multiparous lactating cows immediately after slaughter. These cows were screened for mastitis pathogens prior to slaughter to insure collection of healthy tissues. Cows were removed from feed for 12 h prior to slaughter. A series of tissue slices that weighed 130 ± 30 mg were prepared using a Stadie-Riggs hand-held microtome and placed

individually in 25 ml Erlenmeyer flasks containing 4 ml of treatment media. All slices were prepared within 30 min of animal slaughter.

Random mammary tissue samples from each cow were used to prepare microscopic slides which were stained with hematoxylin and eosin (Sigma, St. Louis, MO) 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 40x magnification. Four images, each representing mammary tissues harvested from each of four cows, are presented in Figure 1.

Treatments: MTS were subjected to the similar treatments as for Expt1 and Exp2 with the exception that the –EAA treatments used EAA concentrations that were 5% of normal DMEM/F12 concentrations instead of the complete absence of EAA. This allowed inclusion of $^{2}H_5$-Phe to achieve a uniform enrichment of 20% across all treatments and provided some substrate for protein synthesis. Prior work with MAC-T cells in our laboratory showed that the 5% EAA level did not induce significant increases in phosphorylation of cellular signaling proteins.

Two flasks containing individual mammary tissue slices from each of the four cows were randomly assigned to each of the six treatment media, purged with 95:5 mix of O_2:CO_2 for 20 s, sealed with rubber stoppers, and placed in a shaking water bath at 37^{o}C. Flasks were opened at 90 min and perged with 0.006 and 0.090 mM $^{2}H_5$-Phe respectively into –AA and +AA media. Incubations were stopped by placing the flasks on ice and immediately adding ice-cold stop buffer containing 1 mM NaF and 10 μM Na_3VO_4 in sterile PBS at 120 min. Tissue slices were retrieved, washed twice in ice cold

stop buffer, mixed 1:7 (w:v) with the lysis buffer used in Expt1 and Expt2, and homogenized (Power Gen 1000; Fisher Scientific, Waltham, MA).

Preparation of cell lysates

Cell lysates from both cell culture and tissue culture experiments were prepared for Western immunoblotting analysis as previously described by Rius et al. (2010). Protein enriched in casein was precipitated from 300 μl aliquots of the cell lysate supernatants from tissue culture experiments by adjusting the pH to 4.6 using 5% phosphoric acid and centrifugation at 4,000×g for 20 min at 4^{o}C. The precipitate was washed twice with PBS and analyzed for $^{2}H_5$-Phe enrichment (**$^{2}H_5$-Phe_PRT**) as described below. The supernatant was analyzed for intracellular $^{2}H_5$-Phe enrichments (**$^{2}H_5$-Phe_INT**) after precipitating the rest of the proteins using 10% (w/v) 5-sulfosalicylic acid.

Western immunoblotting analysis

Western immunoblotting analyses were performed as described by Rius et al. (2010). The blots were first probed with antibodies (Cell signaling, Danvers, MA) that recognized the phosphorylated forms of AMPK (Thr172), mTOR (Ser2448), rpS6 (Ser235/236), 4EBP1 (Thr37/46), and eEF2 (Thr56). Blots were subsequently stripped and reprobed with the antibodies that recognize the total form of each protein. For experiment 1, proteins were visualized by chemiluminescent exposure of radiography film (ECL, Amersham, Piscataway, NJ) after probing with a secondary antibody attached to horseradish peroxidase. Films were scanned and digitized using Un-Scan-It (SRX 101A Konica Minolta; Wayne, NJ). For experiments 2 and 3, proteins were visualized and digitized after probing with a a secondary antibody attached to a florescent tag using

an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NB) as previously described (Reinking et al., 2009). The blots were then stripped (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl buffer at pH 6.8) for 10 min at 75°C while shaking, blocked and re-probed with polyclonal antibodies for the total form of each signaling protein. All the antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Phosphorylation state of each signaling protein was calculated as the ratio of the phosphorylated form to the total form and presented as arbitrary units.

$^{2}H_5$-Phe enrichment analysis

Protein precipitates were hydrolyzed with 6N HCl in 0.1% phenol at 110^{o}C for 20 h. The hydrolysate and the supernatant collected after casein precipitation were desalted by ion exchange chromatography, converted to N-(tert-butyldimethyl) AA derivatives as described previously (Bequette et al., 1999) and analyzed for $^{2}H_5$-Phe enrichment by gas chromatography-mass spectrometry (GC-MS:Trace GC Ultra- DSQII , Thermo Electron Corporation; Waltham, MA) using selected ion monitoring to determine the ratio of the 239 ($^{2}H_5$-Phe) and 234 ($^{1}H_5$-Phe) m/z ions. Calibration curves were generated from gravimetric mixtures of $^{2}H_5$-Phe and $^{1}H_5$-Phe as previously described by (Bequette et al., 2002) and used to convert mass ratios to enrichments.

Statistical Analysis

The effects of acetate, glucose, and EAA on phosphorylation of signaling proteins and $^{2}H_5$-Phe-PRT were analyzed using following statistical model:

$$Y_{ijk} = \mu + EAA_i + EN_j + (AA \times EN)_{ij} + e_{ijk},$$

where Y_{ijk} = Phosphorylation state of a signaling protein or $^2H_5$-Phe-PRT, μ= overall mean of the phosphorylation state or $^2H_5$-Phe-PRT, EAA_i = fixed effect of EAA in media, EN_j = fixed effect of energy substrate; acetate or glucose, $(AA \times EN)_{ij}$ = fixed interactive effect between EAA and energy substate, e_{ijkl} = random error. Treatment effects on $^2H_5$-Phe-PRT were analyzed including $^2H_5$-Phe-INT as a covariate in the model. Statistical significance for the treatment effects and Pearson's correlation coefficients for associations among the phosphorylation state of signaling proteins and $^2H_5$-Phe were obtained by MIXED and CORR procedures in SAS 9.1 (Cary, NC) respectively. Multiple comparisons between treatments were carried out with Tukey-Kramer adjustments test. Moreover, phosphorylation of mTOR was regressed against phosphorylation of AMPK while considering the class effects (presence =1 and absence =0) of EAA on phosphorylation of mTOR using REG procedure in the SAS 9.1.

RESULTS & DISCUSSION

Effects of EAA and acetate on cellular signals in MAC-T cells

AMPK **:** Signaling protein results for Expt1 are presented in Table 2. Regardless of EAA concentrations in media, acetate omission tended to increase (P = 0.094) phosphorylation of AMPK by 42%. Poor cellular energy status indicated by an increased AMP: ATP ratio enhances phosphorylation of AMPK and thereby activates it. The observed acetate deprivation-induced phosphorylation of AMPK in MAC-T cells suggests that acetate was an important energy source in MAC-T cells. On the other hand deteriorating cellular energy status tends to attenuate stimulatory mTOR signals for protein synthesis by

activating AMPK (Gleason et al., 2007). However, as Table 4 shows such an inverse correlation between phosphorylation of AMPK and phosphorylation of mTOR (r = -0.05, P = 0.731) was not observed in MAC-T cells. In present study, phosphorylation of mTOR at Ser2448 was tested. Observed non-significant relationship therefore infers that AMPK did not affect this phosphorylation site of mTOR.

mTOR signals: Irrespective of acetate concentrations in media, omission of EAA significantly (P = 0.006) reduced phosphorylation of mTOR by 62%. Phosphorylation at Ser2448 becomes a good biomarker for mTOR activity (Chiang and Abraham, 2005). Our results indicate that the same site responds to EAA in MAC-T cells regardless of energy substrate supply. In the absence of acetate (+AA-AC) phosphorylation of mTOR decreased by 15% whereas removal of EAA, in the presence of acetate (-AA+AC) reduced phosphorylation of mTOR by 47%. Media devoid of both EAA and energy substrate were associated with a greater decline in phosphorylation of mTOR (82%) than what observed with individual omission of each nutrient (Table 2). This indicates that the individual effects of EAA and acetate on phosphorylation of mTOR could be additive and thus independent of each other. Interaction effects of EAA and acetate (Table 2) on phosphorylation of mTOR were statistically non-significant (P = 0.511). This further testifies that AA and acetate act on mTOR via independent mechanisms in bovine mammary epithelial cells.

Deprivation of EAA from media reduced phosphorylation of rpS6 and phosphorylation of 4EBP1 respectively by 75 and 92% (Table 2). Phosphorylation of 4EBP1 is rate limiting for global mRNA translation whereas phosphorylation of rpS6 enhances ribosome biogenesis and synthesis of some protein factors governing mRNA

translation. Omission of EAA induced significant declines in the phosphorylation of these protein factors show the significance of EAA availability for translation initiation in the mammary cells. The significant ($P < 0.05$) positive correlations of phosphorylation of mTOR with phosphorylation of rpS6 (r = 0.74) and phosphorylation of 4EBP1 (r = 0.79) in Table 4 further verify that 4EBP1 and rpS6 are under tight control of mTOR (Avruch et al., 2009). Phosphorylation of mTOR, rpS6 and 4EBP1 were all unaffected by acetate in MAC-T cell media. As Table 2 shows phosphorylation of 4EBP1 and phosphorylation of rpS6 did not change in response to removal of acetate in the presence of EAA (+AA-AC) but dramatically reduced when EAA were omitted from media irrespective to the presence of acetate. Removal of EAA caused phosphorylation of eEF2 to increase ($P = 0.023$) by 23%. In Eley et al. (2008), phosphorylation of eEF2 was negatively associated with phosphorylation of mTOR in muscle cells. Present study results (Table 4) reveled such a link (r = -0.51, $P = 0.110$) in MAC-T cells suggesting that activated mTOR is able to dephosphorylate and thereby activate eEF2 in bovine mammry epithelial cells.

Effects of EAA and glucose on cellular signals for protein synthesis in MAC-T cells

AMPK: Significance of EAA and glucose in MAC-T cell media on phosphorylation state of the signaling proteins are presented in Table 3. Deprivation of either EAA ($P = 0.118$) or glucose ($P = 0.109$) did not change phosphorylation of AMPK. It was expected that glucose deprivation would significantly activate AMPK by increasing its phosphorylation. However, glucose and EAA deprivation numerically increase phosphorylation of AMPK by about 28%. Interactive effects of EAA and glucose on

phosphorylation of AMPK tended towards significance ($P = 0.060$, Table 3) indicating that EAA and glucose might act on AMPK via a similar mechanism.

A multiple regression analysis accounting for regression effect of phosphorylated AMPK and class effect of EAA (presence = 1 and absence = 0) on phosphorylation of mTOR further revealed non-significant ($P = 0.397$) relationship (regression coefficient = -0.103) between AMPK and mTOR in MAC-T cells. This analysis used phosphorylation state data of AMPK and mTOR from both Expt1 and Expt2.

mTOR signals: Phosphorylation of mTOR decreased ($P = 0.037$) by 28% when EAA was absent in media. In consistent with what observed with acetate in Expt1, glucose deprivation did not significantly ($P = 0.712$) change phosphorylation of mTOR (Table 3). Interactive effects of EAA and glucose on phosphorylation of mTOR was statistically non-significant ($P = 0.649$) indicating that EAA could substantially influence mTOR activity independent of glucose. Consistent with phosphorylation of mTOR, phosphorylation of 4EBP1 significantly ($P < 0.001$) decreased when EAA was absent in MAC-T cell media. Availability of glucose for MAC-T cells did not have considerable impact on phosphorylation of 4EBP1. Increasing mTOR activity was associated ($P = 0.054$) with increasing phosphorylation of 4EBP1 ($r = 0.62$, Table 4). In contrast, phosphorylation of rpS6 significantly decreased by 58 and 31% for deprivation of EAA ($P < 0.001$) or glucose ($P = 0.011$) respectively. Ribosomal protein S6 is phosphorylated by its kinase S6K which is one of the well recognized direct substrates of mTOR. As mentioned before mTOR activity in MAC-T cells was significantly reduced by omission of EAA from media. The observed EAA deprivation-induced 58% reduction in phosphorylation of rpS6 might be basically due to lack of S6K1 stimulation by mTOR.

Glucose deprivation may impair phosphorylation of rpS6 via AMPK which can either negatively affect phosphorylation of mTOR enhancing it activity or impair activity of raptor in MTORC1. Raptor is known to facilitate interactions between mTOR and its substrates such as 4EBP1 and S6K1. Hardie et al. (2008) reported that AMPK can negatively affect the activity of raptor by directly phosphorylating it. Suppression of raptor activity by AMPK can therefore lead to reduced phosphorylation of rpS6 and phosphorylation of 4EBP1. Nonetheless, the non-significant correlation between (r = -0.20, *P* = 0. 359) phosphorylation of rpS6 and phosphorylation of AMPK (Table 4) and non-significant impact of glucose on phosphorylation of 4EBP1 (Table 3) are suggestive of insignificant involvement of glucose-AMPK-raptor pathway in modulating phosphorylation of rpS6 in MAC-T cells. Therefore, glucose appeared to influence rpS6 activity via mTORC1-independent pathway. Salmond et al. (2009) recognized rpS6 as a convergence point for mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathways besides mTORC1 pathway.

Phosphorylation of eEF2 significantly increased (*P* = 0.030) by 13% in response to EAA omission and had a negative correlation (r = -0.52, *P* = 0.013) with phosphorylation of mTOR. When considering the results of both Expt1 and Expt2 (Table2, Table 3, and Table 4) essential amino acid appeared to significantly regulate mTOR-associated cellular signals for protein synthesis in MAC-T cells.

Effects of EAA, acetate, and glucose on cellular signals for protein synthesis in MTS

AMPK: Significance of EAA, glucose, and acetate on cellular signals and $^{2}H_5$-Phe-PRT in MTS are presented in Table 5. Least square means for phosphorylation sate of some

signaling proteins when MTS were incubated in media containing different levels of EAA, acetate and glucose are presented in Table 6. Omission of acetate did not affect (*P* = 0.234) phosphorylation of AMPK although it was associated with numerically increased phosphorylation of AMPK by 47%.

mTOR signals: In agreement with what observed in Expt 1 (Table 2) and Expt 2 (Table 3), phosphorylation of mTOR significantly decreased by 32% (*P* = 0.001) when only EAA was deprived from media. Similarly, irrespective of energy substrate availability, removal of EAA reduced (*P* = 0.053) phosphorylation of rpS6 by 27%. In disagreement with the observed glucose-induced phosphorylation of rpS6 in MAC-T cells (Table 3), glucose did not have significant impact on phosphorylation of rpS6 in MTS. In line with both phosphorylation of mTOR and phosphorylation of rpS6, phosphorylation of 4EBP1 declined by 35% in response to the absence of EAA in media. Neither EAA nor energy substrates were able to significantly change phosphorylation of eEF2 in MTS (). Unlike MAC-T cells, phosphorylation of eEF2 was more strongly (r = 0.53, *P* = 0.011) correlated to phosphorylation of AMPK compared to its correlation with phosphorylation of mTOR (Table 7). Horman et al. (2002) and Chan et al. (2004) reported a positive link between phosphorylation of AMPK and phosphorylation of eEF2. This link appears to occur via eEF2 kinase as phosphorylated AMPK has been shown to directly phosphorylate and thus activate eEF2 kinase (Browne et al., 2004). 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. .

$^{2}H_5$-Phe enrichment in protein precipitated at a pH of 4.6 ($^{2}H_5$-Phe-PRT) was indicative of protein synthesis rates as faster protein synthesis rates lead to greater $^{2}H_5$-Phe incorporation into mammary proteins. Since the MTS were incubated in treatment media for 2 h, $^{2}H_5$-Phe-PRT results in Table 5 and 6 represent the acute responses of protein synthesis rates to EAA and energy substrate availability. As Table 5 shows, EAA had a significant ($P = 0.009$) impact on protein synthesis rates in MTS. Essential amino acids as 5% of normal DMEM/F12 concentrations reduced protein synthesis rates by 53%. This is consistent with the observed declines of phosphorylation of mTOR, phosphorylation of rpS6, and phosphorylation of 4EBP1 for the same treatments and prior signaling protein work in other cell types (Kimball, 2002). The correlation coefficients given in Table 7 further support the relationships between mTOR dependent signaling and protein synthesis as phosphorylation of mTOR (r = 0.57) and phosphorylation of 4EBP1 (r = 0.53) were positively correlated ($P < 0.02$) with $^{2}H_5$-Phe-PRT. However, phosphorylation of rpS6 had a weak relationship (r = 0.15) with casein synthesis rates. Unlike 4EBP1 and eEF2, effects of rpS6 on global protein synthesis seem to be indirect and long-term (Wang and Proud, 2006). As more time would be required to increase ribosome numbers, the poor association between phosphorylation of rpS6 and protein synthesis rates in these short-term cultures are consistent with the purported actions of rpS6. Protein synthesis rates were negatively associated (r = -0.55) with phosphorylation of eEF2 indicating the importance of dephosphorylation and thus activation of eEF2 for casein synthesis.

Across all three experiments, EAA in media had significant stimulatory effects on mTOR-mediated cellular signals for protein synthesis in bovine mammary cells. Elevated

phosphorylation of AMPK in MACT-cells (Table 2) and numerical increases in MTS (Table 6) in response to acetate deprivation suggest that acetate might have some control over activity of AMPK in bovine mammry cells compared to glucose and EAA. Rook (1979) and Lough et al. (1983) reported that oxidation of acetate provides the majority of ATP demand for milk synthesis representing 70% of the tri-carboxylic acid (TCA) cycle flux (Hanigan et al., 2004) in mammary cells. Mammary gland take up branched chain amino acids (Leu, Ile, and Val), and Arg and Lys in excess compared to their requirements for milk protein synthesis (Bequette et al., 2003). Majority of the excess EAA has been found to undergo oxidation. Although EAA showed some effect on phosphorylation of AMPK ($P = 0.118$) in Expt 2 (Table 3), its effects on AMPK activity in the other experiment were insignificant suggesting a slight contribution of EAA oxidation to energy status in BME cells which is consistent with the small amount of carbon that would be contributed to the TCA cycle from these AA. Although, glucose had some impact ($P = 0.109$) on phosphorylation of AMPK in Expt 2 (Table 3), its effect on phosphorylation of AMPK in MTS was insignificant and even less than that of EAA (Table 5 and 6). Compared to the effects of the enrgy substrates, the significantly greater impact of EAA availability on cellular signals for protein indicate that adequacy of substrate for milk protein synthesis is more critical than ATP supply. This finding will not totally disappoint someone regarding the role of glucose in protein synthesis regulation because glucose-induced plasma insulin elevations can significantly stimulate protein synthesis *in-vivo* (Suryawan et al., 2004). Moreover, the significant regulatory effects of EAA on casein synthesis rates are independent of energy substrate supply to

bovine mammary cells. The considerable correlation between activity of the signaling proteins and casein synthesis rates in MTS reflect that reduced casein synthesis rates during EAA deficiency was due to lack of stimulatory signals for protein synthesis. Since mammary tissue slices were grown in treatment media for a short period of 2 h, EAA deprivation might not limit substrate supply for casein synthesis. However it is very difficult to distinguish whether the EAA effects on casein synthesis rates were due to changes in the cellular signals or due to changes in substrate supply.

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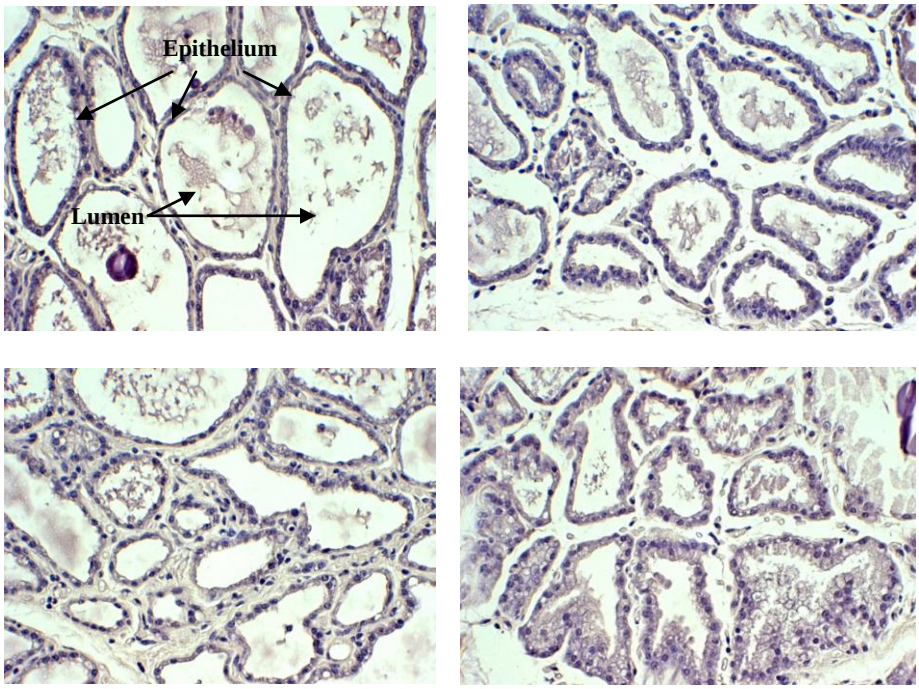

Figure 4-1. Representative images of mammary tissues from four cows at 20x magnification stained with hematoxylin and eosin

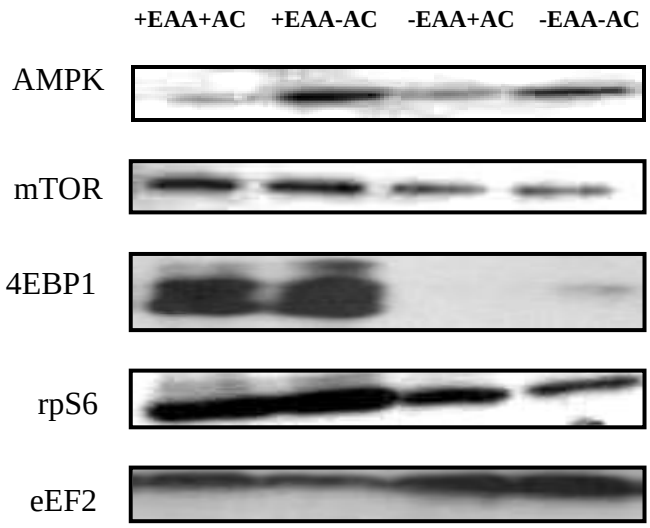

Figure 4-2. Representative Western immunoblot images for phosphorylation state of AMPK at Thr172, mTOR at Ser2448, 4EBP1 at Thr37/46, rpS6 at Ser235/236, and eEF2 at Thr56 in MAC-T cells of experiment 1.

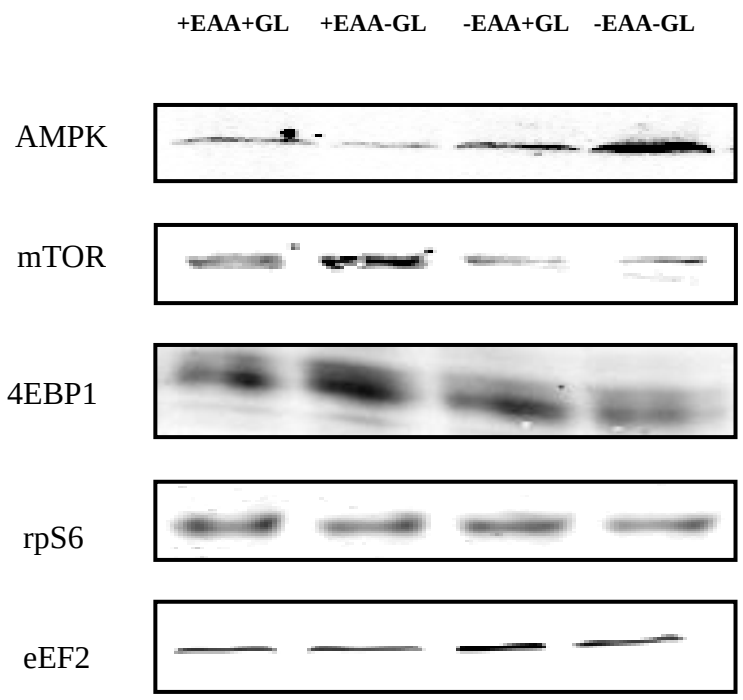

Figure 4-3. Representative Western immunoblot images for phosphorylation state of AMPK at Thr172, mTOR at Ser2448, 4EBP1 at Thr37/46, rpS6 at Ser235/236, and eEF2 at Thr56 in MAC-T cells of experiment 2.

Table 4-1. Treatments for each experiment.

	Experiment 1		Experiment 2		Experiment 3		
	+AC[2]	-AC[2]	+GL[3]	-GL[3]	+AC	+GL	-AC-GL[4]
+EAA[1]	+EAA+AC	+EAA-AC	+EAA+GL	+EAA-GL	+EAA+AC	+EAA+GL	+EAA-AC-GL
-EAA[1]	-EAA+AC	-EAA-AC	-EAA+GL	-EAA-GL	-EAA[4]+AC	-EAA[4]+GL	-EAA[5]-AC-GL

[1]presence (+EAA) or absence (-EAA) of essential amino acids (EAA) in media
[2]presence (+AC) or absence (-EN) of acetate in glucose free media
[3]presence (+GL) or absence (-EN) of glucose in acetate free media
[4]media containing neither glucose nor acetate
[5]EAA were present at 5% of the normal DMEM/F12 concentrations of EAA

Table 4-2. The effects of essential amino acids (EAA) and, acetate (AC) on phosphorylation state of several signaling proteins in MAC-T cells in experiment 1. Data were normalized to the mean observed phosphorylation state of +EAA+AC treatment.

Signaling protein	LSM				SEM	*P* - value		
	+EAA+AC[1]	+EAA-AC[2]	-EAA+AC[3]	-EAA-AC[4]		EAA	AC	EAA x AC
AMPK	1.00	1.73	1.20	1.40	0.17	0.786	0.094	0.308
mTOR	1.00[a5]	0.85[ab]	0.53[ab]	0.18[b]	0.18	0.006	0.175	0.588
4EBP1	1.00[a]	0.94[a]	0.06[c]	0.10[c]	0.07	0.001	0.933	0.495
rpS6	1.00[a]	1.10[a]	0.34[c]	0.19[c]	0.11	<0.0001	0.898	0.240
eEF2	1.00[ab]	0.98[a]	1.15[ab]	1.29[b]	0.10	0.023	0.483	0.371

[1]presence of both EAA and acetate (+EAA+AC), [2]presence of EAA in the absence of acetate (+EAA-AC), [3]presence of acetate alone (-EAA+AC), and [4]absence of both EAA and acetate (-EAA-AC). [5]Different letters in a row indicate significantly ($P < 0.05$) different least square means (LSM) of phosphorylation state of a protein.

Table 4-3. Effects of essential amino acids (EAA) and, glucose (GL) on phosphorylation state of some signaling proteins in MAC-T cells in experiment 2. Data were normalized to the mean observed phosphorylation state of the +EAA+GL treatment.

Signaling protein	LSM of PS				SEM	*P* - value		
	+EAA+GL[1]	+EAA-GL[2]	-EAA+GL[3]	-EAA-GL[4]		EAA	GL	EAA x GL
AMPK	1.00	0.94	0.93	1.55	0.16	0.118	0.109	0.060
mTOR	1.00[a]	0.90[a]	0.68[b]	0.69[b]	0.11	0.037	0.712	0.649
4EBP1	1.00[a]	1.02[a]	0.70[b]	0.58[b]	0.05	<0.0001	0.304	0.115
rpS6	1.00[a]	0.73[ab]	0.45[cb]	0.28[c]	0.07	<0.0001	0.011	0.511
eEF2	1.00[a]	1.02[ab]	1.14[b]	1.13[b]	0.05	0.030	0.893	0.788

[1]presence of both EAA and glucose (+EAA+GL), [2]presence of EAA alone (+EAA-GL), [3]presence of glucose alone (-EAA+GL), and [4]absence of both EAA and GL (-EAA-GL). [5]Different letters in a row indicate significantly ($P < 0.05$) different LSM.

Table 4-4. Pearson's Correlation coefficients (above diagonal) and their statistical significance (*P* – values) for relationships among phosphorylation state of some signaling proteins in MAC-T cells.

	AMPK	mTOR	4EBP1	rpS6	eEF2
AMPK		-0.21	-0.43	-0.20	0.14
mTOR	0.359		0.62	0.53	-0.52
4EBP1	0.434	0.054		0.82	-0.64
rpS6	0.350	0.005	0.002		-0.53
eEF2	0.499	0.013	0.032	0.033	

Table 4-5. Individual and interactive effects of essential amino acids (EAA), acetate (AC), and glucose (GL) on phosphorylation state of some signaling proteins and on $^{2}H_5$-phenylalanine enrichment of protein ($^{2}H_5$-Phe-PRT) in mammary tissue slices of experiment 3.

Signaling protein	*P* – values				
	EAA	AC	GL	EAA x AC	EAA x GL
AMPK	0.616	0.234	0.819	0.812	0.356
mTOR	0.001	0.945	0.723	0.666	0.299
rpS6	0.053	0.516	0.127	0.667	0.415
4EBP1	0.071	0.242	0.311	0.998	0.547
eEF2	0.576	0.345	0.856	0.395	0.814
$^{2}H_5$-Phe-PRT	0.009	0.58	0.22	0.581	0.573

Table 4-6. Least square means (LSM) of phosphorylation state of some signaling proteins and $^{2}H_5$-phenylalanine enrichments in mammary proteins ($^{2}H_5$-Phe-PRT) for interactions of essential amino acids (EAA) with acetate (AC) or glucose (GL) in mammary tissue slices.

	EAA x AC				EAA x GL				
	+EAA+AC[1]	+EAA-EN[2]	-EAA+AC[3]	-EAA-EN[4]	+EAA+GL[5]	+EAA-EN	-EAA+GL[6]	-EAA -EN	MSE
AMPK	1.00	1.64	1.33	1.76	1.00	0.78	0.86	1.23	0.31
mTOR	1.00[a7]	0.97[ab]	0.65[c]	0.69[ab]	1.00[a]	0.94[ab]	0.60[c]	0.72ab	0.11
4EBP1	1.00	1.23	0.61	0.84	1.00	1.36	0.72	0.81	0.21
rpS6	1.00[a]	0.97[ab]	0.79[ab]	0.65[bc]	1.00	1.37	0.81	0.93	0.14
eEF2	1.00	2.07	1.86	1.92	1.00	0.98	1.13	1.30	0.34
$^{2}H_5$-Phe -PRT	1.00	1.24	0.52	0.49	1.00	1.28	0.45	0.57	0.20

[1]presence of both EAA and acetate (+EAA+AC), [2]presence of EAA in the absence of any energy substrate (EN, +EAA-EN), [3]presence of acetate alone (-EAA+AC), and [4]absence of EAA and EN (-EAA-EN), [5]presence of only glucose and EAA (+EAA+GL), [6]presence of only glucose (-EAA+GL). [7]Different letters in a row indicate significantly ($P < 0.05$) different LSM.

Table 4-7. Pearson's Correlation coefficients (above diagonal) and therir statistical significance (*P* – value, below diagonal) for relationships among phosphorylation state of the signaling proteins and $^{2}H_{5}$-Phe enrichments in protein ($^{2}H_{5}$-Phe-PRT) in mammary tissue slices.

	AMPK	mTOR	rpS6	4EBP1	eEF2	$^{2}H_{5}$-Phe-PRT
AMPK		0.19	-0.29	-0.37	0.53	-0.40
mTOR	0.360		0.20	0.34	0.10	0.57
rpS6	0.220	0.508		0.39	0.13	0.15
4EBP1	0.107	0.125	0.098		-0.18	0.52
eEF2	0.011	0.650	0.575	0.448		-0.55
$^{2}H_{5}$-Phe-PRT	0.086	0.007	0.642	0.020	0.015	

CHAPTER 5:

Individual Essential Amino Acids-Induced Cellular Signals Controlling Protein Synthesis Rates in Bovine Mammary Cells

ABSTRACT

Understanding the role of individual amino acids (AA) in regulating milk protein synthesis would help identify the limiting AA for dairy cows with enough precision. Supplementation of these AA can maximize nitrogen utilization efficiencies in dairy cows. The objective of this study was to examine signaling effects of individual essential AA (EAA) on milk protein synthesis rates in bovine mammary cells. Alterations in phosphorylation state of mammalian target of rapamycin (mTOR) at Ser2448, ribosomal protein S6 (rpS6) at Ser235/236, and eukaryotic elongation factor 2 (eEF2) at Thr56 in response to removal of Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val or all EAA (-EAA) from complete DMEM/F12 media (+EAA) was assessed in MAC-T cells and mammary tissue slices (MTS) from lactating cows. Phosphorylation of mTOR, rpS6, eEF2, and initiation factor 4E binding protein 1 (4EBP1) at Thr37/46 was also assessed in response to the addition of all EAA, Arg, Ile, Leu, Met, or Thr to –EAA media using MAC-T cells. Relationships between the phosphorylation of these cellular signaling proteins and mammary protein synthesis rates were also studied in MTS. Omission of all EAA reduced phosphorylation of mTOR and rpS6 in both MAC-T cells and MTS. Omission of only Ile reduced ($P = 0.046$) phosphorylation of mTOR while omission of either Leu or Val tended ($P < 0.070$) to be associated with declined mTOR phosphorylation in MAC-T cells. Deprivation of Ile ($P = 0.027$) or Arg ($P = 0.015$) alone was associated with reduced rpS6 phosphorylation in MAC-T cells. In MTS, omission of

only Leu or Ile reduced phosphorylation of mTOR whereas omission of only Leu or His were associated with reduced phosphorylation of rpS6. Protein synthesis rates in MTS significantly decreased for –EAA treatment. Similar declines in protein synthesis rates were observed when Leu, Ile, Thr, or Met was absent in media. Protein synthesis rates were strongly ($r = 0.87$) associated with phosphorylation of mTOR in MTS. Addition of all EAA to –EAA media significantly enhanced phosphorylation of mTOR, 4EBP1 and rpS6, and reduced phosphorylation of eEF2 in MAC-T cells. Supplementation of Leu, Ile, Arg, or Met alone caused phosphorylation of rpS6 to increase to levels greater than that of –EAA but less than that of +EAA. Addition of Leu reduced phosphorylation of eEF2 so did +EAA. Regardless so of the cell model, Leu and Ile appeared to be predominant in mTOR-mediated control over bovine mammary protein synthesis rates.

INTRODUCTION

The lactating dairy cow is fairly inefficient at converting dietary nitrogen (N) to milk protein. When cows are fed to National Research Council (NRC, 2001) requirements, they convert approximately 25 percent of dietary nitrogen to body proteins such as milk and muscle proteins (Baquette et al. 2003). This nitrogen utilization efficiency is rather low compared to that of 35 - 45% in poultry and swine. In monogastric nutrition, individual amino acid (AA) requirements have been well defined. This allows high N use efficiencies because lower protein diets supplemented with individual AA can maintain similar production compared to that observed with higher protein diets. Defining individual AA requirements for ruminants is a challenging task due to the large variety of feedstuffs used and the extensive remodeling of dietary

nutrients by ruminal microorganisms (Lapierre et al., 2006). Therefore, AA requirements of dairy cows are expressed in aggregate as a metabolizable protein (MP) requirement. Such an approach results in considerable overfeeding of some AA in order to meet requirements of the more limiting AA. Feeding surplus protein leads to significant N losses in urine and feces that have been recognized as significant sources of air and water pollution (EPA 2004). Lapierre (2006) indicated that feeding cows with low CP diets supplemented with AA critical for milk protein synthesis could be a better strategy for improving N use efficiencies without compromising milk production of dairy cows. Therefore, identification of AA potentially critical for milk protein synthesis is important.

Besides being precursors for protein synthesis, AA are able to regulate mRNA translation (Kimball and Jefferson, 2005). Mammalian target of rapamycin (mTOR) complex 1 consisting of mTOR, raptor, mLST8 (GβL), PRAS40 (proline-rich PKB/Akt substrate 40 kDa), and FKBP38 are involved in signaling AA availability (Kim, 2009). Increased AA availability phosphorylates and thus activates mTOR. Activated mTOR in turn induces phosphorylation of ribosomal protein S6 (rpS6) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) which play critical roles in translation initiation (Wang and Proud, 2006). Activation of mTOR also enhances rates of elongation by catalyzing the dephosphorylation of eukaryotic elongation factor 2 (eEF2) which results in an increase in its activity (Browne and Proud, 2004).

The effects of AA on signaling pathways have been extensively explored in muscle, but little work has been completed in bovine mammary epithelial (BME) cells. Toerien and Cant (2007) concluded that protein factors involved in mRNA translation were not maximally activated in bovine mammary glands indicating the possibility of

enhancing milk protein synthesis rates through intensifying potential positive signals. Although Leucine was found to be the chief regulator of muscle protein synthesis (Rieu et al., 2006), Moshel et al. (2006) reported that mTOR signals for protein synthesis in bovine mammary epithelial cells were more sensitive to elimination of all AA than to elimination of Leucine alone. Moreover, Clark et al. (1978) observed that multiple AA: Cystine, Threonine, and Methionine were simultaneously limiting for protein synthesis in bovine mammary tissues. From these previous observations, it was hypothesized that one or more individual AA may be able to induce mTOR signals for protein synthesis. The objective of the present study was to investigate the effects of individual EAA on mTOR-mediated signaling and protein synthesis rates in MAC-T and bovine mammary tissue slices from lactating cows.

MATERIALS & METHODS

Four experiments were conducted. The first experiment (Expt1) examined the impact of the omission individual or all essential AA (EAA) from an EAA rich media (+EAA) on cellular signals for protein synthesis in MAC-T cells. The second experiment (Expt2) was a dose response experiment testing stimulatory effects of graded levels of total EAA on phosphorylation state of rpS6. The third experiment (Expt3) investigated relationships between these cellular signals and protein synthesis rates in bovine mammary tissue slices (MTS) prepared from lactating cows. In the fourth experiment (Expt4), EAA that exhibited substantial effects in Expt1 and Expt3 were tested for their cellular signaling effects in MAC-T cells when individually supplemented to an EAA deprived media (-EAA).

Expt1: Effects of EAA deprivation on mTOR signals in MAC-T cells

An immortalized bovine mammary epithelial cell line, MAC-T, established by Huynh et al. (1991) was used. Cells were seeded into 6-well plates at a density of 5×10^4 and grown to about 90% confluency in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Invitrogen, Carlsberg, CA) supplemented with 8% fetal bovine serum, and 100 U/mL Penicilin, 100 ug/mL Streptomycin and 0.25 ug/mL Amphotericin B (HyClone Laboratories Inc., Logan, UT). Cells were serum starved overnight before applying treatments. Regular DMEM/F12 media without FBS and containing 0.70 arginine (Arg), 0.15 histidine (His), 0.42 isoleucine (Ile), 0.45 leucine (Leu), 0.5 lysine (Lys), 0.17 methionine (Met), 0.45 phenylalanine (Phe), 0.22 threonine (Thr), 0.04 tryptophan (Trp), and 0.45 valine (Val, all in mM) served as the positive control (+EAA). Ten treatment media were constructed to be devoid (0 mM) of a single EAA (Arg, His, Ile, Leu, Lys, Met, Thr, Trp, or Val) with all other AA present at normal DMEM/F12 concentrations. DMEM/F12 media devoid of all EAA served as the negative control (-EAA). All treatment media contained 3.5 g/L glucose and 10ng/mL insulin. Treatments were applied for 1 h. Cells were harvested in a buffer containing protease and phosphatase inhibitors as described previously (McFadden and Corl, 2009). This experiment was repeated five times (n=5) on five different days.

Expt2: Effects of graded levels of total EAA on phosphorylation of rpS6 in MAC-T cells

Serum starved MAC-T cells were grown in the DMEM/F12 media containing 0, 0.18, 0.35, 0.70, 1.05, 1.75, 2.45, and 3.50 mM all EAA for 1 h. These concentration were equivalent to 0, 5, 10, 20, 30, 70, and 100% of the normal EAA concentrations in

regular DMEM/F12 (Invitrogen, Carlsberg, CA). Each treatment media was assigned to each of three wells (n=3) in 6-well plates of MAC-T cells. Cells were harvested in a lysis buffer used in Expt1.

Expt3: Effects of individual EAA deprivation on mTOR signals and protein synthesis rates in mammary tissue slices

Mammary tissues slices were prepared from the rear quarter of 4 multiparous lactating cows immediately after slaughter. These cows were screened for mastitis pathogens prior to slaughter to insure collection of healthy tissues. Cows were removed from feed for 12 h prior to slaughter. A series of tissue slices weighing 130 ± 30 mg were prepared using a Stadie-Riggs hand-held microtome and placed in 25 ml Erlenmeyer flasks containing 4 ml of treatment media. All slices were prepared within 30 min of animal slaughter. Treatments for MTS were the same as for Expt1 except that media concentrations of EAA were reduced to 5% of the corresponding DMEM/F12 concentrations rather than completely removed.

Protein synthesis rates were determined by measuring $^{2}H_{5}$-Phe enrichments in protein recovered from mammary tissue homogenates. A constant $^{2}H_{5}$-Phe enrichment of 20% was maintained across all treatment media. As Figure 1 shows, phosphorylation of rpS6 in response to graded levels of EAA in media of Expt1 revealed at least 1.00 mM of EAA (30% of the EAA concentration in regular DMEM/F12) should be available to induce cellular signals (i. e., phosphorylation of rpS6) similar to that of +EAA. Hence EAA at 5% concentration would not generate significantly greater cellular signals that would otherwise mask the EAA deprivation treatment effects.

Two flasks containing individual mammary tissue slices from each of the four cows were randomly assigned to each of the 12 treatment media, purged with 95:5 mix of O_2:CO_2 for 20 s, sealed with rubber stoppers, and placed in a shaking water bath at 37^{o}C. Flasks were opened at 90 min for dosing $^{2}H_5$-Phe and placed back in the shaking water bath. Incubations were stopped at 120 min by placing the flasks on ice and immediately adding ice-cold stop buffer containing 1 mM NaF and 10 μM Na_3VO_4 in sterile PBS at 120 min. Tissue slices were retrieved, washed twice in the ice cold stop buffer, mixed 1:7 (w:v) with lysis buffer, and homogenized (Power Gen 1000; Fisher Scientific, Waltham, MA).

Expt4: Effects of EAA supplementations on mTOR signals in MAC-T cells.

Cells were grown up to 90% confluence as described in Expt1. After overnight serum and EAA starvation, cells were exposed to treatment media for 1 h. The treatment media were +EAA, -EAA, and –EAA plus Arg, Ile, Leu, Met, or Thr alone. Treated cells were harvested as described in previous experiments.

Preparation of cell lysates

Cell lysates from both cell culture and tissue culture experiments were prepared for Western immunoblotting analysis as previously described (McFadden and Corl, 2009) Protein was precipitated from 300 μl aliquots of the cell lysate supernatants from the tissue culture experiment by adjusting the pH to 4.6 by adding of 12 μl of 5% phosphoric acid which should result in a precipitate enriched in casein. Protein precipitates were centrifuged at 4,000×g for 20 min at 4^{o}C, and the supernatant was carefully recovered. The precipitate was then washed twice with PBS and analyzed for $^{2}H_5$-Phe enrichments

of casein ($^2H_5$-Phe_PRT) as described below. The supernatant was analyzed for intracellular free EAA concentrations and $^2H_5$-Phe enrichment ($^2H_5$-Phe_INT) after precipitating the remaining protein with 10% (w/v) 5-sulfosalicylic acid.

Western immunoblotting analysis

Expt1: Western immunoblotting analysis was performed as described by Rius et al. (2010). The blots were first probed with antibodies recognizing the phosphorylated forms of mTOR (Ser2448), rpS6 (Ser235/236), and eEF2 (Thr56). Blots were developed using an enhanced chemiluminescense kit (ECL Plus, Amersham; Piscataway, NJ), visualized using ECL film and a medical film processor (SRX 101A Konica Minolta; Wayne, NJ) and digitized and integrated using Un-Scan-It (Silk Scientific, Inc., Orem, UT). Blots were then stripped and reprobed with antibodies recognizing the total forms of each protein. All antibodies were purchased from Cell Signaling Technology (Danvers, MA). The phosphorylation state of each protein was calculated as a ratio of phosphorylated form: total form.

***Expt2 ,Expt3, and Expt4*:** Proteins in cell lysates were resolved in 7 – 12% polyacrylamide gels and transferred PVDF membranes (Millipore; Billerica, MA). Blocking was performed using Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NB) for 1 h. The blots were probed with the same antibodies in Expt1 with an antibody recognizing phosphorylation of 4EBP1 at Thr37/46 (Cell signaling, Danvers, MA) in Odyssey Blocking Buffer overnight. After three 5-minute washes in Phosphate-Buffered Saline with 1% Tween-20 (PBS-T), the blots were incubated with secondary antibodies (IRDye 800 goat anti-rabbit, LI-COR Biosciences, Lincoln, NB) for 1 h. After three 5-minute washes in PBS-T, blots were scanned using the Odyssey Infrared Imaging System

(LI-COR Biosciences, Lincoln, NB). Band intensities were quantified using the Odyssey 2.1.12 application software. Stripped blots were then reprobed with the antibodies recognizing the total form of each protein. Phosphorylation state of each protein was calculated as a ratio of phosphorylated: total forms of each protein.

$^{2}H_5$-Phe enrichments in mammary tissue slices

Mammary tissue protein precipitates were hydrolyzed with 6 N HCl in 0.1% phenol at 110^{o}C for 20 h. The protein hydrolysate and the protein free cell supernatant were desalted by ion exchange chromatography, converted to N-(tert-butyldimethyl) AA derivatives as described previously (Bequette et al., 1999) and analyzed for $^{2}H_5$-Phe_PRT and $^{2}H_5$-Phe_INT by gas chromatography-mass spectrometry (GC-MS:Trace GC Ultra-DSQII , Thermo Electron Corporation; Waltham, MA) using selected ion monitoring to determine the ratio of the 239 ($^{2}H_5$-Phe) and 234 ($^{1}H_5$-Phe) m/z ions. Calibration curves were generated from gravimetric mixtures of these two ions as previously described by (Bequette et al., 2002). Intracellular EAA concentrations were determined by isotopic dilution with the GC-MS as previously described El-Kadi et al. (2006)

Statistical analysis

Analysis of variance (ANOVA) was performed using MIXED procedure of SAS 9.1 (SAS Institute Inc., Cary, NC) to test the treatment effects on $^{2}H_5$-Phe_PRT, phosphorylation state of signaling proteins, and intracellular EAA concentrations. Individual EAA treatment means were compared to the control treatment means (+EAA or –EAA) using Dunnetts's test. $^{2}H_5$-Phe_INT was used as a covariate when testing for treatment effects on $^{2}H_5$-Phe_PRT. Pearson's correlation coefficients for relationships among PS and $^{2}H_5$-Phe_PRT were obtained using the CORR procedure in the SAS 9.1.

RESULTS

Expt1: effects of individual EAA deprivation on cellular signals in MAC-T cells

Least square means for phosphorylation state of mTOR, rpS6, and eEF2 are shown in Figure 2. Compared to +EAA, the –EAA treatment reduced phosphorylation of mTOR ($P = 0.025$) and phosphorylation of rpS6 ($P = 0.014$) by 83 and 67%, respectively. Omission of Ile significantly reduced ($P < 0.050$) phosphorylation of rpS6 and mTOR respectively by 58 and 57%. Deprivation of Arg was also associated with a significant decline (by 57%, $P = 0.014$) in the phosphorylation of rpS6. Omission of Leu or Val tended ($P < 0.070$) to cause phosphorylation of mTOR to reduce by more than 40%. Deprivation of individual or all essential AA from media had no significant effects on phosphorylation of eEF2 in MAC-T cells. However, in the absence of branched-chain AA (BCAA); Ile, Leu and Val, and Arg, phosphorylation of eEF2 numerically increased by more than 30%. Pearson's correlation coefficients (r) and their statistical significance (*P*- values) for associations among phosphorylation of mTOR, rpS6, and eEF2 are presented in Table 1. Phosphorylation of mTOR had strong ($P = 0.0003$) positive correlation ($r = 0.86$) with phosphorylation of rpS6 and tended ($P = 0.073$) to have a negative relationship ($r = -0.54$) with phosphorylation of eEF2.

Expt2: Graded levels of EAA affecting phosphorylation of rpS6

Maximum phosphorylation of rpS6 in MAC-T cells was associated with the EAA concentration (3.5 mM) in regular DMEM/F12 media. When EAA concentration decreased less than 1.0 mM (30% of regular DMEM/F12 EAA concentration), phosphorylation of rpS6 declined ($P < 0.010$, Figure 3) significantly.

Expt3: Effects of EAA deprivations on cellular signals and protein synthesis rates in MTS

As results in Table 2 show, -EAA significantly reduced phosphorylation of mTOR ($P = 0.001$) and phosphorylation of rpS6 ($P = 0.005$) by 67 and 76%, respectively whereas omission of Leu alone significantly reduced both phosphorylation of mTOR ($P = 0.009$) and phosphorylation of rpS6 ($P = 0.014$) by 48 and 51% respectively. Deprivation of only Ile or His were respectively associated with significant ($P < 0.040$) declines in phosphorylation of mTOR (by 41%) and phosphorylation of rpS6 (by 48%, Table 2). Withdrawal of individual or all EAA from media did not have statistically significant ($P = 0.156$) impacts on phosphorylation of eEF2 ($P = 0.156$). However, removal of all EAA (-EAA) or removal of only Met numerically increased phosphorylation of eEF2 by more than 60% (Table 2). Omission of all EAA, or omission of only Leu, Ile, Thr, or Met significantly ($P < 0.05$) reduced $^{2}H_5$-Phe-PRT by 61, 55, 60, 55, and 48% respectively. Pearson's correlation coefficients (above diagonal) and the significance of these correlations (*P*-values, below diagonal) for relationships among phosphorylation state of the signaling proteins and $^{2}H_5$-Phe-PRT are given in Table 1. Phosphorylation of mTOR was ($r= 0.87$, $P < 0.001$) positively correlated with $^{2}H_5$-Phe-PRT. Phosphorylation of eEF2 was negatively associated ($r = -0.67$, $P = 0.017$) with both phosphorylation of mTOR and phosphorylation of rpS6. There was a strong positive association ($r = 0.66$, $P = 0.019$) between phosphorylation of mTOR and phosphorylation of rpS6 in MTS.

Least square means for intracellular concentrations of some EAA are given in Table 4. Omissions of Ile, Lys, Met, Phe, Thr, or Val from media were associated with

significantly ($P < 0.05$) reduced (by 80, 75, 83, 70, 64, and 55% respectively) corresponding intracellular concentrations compared to that of +EAA treatment. Omission of leu alone tended ($P < 0.10$) to reduce its intracellular concentration by 48%. Removal of all EAA from MTS media significantly reduced ($P < 0.05$) intracellular concentrations of these EAA by more than 63% compared to that of +EAA treatment.

Expt4: effects of EAA supplementations on the cellular signals in MAC-T cells

Least square means and statistical significance for the effects of EAA supplementation treatments on phosphorylation state of mTOR, rpS6, eEF2, and 4EBP1 are given in Table 3. Addition of all EAA (+EAA) to EAA deprived media (-EAA) significantly increased ($P < 0.001$) phosphorylation of rpS6 by 7 fold (Table 3). Addition of Leu, Ile, or Arg alone significantly ($P < 0.050$) increased phosphorylation of rpS6 by more than 2 fold whereas Met supplementation tended to increase ($P = 0.071$) rpS6 phosphorylation by approximately 2 fold compared to that of -EAA. However, Leu, Ile, or Arg alone failed to raise phosphorylation of rpS6 to the extent that +EAA did. Supplementation of all EAA or only Leu significantly ($P < 0.020$) reduced phosphorylation of eEF2 by more than 25%. When all EAA were present, phosphorylation of mTOR and 4EBP1 increased more than 2 fold ($P < 0.030$). Addition of only Leu was tended ($P = 0.079$) to associate with increased phosphorylation of mTOR compared to that of –EAA.

DISCUSSION

The objective of this study was to investigate the cellular signaling effects of all ten EAA; Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, on protein synthesis in mammary cells. Since cows' milk protein responses to individual AA are highly variable

(50% coefficient of variation, (Clark et al., 1978)), an *in vivo* study testing this many treatments would demand a large number of animals and thus require a large investment of time, labor, and money. Therefore, the present work used an *in vitro* approach to test the effects of individual EAA on cellular signaling and protein synthesis rates in bovine mammary cells.

MAC-T cells are considered a good model for studying nutrient induced changes in mammary growth (Berry et al., 2003). Hence, MAC-T cells were used in Expt1 to test the effects of all and individual EAA on cellular signals for global protein synthesis. The relationships of these cellular signals with protein synthesis rates were examined using MTS in Expt3. As Figure 3 and Table 2 show, complete removal of EAA from cell media or restriction to 5% of normal DMEM/F12 concentrations in MTS media (-EAA) substantially reduced mTOR phosphorylation by 83 and 67% respectively. In line with these cellular signals, $^{2}H_5$-Phe -PRT, which is indicative of protein synthesis rates in MTS, significantly decreased by 61% for the -EAA treatment. Phosphorylation of mTOR at Ser2448 was tested as it has been demonstrated to be a good biomarker for mTOR activation state (Chiang and Abraham, 2005). Increased activity of mTOR has been shown to significantly increase protein synthesis in eukaryotic cells. For example 90% of mRNA translation in *S. cerevisiae* depends on mTOR activity (Fujita et al., 2009). In agreement with this association, the present study revealed significantly positive correlations ($r = 0.87$; Table 1) between phosphorylation of mTOR and $^{2}H_5$-Phe-PRT in MTS indicating substantial potential for regulating cow milk protein synthesis rates via the mTOR pathway.

Nonetheless, it would be more interesting for dairy nutritionists to know which individual EAA might have significant regulatory effects on milk protein synthesis via the mTOR pathway. Fairly consistent cellular signaling responses to the omission of individual EAA were observed across MAC-T cells and MTS experiments as indicated by the strong positive correlation (r = 0.71, Table 1) between phosphorylation of mTOR in MAC-T cells and that in MTS. This moreover suggests that MAC-T cells provide a good model for studying cellular signals for milk protein synthesis.

In MAC-T cells, phosphorylation state of the signaling proteins was predominantly influenced by isoleucine (Figure 3). Omission of Isoleucine significantly reduced phosphorylation of mTOR by 57% whereas omission of only valine or leucine from +EAA tended to cause phosphorylation to decrease by 57 and 43% respectively. In agreement with this observation, Moshel et al. (2006) suggested that AA other than leucine would have greater impact on mTOR-mediated cellular signals in bovine mammary epithelial cells. Mammary tissue slices responded maximally to both leucine and isoleucine with declines in phosphorylation of mTOR of more than 40% (Table 2).

Consistent with the changes in phosphorylation of mTOR, phosphorylation of rpS6 significantly dropped by 67 and 58% respectively when all EAA or only isoleucine were removed from MAC-T cell media (Figure 3). In MTS (Table 2), omission of leucine was associated with significantly less (by 51%) phosphorylation of rpS6. Interestingly, histidine deprivation caused a substantial (by 48%) drop in phosphorylation of rpS6 in MTS. Increasing phosphorylation of mTOR was strongly associated (r = 0.86, P = 0.0003) with increasing phosphorylation of rpS6 in MAC-T cells (Table 1). This was expected as rpS6 kinase (S6K) is a well recognized direct substrate of mTOR (Avruch et

al., 2009). However, this correlation was not as strong (r = 0.66, *P* = 0.019) in MTS indicating the possibility of rpS6 phosphorylation by an mTOR-independent signaling pathway. Salmond et al. (2009) placed rpS6 as a convergence point for mitogen activated protein kinase and phosphatidylinositol-3-kinase pathways besides the mTOR pathways.

Although the correlation between phosphorylation of mTOR and rpS6 was high, protein synthesis was more highly correlated with phosphorylation of mTOR than rpS6 (Table 1). This indicates relatively less involvement of phosphorylation of rpS6 in determining protein synthesis rates in MTS. Phosphorylaton and thus activation of rpS6 specifically enhances translation of mRNAs that are characterized by 5' terminal oligopyridine tract (5' TOP). These 5' TOP mRNAs encode ribosomal proteins and some eukaryotic translation initiation and elongation factors that increase the capacity of cellular translation machinery (Anthony et al., 2001b). Effects of phosphorylation of rpS6 on global protein synthesis could be expected to require time for adoption. Since this study was carried out over a short time period (2 h), the contribution of phosphorylation of rpS6 to increased protein synthetic capacity could not be expected.

Phosphorylation of eEF2 on Thr56 was unaffected by deprivation of all the EAA in both MAC-T cells (*P* = 0.529) and MTS (*P* = 0.156). In MAC-T cells, deprivation of branched chain amino acids; isoleucine, leucine, and valine increased phosphorylation of eEF2 by more than 40%. In MTS, total EAA withdrawal increased phosphorylation of eEF2 by more than 100% while deprivation of either methionine, leucine, or arginine were associated with more than 40% increase in phosphorylation of eEF2. Eukaryotic elongation factor 2 facilitates translocation of ribosome by one codon while peptidyl-tRNA moves from the A-site to the P-site (Soe et al., 2007). Phosphorylation of eEF2 by

eEF2 kinase (eEF2K) impairs its function. Redpath et al. (1996) observed that insulin-induced mTOR activity might regulate the function of eEF2K and thereby affect the phosphorylation of eEF2. A negative correlation between phosphorylation of mTOR and phosphorylation of eEF2 was observed in both MAC-T cells (r = -0.54) and MTS (r = -0.67, Table 1) This indicates a positive link between the activity of mTOR and eEF2 activity in bovine mammary epithelial cells.

Intracellular EAA concentration results presented in Table 4 indicate that individual and all EAA deprivation caused corresponding intracellular concentrations to drop by 48 to 83%. These results indicate that individual EAA deprivation treatments were able to substantially reduce corresponding intracellular EAA concentrations in MTS. It is yet to be clarified whether mTOR signals respond to extracellular or intracellular AA levels. However, several studies (e. g. Beugnet et al. (2003)) have shown that it is most probably the intracellular AA concentrations controlling mTOR signals.

$^{2}H_5$-Phe enrichment in tissue slice proteins ($^{2}H_5$-Phe-PRT) reflects how fast $^{2}H_5$-Phe was incorporated into the proteins so that it is indicative of protein synthesis rates in MTS. Mammary tissue slices were first incubated in $^{2}H_5$-Phe free treatment media for 1.5 h to allow the cells to adapt to the treatment media. $^{2}H_5$-Phe was then added to the media and the incubation was continued for another 30 min to allow incorporation into newly synthesized proteins which would mix with existing protein in the culture. Therefore $^{2}H_5$-Phe incorporation into protein represents the acute effects of EAA treatments on casein synthesis rates. It is possible that intracellular Phe enrichment may have been altered by changes in protein turnover causing more or less dilution of the label. This possibility was negated by using $^{2}H_5$-Phe-INT as a covariate in the statistical

model to evaluate protein bound enrichment. Additionally intracellular free $^{2}H_5$-Phe enrichment was not significantly altered by treatments averaging 0.22 ± 0.05 across all treatments (Table 2).

Deprivation of all EAA significantly reduced mammary tissue protein synthesis rate by 61%. In agreement with mTOR phosphorylation responses, omission of isoleucine or leucine alone had large impact on mammary slice protein synthesis rates (Table 2). Moreover removal of either threonine or methionine also resulted in large reductions in protein synthesis rates (55 and 48%, respectively). The strong correlation between phosphorylation of mTOR and $^{2}H_5$-Phe_PRT indicates that protein synthesis responses to EAA supply are driven primarily by mTOR signaling rather than substrate limitations. Moreover this correlation leads to the conclusion that EAA in particular leucine, isoleucine, methionine, and threonine have substantial mTOR-mediated control on casein synthesis in BME cells.

In Expt1 and Expt3, cellular signaling effects of AA were tested by withdrawing EAA. However, some literature (Proud, 2004) reported that these signaling effects were not the same when AA were individually added to deficient media. The latter approach has more implications to dairy nutrition because a feeding of low protein diet supplemented with individual AA have been recognized to have significant potential for improving N utilization efficiency of dairy cows (Lapierre et al., 2006). In experiment 4, leucine, isoleucine, methionine, threonine, and arginine were individually added to media devoid of all other EAA and tested for signaling protein responses in MAC-T cells. The effects of each EAA on phosphorylation of mTOR, rpS6, eEF2, and 4EBP1 were tested compared to that of –EAA and +EAA media. In addition to S6K, 4EBP1 has also been

recognized as a direct substrate of mTOR (Avruch et al., 2009). Phosphorylation of mTOR in turn phosphorylates 4EBP1 causing it to release eIF4E to form eIF4F translation initiation complex. This initiation complex then scans mRNA through the 5' untranslated region while unwinding secondary structure in the mRNA to expose the translation initiation codon, enabling ribosome loading, and ultimately promoting translation initiation (Graff et al., 2008). Dephosphorylation of 4EBP1 on Thr37/46 has been shown to inhibit formation of eIF4F complex and thus result in blunted mRNA translation (Rose et al., 2009). Through its effects on eIF4F complex, phosphorylation of 4EBP1 is directly involved in translation initiation indicating potentially acute control on protein synthesis rates. Since casein synthesis rates in MTS exhibited significant and acute responses for EAA omissions, phosphorylation of 4EBP1 was also examined in Expt3.

The addition of all EAA (+EAA) resulted in a significant 8 fold (100% vs. 13%, Table 3) increase in phosphorylation of rpS6 in MAC-T cells as compared to cells cultured with media devoid of EAA. The addition of leucine, isoleucine, arginine, or methionine stimulated increased phosphorylation of rpS6 by about two or more than two fold. However, the increased phosphorylation induced by these individual EAA was still significantly less than that of +EAA suggesting a need of supplementing multiple EAA for maximum phosphorylation of rpS6 in bovine mammary cells. This observation does not therefore agree with conventional single limiting amino acid concept where only one AA can be limiting at a time.

Supplementation of all the EAA increased both phosphorylation of mTOR and phosphorylation of 4EBP1 by more than 2 fold. Supplementation of leucine tended to

increase phosphorylation of mTOR compared to that of –EAA (Table 3). Interestingly, supplementation of all EAA significantly reduced phosphorylation of eEF2 while supplementation of Leu alone tended to reduce phosphorylation of eEF2 to a similar extent as +EAA. Considerable impact of leucine on phosphorylation of both mTOR and phosphorylation of eEF2, and the negative correlations between these phosphorylation events in Table 1 clearly revealed a strong link between mTOR and eEF2 activities in bovine mammary cells.

Although several EAA exhibited different degrees of influence on cellular signaling, leucine and isoleucine appeared to be the chief regulators. Signaling responses to these AA were predominantly mediated by the mTOR pathway. Leucine has also been shown to significantly stimulate the mTOR pathway and protein synthesis in muscle (Escobar et al., 2006) and liver (Anthony et al., 2001b) tissues. Recent studies by Hayashi et al. (2009) and Burgos et al. (2010) showed the potential of mTOR pathway to be a significant control point of milk protein synthesis in the bovine mammary gland. This study showed that isoleucine had similar affects as Leu in regulating mTOR activity in BME cells. Richert et al. (1997) observed significant linear increases in milk casein content of lactating sows fed increasing levels of isoleucine. Having an alternative AA stimulating milk protein synthesis is an advantage in feeding dairy cows. Supplementation of leucine into a low protein diet would not only stimulate milk protein synthesis but also muscle protein synthesis in cattle. Enhanced sequestration of AA in muscle tissues can limit AA availability for milk protein synthesis. This disadvantageous partitioning of AA could be minimized and milk protein synthesis efficiencies could possibly be enhanced by supplementing AA having greater stimulatory effects on milk

protein synthesis than peripheral tissue protein synthesis. Leucine has been shown to significantly enhance muscle protein synthesis compared to the other BCAA: isoleucine and valine (Escobar et al., 2006). Therefore, isoleucine supplementation can potentially enhance milk protein efficiencies and thereby reduce N losses in dairy cows.

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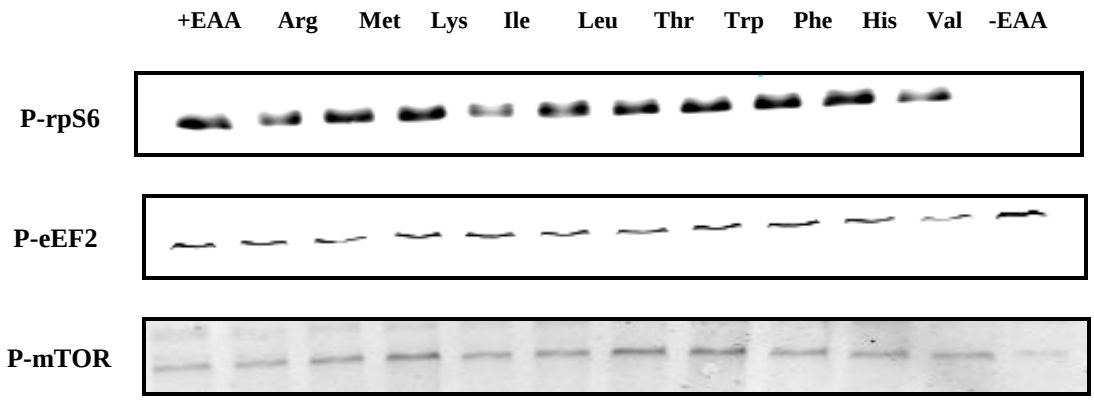
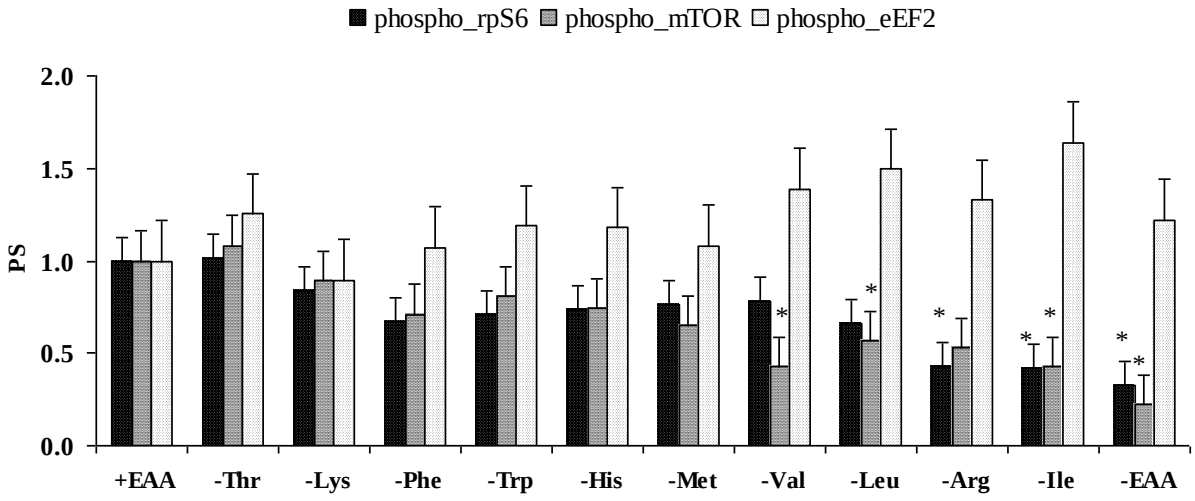
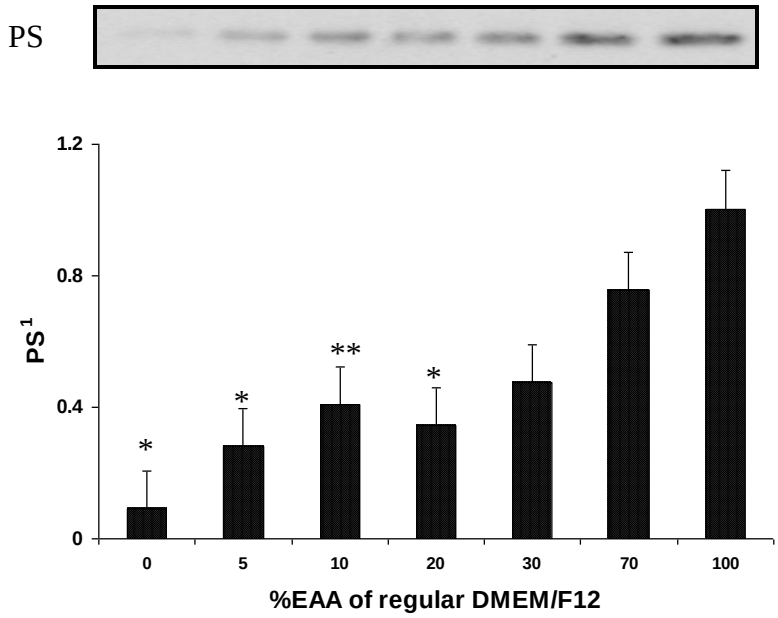


Figure 5-1. Representative Western immunoblot images for phosphorylation of ribosomal protein S6 at ser235/236 (P-rpS6), eukaryotic elongation factor 2 at Thr56 (P-eEF2), and mammalian target of rapamycine at Ser2448 (P-mTOR) in MAC-T cells of experiment 1.



*significantly (P <0.10) less PS compared to that of +EAA

Figure 5-2. Least square means (LSMs) and associated standard errors for phosphorylation of ribosomal protein S6 at Ser235/236 (phospho_pS6), mammalian target of rapamycin at Ser2448 (phospho_mTOR), and eukaryotic elongation factor 2 at Thr56 (phospho_eEF2) in MAC-T cells of experiment 1.



[1]Ratio of phosphorylated : total forms, normalized to that of 100%.
*significantly different from 100% ($P < 0.05$)
**significantly different from 100% ($P<0.10$)

Figure 5-3. Least square means for phosphorylation state of ribosomal protein S6 at Ser235/236 (PS) in MAC-T cells grown in media containing different percentages of the essential amino acid (EAA) in regular DMEM/F12 media of experiment 2.

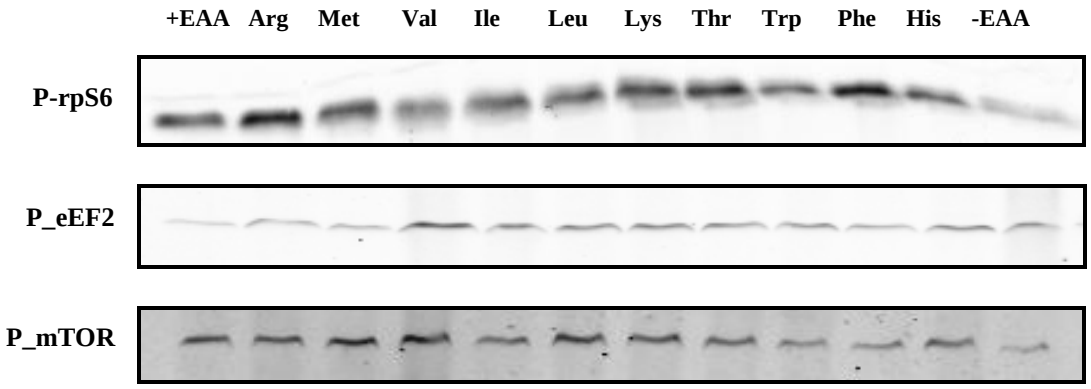


Figure 5-4. Representative Western immunoblot images of phosphorylation of ribosomal protein S6 at ser235/236 (P-rpS6), eukaryotic elongation factor 2 at Thr56 (P-eEF2), and mammalian target of rapamycine at Ser2448 (P-TOR) in mammary tissue slices of experiment 3.

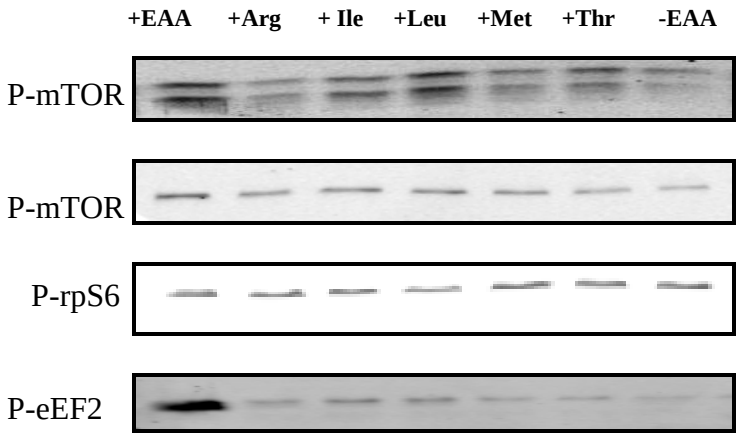


Figure 5-5. Representative Western immunoblot images of phosphorylation of eukaryotic initiation factor 4E binding protein 1 at Thr37/46 (P-4EBP1), mammalian target of rapamycine at Ser2448 (P-mTOR), eukaryotic elongation factor 2 at Thr56 (P-eEF2) and ribosomal protein S6 at ser235/236 (P-rpS6) in MAC-T celss of experiment 4.

Table 5-1. Pearson's correlation coefficients (above diagonal) and their statistical significance (*P*-values, below diagonal) for relationships among phosphorylation state of some signaling proteins and $^2H_5$-Phe enrichments of proteins ($^2H_5$-Phe_PRT) in MAC-T cells and mammary tissue slices (MTS). The last column gives relationships of phosphorylation states of each signaling protein between MAC-T cells and MTS.

	MAC-T			MTS				
	rpS6	eEF2	mTOR	rpS6	eEF2	mTOR	$^2H_5$-Phe	MAC-T vs MTS
rpS6		-0.61	0.86		-0.67	0.66	0.55	0.63 (*0.028*)
eEF2	*0.096*		-0.54	*0.017*		-0.67	-0.38	0.04 (*0.925*)
mTOR	*0.0003*	*0.073*		*0.019*	*0.017*		0.87	0.71 (*0.010*)
$^2H_5$-Phe				*0.062*	*0.227*	*0.0002*		

Table 5-2. Least square means (LSM), associated mean standard errors (mse) and statistical significance for essential amino acid deprivation treatments on phosphorylation of mammalian target of rapamycin (mTOR), ribosomal protein S6 (rpS6), eukaryotic elongation factor 2 (eEF2), $^2H_5$-Phe enrichment in proteins ($^2H_5$-Phe_PRT) and intracellular $^2H_5$-Phe enrichment ($^2H_5$-Phe_INC) in mammary tissue slices of experiment 3.

	rpS6[4]	eEF2[4]	mTOR[4]	$^2H_5$-Phe_PRT[4]	$^2H_5$-Phe_INT
+EAA[1]	1.00	1.00	1.00	1.00	0.25
-Arg[2]	0.63	1.4	0.65	0.66	0.20
-Lys[2]	0.71	1.11	0.79	0.67	0.20
-Met[2]	0.84	1.65	0.66	0.52*	0.25
-Leu[2]	0.49*	1.4	0.52*	0.45*	0.22
-Ile[2]	0.76	1.02	0.59*	0.40*	0.30
-Val[2]	0.83	1.22	0.64	0.66	0.19
-Trp[2]	0.74	1.36	0.68	0.65	0.17
-Thr[2]	0.73	0.97	0.67	0.45*	0.21
-His[2]	0.52*	1.12	0.83	0.67	0.15
-Phe[2]	0.70	1.32	0.89	0.85	0.27
-EAA[3]	0.24*	2.36	0.33*	0.39*	0.18
MSE[4]	0.11	0.35	0.09	0.13	0.06
P-value	0.005	0.156	0.001	0.021	0.32

[1] presence of all EAA, [2]absence of individual EAA, [3]absence of all EAA, [4]Normalized to that of +EAA, *significantly different from +EAA ($P < 0.05$)

Table 5-3. Significance of essential amino acid supplementation treatments on phosphorylation of some signaling proteins in MAC-T cells of experiment 4. Phosporylation state of each protein is presented as the ratio of phosphorylated to total forms and normalized to the observed mean values for the +EAA treatment.

	Least square means								
	+EAA	+Leu	+Ile	+Met	+Arg	+Thr	-EAA	MSE	P-value
rpS6	1.00*	0.37*	0.29*	0.25**	0.31*	0.17	0.13	0.03	<0.0001
eEF2	1.00*	1.03*	1.14	1.24	1.19	1.16	1.35	0.07	0.034
mTOR	1.00*	0.84**	0.79	0.70	0.69	0.75	0.44	0.14	0.066
4EBP1	1.00*	0.85	0.91	0.67	0.67	0.70	0.44	0.10	0.075

*significantly different from -EAA ($P < 0.05$), **significantly different from -EAA ($P < 0.10$)

Table 5-4. Least square means and associated mean standard error (MSE) for intracellular concentrations (μmol/mL) of some essential amino acids across essential amino acid deprivation treatments for mammary tissue slices in experiment 3.

	Ile	Leu	Lys	Met	Phe	Thr	Val
+EAA	21.56	23.21	20.27	7.88	9.30	33.98	30.71
-Arg	16.70	25.28	22.79	5.61	10.30	39.13	33.50
-Lys	15.90	26.97	4.58*	5.76	11.13	40.16	20.87
-Met	15.36	21.84	19.85	1.34*	9.51	33.18	20.69
-Leu	16.06	12.14**	22.72	5.48	11.03	35.74	30.47
-Ile	4.41*	24.76	23.99	5.49	11.12	37.77	24.53
-Val	17.91	22.36	24.11	6.94	11.39	38.27	13.53*
-Trp	14.30	27.67	22.33	5.34	10.30	38.88	33.22
-Thr	18.40	26.18	24.58	6.09	11.71	12.25*	24.54
-His	16.28	22.65	22.75	5.05	10.28	32.76	26.28
-Phe	13.20	25.07	19.75	6.37	2.77*	29.63	65.08
-EAA	2.91*	8.55*	4.63*	1.47*	2.14*	2.89*	9.35*
MSE	2.42	2.40	2.60	0.76	1.12	3.51	10.68

*Significantly different from +EAA ($P < 0.001$)
** Significantly different from +EAA ($P = 0.057$)

CHAPTER 6

Effects of Jugular Infused Lysine, Methionine, and Branched Chain Amino Acids on Milk Protein Synthesis in High Producing Dairy Cows

ABSTRACT

In addition to lysine (Lys) and methionine (Met), current ration balancing programs suggest that branched chain amino acid (BCAA) supply may also be limiting in dairy cows. The objective of this study was to investigate whether BCAA, leucine (Leu), isoleucine (Ile), and valine (Val), become limiting for milk protein synthesis when Met and Lys supply were not limiting. Nine multiparous Holstein cows with average milk production of 53.5 ± 7.11 kg/day were randomly assigned to 7 d continuous jugular infusion treatments of saline (CTL), Met and Lys (ML; 12 g and 21 g /d respectively), and ML plus Leu, Ile, and Val (ML+BCAA; 35 g, 15 g, and 15 g/d respectively) in 3 x 3 Latin square design with three infusion periods separated by 7 d non-infusion periods. The basal diet consisted of 40% corn silage, 14% alfalfa hay, and a concentrate mix and respectively supplied Lys, Met, Ile, Leu, and Val as 6.1, 1.8, 4.7, 8.9, and 5.3% of metabolizable protein. Dry matter intake (24.8 kg/d), milk yield (52.8 kg/d), fat content (2.8%), fat yield (1.37 kg/d), lactose content (4.7%), and lactose yield (2.5 kg/d) were similar across treatments. Protein yield and protein content were not significantly different between ML (1.53 kg/d and 2.85% respectively) and ML+BCAA (1.48 kg/d and 2.80% respectively), but they were significantly greater than that of CTL (1.38 kg/d and 2.69%). Protein efficiency, expressed as milk protein yield divided by total crude protein

intake (feed plus infusate), was not significantly different between ML (0.41 ± 0.02) and ML+BCAA (0.38 ± 0.03) but that of CTL (0.37± 0.02) was significantly less than that of ML. Cows that received ML+BCAA had significantly reduced MUN yield (5.9 kg/d) and content (10.9 mg/dl) compared to milk of CTL cows (6.5 kg/d and 12.1 mg/dl respectively), while milk of ML cows were intermediate (6.4kg/d and 11.5 mg/dl). Similar plasma 3-methylhistidine concentrations across treatments suggest non-significant treatment effects on AA mobilization from muscle. While high producing cows responded positively to Met and Lys supplementation, there were no apparent benefits of BCAA supplementation in milk protein synthesis. Infusion of BCAA may have stimulated synthesis of other body proteins, probably muscle proteins, as evidenced by decreased MUN.

INTRODUCTION

Branched-chain amino acids (**BCAA**) are taken up by the mammary gland and used for cellular and milk protein synthesis, de novo milkfat synthesis, and provision of metabolic intermediates and energy. Branched-chain amino acids comprise 44% of the essential amino acids in milk proteins. Current ration balancing software (NRC 2001, Washington, DC and CPMDairy V5.0, Ithaca, NY) suggest that the BCAA may be as limiting as methionine and lysine for high producing dairy cows (>40 kg. milk/d) fed rations with large proportions of corn silage, corn grain, and corn by-products.

Several research studies have been conducted which examined the role of BCAA in milk and milk protein synthesis. Weekes et al. (2006), fed a 9% crude protein diet supplemented with either casein or an amino acid mixture devoid of BCAA via abomasal infusion. While there was no decrease in milk yield or milk protein concentration after

six days of infusion with the incomplete mixture, serum levels of the BCAA were 60% of the levels when the cows were fed the complete mixture, suggesting that there may be a limit to how much BCAA can be mobilized from muscle to supply the mammary glands. If the infusions had been longer in duration, there may have been an impact on milk synthesis. Similar decreases in serum BCAA concentrations have been observed with increasing milk protein synthesis in response to hyperinsulinemic-euglycemic clamp studies (Griinari et al., 1997, McGuire et al., 1995). It is unclear whether the observed reductions in concentrations of BCAA associated with hyperinsulinemia were due to withdrawal of AA for milk protein production as the hind-limb clearance rates for the BCAA are responsive to insulin (Bequette et al., 2001). Therefore, the reductions in blood BCAA could have been due to increased catabolism by muscle and not reflective of the overall BCAA status in the animal.

Mackle et al. (1999) observed no milk yield or milk composition responses to abomasal infusions of BCAA when cows were fed diets to exceed ME and MP requirements,. However, the response to sodium caseinate infusion in this study was also modest, suggesting that increasing the supply of amino acids to the mammary glands above the requirements for milk synthesis and tissue metabolism does not further increase milk protein yields. Hopkins et al. (1994) infused a mixture of BCAA and arginine intraperitoneally into lactating cows fed low CP diets and observed no effects on milk yield or milk composition. An evaluation of those diets suggests that lysine, methionine, and histidine in addition to the BCAA and arginine may have been limiting for milk synthesis. Research with cows fed diets based on grass silage have shown no response to leucine or BCAA infusions (Huhtanen et al., 2002, Korhonen et al., 2002), whereas in

cows fed a corn silage based diet, duodenal infusions of leucine resulted in a quadratic response in milk protein content and yield (Rulquin and Pisulewski, 2006b).

In summary, while current ration balancing programs suggest that BCAA supply may be limiting for milk protein synthesis, the scientific evidence is mixed. This experiment was designed to determine whether BCAA become limiting for milk protein synthesis in high producing dairy cows (>40 kg milk/d) fed rations based on corn silage and corn.

MATERIALS & METHODS

Animals and Treatments

All animal procedures in this study were conducted under approval of Virginia Tech Animal Care and Use Committee. Nine multiparous Holstein cows with average milk production of 53.5 kg/day and average body weight of 611 kg at 43 DIM were used. Cows were housed in a free-stall barn when not receiving infusions and in individual metabolic stalls when receiving infusions. Cows were fed ad libitum to achieve a minimum of 5% weighbacks on an as fed basis and were given free access to water. Individual daily feed intake and refusals were measured throughout the study. Cows were milked 2x/d. Body weights were recorded 2x/d as cows exited the parlor when they were housed in the freestall barn. Body weights were not monitored in the metabolism barn.

Cows were fed a common diet (Table 1) throughout the study and were adjusted to the diet for 14 d prior to the first infusion. The diet was formulated to meet or exceed all nutrient requirements except for methionine, lysine, and BCAA using CPMDairy 5.0 and was evaluated using NRC 2001. Cows were randomly assigned to an infusion

sequence with treatments arranged in a 3 x 3 Latin square design. Treatments were 7 d continuous jugular infusions of either 0.9% saline (Baxter, Deerfield, IL) as the control treatment (**CON**), Met plus Lys (**ML**), and ML plus leucine, isoleucine, and valine (**ML+BCAA**). In order to minimize carryover effects, treatment periods were separated by 7 d non-infusion periods. Milk, blood, and feed samples were obtained after 4 d of infusion. Rulquin and Pisulewski (2006b) reported that milk protein responses to amino acid supplementations can be detected within 3 d of the start of infusion.

Pharmaceutical-grade L-lysine, L-methionine, L-leucine , L-isoleucine, and L-valine was obtained from Ajinomoto USA Inc., Raleigh, NC. Solutions were prepared by dissolving 21 g of L-lysine and 12 g of L-methinine in 2 L of 0.9.% saline for ML the treatment and 21 g L–lysine, 12 g L-methionine, 35 g of leucine, 15 g of isoleucine, and 15 g of valine in 2 L of 0.9.% saline for the ML+BCAA treatment. Solutions were filtered sterilized through 0.22 µm membrane filters (Millipore, Billercia, MA). Infusates for each period were prepared 2 d before the start of the period and refrigerated until use. On day 1 of each treatment period, cows were aseptically fitted with indwelling jugular polyvinyl chloride catheters (1.5 mm o.d.; Ico-Rally Corp., Palo Alto, CA; catalogue no. SVL 105-18CLR). Infusates were delivered at a rate of 2 L/d using clinical infusion pumps (Abbott Lifecare, San Antonio, TX: model: RF-5000). Exact weights of bags before and after infusions and bag change times were recorded.

Sampling and Chemical Analysis of Feed and Milk

Milk samples were collected from each milking during the last 3 d of each treatment period. Milk samples were analyzed in United Federation of DHIA Laboratory (Blacksburg, VA) for true protein, fat, lactose and SCC using a Fossomatic 4000 Combi infrared analyzer (Eden Prairie, MN). MUN concentrations were determined using a

modified Berthelot procedure (ChemSpec 150 Analyzer; Bentley Instruments, Chaska, MN).

Samples of corn silage, alfalfa hay, concentrate mix, and TMR of individual cows were collected on 3 consecutive days at the end of each period and stored at -20^{o}C until analysis. All feed samples were dried at 60^{o} C until obtained a constant dry-weight. Composites were ground through a 1-mm screen of a Wiley mill (Arthur H, Thomas, Philadelphia, PA) for analyses at Dairyland Laboratory Inc. (Arcadia, WI). Kjeldahl N, ether extract, ash and DM contents were determined according to AOAC methods (AOAC, 1997). Acid detergent fiber and lignin were determined according to AOAC (1997; method 973.18) and NDF according to Van Soest et al. (1991). Starch was measured as dextrose after treating samples with glucoamylase using a YSI 2700 Select Biochemistry Analyzer (Application Note #319, Yellow Springs, OH) and ether extract by AOAC (1997). Minerals were quantified according to AOAC methods (1997; method 985.01) using an inductively coupled plasma spectrometer (Thermo Jarrell Ash, Franklin, MA). Nutrient composition values presented in Table 1 as average values of TMR sample analyses and calculated nutrient composition values based on the individual ingredient analyses and dietary inclusion rates.

Blood plasma analysis for essential AA and 3-methyl-histidine

Blood samples were obtained from coccygeal vessels into heparinized vaccutainers (Preanalytical Solutions, Franklin Lakes, NJ) at 0800 and 2000 h on the last 2 d of each infusion period. Blood samples were stored on ice, centrifuged (1000 x g, 10 min, and 4^{o}C) and the plasma was stored at -20^{o}C. Plasma essential AA (**EAA**) concentrations were determined by isotopic dilution with a gas chromatograph coupled to

a mass spectrometer (GC-MS, Thermo Scientific, Waltham, MA: GC model: Focus; MS model: PlolarisQ) as previously described (El-Kadi et al., 2006).

Muscle protein mobilization was determined by measuring plasma 3-methylhistidine concentrations using high performance liquid chromatography (HPLC). Briefly, glass vials (*Kimble* Glass INC., Vineland, NJ, USA) containing 25 µL of plasma were deproteinized using spin columns with a 10, 000 kDa molecular weight cut-off (PALL Life Sciences, Hauppauge, NY) at 14,000 g for 60 min and then deacetylated by vapour-phase acid hydrolysis with 6 N HCl (1 ml) under N_2 at 110^o C for 24 h. Acid hydrolysates were freeze dried in a SpeedVac (Labconco, Kansas City, MO) after addition of 12.5 µL of 0.4 Mm methionine sulfone (MetSO2) and 12.5 µL of 0.4 mM norleucine as internal standards. The dried samples were resolubilized in methanol, sodium acetate, and triethylamine (10 µL; 2: 2: 1) and redried in the SpeedVac before they were derivatized with phenylisothiocyanate, freeze dried, and subjected to HPLC analysis using Waters 2695 separation module with a Waters 2487 absorbance detector (Milford, MA).

Calculations and Statistical Analysis

Milk protein efficiency (**MPE**) was calculated as:

MPE = Daily milk protein yield (kg) / Daily crude protein intake (kg)

where daily crude protein intake included crude protein from feed plus infused AA. Feed intake and milk production data from the last 3 d of each infusion period were used for statistical analysis. Significance of treatment effects on milk component content (%) and yield (kg/d), feed and nutrient intake (kg/d), and MPE were tested with the following statistical model using the MIXED procedure of SAS 9.1 (SAS Inst. Inc., Cary, NC).

$$Y_{ijklm} = \mu + T_i + L_j + P_k + C(L)_{(j)l} + D_m + \beta_1 COV_{ijklm} + e_{ijklm}$$

where Y_{ijklm} = response variable value in the m^{th} sampling day of the l^{th} cow in the j^{th} parity, subjected to the i^{th} treatment in the k^{th} period. μ = the mean, T_i = fixed effect of i^{th} treatment (i = ML, ML+BCAA, and CONT), L_j = fixed effect of j^{th} parity (j = 2, 3, and 4), P_k = fixed effect of k^{th} treatment period (k = 1, 2, and 3), $C(L)_{(j)l}$ = random effect of l^{th} cow nested within j^{th} parity (l = 1, 2, 3, and 4), D_m = fixed effect of m^{th} sampling day (m = 1, 2, and 3), β_1 = the regression effect of the corresponding covariate (COV), and e_{ijklm} = random error ~ N (0, $I\sigma_e^2$). Values from the sampling days before commencing each infusion period were used as COV for each response variable. Repeated measures analyses were conducted on period and time with an autoregressive covariate structure. Treatment effects on individual plasma EAA and Plasma 3M-His were analyzed using aforementioned statistical model without considering repeated measures because plasma samples of last two days of each period were combined to have a pooled plasma samples for each cow in each period. Multiple comparisons between treatments were carried out with Tukey-Kramer adjustments test.

RESULTS & DISCUSSION

The objective of present study was to test the effects of BCAA on milk protein synthesis when Met and Lys requirements were met or exceeded in high producing dairy cows fed a corn-based diet. Amino acids were supplemented via jugular infusion rather than by post-ruminal infusion because jugular canulation can be easily carried out, and it eliminates uncertainties of digestion and gastrointestinal metabolism of AA ensuring that all the infused AA enter the general circulation and are available for delivery to the mammary glands. Thivierge et al. (2002) observed that jugular infusion of AA tended to

increase plasma EAA concentrations by 11% compared to that of abomasal infusion. Furthermore, Schei et al. (2007) observed significantly greater milk protein synthesis for jugular infusion of AA than that for post-ruminal infusion of AA. In the present study the AA were infused continuously for 7 d. However Aikman et al. (2002) did not observe any differences in milk protein yield responses between mesenteric arterial infusions and abomasal infusions.

Diet, predicted EAA supply, and DM and nutrient intake

Ingredient composition of the basal diet is given in Table 1. Two thirds of the experimental diet was composed of corn based ingredients: corn silage (39.6%), ground corn grain (18.0%), and dry corn distiller's grain with solubles (9.0%). The experimental diet supplied 95 and 94% of predicted (NRC-2001 model, Washington, DC) net energy for lactation and MP requirements respectively. Predicted (NRC, 2001) duodenal flows of digestible EAA from the basal diet (when mean dietary intakes were used as inputs) and the mean daily Met, Lys, and BCAA infusion rates (g/d) are presented in Table 2. Estimates for duodenal flows of digestible Lys and Met from the basal diet respectively were 6.1 and 1.8% of MP which is considerably less than the corresponding target values for Lys (7.2%) and Met (2.3%) to maximize milk protein synthesis in dairy cows (NRC, 2001). This suggests that Lys and Met were co-limiting for milk protein synthesis. Jugular infusions of Lys (21 g/d) and Met (12g/d) were predicted to raise the total digestible Lys and Met to 6.8% and 2.2% of MP, respectively which is near the NRC 2001 recommendations.

Least square means (**LSM**) of DMI, ADF and NDF intake, oral CP intake, AA intake, and total CP intake were not affected by treatment (Table 3). Dry matter intake averaged of 24.8 kg/d and was similar across treatment groups. Donkin et al. (1989) and

Armentano et al (1997) also observed non-significant changes in DMI when dairy cows were fed ruminally protected Met (**RPM**, 11.5 and 15.0 g/d respectively) and Lys (**RPL**, 14.7 and 40.0 g/d respectively) in experimental diets with 16.4 and 19.5% CP, respectively. Although, Met supplementation can cause reduced feed intake presumably due to an AA imbalance, such negative effects are not consistent across studies. For example, Robinson et al. (2000) observed a significant reduction in DMI in response to an abomasal infusion of 16.0 g of Met per day when cows were fed a corn and timothy silage diet with 14.4% CP, whereas Varvikko et al. (1999) did not observe any negative effects on DMI when 40 g Met/d was infused abomasally in cows fed grass silage diet with 14.2% CP. However, Weekes et al. (2006) observed that abomasal infusion of an AA mixture deficient in Met and Lys reduced DMI by 35% compared to infusion of a complete AA mixture during d 2 and 3 of infusion periods but, by d 4, DMI became similar between both treatments. Feed intake was assessed in the current study using only the data d 5, 6, and 7 of each infusion period.

Dry matter intake was also unaffected by infusion of BCAA. These results are in agreement with the observations of Korhonen et al. (2002) and Huhtanen et al. (2002) when BCAA or Leu were infused into dairy cows fed grass silage diets. Weekes et al. (2006) also observed no significant change in DMI between abomasal infusion of an AA mix devoid of BCAA and abomasal infusion of a complete AA mix.

Milk yield and milk components

High producing cows (greater than 45 kg/d) in early lactation (mean of 43 DIM) were chosen for the study to maximize the daily requirement for AA and provide as responsive of a model as possible. Milk production data are presented in Table 3. Milk yield was not affected by treatment and averaged 52.6 kg/d across treatments. These

results are similar to previous observations (Aldrich et al., 1993, Donkin et al., 1989, Lynch et al., 1991, Ouellet et al., 2003), Korhonen et al, 2002, and Weekes et al., 2006) . In line with milk yield, lactose yield (2.5 kg/d) and lactose content (4.7%) were also similar across treatments. The literature rarely reports supplementation of Met and Lys having significant impact on lactose synthesis suggesting dietary limitation of these AA does not influence lactose synthesis. In the present study, cows that received different treatments did not significantly differ in content and yield of milk fat. This is consistent with the literature often reporting small, non-significant increases in fat yield and content in response to AA supplementation.

Amino acid infusions significantly affected milk protein yield ($P = 0.001$) and protein content ($P < 0.0001$, Table 3). Cows infused with ML produced 142 g more milk protein and had milk with 0.17% units greater milk protein content than cows infused with saline. several studies have shown that supplementation of Met and Lys, either separately or together, significantly improved milk protein synthesis in dairy cows fed corn based diets (Weekes et al., 2006). As the common experimental diet of the present study was predicted to be responsive to Met and Lys supply when assessed as a percentage of MP, the observed milk protein responses were consistent with model prediction. Although ML+ BCAA significantly increased milk protein yield (1.48 vs. 1.38 kg/d) and milk protein content (2.80 vs. 2.69%) compared to CTL treatments, there were no significant differences in either when compared to the ML treatment. This indicates that enhanced milk protein synthesis associated with the ML+BCAA treatment was due solely to provision of Met and Lys with no effect of BCAA. Hopkins et al (1994), Korhonen et al. (2002), and Weekes et al. (2006) also reported non-significant

effects of BCAA supplementations on milk protein synthesis in dairy cows, but Rulquin and Pisulewski (2006) observed significant milk protein yield responses when 40 g Leu/d or more was duodenally infused into cows. Since the diet in this study was largely corn-based, Lys and Met were additionally infused in order to make sure that these AA were not limiting for milk protein synthesis. Milk protein content of cows infused with 80 g and 120 g/d of Leu were similar to that of 40 g/d suggesting that the Leu requirement for maximum milk protein synthesis was met with the dose of 40 g/d for those dietary circumstances. Rulquin and Pisulewski (2006), estimated duodenal flows of digestible Val, Ile, and Leu (% of MP) from the basal diet were 4.5, 4.1, and 6.5% respectively and less than that of the present study (5.3, 4.7, and 8.9% respectively; Table 2). When Rulquin and Pisulewski (2006) infused 40 g of Leu/d, duodenal flow of digestible Leu increased to 8.9% of MP which is equivalent to the mean predicted digestible Leu from the basal diet of the present study. These results would indicate that the basal diet of the present study supplied sufficient Leu for maximum milk protein synthesis and may explain the lack of a response to infused BCAA.

Infusion of ML+BCAA significantly reduced MUN yield and MUN content by 9 and 10% respectively ($P < 0.05$) compared to the saline infusion (CTL, Table 3). Infusion of only ML had no significant impact on MUN compared to that of CTL. Milk urea nitrogen measures are considered to be a useful tool for monitoring nitrogen utilization efficiency of dairy cows (Godden et al., 2001). Lower MUN concentrations are indicative of improved whole body N utilization efficiency (Broderick et al., 2008) suggesting that more dietary protein was utilized to synthesize protein in the body, i.e. milk and muscle protein. Significantly reduced MUN without increased mik protein synthesis associated

with the ML+BCAA treatment suggests that infusion of BCAA promoted more muscle protein synthesis than of the ML or CTL treatments.

Milk protein efficiency and body weight

Poor conversion efficiencies of dietary protein to productive proteins, i.e. milk and muscle, in dairy cows lead to significant losses of nitrogen in feces and urine. These dairy farm wastes have been recognized as critical sources of environmental pollution (Cyriac et al., 2008, EPA, 2004). Improving efficiency of dietary protein utilization for milk protein synthesis is therefore of great importance for reducing the negative effects of dairy farm waste on the environment. The milk protein efficiencies presented in Table 3 were calculated as milk protein yield (kg/d) divided by total CP intake (kg/d). Total CP intake consisted of dietary CP and infused AA. Protein efficiency averaged 0.38 which is greater than values commonly reported in the literature. Elisabet et al. (2007) reported milk protein efficiencies ranging from 0.18 to 0.40 in dairy cows fed diets containing 14 to 18% CP and producing 13 to 57 kg/d fat corrected milk, and efficiency improved as milk production increased. The average milk protein efficiency of 0.38 in the present study is consistent with those observations given daily production of 53 kg of milk and dietary protein of 16.1% CP. Infusion of ML significantly increased milk protein efficiency by 9% (0.401 vs. 0.365) compared to infusion of saline. Socha et al. (2005) also observed efficiency increase from 0.29 to 0.32 when supplementing an 18.5% CP diet with rumen protected Met and Lys.

Least square means for final BW after each infusion treatment and LSM for difference between BW (BW_diff) at the beginning and the end of the infusion periods are presented in Table 3. The final body weight of ML+BCAA cows tended ($P = 0.095$) to be greater than that of CTL cows. Moreover, infusion of BCAA besides Met and Lys

increased BW by 7.3 kg. These observations lead to a conclusion that although BCAA supplementation did not influence milk protein synthesis, it may have stimulated muscle protein synthesis. Supporting this conclusion, a number of studies in other species have demonstrated that BCAA, particularly Leu, stimulates muscle protein synthesis (Escobar et al., 2006, Suryawan et al., 2008)

Plasma concentrations of EAA

Least square means for plasma essential AA concentrations are given in Table 4. Plasma Met concentrations significantly increased by 29% when cows were infused with ML. Although not statistically significant (P = 0.182, Table 4), infusion of ML increased plasma Lys concentrations by 13% compared to that of saline infusions. Increased plasma concentrations are a classical method for detecting an AA excess (Harper et al., 1970). Plasma AA concentrations reflect balance between AA supply and utilization (Meijer et al., 1995). Increasing and decreasing plasma AA concentration are therefore indicative of excessive and deficient AA supply relative to their requirements. Increased plasma Met and Lys concentrations thus explained that ML cows received excess Met and Lys compared to their requirements for elevated milk protein synthesis. There were no significant differences in plasma Lys between the CTL and ML+BCAA treatments. Plasma Met concentration of ML+BCAA tended (P = 0.072) to decrease, compared to that of ML. Infusion of Met and Lys plus BCAA appeared to make Met limiting for other tissue protein synthesis than milk protein synthesis because similar milk yield responses were observed between ML and ML+BCAA. Despite the observed increase in milk protein synthesis, ML did not cause any changes in plasma concentration of other EAA. Donkin et al. (1989) also reported no change in plasma EAA except Met and Lys when cows were fed 15 g of rumen protected Met (15 g/d) and Lys (40g/d).

Interestingly, infusion of BCAA did not significantly increase plasma BCAA concentrations. Compared to the average plasma BCAA concentrations of CTL and ML treatments, ML+BCAA treatment increased plasma Leu, Ile, and Val concentrations only by 10, 2, and 14% respectively. In contrast to these observations, Rulquin and Pisulewski (2006) observed that daily duodenal infusion of 40 g Leu increased plasma Leu concentration by 55%. Moreover, infusion of a BCAA-deficient AA mixture resulted in 58% plasma BCAA reduction compared to that of a complete AA mix infusion (Weekes et al., 2006). Both of these prior studies used low-protein diets (12.5 and 9.0% of CP respectively) which presumably would have caused a BCAA deficiency. The increased concentrations observed by Rulquin and Pisulewski (2006b) thus suggest those animals were not BCAA deficient. The lack of a plasma BCAA response in the present study suggests they were indeed limiting, although it would appear the limit was for muscle protein synthesis rather than for milk protein.

High producing dairy cows can mobilize AA from muscle to compensate for deficiencies in dietary AA supply compared to demand for milk protein synthesis. 3-Methylhistidine is formed by methylation of histidine in actin and myosin (Blum et al., 1985). Moreover, upon release following actin and myosin degradation, 3M-His is not subjected to tissue metabolism and directly enters the systemic circulation. Therefore, blood 3M-His is a sensitive measure of muscle breakdown (Akamatsu et al., 2007). There are two forms of 3M-His, N-acetyl-3M-His and the deacetylated parent compound. The proportion of these 2 forms in plasma varies widely across species, i. e. 5% N-acetyl-3M-His in adult humans compared to 80-90% in rats (Kuhl et al., 1996). To detect 3M-His using classical methods, it must be in the deacetylated form which is accomplished by

acid hydrolysis. Average 3M-His concentrations of 10.6 μM were observed in the present study. These concentrations fall within the range of blood 3M-His concentrations (7.1 to 14.0 μM) reported in the literature (Akamatsu et al., 2007, Blum et al., 1985) for dairy cows at peak lactation. In (Akamatsu et al.) serum samples were subjected to acid hydrolysis (in perchloric acid at 80^{o}C for 1 h) before HPLC analysis whereas Blum et al. (Blum et al., 1985) used a radioimmunoassay system to measure plasma 3M-His concentrations. While numerically greater for ML and ML+BCAA treatments, there were no significant differences in plasma 3M-His concentrations across treatments (). Thus the numerical reductions in MUN, no change in plasma BCAA concentrations, no change in muscle breakdown, and a trend for BW gain in association with infusion of BCAA are all consistent with an increase in muscle protein retention. If true, this suggests that muscle protein synthesis requires greater concentrations of BCAA than milk protein synthesis does.

CONCLUSIONS

Results of this study show that supplementation of a corn-based diet with Met plus Lys resulted in significantly increased milk protein yield and content and dietary protein utilization efficiency for milk protein synthesis in high producing dairy cows. When Met and Lys were not limiting for milk protein synthesis, BCAA infusion did not increase milk protein synthesis under the dietary circumstances of the present study. Significantly reduced MUN, increased BW gain, and no change in plasma 3-methylhistidine of BCAA-infused cows suggest that BCAA may have been limiting for synthesis of other body tissue proteins. Similar plasma 3-methylhistidine concentrations

across treatments also indicate that AA mobilization from muscle tissues did not significantly support increased milk protein synthesis when Lys and Met were infused.

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Table 6-1. Ingredient and chemical composition, and predicted supplied and required amounts of net energy for lactation (NE_L) and metabolizable protein (MP) of the basal ration fed to all cows.

Diet	Quantity
DM g/kg	556.7
Ingredients g/kg of DM	
Corn silage	396
Alfalfa hay	144
Corn grain ground	180
Corn dry distiller grain	90
Wheat middlings	63
Soyplus[1]	36
Soybean meal	36
Soybean hulls	36
Calcium carbonate	9.0
Sodium bicarbonate	4.5
salt	4.0
Urea	0.8
Vitamins and minerals[2]	0.7
Nutrient composition[1] g/kg of DM	
CP[2]	160.8
ADF	179.9
NDF	316.3
Lignin	22.13
Starch	283.3
Fat	36.45
Ash	53.56
Nutrient supply[3]	
NE_L, Mcal/d	38.3
MP, g/d	2733
Nutrient requirement[3]	
NE_L, Mcal/d	40.4
MP, g/d	2918

[1]Ralston, Iowa
[2]Vitamin A, D, E, magnesium oxide, manganous oxide, zinc oxide, hydrated sulfate, cobalt carbonate, ferrous sulfate, copper sulfate.
[3]Calculated using the NRC model (2001) and observed input values

Table 6-2. Digestible essential amino acid flows as predicted by NRC 2001/CPM Dairy 5.0 from the basal diet at observed mean DM intake, mean infused AA , and total digestible AA expressed as g/d and percentages of metabolizable protein (%MP) of cows infused with saline (CTL), Met and Lys (ML), and Met and Lys plus BCAA (ML+BCAA).

		CTL	ML			ML+BCAA		
EAA	Diet (g/d)	%MP	Infusate (g/d)	Total (g/d)	Total %MP	Infuste (g/d)	Total (g/d)	Total %MP
Arg	127	4.6		127	4.5		127	4.4
His	61	2.2		61	2.2		61	2.1
Ile	131	4.7		131	4.7	14	145	5.1
Leu	245	8.9		245	8.8	32	277	9.7
Val	147	5.3		147	5.3	14	161	5.6
Lys	168	6.1	21	189	6.8	20	188	6.6
Met	50	1.8	12	62	2.2	11	61	2.1
Phe	137	4.9		137	4.9		137	4.8
Thr	130	4.7		130	4.7		130	4.6
Total EAA[1]	2761		33	2794		91	2852	

[1]Except Trp

Table 6-3. Statistical significance of treatment differences and least square means and associated mean standard error (MSE) of intake measures, milk protein and milk component measures, milk protein efficiency, and BW measures for saline (CTL), Met and Lys (ML), and Met, Lys plus BCAA (ML+BCAA) infusion treatments.

Variable	CTL[1]	ML	ML+BCAA	MSE	P-value
Intake					
DM Intake (kg/d)	24.5	24.9	25.0	1.10	0.732
ADF Intake (kg/d)	4.28	4.21	4.32	0.11	0.870
NDF intake (kg/d)	7.53	7.45	7.59	0.20	0.496
Dietary CP intake kg/d	3.89	3.85	3.92	0.10	0.638
Infused AA g/d	0.0[b]	32.5[b]	91.0[a]	3.01	<.0001
Total CP intake	3.89	3.85	3.92	0.10	0.764
Milk production					
Milk yield (kg/d)	51.5	53.7	52.8	0.94	0.212
Protein yield (kg/d)	1.38[b]	1.53[a]	1.48[a]	0.03	0.001
Protein %	2.69[b]	2.85[a]	2.80[a]	0.02	<.0001
Fat yield (kg/d)	1.36	1.42	1.34	0.05	0.504
Fat %	2.67	2.79	2.78	0.10	0.605
Lactose yield (kg/d)	2.45	2.55	2.50	0.04	0.198
Lactose %	4.75	4.75	4.70	0.02	0.255
MUN yield (kg/d)	6.49[b]	6.44[ab]	5.90[a]	0.19	0.026
MUN content (mg/dL)	12.1[b]	11.5[ab]	10.9[a]	0.63	0.042
Efficiency					
Milk protein efficiency[2]	0.36[a]	0.40[b]	0.37[ab]	0.02	0.025
Body weight					
BW (kg)	567	584	598	18.3	0.109
BW_diff (kg)	-18.0[a*]	-2.60[ab]	7.26[b]	6.90	0.049

[1] different letters within a row indicate significantly different LSM (P<0.05)
[2]Milk protein efficiency= milk protein yield (kg/d) / total CP intake (kg/d)
*LSM significantly different (P < 0.05) from 0

Table 6-4. Statistical significance (*P*-value) of and Least square means of plasma essential amino acid (EAA, µmol/L) and 3-Methyl Histidine (3M-His, µmol/L) concentrations for saline (CTL), Met and Lys (ML), and Met, Lys plus BCAA (ML+BCAA) infusion treatments.

EAA	CTL[1]	ML	ML+BCAA	MSE[2]	*P*-value
Arg	77.50	70.30	78.16	8.398	0.742
Ile	99.52	91.11	97.07	4.840	0.451
Leu	171.6	174.2	189.9	10.67	0.441
Lys	70.92	80.30	70.49	4.160	0.181
Met	23.57[a]	30.36[b]	25.75[ab]	1.430	0.010
Phe	53.89	51.67	51.02	3.980	0.840
Thr	105.3	108.1	102.6	2.027	0.160
Trp	30.06	31.87	32.95	2.993	0.798
Val	215.2	210.4	242.9	18.02	0.359
3M-His	9.972	10.61	11.22	0.550	0.215

[1] different letters within a row indicate significantly different LSM ($P<0.05$)
[2]Mean standard error

CHAPTER 7

Modeling the Effects of Insulin and Amino Acids on the Phosphorylation of mTOR, Akt, and 4EBP1 in Mammary Cells

ABSTRACT

The objective of this work was to develop a mathematical representation of the effects of insulin and essential amino acid (EAA) on phosphorylation of protein kinase B (Akt), mammalian target of rapamycin (mTOR) and eukaryotic initiation factor 4E binding protein 1 (4EBP1), the latter being critical for initiation of protein synthesis. The model included six protein pools (Q) representing phosphorylated (P) and unphosphorylated (U) forms of Akt, mTOR, and 4EBP1. Mass action equations were used to represent phosphorylation ($F_{U,P(X)}$) and dephosphorylation ($F_{P,U(X)}$) reactions. The $F_{U,P(X)}$ for Akt and mTOR were regulated by extracellular insulin (C_{Ins}) and EAA (C_{EAA}) concentrations, respectively. Exponents were used to adjust the sensitivity of the fluxes to the regulators. Changes in pool size with respect to time were calculated as the difference between $F_{U,P(X)}$ and $F_{P,U(X)}$. The $Q_{U(X)}$ were determined by numerical integration of the differentials starting from specified initial pool sizes. $Q_{P(X)}$ were calculated by subtracting $Q_{U(X)}$ from the fixed total protein mass ($Q_{T(X)}$). The model was fitted to observed phosphorylation data obtained from a bovine mammary epithelial cell line treated with four C_{Ins} (0, 5, 10, and 100 ng/mL) and four C_{EAA} (0, 0.35, 1.00, and 3.5 mM) arranged in a 4 x4 factorial design. Model optimization and sensitivity analyses were carried out in ACSLXtreme. The data were adequate to describe the model parameters as standard deviations of model

parameters were <20% of the parameter estimates. Sensitivity exponent estimates were greater than 1 indicating EAA and insulin signal loss associated with transmission down the cascade was partially mitigated. Phosphorylation of Akt was highly sensitive to insulin compared to mTOR and 4EBP1. Phosphorylation of mTOR and 4EBP1 responded similarly to insulin and EAA. The model was able to predict $Q_{P(X)}$ with root mean square prediction errors less than 10% of the observed means. There appeared to be a slight negative slope bias for $Q_{P(Akt)}$, indicating the model tended to overpredict $Q_{P(Akt)}$ as predicted $Q_{P(Akt)}$ increased.

Key words: amino acid, cellular signals, insulin, mathematical representation

INTRODUCTION

Amino acids (AA) and insulin are able to stimulate translation initiation in mammalian cells. These stimulatory signals have been shown to converge on the protein kinase, mammalian target of rapamysin (mTOR) (Proud, 2007). Insulin and AA activate mTOR by stimulating its phosphorylation via two independent pathways (Suryawan *et al.*, 2007). Insulin actions are mediated through phosphorylation of the upstream kinase, Akt (Hinault *et al.*, 2004). The exact pathway through which amino acids stimulate mTOR phosphorylation is still not clear. Eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1) is one of the best studied direct substrates of mTOR (Avruch *et al.*, 2009). The eIF4E is able to bind the 5'cap of mRNA and thus enhances translation initiation by recruiting methionyl-tRNA to mRNA. The 4EBP1 competitively binds eIF4E thus inhibiting its function. mTOR mediated hyper-phosphorylation of 4EBP1

leads to inactivate 4EBP1 thereby releasing eIF4E and enhancing rates of translation initiation (Richter & Sonenberg, 2005).

Understanding mTOR mediated signals is important for continued development of nutrient requirement systems that allow construction of sound nutritional and feeding management programs for human and livestock species. Since it has been well recognized that amino acids play an important role in controlling protein synthesis in addition to its role as a substrate (Proud, 2007), mathematical representation of these signaling effects is critical to improving amino acid requirement predictions for livestock species. Moreover, quantification of regulatory effects of amino acids and their interactions with other signals such as insulin, could also be important in human health studies given the involvement of mTOR dependent cellular signals in obesity, type 2 diabetes, and cancer (Marshall, 2006). The objective of this work was to develop a mathematical model representing the effects of insulin and amino acids on phosphorylation of Akt, mTOR, and 4EBP1 and to parameterize the model using a data set derived from a bovine mammary epithelial cell line (MAC-T cells).

MATERIAL & METHODS

Model development

The model was constructed as a dynamic, deterministic, mechanistic representation of the Akt/mTOR/4EBP1 signalling cascade as depicted in Figure 1. The model considered 3 proteins each in the *P* and *U* forms: Akt ($Q_{P(Akt)}$ and $Q_{U(Akt)}$), mTOR ($Q_{P(mTOR)}$ and $Q_{U(mTOR)}$), and 4EBP1 ($Q_{P(4EBP1)}$ and $Q_{U(4EBP1)}$), respectively for a total of 6 protein pools. Mass action equations were used to represent kinase ($F_{U,P}$) and

phosphatase ($F_{P,U}$) reactions for each signaling protein. The time unit for the model was minutes.

For purposes of model development, a reference model state was defined as having inputs of 10 ng/ml insulin concentration (C_{Ins}) and 1 mM total EAA concentration (C_{EA}). In preliminary *in vitro* experiments using a bovine mammary epithelial cell line, MAC-T, these concentrations fell in the middle of dose response curves of total protein synthesis. At this reference state, the total pool size (Q_T) and Q_P of each signaling protein were set to 1 and 0.5 (arbitrary units) respectively assuming 50% of the total protein in each pool was in the phosphorylated state.

Kinase reactions ($F_{U,P}$) for Akt ($F_{U,P(Akt)}$), mTOR ($F_{U,P(mTOR)}$), and 4EBP1 (($F_{U,P(4EBP1)}$) were represented respectively as:

$$F_{U,P(Akt)} = Q_{U(Akt)} \times K_{U,P(Akt)} \times \left(\frac{C_{Ins}}{J_{Ins}} \right) \tag{1}$$

$$F_{U,P(mTOR)} = Q_{U(mTOR)} \times K_{U,P(mTOR)} \times \left(\left(\frac{Q_{P(Akt)}}{J_{QP(Akt)}} \right)^{a} + \left(\frac{C_{EAA}}{J_{EAA}} \right)^{b} \right) \tag{2}$$

$$F_{U,P(4EBP1)} = Q_{U(4EBP1)} \times K_{U,P(4EBP1)} \times \left(\frac{Q_{P(mTOR)}}{J_{QP(mTOR)}} \right)^{c} \tag{3}$$

where a, b, and c represented the sensitivity exponents, and J_{Ins}, $J_{EAA,}$. $J_{QP(Akt)}$, and $J_{QP(mTOR)}$ were constants representing the regulatory effects of C_{Ins}, C_{EAA}, $Q_{P(Akt)}$, and $Q_{P(mTOR)}$ respectively. They were set equal to the reference values of C_{Ins} and C_{EAA}, respectively and the steady state values of $Q_{P(Akt)}$, and $Q_{P(mTOR)}$ respectively when the model was in the reference state. This resulted in the ratios assuming a value of 1 in the reference state which allowed a, b, and c to assume any positive value without affecting

model balance in the reference state. $K_{U,P(Akt)}$, $K_{U,P(mTOR)}$, and $K_{U,P(4EBP1)}$ were the mass action rate constants for phosphorylation of Akt, mTOR, and 4EBP1 respectively. Since it has been demonstrated that insulin and AA stimulate kinase activity resulting in the phosphorylation of mTOR via independent pathways in mammalian cells (Hinault *et al.*, 2004 and Suryawan *et al.*, 2007), the effects of insulin and EAA on $F_{U,P(mTOR)}$ were represented as additive factors in Equation 2.

Phosphotase reactions for Akt ($F_{P,U(Akt)}$), mTOR ($F_{P,U(mTOR)}$), and 4EBP1 ($F_{P,U(4EBP1)}$) were represented as:

$$F_{P,U(Akt)} = Q_{P(Akt)} \times K_{P,U(Akt)} \tag{4}$$

$$F_{P,U(mTOR)} = Q_{P(mTOR)} \times K_{P,U(mTOR)} \tag{5}$$

$$F_{P,U(4EBP1)} = Q_{P(4EBP1)} \times K_{P,U(4EBP1)} \tag{6}$$

where $K_{P,U(Akt)}$, $K_{P,U(mTOR)}$, and $K_{P,U(4EBP1)}$ represented the respective reaction rate constants. No regulation of dephosphorylation was represented.

The differential equations defining the rate of change (units/min.) for each unphosphorylated signaling protein were calculated from the balance of the phosphorylation and dephosphorylation fluxes:

$$\frac{dQ_{U(Akt)}}{dt} = F_{P,U(Akt)} - F_{U,P(Akt)} \tag{7}$$

$$\frac{dQ_{U(mTOR)}}{dt} = F_{P,U(mTOR)} - F_{U,P(mTOR)} \tag{8}$$

$$\frac{dQ_{U(4EBP1)}}{dt} = F_{P,U(4EBP1)} - F_{U,P(4EBP1)} \tag{9}$$

The Q_U for each signaling protein was calculated by numerical integration of Equations 7, 8, and 9 given specified initial pool sizes for Q_U (iQ_U).

$$Q_{U(Akt)} = Integ\left(dQ_{U(Akt)}, iQ_{U(Akt)}\right) \tag{10}$$

$$Q_{U(mTOR)} = Integ\left(dQ_{U(mTOR)}, iQ_{U(mTOR)}\right) \tag{11}$$

$$Q_{U(4EBP1)} = Integ\left(dQ_{U(4EBP1)}, iQ_{U(4EBP1)}\right) \tag{12}$$

The total mass for each signaling protein (Q_T) which represents the sum of Q_P and Q_U was fixed in size and used to calculate Q_P of each signaling protein.

$$Q_{P(Akt)} = Q_{T(Akt)} - Q_{U(Akt)} \tag{13}$$

$$Q_{P(mTOR)} = Q_{T(mTOR)} - Q_{U(mTOR)} \tag{14}$$

$$Q_{P(4EBP1)} = Q_{T(4EBP1)} - Q_{U(4EBP1)} \tag{15}$$

Because phosphorylated and total quantities of signaling proteins are measured independently in arbitrary units using Western blotting techniques, it is possible that the signal intensity for the phosphorylated protein assay could exceed the signal intensity for the total assay. Of course this simply reflects the relative binding affinity of the 2 sets of antibodies given that the phosphorylated form of the protein cannot exceed the total form in reality. To accommodate this in the model and provide for a model variable that could be compared to the observed data, a scaled value (rQ_P) was calculated from Q_P using a reference scalar for each Q_P (ref_Q_P) in the same manner that the Western results were scaled:

$$rQ_{P(Akt)} = Q_{P(Akt)} / ref\_Q_{P(Akt)} \tag{16}$$

$$rQ_{P(mTOR)} = Q_{P(mTOR)} / ref\_Q_{P(mTOR)} \tag{17}$$

$$rQ_{P(4EBP1)} = Q_{P(4EBP1)} / ref\_Q_{P(4EBP1)} \quad (18)$$

Model optimization

MAC-T cells were grown to approximately 90% confluence in complete DMEM/F12 media supplemented with 8 % serum. Treatments were applied for 1h in serum free DMEM/F12 media containing 3.51 g/L D-glucose. Treatments were four concentrations of EAA (0.0, 0.35, 0.70, and 3.50 mM) and four concentrations of insulin (0, 5, 10, and 100 ng/mL) arranged in a 4 x 4 factorial design. Protein homogenates obtained from treated cells were subjected to Western immunoblotting analysis. Briefly, proteins were electroporetically separated in 8 – 14% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5% non-fat dry milk in a tris-base Tween buffer (10 mM tris-base, 150 mM NaCl, and 1% Tween 20) and first probed with antibodies (1: 1000 dilutions) specific to the phosphorylated forms (P) of Akt (Ser473), mTOR (Ser2448), and 4EBP1 (Thr37/46). Blots were then stripped and reprobed with antibodies (1: 2000 delusions) that recognized the total form (T) of each. All antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Protein abundance was visualized by chemiluminescence (ECL Plus, Amersham; Piscataway, NJ) and quantified using Un-Scan-It software (Silk Scientific, Inc., Orem, UT). The phosphorylation state of each signaling protein was expressed as a ratio of P: T that was then standardized to the observations from cells treated with media containing highest concentrations of insulin (100 ng/mL) and EAA (3.50 µM). This standardized ratio was equivalent to $rQ_{P(X)}$ in the model.

The model was fitted to the observed P: T ratio data by solving for $K_{U,P(Akt)}$, $K_{U,P(mTOR)}$, $K_{U,P(4EBP1)}$, $K_{P,U(Akt)}$, $K_{P,U(mTOR)}$, $K_{P,U(4EBP1)}$, J_{Ins}, J_{EAA}, $J_{QP(mTOR)}$, $J_{QP(Akt)}$, a, b, c, $Ref\_\ Q_{P(Akt)}$, $Ref\_\ Q_{P(mTOR)}$, $Ref\_\ Q_{P(4EBP1)}$, $iQ_{P(Akt)}$, $iQ_{P(mTOR)}$, and $iQ_{P(4EBP1)}$ while maximizing a log-likelihood function using the Nelder Mead search algorithm in ACSLXtreme (AEgis Technologies, Huntsville, AL). The residual errors of $Q_{P(Akt)}$, $Q_{P(mTOR)}$, and $Q_{P(4EBP1)}$ were regressed on corresponding predicted values using SAS 9.1 (SAS Inc., Cary, NC). Sensitivities of Akt, mTOR, and 4EBP1 to C_{Ins} and C_{EAA} were also determined.

RESULTS & DISCUSSION

Parameter estimates are presented in Table 1. As evidenced by standard deviations for parameter estimates that were less than 20% of the estimates in all cases, the data were adequate to describe the model parameters. Correlations among parameters did not appear to significantly contribute to variance inflation in the parameter estimates. The final parameter estimates for J_{Ins} (8.390), J_{EAA} (1070), $J_{QP(Akt)}$ (0.494), and $J_{QP(mTOR)}$ were close to their values at the reference state; 8, 1000, and 0.5 respectively.

The sensitivity exponents, a and b were 1.450 and 1.490, respectively for the effects of phosphorylated Akt and EAA on mTOR phosphorylation (Equation 2). The sensitivity exponent, c, for the effect of active mTOR on 4EBP1 phosphorylation (Equation 3) was 2.370. A value greater than one is needed to maintain signal intensity as it is transmitted down the cascade. Setting c to a value of one results in loss of signal range, i.e., low to high given varying inputs, which was inconsistent with the observed data. This propensity for signal decay contradicts the suggestion that multiple steps in a cascade would propagate and magnify phosphorylation signals as distance from signal source

increased (Kholodenko, 2006). However it has also been suggested that that there can be a gradient of kinases and phosphatases within cells where kinases are concentrated near the cell membrane while phosphatases are uniformly distributed. Such a gradient would dampen the range in signal as distance from the kinase location increased (Muñoz-García *et al.*, 2009). Phosphorylation of Akt has been found to be phosphorylated in close proximity to the plasma membrane (Huang & Kim, 2006). If downstream elements of the pathway are located in close proximity to the Akt phosphorylation site, the range in signal as determined from cell homogenates may be less than the local concentration range, which would explain the need for an exponent greater than 1 in Equations 2 and 3. Unlike phosphorylattion of mTOR and phosphorylation of 4EBP1, phosphorylation of Akt did not necessitate a sensitivity exponent to amplify the effects of insulin (Equation 1).

As mentioned before, the model was fitted to the observed phospho: total ratios that were standardized to values observed for the highest concentrations of insulin (100 ng/mL) and EAA (3500 μM). This resulted in ratios of 1 for those treatments which has an implicit assumption of complete phosphorylation of $Q_{T(X)}$. Additionally, it was assumed that the reference insulin and EAA concentrations of 10 ng/mL and 1000 μM, respectively represented 50% phosphorylation. Neither assumption can be tested using Western blotting data given the semi-quantitative nature of the assay. However, the relative responses in signal intensities are representative of concentration changes in the samples. In order to maintain the assumption of 50% phosphorylation in the reference state, an additional parameter was required during fitting to scale the phosphorylation ratios in the same manner the observed data were scaled. This was achieved through the

use of the variable *Ref_* $Q_{P(X)}$ in the model which has the same role in the model as the scalar applied to standardize the observed ratios. Estimates of *Ref_* $Q_{P(X)}$ were very close to 1 (supporting the original assumption of complete phosphorylation when both insulin and EAA concentrations are very high.

The sensitivity analysis results are given in Table 2. These results represent the average increase in $Q_{P(Akt)}$, $Q_{P(mTOR)}$, and $Q_{P(4EBP1)}$ in response to unit increases in C_{Ins} (1.0 ng/mL) and C_{EAA} (1.0 µM). The C_{Ins} related sensitivity coefficients of $Q_{P(Akt)}$ (0.0083) that is more than 4 fold greater than that of $Q_{P(mTOR)}$ (0.0019), and $Q_{P(4EBP1)}$ (0.0017) reflect that phosphorylation of Akt is mainly mediated by extracellular insulin compared to phosphorylation of mTOR and phosphorylation of 4EBP1. These results pertained to MAC-T cells.

Similar significant effects of insulin on phosphorylation of Akt have been observed in muscle cells (Alessi *et al.*, 1996). The comparatively lower sensitivity of $Q_{P(mTOR)}$ (0.0019), and $Q_{P(4EBP1)}$ (0.0017) to insulin reflects the degeneration of the signal as it is propagated down the cascade and indicate that mTOR and 4EBP1 have available phosphorylation range to respond to other signals, i.e. insulin does not overwhelm the responsiveness of other effectors. It is widely accepted that additional signals such as energy, regulate mTOR phosphorylation (Sofer *et al.*, 2005). Both $Q_{P(mTOR)}$ and $Q_{P(4EBP1)}$ had similar sensitivities to insulin (0.0019 and 0.0017 respectively) and EAA (0.0008 and 0.0007 respectively) indicating responses are of similar magnitude to unit changes in insulin and EAA similarly. This observation is not surprising as 4EBP1 has been found to be a direct substrate of mTOR in several mammalian cells (Avruch *et al.*, 2009).

Root mean square prediction errors (RMSPE) associated with $Q_{P(Akt)}$, $Q_{P(mTOR)}$ and $Q_{P(4EBP1)}$ are presented in Table 2. The RMSPE are less than 10% in all cases reflecting good model accuracy and precision in predicting $Q_{P(Akt)}$, $Q_{P(mTOR)}$ and $Q_{P(4EBP1)}$. Residual errors are plotted in Figure 2. Mean and slope bias analyses indicated non significant mean (intercept) bias and slope bias for $Q_{P(mTOR)}$ ($P < 0.47$ and $P < 0.18$ respectively) and $Q_{P(4EBP1)}$ ($P < 0.32$ and $P < 0.25$ respectively). However model predictions of $Q_{P(Akt)}$ appeared to have slope bias ($P < 0.07$) indicating the model tended to over predict $Q_{P(Akt)}$ as C_{Ins} increased.

The effects of insulin and EAA on mTOR phosphorylation were represented additively in the model which supports the hypothesis that AA and insulin stimulate protein synthesis by independent signaling through mTOR. As the model was well defined by the data and appeared to reasonably predict the effects of insulin and EAA on phosphorylation of AKT, mTOR, and 4EBP1, this modeling work further strengthens the idea of the differential effects of insulin and EAA on protein synthesis. Since the model was fitted to data from a bovine mammary cell line, the estimates are more applicable to dairy and beef cattle. The work also supports the idea of independent effects of insulin and EAA on protein synthesis.

Model described in this work could be used to improve mathematical representations of amino acid requirements for milk protein synthesis in dairy cows. Since the mTOR pathway is highly conserved, this representation may also be directly applicable to other species.

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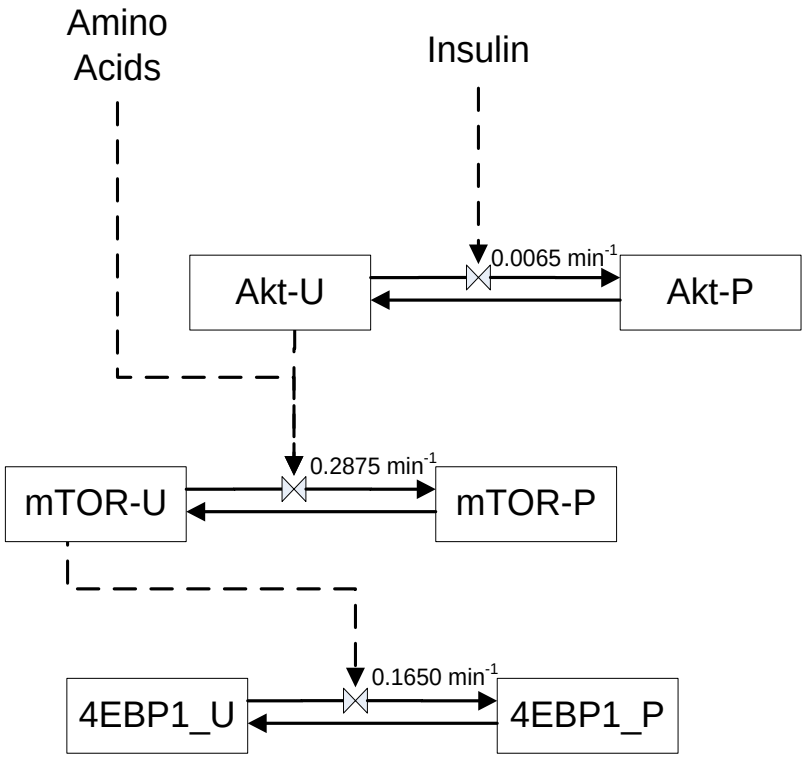

Figure 7-1. A schematic of the model. The arrows represent fluxes. Dashed arrows represent the upstream effects on phosphorylation fluxes. Boxes with solid lines represent protein mass pools. Values of phosphorylation fluxes (min^{-1}) for each protein at reference state (insulin = 10 ng/mL and EAA = 1 mM) are given.

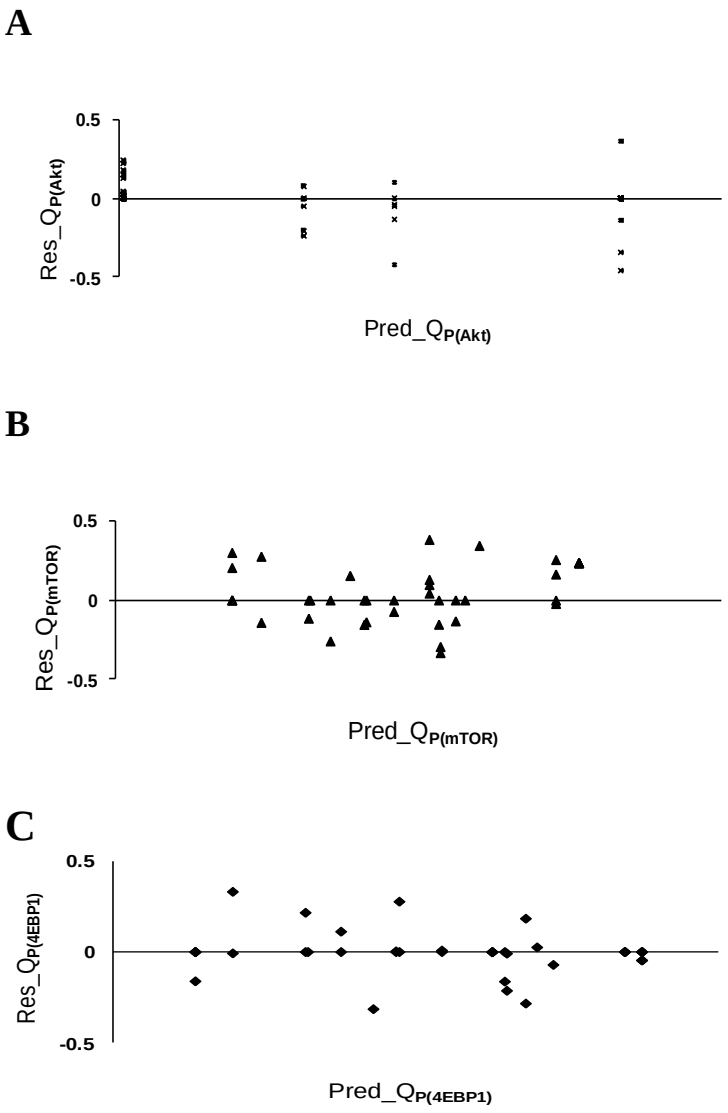

Figure 7-2. Residuals plots representing relationships between residual error ($Res\_Q_{P(X)}$) and predicted values of phosphorylated masses ($Pred\_Q_{P(X)}$) of Akt (A), mTOR (B), and 4EBP1(C).

Table 7-1. Model parameter estimates and associated standard deviations (SD). The SD are presented as percentages of the parameter estimate (SD%).

Parameter	Estimate	SD	SD%
Kinase reaction rate constants			
$K_{U,P(Akt)}$	0.013	5.2×10^{-6}	0.000
$K_{U,P(mTOR)}$	0.575	1.2×10^{-6}	0.000
$K_{U,P(4EBP1)}$	0.330	9.3×10^{-7}	0.003
Phosphotase reaction rate constants			
$K_{P,U(Akt)}$	1.220	9.5×10^{-5}	0.008
$K_{P,U(mTOR)}$	2.219	4.6×10^{-5}	0.002
$K_{P,U(4EBP1)}$	1.206	2.6×10^{-5}	0.002
Regulatory effect constants			
J_{Ins}	8.390	7.5×10^{-4}	0.001
J_{EAA}	1070	2.2×10^{-2}	0.002
$J_{QP(mTOR)}$	0.494	8.1×10^{-6}	0.002
$J_{QP(Akt)}$	0.487	7.7×10^{-6}	0.002
Sensitivity exponenets			
a	1.450	2.5×10^{-5}	0.002
b	1.490	3.1×10^{-5}	0.002
c	2.370	3.9×10^{-5}	0.002
Reference scalars for phosphorylated protein pools			
$Ref\_\ Q_{P(Akt)}$	0.910	5.2×10^{-6}	0.001
$Ref\_\ Q_{P(mTOR)}$	1.037	1.2×10^{-2}	1.157
$Ref\_\ Q_{P(4EBP1)}$	0.828	9.3×10^{-7}	0.000
Initial phosphorylated protein pools			
$iQ_{P(Akt)}$	0.512	2.7×10^{-6}	0.000
$iQ_{P(mTOR)}$	0.668	6.6×10^{-2}	9.880
$iQ_{P(4EBP1)}$	0.566	9.4×10^{-2}	16.61

Table 7-2. Average observed phosphorylated Akt ($Q_{P(Akt)}$), mTOR ($Q_{P(mTOR)}$), and 4EBP1 ($Q_{P(4EBP1)}$) masses, root mean square prediction error (RMSPE) and sensitivity coefficients of insulin (S_{Ins}) and EAA (S_{EAA}).

	Observed	RMSPE	S_{Ins}	S_{EAA}
$Q_{P(Akt)}$	0.535	0.043	0.0083	N/A
$Q_{P(mTOR)}$	0.638	0.050	0.0019	0.0008
$Q_{P(4EBP1)}$	0.603	0.053	0.0017	0.0007

CHAPTER 8

Overall Conclusions

Essential amino acids had significant impact on the mTOR signaling pathway in both MAC-T cell and mammary tissue slice cultures. These responses were consistent regardless of whether essential amino acids were deprived from complete media or supplemented to a deficient media. Increasing amino acid availability was strongly associated with increasing phosphorylation of mTOR, 4EBP1, S6K1 and decreasing phosphorylation of eEF2 and eIF2α. Similar alterations in phosphorylation status of these proteins have been previously observed in muscle celss. Moreover, phosphorylation of mTOR was positively correlated with phosphorylation of S6K1, 4EBP1, S6K1, and rpS6 and negatively correlated with phosphorylation of eEF2. Similar correlations have been observed in muscle cells. In line with essential amino acids, insulin also substantially changed the phosphorylation of these proteins in a similar pattern as observed in muscle cells. Interactive effects of essential amino acid and insulin on mTOR signals were non-significant. A summary of the effects of essential amino acids, insulin and the energy substrates; glucose and acetate on phosphorylation status of the cellular signaling proteins are depicted in Figure 1. Insulin in media significantly enhanced (by 83%) phosphorylation of Akt which had a positive relationship with phosphorylation of mTOR. Essential amino acids did not impact phosphorylation of Akt suggesting they stimulate the mTOR pathway through a different mechanism in the mammary cells. All these observations allow us to confirm our hypothesis that the cellular signaling mechanisms operating in muscle cells exist in mammary epithelial cells as well.

Since EAA and insulin utilize independent mechanisms to stimulate mTOR, their stimulatory effects on mammary protein synthesis should be additive. Since glucose or propionate can stimulate insulin secretion, feeding dairy cows a high protein diet supplemented with energy should produce more protein than either a high protein or high energy diet. This disagrees with the single limiting nutrient concept used in the NRC.

Leucine, isoleucine, methionine, and threonine were the primary regulators of the mTOR pathway as shown in Figure 1. Deprivation of Leu and Ile basically reduced protein synthesis rates that were strongly associated with reduced mTOR signals. Results of the amino acid supplementation experiment showed that maximum stimulation of some Mtor-mediated signals (e. g. phosphorylation of rpS6) could not be achieved through the addition of a single amino acid suggesting involvement of multiple amino acids in regulating mTOR signals in the mammary cells. This disagrees with the single limiting amino acid concept in our current amino acid requirement models.

Acetate availability improved energy status of the mammary cells as acetate deprivation increased phosphorylatyion of AMPK which is indicative of deteriorating cellular energy status. Conversely, glucose did not have significant impact on phosphorylation of AMPK indicating a lesser contribution to ATP synthesis in bovine mammary epithelial cells. Neither glucose nor acetate had substantial control over mTOR signals and protein synthesis rates in the mammary cells. However, glucose was associated with increased phosphorylation of rpS6 suggesting that glucose may modulate rpS6 activity via an mTOR-independent pathway. rpS6 has been found to be a convergence point for many signaling pathways such as MAPK pathway. Although mTOR has been identified as a target of AMPK in muscle cells, such a link was not seen

in our experiments. AMPK may regulate mTOR via a different phosphorylation site from the one tested in present study. AMPK phosphorylation had a positive relationship with phosphorylation of eEF2. This relationship, in part provides a basis for the observed negative relationship between AMPK and protein synthesis rates as phosphorylation of eEF2 impairs global protein synthesis.

Although in-vitro experiments revealed significant stimulatory effects of branched chain amino acids (BCAA), particularly leucine and isoleucine, on protein synthesis rates in the mammary cells, their supplementation did not improve milk yield of dairy cows fed a corn-based diet with added methionine and lysine. However, supplementation of methionine and lysine significantly increased milk protein yields. Corn-based diets are known for methionine and lysine deficiencies. However, it is questionable whether these deficiencies may cause substrate limitation or stimulatory signal limitation for milk protein synthesis because in-vitro experiments also recognized methionine having considerable control over protein synthesis rates in the mammary cells. On the other hand, BCAA supplementation reduced milk urea nitrogen indicating an improved total body protein synthesis efficiency. However it did not improve milk protein efficiencies suggesting that BCAA might improved protein synthesis in non-mamamry tissues probably in muscle tissues because cows received BCAA tended to gain weight. Moreover, BCAA infusion did not significantly increase their plasma concentrations indicating infused BCAA doses might not be adequate to stimulate milk protein synthesis. The infused BCAA mix contained leucine and isoleucine in a 2: 1 ratio. Since our in vitro experiments revealed that isoleucine had stimulatory effects on mammary protein synthesis similar to leucine, infusion of isoleucine at a higher dose may produce

different results. Leucine can significantly enhance muscle protein synthesis compared to the other amino acids including isoleucine. . Therefore leucine supplementation may induce greater utilization of amino acid by muscle tissues resulting in less amino acid supply to the other tissues including the mammary glands. Infusion of isoleucine may minimize this risk associated with amino acid partitioning. Regulatory effects of amino acids and their interaction with other stimuli can adequately be represented in a mathematical model using western immunoblotting data. Such a mathematical representation may improve our current AA requirement models to predict AA requirements of dairy cows with increased accuracy.

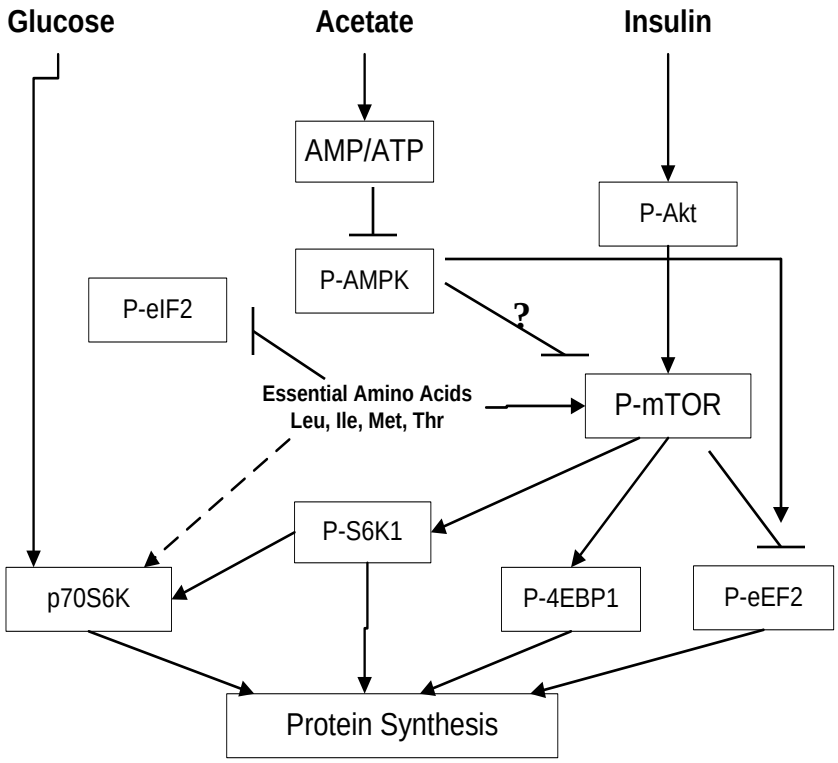

Figure 8-1. Cellular signals associated with essential amino acids, insulin, glucose, and acetate supplementation to bovine mammary epithelial cells and bovine mammary tissue slices.