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Isoleucine, leucine, methionine, and threonine effects on mammalian target of rapamycin signaling in mammary tissue

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ABSTRACT

Improved representation of postabsorptive N metabolism in lactating dairy cows requires a better understanding of protein synthesis regulation in the mammary glands. This study aimed to determine the quantitative effects of Ile, Leu, Met, and Thr on the phosphorylation state of signaling proteins that regulate protein synthesis. The experiment used a composite design with a central point, 2 axial points per AA, and a complete 2^4 factorial. All of the other AA were provided at the concentrations in Dulbecco's modified Eagle's medium. The experiment was replicated with tissues from 5 lactating cows. Mammary tissue slices (0.12 ± 0.02 g) were incubated for 4 h. Total and site-specific phosphorylated mammalian target of rapamycin (mTOR; Ser2448), eukaryotic elongation factor (eEF) 2 (Thr56), ribosomal protein S6 (Ser235/236), and eukaryotic initiation factor 2α (Ser51) were determined by western immunoblotting. Tissue concentrations of the 4 AA studied responded linearly to media supply. Addition of Ile, Leu, Met, or Thr had no effect on eukaryotic initiation factor 2α phosphorylation. Isoleucine and Thr positively affected mTOR phosphorylation. However, the 2 AA had an antagonistic relationship. Similarly, Ile linearly increased ribosomal protein S6 phosphorylation, and Thr inhibited the Ile effect. In addition, eEF2 phosphorylation was linearly decreased by Ile and Leu. Threonine curvilinearly decreased eEF2 phosphorylation, Ile and Leu negatively interacted on eEF2, and Thr tended to inhibit Leu effects on eEF2. This work demonstrated saturable responses and interactions between AA on activation of the mTOR pathway. Incorporation of these concepts into milk protein response models will help to improve milk and milk protein yield predictions and increase postabsorptive N efficiency and reduce N excretion by dairy cows.

Key words: essential amino acid, mammalian target of rapamycin (mTOR), translation regulation, mammary gland

INTRODUCTION

Among the dairy industry pollutants, N excretion is one of the major concerns because of its impact on air and water quality, ecosystem biodiversity, and human health (Wolfe and Patz, 2002). There are approximately 9 million dairy cattle in the United States (Johnson, 2012). A representative survey across dairy nutritionists estimated that average diets contain 17.8 ± 0.1% CP (Caraviello et al., 2006). A meta-analysis of 846 experimental diets determined that when feeding an average diet of 17.8% CP, only one-quarter of dietary N is recovered in milk (Hristov et al., 2004). These observations indicate that the dairy industry releases about 1.3 million metric tonnes of dietary N per year in the United States.

Among N losses, splanchnic catabolism of AA is the most significant source, accounting for approximately half of the excreted N and two-thirds of the absorbed NAN (Hanigan et al., 2004). Amino acid catabolism by splanchnic tissues is proportional to supply (Hanigan et al., 2004; Raggio et al., 2004). However, compared with the mammary glands, splanchnic affinity for AA is low (MacRae et al., 1997; Hanigan et al., 2004). Conversely, mammary extraction of AA is not 100%, and AA not extracted by the mammary glands in each pass return to peripheral circulation. Because splanchnic blood flow is about 50% of cardiac output (Davis et al., 1988; Hanigan et al., 2004), it receives a large proportion of AA recycled from peripheral tissues. Therefore, if mammary extraction of AA were increased, recycling and splanchnic catabolism would decrease.

Amino acids not only serve as substrates for protein synthesis but also function as signaling molecules that regulate the process (Kimball, 2002). Specific AA are known to affect translation initiation and elongation rates through 2 main pathways: the integrated stress response (**ISR**) and the mammalian target of rapamycin (**mTOR**) pathways. The former regulates methionyl-

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transfer RNA recruitment to the 40 S ribosomal subunit by eukaryotic initiation factor (**eIF**) 2 (Kimball et al., 1989). Depletion of AA indirectly activates general control nonderepressable 2 protein, which phosphorylates eIF2α (Ser51) and blocks translation initiation (Wek et al., 1995). In bovine mammary alveolar cells (**MAC-T**), removing all essential AA (**EAA**) from Dulbecco's modified Eagle's medium (**DMEM**) increased eIF2α phosphorylation (Appuhamy et al., 2011a), and addition of His, Phe, and Val back to depleted medium reduced the phosphorylation level (Arriola Apelo et al., 2010). Similarly, in mammary tissue slices, subtracting all EAA from DMEM significantly increased eIF2α phosphorylation (Appuhamy et al., 2012). However, individual EAA had only numerical effects on eIF2α phosphorylation.

The mTOR pathway revolves around mTOR complex 1 [see Mahoney et al. (2009) for review]. In complex 1, mTOR phosphorylates downstream proteins that regulate the rate of translation initiation and elongation (Dunlop and Tee, 2009; Mahoney et al., 2009). In bovine mammary epithelial cells, deprivation of all AA or Leu affected phosphorylation of mTOR downstream proteins S6K1 and 4E-BP1, and fractional synthesis rates of β-LG (Moshel et al., 2006). Similarly, in mammary tissue, provision of EAA Ile or Leu alone increased mTOR phosphorylation and casein fractional synthesis rate (**CFSR**; Appuhamy et al., 2011a, 2012).

Individual AA have different effects on signaling proteins and CFSR. A reasonable strategy to increase postabsorptive N efficiency would be to decrease current percentages of dietary N and supplement with those specific AA that have the largest effect on translation regulation and protein synthesis. That would increase or maintain the mammary glands' demand and extraction of AA, and reduce recycling to splanchnic tissues (Hanigan et al., 1998). To incorporate this strategy in nutrient requirement systems, it is necessary to understand the quantitative effects of AA on cell signaling and protein synthesis, as well as identification of interactions. Therefore, this study was designed to determine the effects of the 4 EAA (Ile, Leu, Met, and Thr) known to stimulate CFSR (Appuhamy et al., 2012) on phosphorylation of signaling proteins that regulate translation initiation and elongation in mammary tissue slices. A companion study will examine the effects of these treatments on casein fractional synthesis rate.

METHODS

Tissue Collection

Mammary tissue was obtained from 5 multiparous dairy cows belonging to the Virginia Tech (Blacksburg) dairy herd. Four Holstein and 1 Holstein × Jersey crossbred cows were removed from feed for 12 h and milked 2 h before slaughter to induce a responsive and homogenous phosphorylation state of signaling proteins. Slaughter was performed at the Virginia Tech Meat Laboratory Center. Milk from each quarter was tested for SCC before slaughter to ensure that the harvested tissue was not from an infected quarter. Mammary tissue from the upper part of rear quarters was excised and transported in ice-cold PBS to the laboratory where tissue slices were prepared within 2 h of slaughter.

Incubation

A base medium was prepared the day before the experiment from DMEM devoid of EAA (custom formula no. 08-5072EL; Gibco Invitrogen, Grand Island, NY) and containing 3.7 mmol of nonessential AA, 0.7 mmol of L-arginine, 0.15 mmol of L-histidine, 0.5 mmol of L-lysine, 0.45 mmol of $^2H_5$-L-phenylalanine, 0.04 mmol of L-tryptophan, 0.45 mmol L-valine, 17.5 mmol of D-glucose, 14.3 mmol of sodium pyruvate, 15 mmol of HEPES, 0.01 mg of insulin, 100,000 units of penicillin, 100 mg of streptomycin, 0.250 mg of amphotericin B, and 0.0215 mmol of phenol red per liter of medium. The medium was adjusted to pH 7.4 and filtered through a 0.22-μm filter. Individual treatment media were prepared by addition of variable amounts of L-isoleucine, L-leucine, L-methionine, and L-threonine.

Mammary tissue slices (0.121 ± 0.016 g) were prepared using a Stadie-Riggs hand-held microtome (Thomas Scientific, Swedesboro, NJ). All slices were weighed and randomly assigned to 25-mL Erlenmeyer flasks containing 5 mL of treatment medium. Flasks were purged for 30 s with 95:5 O_2:CO_2, sealed with a rubber stopper, and incubated in a shaking water bath (37°C and 60 oscillation/min) for 240 min. Preliminary data showed that [$^2H_5$]Phe enrichment of the pH 4.6 intracellular protein precipitate increased linearly from 0.5 to 8 h (0.43 ± 0.04% enrichment increase/h; R^2 = 0.93), indicating tissue viability for the incubation time selected.

Following incubation, slices were washed with 1 m*M* NaF and 10 μ*M* Na_3VO_4 in PBS, homogenized in lysis buffer [7:1, vol/mass; 50 mmol Tris-HCl (pH 7.4), 150 mmol of NaCl, 1 mmol of EDTA, 1 mmol of phenylmethylsulfonyl fluoride, 1 mmol of Na_3VO_4, 1 mmol of NaF, 0.001 μg of aprotinin, 0.001 μg of leupeptin. 0.001 μg of pepstatin, 10 mL of NP-40, and 2.5 g of Na-deoxycholate per liter) and centrifuged at 16,000 × *g* for 5 min at 4°C. The pellet was discarded.

Protein Immunoblotting

Aliquots of cell homogenate were combined 5:1 (vol/vol) with 6× sample buffer (Laemmli, 1970), incubated