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Increasing the availability of threonine, isoleucine, valine, and leucine relative to lysine while maintaining an ideal ratio of lysine:methionine alters mammary cellular metabolites, mammalian target of rapamycin signaling, and gene transcription

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ABSTRACT

Amino acids not only serve as precursors for protein synthesis but also function as signaling molecules that can regulate the mammalian target of rapamycin (mTOR) pathway. Methionine and Lys are the most-limiting AA for milk production and a ratio of ~3:1 Lys:Met in the metabolizable protein has been determined to be ideal. Besides Met and Lys, recent studies have evaluated Ile, Leu, Val, and Thr as potentially limiting for milk protein synthesis. The objective of this experiment was to determine if varying the ratio of Lys:Thr, Lys:Ile, Lys:Val, and Lys:Leu while maintaining an ideal ratio of Lys:Met and fixed ratio of other essential AA (IPAA) elicits changes in intracellular metabolites, gene transcription related to protein synthesis, and phosphorylation status of mTOR pathway proteins. Immortalized bovine mammary epithelial cell line (MAC-T) cells were incubated for 12 h (n = 5 replicates/treatment) with IPAA (2.9:1 Lys:Met; 1.8:1 Lys:Thr; 2.38:1 Lys:His; 1.23:1 Lys:Val; 1.45:1 Lys:Ile; 0.85:1 Lys:Leu; 2.08:1 Lys:Arg) or IPAA supplemented with Thr, Ile, Val, and Leu to achieve a Lys:Thr 1.3:1 (LT1.3), Lys:Ile 1.29:1 (LI1.29), Lys:Val 1.12:1 (LV1.12), or Lys:Leu 0.78:1 (LL0.78). Compared with IPAA, metabolomics via gas chromatography-mass spectrometry revealed that increases in availability of Thr, Ile, Val, and Leu led to greater concentrations of essential AA (Leu, Ile, Thr), nonessential AA (Gly, Glu, Gln, Ser, Pro, Asp), and various metabolites including uric acid, phosphoric acid, *N*-acetylglutamic acid, and intermediates of glycolysis and the tricarboxylic acid cycle. Compared with other treatments, LV1.12 led to greater phosphorylation status of serine/threonine kinase B (Akt), mTORC1, and ribosomal protein S6 and lower phosphorylation of α subunit of eukaryotic translation initiation factor 2. In addition, LV1.12 upregulated abundance of *CSN2* and both the abundance and promoter methylation of *CSN1S1*. Although LI1.29 led to the second highest response in mTORC1 phosphorylation status, it resulted in the lowest phosphorylation of Akt and eEF2 and mRNA abundance of *CSN2* and various AA transporters (*SLC7A5*, *SLC36A1*, *SLC38A2*, *SLC38A9*, *SLC43A1*). Overall, data indicate that an increase in Val at an ideal ratio of Lys:Met could further enhance milk protein synthesis by altering intracellular concentrations of essential AA and metabolites that could play a regulatory role, increasing phosphorylation status of mTORC1 and key signaling proteins, and upregulation of AA transporters.
Key words: essential amino acid ratio, metabolomics, milk protein synthesis, lactation

INTRODUCTION

It has been estimated that only up to 38% of the total AA flux across the mammary gland is used for milk protein synthesis; hence, additional capture of dietary N is a challenge of economic and environmental importance facing the modern dairy industry (Huhtanen and Hristov, 2009; Toerien et al., 2010; Wang et al., 2010). Recent studies demonstrated that balancing EAA profiles could improve milk protein synthesis (Haque et al., 2015). Methionine and Lys are the most-limiting AA for milk protein synthesis, and a ratio of ~3:1 of Lys to Met was determined to be ideal in the context of in vitro casein protein synthesis through alterations in mTOR pathway phosphorylation status and mRNA abundance (Nan et al., 2014). In addition to Lys and Met, recent studies revealed that Ile, Leu, Val, and Thr

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could be limiting for optimal protein synthesis (Appuhamy et al., 2011b, 2012; Doelman et al., 2015; Zhou et al., 2015).

It is now well known that AA not only serve as precursors for milk protein but also function as signaling molecules to regulate the mammalian target of rapamycin (**mTOR**) pathway and, in turn, protein synthesis (Yang et al., 2006; Xie and Proud, 2014). Upon activation by AA, mammalian target of rapamycin complex 1 (**mTORC1**) phosphorylates eukaryotic initiation factor 4E binding protein 1 (**4EBP1**) and ribosomal protein S6 kinase (**S6K1**; Appuhamy et al., 2011a) followed by ribosomal protein S6 (**RPS6**), α subunit of eukaryotic translation initiation factor 2 (**eIF2α**), and eukaryotic translation elongation factor 2 (**eEF2**) with the end result being the stimulation of mRNA translation initiation and elongation (Doerks et al., 2002; Browne and Proud, 2004; Kimball and Jefferson, 2006; Shimobayashi and Hall, 2014).

The serine/threonine kinase Akt plays an important role in the regulation of mTORC1 activity and forms part of the phosphatidylinositol 3-phosphate kinase (**PI3K**)-Akt-mTOR pathway (Inoki et al., 2002; Xie and Proud, 2014). The most consistent response of mTORC1 to AA is an increase in activity with Leu (Jewell et al., 2013). It is still unclear, however, whether bovine mammary cells respond to increases in the availability of other EAA when the Lys:Met ratio is maintained at the ideal level.

In this study, the general hypothesis was that increasing the in vitro availability of Ile, Leu, Val, and Thr while maintaining an ideal ratio of Lys:Met in mammary cells could influence the phosphorylation status of the mTOR signaling pathway along with gene transcription and intracellular metabolites. Thus, to address this hypothesis we cultured the immortalized bovine mammary epithelial cell line (**MAC-T**), a nonlactating mammary cell line, with various ratios of Lys:Thr, Lys:Ile, Lys:Val, or Lys:Leu and used metabolomics via GC-MS, Western blot, gene transcription, and casein promoter methylation analyses to determine holistic biological responses (Loor et al., 2013).

MATERIALS AND METHODS

Cell Culture

The cell culture was performed using the immortalized MAC-T cell line. The MAC-T cells were incubated in culture dishes (1.8×10^6 cells, 100 mm) to generate enough biological material for metabolomics, and on 6-well plates (0.2×10^6 cells/well) to isolate RNA and protein for Western blot. In both instances, cells were incubated at 37°C in an atmosphere of 5% CO_2 and air, and grown to approximately 90% confluence before imposing treatments exactly as described by Kadegowda et al. (2009).

Treatments and Experimental Design

Details of treatments and experimental design are included in Supplemental Methods and Table S1 (https://doi.org/10.3168/jds.2017-13707). Three independent batches of cell culture were performed to generate material for metabolomics, mRNA abundance, protein abundance, and phosphorylation status. In brief, MAC-T cells were cultured overnight without serum and then treated for 12 h (Li et al., 2017) with 5 treatments (n = 5 replicates/treatment) in medium without serum. A lactogenic medium was used for treatment analyses according to a previous publication from one of our laboratories (Kadegowda et al., 2009), except that minimum essential medium/Earle's balanced salts HyQ was replaced by high-glucose Dulbecco's modified Eagle's medium without EAA (custom made by Gibco, Thermo Fisher Scientific, Waltham, MA). Individual EAA (L-isomer, Sigma-Aldrich, St. Louis, MO) were added into the custom high-glucose Dulbecco's modified Eagle's medium.

The control medium contained the ideal profile of Lys:Met and fixed ratios of other EAA (IPAA, Lys:Met 2.9:1; Lys:Thr 1.8:1; Lys:His 2.38:1; Lys:Val 1.23:1; Lys:Ile 1.45:1; Lys:Leu 0.85:1; Lys:Arg 2.08:1) and treatment medium was prepared using IPAA plus different concentrations of Thr, Ile, Val, and Leu to achieve Lys:Thr 1.3:1 (**LT1.3**), Lys:Ile 1.29:1 (**LI1.29**), Lys:Val 1.12:1 (**LV1.12**), and Lys:Leu 0.78:1 (**LL0.78**; Table 1). The IPAA was designed to resemble recommendations from NRC (2001) and Haque et al. (2012, 2013). The lower ratios of Lys to Val, Lys to Ile, and Lys to Leu were to enhance Val, Ile, and Leu supply to a level demonstrated to elicit positive responses in milk protein (Haque et al., 2012, 2015). Similarly, the chosen ratio of Thr to Phe was to maintain the ratio as close as possible to the theoretical optimal (~1.0:1.0) as discussed in Haque et al. (2012). The Lys to Thr ratios were chosen based on data from Prizant and Barash (2008). By changing the supply of these AA while holding Lys constant, we sought to determine individual effects that might help explain in vivo responses in the literature (Haque et al., 2012, 2013, 2015).

RNA Extraction and Real-Time PCR Analysis

The RNA extraction and real-time PCR was performed exactly as described previously (Bionaz and Loor, 2008). In brief, total RNA was obtained using the RNeasy Mini columns (Qiagen, Valencia, CA). The