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Exogenous essential amino acids stimulate an adaptive unfolded protein response in the mammary glands of lactating cows

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ABSTRACT

The phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) components and integrated stress response networks in the mammary glands of lactating cows have not accounted for the stimulation of milk protein yield by chronic supplementation with AA or glucose. Faster milk protein synthesis could be a consequence of increased milk protein mRNA per cell, the number of ribosomes per cell, the secretory capacity of cells, or the mammary cell number. To investigate these 4 possibilities using a translational and transcriptional approach, we performed protein and gene expression analyses of mammary and longissimus dorsi tissue collected from lactating dairy cows after 5 d of abomasal infusion with saline or 844 or 1,126 g/d of an essential AA (EAA) mixture, with and without 1,000 g/d glucose. Infusion with EAA increased milk protein yield but did not affect the phosphorylation of mTORC1-related proteins in the mammary gland. In skeletal muscle, phosphorylation of 4EBP1 (eIF4E-binding protein 1) increased in response to both EAA and glucose, and phosphorylated S6K1 (70-kDa ribosomal protein S6 kinase) increased with glucose. In response to EAA, mammary mRNA expression of the marker genes for milk proteins, ribosome biogenesis, and cell proliferation were not upregulated. Instead, reciprocal regulation of 2 arms of the unfolded protein response occurred. Infusion of EAA for 5 d activated *XBP1* (X-box binding protein 1) mRNA, encoding a transcription factor for endoplasmic reticulum biogenesis, and it decreased the mRNA expression of genes encoding pro-apoptotic protein CHOP (C/EBP homologous protein) and downstream GADD34 (growth arrest and DNA damage-inducible 34). These findings implicate non-stress-related, adaptive capabilities of the unfolded protein response in the long-term nutritional regulation of milk protein yield in lactating dairy cows.

Key words: mammary, mammalian target of rapamycin complex 1 (mTORC1), unfolded protein response, milk synthesis

INTRODUCTION

The synthesis of secretory milk proteins in the mammary glands of lactating dairy cows is highly regulated, energy demanding, and responds to nutritional status. Increases in either dietary energy or protein intake can stimulate milk protein yield (Rius et al., 2010) by improving the supply of EAA and relaying anabolic signals such as insulin to the mammary glands (Metcalf et al., 1996; Bequette et al., 2002; Doepel and Lapierre, 2010). The intracellular mechanisms by which circulating AA, glucose, and insulin stimulate milk protein yield from the mammary glands remain incompletely understood.

It is well established that signaling through mammalian target of rapamycin complex 1 (**mTORC1**) is responsible for EAA- and insulin-mediated upregulation of protein synthesis in the skeletal muscle and liver of young, growing animals after a meal (Kimball et al., 2000; O'Connor et al., 2003; O'Connor et al., 2004). In mammary epithelial cells in vitro, mTORC1 is activated by EAA and the intracellular kinase Akt (protein kinase B) upon insulin or insulin-like growth factor 1 stimulation (Moshel et al., 2006; Burgos et al., 2010; Appuhamy et al., 2011) and is inhibited by AMP-activated protein kinase (Burgos et al., 2013). In vivo, in underfed dairy cows that have been intensively selected for high milk production, mammary mTORC1 is stimulated by a combination of EAA plus glucose within 36 h (Rius et al., 2010; Toerien et al., 2010). Infusion of glucose without AA into fasted cows for 9 h enhanced milk protein yield but failed to activate mTORC1, instead inhibiting phosphorylation of eukaryotic initiation factor (**eIF**)2α (Toerien et al., 2010), a second messenger of the integrated stress response (**ISR**), which is an alternative pathway that exerts translational control (Proud, 2005).

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In contrast to these acute mammary responses, the effects of chronic perturbations of cows' nutritional status by several days of EAA infusion have not proven to be consistent with the canonical translation regulation pathways. Infusion of complete EAA mixtures for 5 d increased mammary mTORC1 signaling in one study (Doelman et al., 2015a) but not in another (Doelman et al., 2015b). Imbalanced EAA mixtures lacking 1 EAA were expected to increase eIF2α phosphorylation via the ISR that downregulates protein synthesis during nutrient insufficiency, but the imbalances had no effect on the phosphorylation state of mammary eIF2α and they stimulated mTORC1 signaling without increasing milk protein yield (Doelman et al., 2015a; Doelman et al., 2015b). These responses suggest that factors other than mTORC1 and ISR signaling may be responsible for the control of milk protein synthesis. We hypothesized that acute and transient phosphorylation-level control of the mRNA translation rate is replaced by transcriptional control in mammary cells during chronic elevation of nutritional status.

To elucidate potential pathways that mediate the long-term effects of nutritional status on milk protein yield, we measured the abundance and phosphorylation states of proteins involved in the regulation of mRNA translation, and the expression of genes related to translation, ribosome biogenesis, endoplasmic reticulum (**ER**) homeostasis, and cell turnover in mammary tissue of cows infused abomasally with EAA and glucose for 5 d. We found that components of the unfolded protein response (**UPR**) were involved in the nutritional regulation of milk protein yield in a manner consistent with non-stress-related differentiation of the secretory phenotype.

MATERIALS AND METHODS

Experimental Protocol and Sampling

All experimental procedures were approved by the Animal Care and Use Committee at Trouw Nutrition Agresearch, adhering to guidelines set forth by the Canadian Council on Animal Care (2009). Five rumen-fistulated, multiparous (2.4 ± 0.5 lactations) Holstein cows producing an average of 33.0 kg/d milk at 78 ± 13 DIM and 576 ± 70.3 kg of BW were randomly assigned to a 5 × 5 Latin square design in which each period consisted of a 5-d continuous abomasal infusion followed by 2 d of rest. Cows were housed in tie stalls with individual free access to water and milked twice daily at 0500 and 1600 h. Cows were acclimated to a corn-silage-based TMR (6.94 MJ/kg of net energy and 12.4% CP on a DM basis) for 14 d before the start of the experiment and were fed once daily at 0700 h for the duration of the experiment at a fixed intake. Details of the diet are given in a companion paper (Nichols et al., 2016).

Infusion lines were placed in the abomasum via the rumen cannula 1 d before the first experimental period and were checked daily for patency. Infusion treatments were 0.9% saline (**SAL**) or complete mixtures of EAA with the same profile and amount as found in 1,500 and 2,000 g/d casein according to Metcalf et al. (1996), with or without the inclusion of 1,000 g/d of glucose (**1.5EAA**, **2EAA**, **1.5+GLC**, and **2+GLC**, respectively). Treatment solutions were prepared daily in 10-L batches and were infused using a Watson-Marlow 205U/CA multi-channel peristaltic pump (Wilmington, MA) at a rate of 6.95 mL/min, delivering 884 and 1,126 g/d EAA for the 1.5EAA and 2EAA treatments, respectively. Amino acids were infused at the following rates (g/d) for the 1.5EAA and 2EAA treatments, respectively: L-Arg (59 and 78), L-His (48 and 64), L-Ile (86 and 115), L-Val (96 and 128), L-Leu (141 and 188), L-Phe (141 and 188), DL-Met (41 and 55), L-Lys (147 and 196), L-Thr (63 and 85), and L-Trp (21 and 28). Milk sampling and composition analysis were as described by Nichols et al. (2016).

On d 5 of each infusion period, muscle tissue was collected by biopsy according to Nichols et al. (2016) from the left or right longissimus dorsi muscle. Immediately following the muscle biopsies, approximately 500 mg of mammary tissue were also collected by biopsy according to Toerien et al. (2010) from the left or right rear quarters of the udder. The first quarter for biopsy was randomly selected, and quarters were alternated in each successive period. Before incision, 5 mL of lidocaine was injected subcutaneously at the biopsy site. Mammary and muscle samples were immediately rinsed in saline, snap frozen in liquid N_2, and stored at −80°C until analysis. A nonsteroidal anti-inflammatory drug (ketoprofen, 3 mg/kg of BW) was administered intramuscularly after both biopsy procedures to provide analgesia and minimize inflammation.

Western Blot Analysis

Approximately 50 mg each of muscle and mammary tissue were homogenized for 10 s in 0.5 mL of lysis buffer (1% Triton X-100, 0.1% SDS, 50 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl, and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (Thermo Scientific, Nepean, ON, Canada). Homogenates were inverted for 1 h at 4°C, and then centrifuged at 13,000 × *g* for 20 min at 4°C. Mammary and muscle supernatants were diluted with lysis buffer to 1.5 μg/μL and 3 μg/μL, respectively, and diluted to a final concentration of 0.75 μg/μL and 1.5 μg/μL, respectively, with sample