Essential amino acid infusions stimulate mammary expression of eukaryotic initiation factor 2B ϵ but milk protein yield is not increased during an imbalance

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ABSTRACT

Essential amino acid (EAA) deficiencies and imbalances were created in lactating cows by using an infusion subtraction protocol to explore effects on milk protein yield and activity state of regulators of mRNA translation in the mammary glands. Six lactating cows on a diet of 11.2% protein were infused abomasally for 5 d with saline, 563 g/d of a complete EAA mix, or EAA without His, Met, Phe, or Trp in a 6 $\times$ 6 Latin square design. Infusion of complete and imbalanced EAA solutions increased mammalian target of rapamycin (mTOR) signaling in the mammary glands, as evidenced by higher ribosomal S6 kinase 1 (S6K1) phosphorylation compared with saline infusion. Total S6K1 abundance was decreased by imbalanced AA infusions. Except for the mixture lacking Phe, infusion of EAA, whether imbalanced or not, increased abundance of total eukaryotic initiation factor 2B ϵ (eIF2B ϵ). A correlation of 0.33 between phosphorylation state of S6K1 and total eIF2B ϵ abundance suggests that an mTOR-mediated upregulation of eIF2B ϵ translation occurred. Despite increased mTOR/eIF2B ϵ signaling, milk protein yields increased only with the complete EAA mixture compared with saline. Low plasma concentrations of His, Met, and Phe during their respective imbalances likely interfered with protein synthesis. Total abundance and phosphorylation state of eukaryotic initiation factor 2 α were not responsible for the interference. Further study of eIF2B ϵ as a regulator of milk protein yield is warranted.

Key words: mammary, milk protein synthesis, mammalian target of rapamycin (mTOR) signaling, amino acid

INTRODUCTION

The rates of synthesis and secretion of milk protein by the mammary glands of a lactating animal are influenced by dietary EAA supply (Mephram, 1982; DePeters and Cant, 1992). Predicting the magnitude of the milk protein response, however, has proven difficult. In the lactating dairy cow, for which protein nutrition and milk protein yield carry economic weight, the limiting EAA concept, where the supply of one EAA can prevent responses to all other EAA, does not appear to hold (Hanigan et al., 2000; Cant et al., 2003). Recently, researchers have investigated the potential role of regulation of mammary mRNA translation in the response of milk protein yield to nutrition of the dam.

The increase in protein synthesis in skeletal muscle following consumption of a meal is mediated by the mammalian target of rapamycin (mTOR) signaling cascade (Kimball et al., 2000; Hulmi et al., 2009). This signaling pathway is conserved in eukaryotes, from yeast to fruit flies to mammals. When activated by the insulin-dependent kinase Akt, AA (particularly Leu), or low intracellular concentrations of AMP, mammalian target of rapamycin complex 1 (mTORC1) phosphorylates eukaryotic initiation factor (eIF) 4E binding protein 1 (4EBP1) and ribosomal S6 kinase 1 (S6K1) to accelerate initiation of global mRNA translation (Wullschleger et al., 2006). Single EAA deficiencies can affect mRNA translation in eukaryotes through unacylated transfer (t)RNA-mediated phosphorylation of the α subunit of eIF2 that inhibits eIF2 activation and recruitment of the initiator Met-tRNA to the small ribosomal subunit (Hinnebusch, 2000; Proud, 2005). In addition, Akt-mediated inhibition of glycogen synthase kinase 3 (GSK3) independent of mTORC1 causes dephosphorylation of eIF2B ϵ , which is the enzyme responsible for eIF2 activation, and this contributes to the acceleration of Met-tRNA recruitment by growth factors (Proud, 2005). In mammary epithelial cells in culture, the mTORC1 and eIF2 signaling pathways have been shown to respond within minutes to insulin, IGF-

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Table 1. Ingredient and chemical composition (% of DM unless otherwise noted) of feedstuffs and TMR

Component	Content
Ingredient	
Corn silage	41.8
Alfalfa silage	11.1
Mixed hay	3.6
Wheat straw	7.4
Dairy supplement ¹	36.1
Nutrient	
CP	11.2
NDF	31.2
ADF	21.2
NE _L , Mcal/kg	1.44
Ca	0.92
P	0.34

¹Obtained from Shur-Gain Feed Mill (St. Mary's, ON, Canada) and contained 1.41 Mcal/kg NE_L and 9.89% CP. Supplement consisted of barley (87%), limestone (4.8%), molasses (2.6%), salt (2.2%), magnesium sulfate (0.95%), potassium sulfate (0.95%), dicalcium phosphate (0.63%), magnesium oxide (0.4%), vitamin and mineral premix (0.27%), selenium (0.13%), and vitamin E (0.01%).

I, AA, and intracellular energy status (Barash, 1999; Moshel et al., 2006; Burgos and Cant, 2010; Appuhamy et al., 2011, 2012; Burgos et al., 2013), as expected from other cell types. Effects of hormones and nutrients on eIF2Bε phosphorylation state in mammary epithelial cells have not been studied. Furthermore, long-term effects of nutrient supply on mRNA translational control in the mammary glands of lactating animals are not well characterized. Nine hours of i.v. infusion of EAA plus glucose into fasted cows increased milk protein yield, increased mammary abundance of phosphorylated S6K1 (**pS6K1**), and decreased phosphorylation state of mammary eIF2α (Toerien et al., 2010). Thirty-six hours of abomasal infusion of starch plus casein into lactating cows increased phosphorylation state of mammary mTOR and ribosomal S6, consistent with an increase in milk protein yield (Rius et al., 2010). In contrast, 900 g/h of i.v. glucose for 5 d decreased mammary abundances of pS6K1 and phosphorylated 4EBP1 but milk protein yields were not improved (Curtis et al., 2014). Effects of dietary EAA profile on translation signaling in the mammary glands have not been reported.

Controlled manipulation of EAA supply to the lactating dairy cow is complicated by pregastric microbial synthesis of EAA from NEAA and NPN. A subtraction protocol has been developed (Fraser et al., 1991; Weekes et al., 2006) in which a single EAA is subtracted from a complete mix of EAA infused into the abomasum of cows fed a low-protein diet, so that a single EAA deficiency can be created and corrected with confidence. Comparison of the subtracted infusate to the complete infusate represents effects of an EAA deficiency,

whereas comparison of the subtracted infusate to an AA-free infusate represents effects of an AA imbalance. Subtraction of Met, Lys, or His has caused milk protein yields to decrease (Fraser et al., 1991; Kim et al., 1999; Weekes et al., 2006; Lapierre et al., 2009), whereas subtraction of the branched-chain AA caused milk protein yield to decrease in one experiment (Rulquin and Pisulewski, 2006) but not another (Weekes et al., 2006), and Arg subtraction did not affect protein yield (Doepel and Lapierre, 2011). To our knowledge, Phe and Trp have not been studied under the subtraction protocol but they are used in the mammary gland for protein synthesis only and, therefore, may be in limited supply for milk protein production. The purpose of the current experiment was to determine the effects of total EAA supply, deficiencies of His, Met, Phe, or Trp, and EAA imbalances on milk protein yields and regulation of mRNA translation in the mammary glands of lactating cows.

MATERIALS AND METHODS

Experimental Protocol and Sampling

All animal procedures were approved by the Animal Care and Use Committee at Nutreco Canada Agresearch (Guelph, ON, Canada), adhering to guidelines set forth by the Canadian Council for Animal Care (2009). Six multiparous (2.3 ± 0.5 lactations) rumen-cannulated lactating dairy cows producing an average of 30.3 kg/d at 102 ± 4 d of lactation and 630 ± 78 kg of BW were randomly assigned to a 6×6 Latin square design, in which each period consisted of 5 d of infusion followed by 2 d of rest. A TMR was formulated to provide an NE_L of 1.44 Mcal/kg of DM and 11.2% CP (Table 1) to meet 100% and 70% of net energy and MP requirements, respectively (NRC, 2001). Production targets were 32 kg/d of milk yield consisting of 3.2% fat and 2.8% protein. Cows were given ad libitum access to the ration 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 amount based on the daily average as calculated using the last 7 d of the acclimation period. Cows were weighed 2 d before the first period and at the end of each period. Feed refusals were measured daily and feed samples were taken on a weekly basis, stored at -20°C , pooled, and subsampled for proximate analysis. Silages were monitored weekly for DM content and the TMR was adjusted accordingly.

Infusion lines were inserted into the abomasum via rumen cannulas 1 d before the first experimental period and checked daily for patency. A Watson-Marlow 205U/CA multi-channel peristaltic pump (Watson

Marlow, Wilmington, MA) was used to abomasally infuse treatments of saline (**SAL**), all 10 EAA (EAA), and EAA without Met (**Met**–), Phe (**Phe**–), His (**His**–) or Trp (**Trp**–). Amino acids were infused at the following rates (g/h), equivalent to their secretion in 1,000 g/d casein according to Metcalf et al. (1996) and estimated to meet 100% of the MP requirements with the basal diet: L-Arg (1.64), L-His (1.34), L-Ile (2.39), L-Val (2.66), L-Leu (3.92), L-Phe (3.92), L-Met (1.14), L-Lys (4.09), L-Thr (1.76), and L-Trp (0.59). The EAA solutions of 10 L were prepared daily and infused continuously into cows at a rate of 6.95 mL/min (563 g/d of EAA).

Cows were milked twice daily at 0600 and 1500 h. Milk was collected, weighed, and sampled separately from the front and rear quarters at each milking. Milk samples were stored at 4°C and analyzed within 3 d.

Blood samples were taken on the final day of each period at 1000 h from a coccygeal vessel into sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Rutherford, NJ). Samples were centrifuged at 1,500 $\times g$ for 20 min, and plasma was withdrawn into polypropylene vials and frozen at –20°C until analysis.

On d 5 of each period, approximately 500 mg of mammary tissue was obtained from a randomly selected rear quarter by biopsy (Toerien et al., 2010). Rear quarters selected for biopsy were alternated in successive periods. Each sample was rinsed in saline, blotted dry, frozen in liquid N₂, and stored at –80°C until analysis.

Sample Analysis

Milk samples were analyzed for fat, protein, and lactose contents by infrared spectroscopy (Foss System 4000, Foss Electric, Hillerød, Denmark). Proximate composition of diet samples was determined by near-infrared spectroscopy at a commercial laboratory (Nutreco Canada Inc., St. Hyacinthe, QC, Canada). Plasma samples were analyzed spectrophotometrically for glucose, NEFA, and BHBA as previously described (Weekes et al., 2006). Plasma AA were quantified by reversed-phase ultra-performance liquid chromatography using the AccQ-Tag derivatization method (Waters Chromatography Division, Milford, MA) as described by Boogers et al. (2008).

Gel Electrophoresis and Western Blot Analysis

Approximately 500 mg of mammary tissue was homogenized with 1 mL of lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (Thermo Scientific, Nepean, ON, Canada) for 10 s before inversion for 1 h at 4°C.

Lysates were centrifuged at 13,000 $\times g$ for 15 min at 4°C, and supernatants were extracted and stored at –20°C. The concentration of DNA was measured using the Qubit 2.0 Fluorometer (Life Technologies Inc., Burlington, ON, Canada) with the Qubit dsDNA BR Assay.

Western blot analysis of mammary protein abundance was performed as described previously (Curtis et al., 2014). Briefly, duplicate samples of mammary supernatant were diluted in lysis buffer and sample buffer and boiled at 95°C for 5 min. Samples containing 10 to 30 μ g of protein were separated by 10–12% SDS-PAGE at 120 V for approximately 90 min. Proteins were electrotransferred (Bio-Rad Laboratories Inc., Mississauga, ON, Canada) onto polyvinylidene difluoride membranes and were incubated in 5% skim milk blocking buffer solution for 1 h at room temperature, followed by three 5-min washes in Tris-buffered saline-Tween (**TBST**) buffer. Membranes were incubated for 1 h at room temperature with rabbit monoclonal antibodies to either S6K1 (1:800, #ab9366; Abcam, Cambridge, MA), pS6K1 (Thr389; 1:800, #ab2571; Abcam), eIF2B ϵ (1:1,000, #ab32713; Abcam), phosphorylated eIF2B ϵ (peIF2B ϵ ; Ser539; 1:1,000, #ab4775; Abcam), eIF2 α (1:600, #3597; Cell Signaling, Danvers, MA), phosphorylated eIF2 α (peIF2 α ; Ser51; 1:1,000, #3597S; Cell Signaling), or β -actin (1:40,000, #ab6276; Abcam). All antibodies were diluted using 1% milk TBST buffer. Membranes were washed with TBST buffer and incubated at room temperature for 1 h with horseradish peroxidase-linked anti-rabbit IgG (1:2,000, #7074; Cell Signaling). Membranes were washed and proteins were visualized by autoradiography using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Images from radiographic film (VWR International, Pittsburgh, PA) were scanned and the integrated density was determined by ImageJ software (<http://rbs.info.nih.gov/ij>). A common reference sample was included on each of 3 gels required to separate all samples. Densities were normalized first to β -actin density to account for protein loading differences and then to normalized reference sample density to account for differences between gels.

Calculations and Statistical Analysis

Milk production and composition were averaged over the last 2 d of each period. Protein abundance values were natural log-transformed. Variance in each observation (Y_{ijk}) was analyzed using the MIXED procedure in SAS according to the following model:

$$Y_{ijk} = \mu + \text{cow}_i + \text{per}_j + \text{trt}_k + \epsilon_{ijk},$$

Table 2. Performance responses to abomasal infusion of EAA mixtures in lactating dairy cows ($n = 6$)¹

Item	Treatment						SEM	P-value
	Saline	EAA	His–	Met–	Phe–	Trp–		
DMI, kg/d	17.6	17.4	18.1	17.4	17.5	18.5	1.4	0.922
Milk, kg/d	27.8	31.2	26.5	30.0	27.2	29.2	2.1	0.061
Fat, g/d	870	919	982	945	837	812	96	0.208
Protein, g/d	806 ^{ab}	981 ^c	760 ^{ab}	826 ^{abc}	750 ^a	915 ^{bc}	67	0.002
Lactose, g/d	1,275	1,394	1,233	1,377	1,257	1,308	107	0.307
Fat, %	3.12 ^{ab}	2.94 ^{ab}	3.67 ^b	3.13 ^{ab}	3.16 ^{ab}	2.78 ^a	0.29	0.091
Protein, %	2.90 ^a	3.13 ^b	2.89 ^a	2.77 ^a	2.76 ^a	3.15 ^b	0.10	<0.001
Lactose, %	4.58	4.46	4.62	4.58	4.62	4.46	0.06	0.116

^{a–c}Means in a row with different superscripts are significantly different, $P \leq 0.05$.

¹Data are least squares means. Treatments were 5 d of abomasal infusion with saline, EAA, EAA less His (His–), EAA less Met (Met–), EAA less Phe (Phe–), or EAA less Trp (Trp–).

where μ = mean, cow_i = i th random cow effect ($i = 1$ to 6); per_j = j th fixed period effect ($j = 1$ to 6); trt_k = k th fixed treatment effect ($k = 1$ to 6); and ε_{ijk} = random variation. Treatment means were separated by Tukey's test. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.1$. Milk production and composition data from the front, nonbiopsied mammary glands and the whole udder were each subjected to statistical analysis separately, but treatment effects were not different. The results reported herein are from the front and rear mammary glands combined.

RESULTS

Animal Health

Body weight fluctuated only slightly over the experiment and was maintained within 98% of prestudy values. Mammary biopsies, taken at the end of each period, resulted in a transient decrease in milk yield that recovered within 72 h.

Milk Component Production

Milk yields and DMI were not affected by treatment (Table 2). Abomasal infusion of the EAA mixture increased milk protein concentration ($P = 0.003$) and increased milk protein yield by 175 g/d ($P = 0.03$) compared with SAL (Table 2). The infusion of Trp– did not alter milk protein yield compared with EAA ($P = 0.81$) and SAL ($P = 0.34$). However, Trp– increased milk protein concentration relative to SAL ($P = 0.003$). Treatments Met–, Phe–, and His– resulted in significant decreases in milk protein concentration compared with EAA and Trp– (Table 2). Milk protein yields with His– and Phe– were 24 ($P = 0.003$) and 23% ($P = 0.005$) lower than with EAA and tended to be lower with Met– ($P = 0.07$). Omission of His from the EAA infusate increased milk fat concentration over

the Trp deletion ($P = 0.05$) but there were no effects of treatment on fat or lactose yields ($P > 0.20$).

Plasma Constituents

Abomasal infusion of EAA mixtures had no effect on plasma glucose, BHBA, or NEFA concentrations (Table 3). The EAA infusion increased concentrations of all EAA in plasma (Table 4) except Thr ($P = 0.06$) and Trp ($P = 0.29$). Subtraction of each individual EAA significantly reduced its respective concentration in plasma back to the level observed with SAL, except for Trp, which declined to a level lower than that of SAL ($P = 0.01$). Other EAA concentrations were not different between EAA and subtraction treatments. Of the NEAA in plasma, Tyr was decreased by Phe– compared with EAA ($P < 0.01$), and Gly was decreased by Phe– compared with SAL ($P < 0.01$). The sum of NEAA concentrations was lower on Phe– compared with SAL ($P = 0.05$).

Translational Signaling Pathways

Abundance of pS6K1 in mammary tissue was not affected by EAA infusion (Figure 1A), but was increased by infusion of imbalanced Met– or His– mixtures ($P < 0.05$), and tended to be increased by Phe– ($P = 0.08$), compared with SAL. Abundance of total S6K1 in mammary tissue was not affected by EAA infusion (Figure 1B) but was decreased by infusion of imbalanced His–, Met–, Phe–, or Trp– mixtures ($P < 0.05$), compared with SAL. The phosphorylation state of S6K1, normalizing pS6K1 abundance to total S6K1 (Figure 1C), was increased by EAA infusion ($P < 0.05$) and elevated even further by His– infusion ($P < 0.05$). Infusion of Met–, Phe–, or Trp– produced S6K phosphorylation states intermediate between those of EAA and His– infusions.

Table 3. Plasma concentrations of metabolites in lactating dairy cows (n = 6) abomasally infused with EAA mixtures¹

Item	Treatment						SEM	P-value
	Saline	EAA	His–	Met–	Phe–	Trp–		
Glucose, mmol/L	3.66	3.58	3.64	3.70	3.51	3.56	0.14	0.935
BHBA, mmol/L	0.77	0.64	0.67	0.62	0.6	0.68	0.06	0.387
NEFA, μmol/L	272	307	217	183	244	283	58	0.679

¹Data are least squares means. Treatments were 5 d of abomasal infusion with saline, EAA, EAA less His (His–), EAA less Met (Met–), EAA less Phe (Phe–), or EAA less Trp (Trp–).

Mammary abundance of peIF2α increased on EAA compared with SAL ($P < 0.05$; Figure 2A). We detected no significant effects of treatment on mammary abundance of total eIF2α ($P > 0.13$; Figure 2B). Phosphorylation state of eIF2α was increased by EAA compared with SAL ($P < 0.05$; Figure 2C). The other infusions did not affect eIF2α phosphorylation state ($P > 0.24$).

Abundance of peIF2Bε was not affected by treatment ($P > 0.13$). Abundance of total eIF2Bε was elevated on His–, Met–, and Trp– ($P < 0.05$; Figure 3B) and tended to be elevated on EAA ($P = 0.09$) compared with SAL. The phosphorylation state of eIF2Bε tended to be depressed during His– infusion compared with SAL ($P = 0.06$; Figure 3C) and was not affected by other treatments ($P > 0.11$).

DISCUSSION

Duodenal flows of MP from the diet were estimated using NRC (2001) to be 871 g/d on average and 1,434 g/d including the EAA infusate (563 g/d). Estimated duodenal supplies of Met, Phe, His, and Trp from the diet were 38, 97, 43, and 23 g/d, respectively. The EAA infusate provided 39% of the total duodenal flow, whereas subtractions removed 43, 42, 49, and 38% of the duodenal His, Met, Phe, and Trp supply, respectively. Subtractions of His, Met, and Phe produced deficiency states as evidenced by the decreases in milk protein yields compared with EAA. The concentration of His in plasma decreased to 13 μM, which is similar to concentrations of 8 to 13 μM previously reported when His subtraction depressed milk protein yield in

Table 4. Plasma concentrations of AA in lactating dairy cows (n = 6) abomasally infused with EAA mixtures¹

AA, μM	Treatment						SEM	P-value
	Saline	EAA	His–	Met–	Phe–	Trp–		
Arg	62 ^a	102 ^b	86 ^{ab}	88 ^{ab}	94 ^{ab}	88 ^{ab}	9	0.089
His	21 ^a	58 ^b	13 ^a	47 ^b	53 ^b	54 ^b	5	<0.001
Ile	59 ^a	121 ^b	102 ^{ab}	100 ^{ab}	103 ^{ab}	102 ^{ab}	11	0.017
Leu	61 ^a	140 ^b	137 ^b	129 ^b	133 ^b	116 ^{ab}	15	0.004
Lys	53 ^a	135 ^b	108 ^b	116 ^b	125 ^b	117 ^b	13	0.002
Met	15 ^a	49 ^b	38 ^b	10 ^a	37 ^b	40 ^b	3	<0.001
Phe	33 ^a	71 ^b	73 ^b	62 ^b	25 ^a	60 ^b	7	<0.001
Thr	95	152	120	117	127	139	16	0.101
Trp	49 ^b	61 ^b	59 ^b	54 ^b	51 ^b	27 ^a	4	<0.001
Val	133 ^a	306 ^b	302 ^b	282 ^b	305 ^b	288 ^b	31	<0.001
Ala	211	198	186	185	174	191	19	0.676
Asn	45	36	36	30	32	30	4	0.152
Asp	6	7	6	5	7	7	1	0.244
Glu	68	94	69	93	70	91	16	0.627
Gly	496 ^b	441 ^{ab}	366 ^{ab}	402 ^{ab}	298 ^a	403 ^{ab}	44	0.016
Pro	63	58	54	59	49	55	6	0.600
Ser	120	100	114	119	83	91	11	0.046
Tyr	35 ^{ab}	49 ^b	45 ^b	43 ^b	20 ^a	40 ^b	4	<0.001
EAA ²	576 ^a	1,195 ^b	1,038 ^b	992 ^b	1,054 ^b	1,031 ^b	93	0.002
NEAA ³	1,043 ^b	984 ^{ab}	877 ^{ab}	935 ^{ab}	734 ^a	910 ^{ab}	74	0.079
TAA ⁴	1,620	2,179	1,915	1,927	1,787	1,940	144	0.122

^{a,b}Means in a row with different superscripts are significantly different, $P \leq 0.05$.

¹Data are least squares means. Treatments were 5 d of abomasal infusion with saline, EAA, EAA less His (His–), EAA less Met (Met–), EAA less Phe (Phe–), or EAA less Trp (Trp–).

²EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

³NEAA = Ala, Asn, Asp, Glu, Gly, Pro, Ser, and Tyr.

⁴TAA = EAA + NEAA.

Figure 1. Abundances of phosphorylated ribosomal S6 kinase 1 normalized to β -actin (A), total ribosomal S6 kinase 1 (S6K1) normalized to β -actin (B), and phosphorylated S6K1 normalized to total S6K1 (C) in mammary tissue of lactating dairy cows ($n = 6$) after 5 d of abomasal infusion with saline, EAA, EAA less His (His-), Met (Met-), Phe (Phe-), or Trp (Trp-). Values are presented as least squares means $\pm$ SE. Means without a common letter differ, $P \leq 0.05$.

Figure 2. Abundances of phosphorylated eukaryotic initiation factor 2 α normalized to β -actin (A), total eukaryotic initiation factor 2 α (eIF2 α) normalized to β -actin (B), and phosphorylated eIF2 α normalized to total eIF2 α (C) in mammary tissue of lactating dairy cows ($n = 6$) after 5 d of abomasal infusion with saline, EAA, EAA less His (His-), Met (Met-), Phe (Phe-), or Trp (Trp-). Values are presented as least squares means $\pm$ SE. Means without a common letter differ, $P \leq 0.05$.

Figure 3. Abundances of phosphorylated eukaryotic initiation factor 2B ϵ normalized to β -actin (A), total eukaryotic initiation factor 2B ϵ (eIF2B ϵ) normalized to β -actin (B), and phosphorylated eIF2B ϵ normalized to total eIF2B ϵ (C) in mammary tissue of lactating dairy cows (n = 6) after 5 d of abomasal infusion with saline, EAA, EAA less His (His-), Met (Met-), Phe (Phe-), or Trp (Trp-). Values are presented as least squares means $\pm$ SE. Means without a common letter differ, $P \leq 0.05$.

dairy cattle (Kim et al., 1999; Weekes et al., 2006) and goats (Bequette et al., 2000). Plasma Met concentration decreased to 10 μ M, also similar to the 7 and 15 μ M reported for Met deficiencies produced by subtraction from an AA mixture (Kim et al., 2000; Weekes et al., 2006). To our knowledge, Phe subtractions have not been conducted, but our findings suggest that 25 μ M Phe in plasma indicates a deficiency state. Tryptophan concentrations decreased from 61 to 27 μ M when Trp was subtracted but milk protein yield was not affected. We conclude that 27 μ M Trp was not low enough to affect milk protein synthesis. Similarly, when Trp was subtracted from a mix of His, Met, Lys, and Trp infused i.v. into lactating cows, plasma Trp only decreased to 40 μ M and milk protein yield was not affected (Kim et al., 1999). In the current study, the estimated duodenal flow of Trp was 2% of the total EAA flow during the Trp- treatment, whereas estimated His, Met, and Phe flows on His-, Met-, and Phe- treatments were 3, 3, and 7% of EAA flow, respectively, and were deficient proportions.

Infusion of either EAA or Trp- resulted in similar milk protein yields but we observed no consistent change in mammary translation factor abundance or phosphorylation state uniquely associated with these 2 treatments. The signaling factors we examined were S6K1 to indicate mTORC1 activity, eIF2 α to indicate uncharged tRNA signaling, and eIF2B ϵ to indicate alternative effects. We found that infusion of all EAA solutions, whether imbalanced or not, increased mammary mTORC1 signaling and total eIF2B ϵ abundance, but that S6K1 abundances were reduced by imbalanced mixtures.

The mTORC1 network responds to many signals but the best characterized are insulin, AA, and intracellular ATP (Wullschlegel et al., 2006). All 3 of these stimuli have been shown to rapidly activate mTORC1 in mammary epithelial cells in culture (Barash, 1999; Moshel et al., 2006; Burgos and Cant, 2010; Appuhamy et al., 2011, 2012; Burgos et al., 2013). Infusion of EAA into cows elevates both insulin and EAA concentrations (Weekes et al., 2006) and therefore has 2 potential ways to stimulate mTORC1 activity. Plasma concentrations of total AA and the particularly strong mTORC1 activators Leu and Ile (Appuhamy et al., 2012) were elevated on all imbalanced and complete treatments, and a previous experiment found that insulin concentrations were elevated equally on complete and imbalanced treatments compared with saline (Weekes et al., 2006). Our finding that EAA mixtures stimulate mammary mTORC1 activity after 5 d of infusion corroborates and extends our earlier finding that Leu infusion for 9 h into feed-deprived cows increases mammary pS6K (Toerien et al., 2010).

The decreased abundance of mammary S6K1 during AA imbalance is interesting. Translational control via phosphorylation cascades is a mechanism to produce short-term changes in protein synthesis such as those that occur in skeletal muscle and liver after a meal (Kimball et al., 2000). The role of the mTORC1/S6K1 cascade in mediating a chronic elevation of protein synthesis, such as would occur after several days of improved protein nutrition, remains unclear. For example, administering Leu for 60 min increased phosphorylation state of S6K1 in skeletal muscle (Escobar et al., 2005) but Leu supplementation for 10 d did not affect muscle S6K1 phosphorylation, even though it stimulated protein synthesis (Lynch et al., 2002). In cases where protein synthesis is chronically depressed or elevated for several days by dietary treatment, such as the Leu supply experiment of Lynch et al. (2002) or the current infusion experiment, it may be important to consider the total abundance of the signaling proteins in addition to their phosphorylation state. The decline in total S6K1 abundance in mammary tissue during AA imbalance compared with SAL may have contributed to the lack of a response in milk protein yield despite elevated S6K1 phosphorylation and abundance of pS6K1. Regulation of expression of the gene encoding S6K1 has been studied little (Maruani et al., 2012), but we previously found that i.v. infusion of 900 g/d glucose into lactating cows decreased mammary abundance of S6K1 (Curtis et al., 2014), and others have shown that overexpression of certain insulin signaling components in mammary epithelial cells depresses total S6K1 abundance (Wang et al., 2014; Zhang et al., 2014). Mechanisms of transcriptional control of S6K1 expression and their role in nutritional control of milk protein production remain poorly understood.

All AA infusions increased total eIF2B ϵ abundance without significantly changing peIF2B ϵ . Although eIF2B ϵ expression has received more attention in relation to feeding and exercise in skeletal muscle, this is the first report, to our knowledge, of eIF2B ϵ expression in mammary glands of lactating animals. The main mechanism by which resistance exercise increases muscle mass is thought to be through expression of eIF2B ϵ (Mayhew et al., 2011). Phosphorylation state of eIF2B ϵ is depressed, and activity thereby increased, by insulin through inhibition of GSK3, independent of mTORC1 (Welsh et al., 1998). In addition, translation of eIF2B ϵ mRNA into protein is accelerated by mTORC1 (Kubica et al., 2008). The increase in eIF2B ϵ abundance we observed during AA infusions may have been related to mTORC1 activation. The correlation between S6K1 phosphorylation state, representing mTORC1 activity, and total eIF2B ϵ abundance across all biopsy samples was 0.33 ($P < 0.01$). Phosphorylation state of peIF2B ϵ

tended to be lower on the His– treatment. Kubica et al. (2005) found a similar effect during recovery of skeletal muscle from acute resistance exercise; abundance of eIF2B ϵ increased in a rapamycin-sensitive manner indicating mTORC1 involvement, and its phosphorylation state was reduced accordingly without any change in phosphorylation state of the eIF2B ϵ kinase GSK3.

Phosphorylation of S6K1 and expression of eIF2B ϵ are both stimulatory to global protein synthesis. The exact mechanism by which S6K1 acts to increase protein synthesis remains unclear but it phosphorylates a diverse array of proteins involved in mRNA translation, including ribosomal protein S6, eIF4B, eEF2 kinase, and programmed cell death 4 (Magnuson et al., 2012). In addition, phosphorylation of S6K1 at Thr389 is indicative of mTORC1 activity that also phosphorylates 4EBP1 to accelerate formation of the 5' cap-binding complex on mRNA and thereby facilitates initiation of translation (Wullschlegel et al., 2006). The eIF2B effect is due to its catalysis of the exchange of GDP on eIF2 for GTP so that eIF2 becomes active and forms a pre-initiation complex with Met-tRNA and the small ribosomal subunit (Proud, 2005). Overexpression of eIF2B ϵ by itself causes protein synthesis to increase (Hardt et al., 2004).

Despite increases in mammary mTORC1 activity and increases in eIF2B ϵ abundance during all AA infusions, milk protein yields increased only on EAA. It is likely that the low concentrations of His, Met, and Phe in the His–, Met–, and Phe– treatments, respectively, prevented mTORC1/eIF2B-mediated increases in milk protein yield. A similar phenomenon has been reported where more than 1 h of i.v. Leu infusion into neonatal pigs stimulated mTORC1 activity in skeletal muscle but low concentrations of EAA prevented protein synthesis from increasing (Escobar et al., 2005; Wilson et al., 2010). Amino acid deficiencies are hypothesized to inhibit protein synthesis via accumulation of uncharged tRNA that activates GCN2 and causes phosphorylation of eIF2 α , which is a competitive inhibitor of eIF2B (Hinnebusch, 2000). In support of this hypothesis, phosphorylation state of eIF2 α in liver of rats consuming a diet 30% deficient in Met was elevated compared with those fed a Met-sufficient diet (Sikalidis and Stipanuk, 2010). However, we did not observe any effect of His, Met, or Phe deficiency on mammary eIF2 α phosphorylation state, despite 24% decreases in milk protein yields. Similarly, Wilson et al. (2010) did not find an elevation of eIF2 α phosphorylation state in skeletal muscle of piglets when low EAA concentrations during Leu infusion prevented mTORC1 stimulation of protein synthesis. In fact, those authors reported a decline in peIF2 α that would have been expected to contribute to an acceleration of muscle protein synthesis. Removal of

Met from the medium in which avian myoblasts were cultured also caused a decline in p ϵ IF2 α while protein synthesis decreased (Métayer-Coustard et al., 2010). In that study, dephosphorylation of S6K1 was implicated in the protein synthesis decline. The mechanism by which low EAA concentrations interfered with stimulation of protein synthesis in our study may be related to the decreased abundance of total S6K1 in mammary tissue.

CONCLUSIONS

In summary, we created EAA deficiencies and imbalances in lactating dairy cows by using an abomasal infusion subtraction protocol to explore the involvement of mammary translational control in milk protein yield responses to AA. Infusion of a complete EAA mixture and a Trp-lacking mixture both stimulated milk protein yields to a similar extent. Infusion of mixtures lacking His, Met, or Phe had no effect on milk protein yields. With the exception of Phe–, all of the AA infusions caused an upregulation of mammary expression of eIF2B ϵ , the enzyme responsible for eIF2 activation and subsequent delivery of the initiator AA to the small ribosomal subunit. Expression of eIF2B ϵ may have been mediated by mTORC1, which was activated on all AA treatments, according to increases in S6K1 phosphorylation state. Low plasma concentrations of His, Met, and Phe during infusion of their imbalanced mixtures prevented an mTORC1/eIF2B ϵ -mediated stimulation of milk protein yield. Phosphorylation of eIF2 α was not responsible for this interference but a decline in mammary S6K1 expression may have been, although the mechanism remains unidentified. Further study of eIF2B ϵ as a regulator of milk protein yield is warranted.

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