Glucose supplementation stimulates peripheral branched-chain amino acid catabolism in lactating dairy cows during essential amino acid infusions

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ABSTRACT

To determine how glucose modulates protein synthesis when essential AA are in abundant supply, 5 early-lactation, rumen-fistulated Holstein dairy cows were fed a diet containing 6.95 MJ/kg of net energy for lactation and 12.4% crude protein and abomasally infused for 5 d with saline, 844 or 1,126 g/d of a complete essential AA mix, with and without the inclusion of 1,000 g/d of glucose, in a 5 × 5 Latin square design. Infusion of essential AA increased milk yield by 4.1 kg/d, milk protein by 256 g/d, milk fat by 95 g/d, and milk urea nitrogen by 70% compared with saline, with no differences between the level of essential AA infusion. The addition of glucose to essential AA infusate did not stimulate milk protein yield or concentration, but reduced milk urea nitrogen by 17% and decreased milk fat yield. Arterial concentrations of total essential AA increased 3- to 4-fold, mammary clearance decreased 61%, and mammary uptake of essential AA increased 65% in response to essential AA infusion. Arterial branched-chain AA concentrations declined 29% in response to glucose and mammary clearance increased 48%, but mammary AA uptake was unchanged. Essential AA infusion increased plasma 3-methylhistidine by 50% and reduced muscle branched-chain α -keto acid dehydrogenase kinase abundance by 14%, indicating stimulation of muscle protein turnover and branched-chain AA catabolism, respectively. Glucose had no further effect on muscle branched-chain α -keto acid dehydrogenase kinase abundance but decreased mRNA expression of branched chain aminotransferase 1. Lack of further increases in plasma 3-methylhistidine or greater stimulation of muscle branched-chain AA catabolism indicates that muscle protein degradation was unchanged with glucose but that accretion may have been stimulated. The decrease in circulating branched-

chain AA concentrations and nitrogen excretion in response to glucose suggests that surplus essential AA were redirected to peripheral, extra-mammary tissues.

Key words: amino acid, glucose, protein synthesis, mammary, muscle

INTRODUCTION

Milk protein yield from dairy cows can be manipulated by both protein and energy supply, but these dietary components appear to exert different effects on whole-body metabolism and AA partitioning. It is well known that more than 50% of dietary protein may not be captured in milk protein; most of the N loss occurs at the postabsorptive level, where AA not used for milk protein are partitioned into skeletal muscle, other labile protein pools, or catabolic pathways (Hanigan et al., 1997, 1998a). Increased mammary uptake and use of AA for milk protein synthesis would improve efficiency of use, lowering ingredient cost and reducing environmental N loss. Because protein synthesis is an energy-demanding process, increasing energy supply to the mammary gland may enhance milk protein yield and efficiency of N capture. Many groups have observed positive effects of energy on lactation performance and postabsorptive N efficiency. Cows fed 16.6 and 14.6% CP diets, both with 6.7 MJ of NE_L/kg of DM, produced 220 and 160 g/d more milk protein, respectively, than cows fed 5.9 MJ/kg (Rius et al., 2010). Efficiency of conversion of absorbed N into milk N increased when glucose was added to abomasal casein infusions (Clark et al., 1977). Raggio et al. (2006a,b) stimulated milk protein synthesis with infusions of casein into the duodenum or propionate into the rumen, with a greater increase observed for casein infusion and an additive effect when the 2 were infused together. The stimulatory effect of glucose or glucose precursors on AA partitioning to the mammary glands may be mediated in part by insulin, as shown from the increased mammary blood flow and milk protein yield response to a hyperinsulinemic-euglycemic clamp (Grinari et al., 1997; Mackle et al., 2000).

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Although several studies report positive milk yield responses to glucose or glucose precursor infusions (Hurtaud et al., 2000; Rulquin et al., 2004; Raggio et al., 2006a,b), in some instances glucose did not stimulate milk protein yield (Clark et al., 1977; Vanhatalo et al., 2003a,b; Curtis et al., 2014). It is possible that EAA supply to the mammary gland limits the effect of postruminal glucose on milk protein yield. Glucose infusions have been shown to decrease plasma concentrations of EAA, primarily the branched-chain AA (BCAA; Hurtaud et al., 1998, 2000; Lemosquet et al., 2004). In response to propionate + casein infusion, mammary uptake of EAA increased, but during propionate infusion alone, NEAA uptake increased more than EAA uptake (Raggio et al., 2006a), probably because EAA were in short supply. Supply of energy precursors can alter the profile of mammary uptake depending on the concomitant supply of EAA available to the gland. Additionally, if insulin is mediating the protein synthetic response at the mammary glands, it is possible that variation in the insulin response to glucose results in inconsistent effects on milk protein yield (Rulquin et al., 2004). Furthermore, the reductions in plasma EAA concentrations and N excretion have been interpreted to suggest that glucose partitions AA toward extra-mammary tissues, such as skeletal muscle (Clark et al., 1977; Curtis et al., 2014).

The objective of this study was to determine the effects of supplemental glucose on milk protein production in lactating dairy cows during EAA infusion. A high level of AA infusion, in comparison with previous studies (Metcalf et al., 1996; Doepel and Lapierre, 2010; Galindo et al., 2011; Doelman et al., 2015a,b), was used to ensure that AA would not limit milk protein yield when glucose was infused. In previous experiments, we infused EAA in equivalent amounts found in 1 kg/d of casein into cows fed a low-protein diet, and observed an average increase of 164 g/d in milk protein yield compared with a saline control (Doelman et al., 2015a,b). In the current experiment, we applied the same infusion model with 1.5 and 2 times the EAA supply.

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; second lactation $n = 3$, third lactation $n = 2$) Holstein cows producing an average of 33.0 kg/d 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. A TMR (Table 1) was formulated to provide an NE_L of 6.94 MJ/kg of DM and 12% CP to meet 100 and 70% of NE_L and MP target requirements, respectively. Production targets were 32.5 kg/d of milk yield consisting of 3.8% fat and 2.8% protein. Cows were acclimated to the diet 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 14-d acclimation period. Cows were weighed 2 d before the start of the experiment and at the end of each period. Feed refusals were measured daily and TMR 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 mix was adjusted accordingly.

Infusion lines were placed in the abomasum via the rumen cannula 1 d before the first experimental period and were checked daily for patency and position. 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg 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

Table 1. Ingredient and chemical composition of TMR

Component	Content (% of DM)
Ingredient composition	
Corn silage	49.8
Haylage	17.0
Corn, ground	15.7
Wheat shorts	6.1
Straw, chopped	5.3
Mixed hay	2.6
Tallow	1.2
Rumen-protected soybean meal ¹	1.1
Vitamin/mineral mix ²	1.1
Urea	0.003
Nutrient analysis	
CP	12.4
NDF	28.7
ADF	18.6
NFC	46.8
Crude fat	5.3
Ash	6.8
Ca	0.71
P	0.39
NE _L (MJ/kg)	6.94

¹TopSoy obtained from Shur-Gain Feed Mill (St. Mary's, ON, Canada).

²Contained 42% limestone, 32% K, 14% Mg, 11% Ca, 0.3% vitamin E, 0.3% NaCl, 0.3% Se.

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 of EAA for the 1.5EAA and 2EAA treatments, respectively. Amino acids were infused at the following rates (g/d) for the 1.5 and 2 EAA 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 was collected, weighed, and sampled separately from the front and rear mammary glands at each milking during the entire 5-d infusion period. Milk samples were stored at 4°C and analyzed within 3 d. Mammary biopsies were collected at the end of each period (data not shown), necessitating separate milk collection from the front and rear mammary glands.

Blood samples were taken concurrently from the coccygeal vessels and the subcutaneous abdominal vein into sodium heparin and potassium EDTA Vacutainers (Becton Dickinson, Rutherford, NJ) on d 4 of the infusion period at 0800, 1000, 1200, and 1400 h. Samples were collected from the left and right subcutaneous abdominal veins, alternating sides with each time point to avoid oversampling a particular area and to account for differences between sides. Collection tubes were immediately placed in ice and centrifuged at $3,000 \times g$ for 15 min at room temperature. Plasma was transferred into polypropylene tubes and frozen at -20°C until analysis.

After the morning milking at the end of each 5-d infusion period, muscle tissue was collected by biopsy from the left or right longissimus dorsi muscle in the L1 to L6 vertebral region, alternating sides in successive periods. Cows were sedated with 0.5 mL of intravenous xylazine, and 10 mL of lidocaine was injected anterior to the biopsy site as an intercostal nerve block. An incision approximately 3 cm in length was made through the skin in the intercostal space perpendicular to the vertebrae, and approximately 500 mg of muscle tissue was removed with a 6-mm biopsy punch (Integra Miltex, Burlington, ON, Canada). Muscle samples were immediately rinsed in saline, snap frozen in liquid N₂, and stored at -80°C until analysis.

Milk and Plasma Analysis

Milk samples were analyzed for fat, protein, lactose (Laboratory Services Division, University of Guelph, Guelph, ON), and milk urea nitrogen (CanWest DHI Ontario, DHI Milk Analysis Centre, Guelph, ON) by infrared spectroscopy. Arterial or venous plasma samples were pooled over time for each cow by period and analyzed for glucose, BHB, nonesterified fatty acids (NEFA), triacylglycerol (TAG), acetate, and

urea as described by Weekes et al. (2006), and free glycerol according to Bucolo and David (1973). Insulin was analyzed by immunoassay (Crystal Chem Inc., Downers Grove, IL). Long-chain fatty acid concentrations were calculated on a molar basis as $3 \times \text{TAG} + \text{NEFA}$. Amino acid concentrations in plasma samples collected at 1000, 1200, and 1400 h were analyzed using ultra-performance liquid chromatography in conjunction with Empower Chromatography Data Software (Waters Corporation, Milford, MA) according to the protocol described by Boogers et al. (2008). A 250 μM 3-methyl-L-His standard solution (Sigma-Aldrich, St. Louis, MO) was run to quantify plasma 3-methylhistidine (3M-His) concentrations.

Western Blot Analysis and Quantitative Real-Time PCR

Approximately 50 mg of muscle tissue was homogenized for 10 s in 0.5 mL of lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 8.0, 150 mM 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 $13,000 \times g$ for 20 min at 4°C. Muscle supernatants were diluted with lysis buffer to 3 $\mu\text{g}/\mu\text{L}$, and further diluted to 1.5 $\mu\text{g}/\mu\text{L}$ with sample buffer (4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.125 M Tris HCL, and 0.004% bromophenol blue), before boiling at 90°C for 5 min. Ten microliters of boiled muscle sample containing 15 μg of total protein and BLUeye Prestained Protein Ladder (FroggaBio, Toronto, ON, Canada) was separated by 8% SDS-PAGE at 120 V for approximately 90 min. Proteins were electrotransferred (Mini Trans-Blot, Bio-Rad Laboratories Inc., Mississauga, ON, Canada) onto polyvinylidene difluoride membranes (Millipore, Mississauga, ON, Canada) at 100 V for 60 min. Membranes were incubated at room temperature for 1 h in Tris-buffered saline-Tween (TBST) buffer containing 5% nonfat dry milk before incubation at room temperature with rabbit monoclonal antibodies to branched-chain aminotransferase 1 (BCAT1; #12822, Cell Signaling, Danvers, MA) and branched-chain α -keto acid dehydrogenase kinase (BCKDH-K; ab128935, Abcam, Cambridge, MA). All primary antibodies were diluted using 1% milk TBST buffer. Membranes were rinsed with TBST buffer and incubated at room temperature for 1 h with horseradish peroxidase-linked anti-rabbit IgG (NA934V, GE Healthcare Life Sciences, Mississauga, ON, Canada) diluted in TBST buffer. Following six 5-min washes of the membranes in TBST buffer, proteins were developed using chemiluminescence (Clarity Western ECL Substrate, Bio-Rad). All membranes first

probed with proteins of interest underwent three 5-min washes in TBST buffer and appropriate portions were re-probed with antibody against glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**; ab8245, Abcam) as a loading control. Blot densities in scanned images were determined by ImageLab software (BioRad) and normalized to the corresponding GAPDH blot density.

Total RNA was isolated from approximately 100 mg of muscle tissue using 1 mL of TRizol Reagent (Invitrogen, Life Technologies, Burlington, ON, Canada), according to the manufacturer's instructions. Quality of RNA was determined with a 1% agarose TBE gel (40 mM Tris base, 40 mM boric acid, 0.2% 0.5 M EDTA pH 8.0) by staining with ethidium bromide. Primers were designed to yield PCR amplification products of 100 to 200 bp for BCAT1 and the branched-chain α -keto acid dehydrogenase complex (**BCKDH**; Table 2). The cDNA was synthesized from 500 ng of extracted total RNA with random hexamers using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), following the manufacturer's instructions. The qPCR was carried out using PerfeCta SYBR Green FastMix (Quanta BioScience, Gaithersburg, MD) with an Applied Biosystems 7300 Real Time PCR instrument. Fold changes in gene expression relative to SAL were calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) after normalizing to GAPDH.

Calculations and Statistical Analysis

Milk crude protein yield was adjusted to true protein yield using a factor of 5.5% (DePeters and Ferguson, 1992). All following calculations were based on this estimate of true protein yield. Mammary plasma flow (**MPF**) across the whole udder was estimated according to the Fick principle using Phe and Tyr as internal markers (Cant et al., 1993), where $MPF (L/h) = \text{milk Phe} + \text{Tyr output } (\mu\text{mol/h}) / \text{arteriovenous Phe} + \text{Tyr difference } (\mu\text{mol/L})$, with an allowance for 3.5% contribution from blood-derived proteins. Milk output of Phe + Tyr was estimated from the d-4 afternoon milk protein yield corresponding to the blood samples taken that day using Phe and Tyr contents reported by Mephram (1987). Mammary clearances (k) of AA and me-

tabolites were calculated from the model of Hanigan et al. (1998b), where $k (L/h) = (\text{arteriovenous difference} \times MPF) / \text{venous concentration}$. Uptakes (mmol/h) of AA and metabolites across the mammary glands were calculated as the product of their plasma arteriovenous differences and MPF. Positive arteriovenous differences and uptakes indicate AA removal from plasma, whereas negative values indicate net AA release from the mammary glands. Energy balance was calculated as $NE_{\text{intake}} - NE_L - NE_M$, where NE_{intake} was calculated from observed DMI and its estimated net energy content plus the net energy content of EAA and glucose infusions, which were 8.5 MJ/kg (calculated based on 64% of the heats of combustion of individual EAA in the infusate) and 11.5 MJ/kg (Hurtaud et al., 1998), respectively. The NE_L was obtained from the regression equation of Tyrrell and Reid (1965) against observed milk fat, protein, and lactose yields, and $NE_M = 0.08BW^{0.75}$ (NRC, 2001). Metabolizable protein balance was calculated as $MP_{\text{intake}} - MP_L - MP_M$, where MP_{intake} was calculated from the observed DMI and its estimated MP content (NRC, 2001) plus the EAA infusates, and MP_L and MP_M were calculated using NRC (2001) requirement equations.

Milk yield, milk composition, and DMI were averaged over the final 2 d of each period. Plasma AA concentrations were averaged over the 3 sampling times on d 4 of each period. Variances in lactation performance, plasma concentrations, arteriovenous differences, mammary clearances and uptakes, protein abundance, and gene expression were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) according to the following model:

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

where μ = mean, cow_i = i th random cow effect ($i = 1$ to 5), per_j = j th fixed period effect ($j = 1$ to 5), trt_k = k th fixed treatment effect ($k = 1$ to 5), and ε_{ijk} = random variation. Effects of EAA were estimated as linear contrasts of 0, 1.5EAA, and 2EAA without glucose, using coefficients calculated with PROC IML of SAS. Effects of 1.5EAA+GLC and 2EAA+GLC (GLC) were estimated from orthogonal contrasts between

Table 2. Primer sequences for quantitative real-time PCR in bovine mammary and muscle tissue

Database	Gene	Protein	Primer sequence	Product size
NM_001083644	<i>BCAT1</i>	BCAT1	5'-GGCCCCACGATGAAGGATT 3'-AACGGTGGCTCGTGTGATTA	120
NM_174506	<i>BCKDHA</i>	BCKDH	5'-TTTGAGACCAAGTCGAGGC 3'-GAAATCTAGCCAGCCACGA	81
NM_001034034	<i>GAPDH</i>	GAPDH	5'-GGGTCATCATCTCTGCACCT 3'-GGTCATAAGTCCCTCCACGA	176

1.5EAA and 2EAA (EAA) treatments with and without glucose. Treatment means were also separated by Tukey's means separation procedure. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.15$. No significant differences were found between 1.5EAA and 2EAA by Tukey's test. Interactions between EAA and GLC were not estimable due to the incomplete factorial design of the experiment. Milk production and composition data from the front and rear mammary glands were subjected to statistical analysis separately, but treatment effects were not different. The results presented herein are from the front and rear mammary glands combined.

RESULTS

Dry Matter Intake and Milk Yield

Dry matter intake was 17 kg/d during EAA infusions and decreased 0.6 kg/d with GLC ($P = 0.04$; Table 3). Milk yield increased 4.1 kg/d in response to EAA compared with SAL ($P < 0.001$) and tended to further increase 1.5 kg/d with GLC ($P = 0.057$). The EAA tended to increase milk fat yield ($P = 0.123$), whereas GLC infusion decreased milk fat yield by 99 g/d ($P = 0.038$) and concentration by 0.42 units ($P = 0.007$). Compared with SAL, EAA stimulated an increase in milk CP yield by 256 g/d ($P < 0.001$) and concentration by 0.40 units ($P < 0.001$). Infusion of GLC had no effect on protein yield ($P = 0.318$) and tended to decrease protein content ($P = 0.097$). Lactose yield was increased with EAA infusion by 179 g/d ($P = 0.002$) and by 262 g/d with GLC ($P = 0.046$). Milk urea nitrogen content increased 70% ($P < 0.001$) with EAA

compared with SAL and subsequently decreased 17% in response to GLC ($P = 0.021$).

Arterial AA Concentrations, Mammary Plasma Flow, and AA Kinetics

Infusion of EAA increased the arterial concentration of total EAA to 3.5 times that of the SAL treatment ($P < 0.001$; Table 4). Nonessential AA concentrations decreased 20% ($P < 0.001$) except Asp ($P = 0.746$) and Gln, which only tended to decrease ($P = 0.114$), and Glu and Tyr, which increased ($P < 0.015$). The addition of glucose to EAA infusates decreased arterial BCAA concentrations by 29% ($P < 0.001$), tended to decrease Arg and Lys by 18% each ($P < 0.150$), and increased Thr by 38% ($P < 0.001$). Of the NEAA, Ser increased with GLC ($P < 0.001$), Pro decreased ($P = 0.028$), and Gln and Glu tended to decrease by 14 ($P = 0.098$) and 7% ($P = 0.076$), respectively. Plasma concentration of 3M-His, a marker of muscle protein turnover, tended to increase 50% in response to EAA compared with SAL ($P = 0.054$; Table 4). Addition of glucose had no effect on circulating 3M-His concentration.

Infusion of EAA increased the arteriovenous (AV) difference of total EAA ($P = 0.001$; Table 5) by 83% compared with SAL. Individually, AV differences for all EAA increased significantly ($P < 0.035$) except Arg and Trp, which only tended to increase ($P \leq 0.61$). Arteriovenous difference for total NEAA was unaffected by EAA infusion ($P = 0.274$), but AV difference for Asp ($P = 0.003$), Glu ($P = 0.017$), and Ser ($P = 0.088$) decreased. Addition of glucose to EAA infusates tended to decrease total EAA AV difference by 17% ($P = 0.093$), with individual decreases seen for His, Lys,

Table 3. Performance of lactating dairy cows ($n = 5$) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
DMI (kg/d)	17.1	17.0	17.1	16.7	16.1	0.9	0.979	0.040
Yield								
Milk (kg/d)	30.0	33.8	34.4	35.4	35.8	1.3	<0.001	0.057
Fat (g/d)	914	1,023	995	914	907	120	0.123	0.038
Protein (g/d)	868	1,118	1,130	1,139	1,156	54	<0.001	0.318
Lactose (g/d)	1,418	1,589	1,604	1,677	1,682	53	0.002	0.046
Composition (%)								
Fat	3.08	3.03	2.91	2.56	2.54	0.35	0.447	0.007
Protein	2.90	3.31	3.28	3.22	3.23	0.09	<0.001	0.097
Lactose	4.73	4.71	4.67	4.74	4.70	0.08	0.136	0.280
MUN (mg/dL)	4.1	6.7	7.3	5.6	6.0	1.0	<0.001	0.021
BW (kg)	581	582	586	585	584	31	0.406	0.868
BCS	2.45	2.45	2.48	2.53	2.48	0.09	0.594	0.207

¹Data are least squares means from the final 2 d of each period.

²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).

³ P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.

Table 4. Arterial plasma concentrations of AA and 3-methylhistidine (3M-His; μM) in lactating dairy cows ($n = 5$) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
Arg	62	128	152	104	126	16	<0.001	0.074
His	19	108	121	100	139	18	<0.001	0.671
Ile	105	286	348	181	252	22	<0.001	<0.001
Leu	107	391	499	240	365	33	<0.001	<0.001
Lys	60	185	243	144	208	27	<0.001	0.142
Met	17	92	117	94	125	10	<0.001	0.530
Phe	41	152	191	142	204	17	<0.001	0.936
Thr	84	213	241	294	334	22	<0.001	<0.001
Trp	32	53	58	50	60	3	<0.001	0.872
Val	183	643	833	429	656	42	<0.001	<0.001
Ala	234	164	133	169	154	12	<0.001	0.122
Asn	38	32	27	27	27	2	0.001	0.233
Asp	9	9	9	9	9	0.4	0.746	0.617
Gln	185	146	165	129	139	12	0.114	0.098
Glu	125	141	146	133	135	13	0.007	0.076
Gly	418	352	278	378	308	39	0.005	0.332
Pro	80	62	54	54	51	3	<0.001	0.028
Ser	102	69	65	88	79	4	<0.001	<0.001
Tyr	42	66	63	68	80	10	0.015	0.146
BCAA ⁴	394	1,319	1,681	849	1,274	94	<0.001	<0.001
EAA ⁵	708	2,250	2,803	1,779	2,471	167	<0.001	0.033
Non-BCAA ⁶	315	931	1,122	929	1,197	101	<0.001	0.681
NEAA ⁷	1,234	1,042	940	1,055	982	37	<0.001	0.313
TAA ⁸	1,942	3,292	3,743	2,833	3,453	170	<0.001	0.040
3M-His	17	27	24	23	23	4	0.054	0.406

¹Data are least squares means from d 4 of each period.²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).³ P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.⁴BCAA = branched-chain AA (Ile, Leu, and Val).⁵EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.⁶Non-BCAA = EAA – BCAA.⁷NEAA = Ala, Asn, Asp, Glu, Gly, Gln, Pro, Ser, and Tyr.⁸TAA = EAA + NEAA.

Met, and Trp ($P = 0.028$ – 0.135). Of the BCAA, AV difference for Val tended to decrease with GLC infusion ($P = 0.096$), whereas Ile and Leu were unaffected ($P > 0.2$). Infusion of GLC had no effect on AV difference of NEAA, except Ala, which tended to decrease 35% ($P = 0.113$).

Mammary plasma flow was unaffected by treatment ($P > 0.241$; Table 6). Mammary clearances of individual EAA decreased 43 (Arg) to 89% (His) with EAA infusion compared with SAL ($P < 0.05$; Table 6), except Trp clearance, which was unchanged ($P = 0.617$). The clearance of total NEAA was unaffected by EAA infusion ($P = 0.979$), but Asp, Glu, and Tyr clearances were decreased individually ($P \leq 0.026$). The addition of glucose to EAA infusates decreased clearance of Thr ($P = 0.021$) and Trp ($P = 0.107$) by 41 and 28%, respectively, and the clearance of total BCAA was increased 48% ($P = 0.009$) by GLC infusion. Although Val clearance was not affected, Ile and Leu clearances increased 79 ($P = 0.005$) and 85% ($P =$

0.004), respectively. Of the NEAA, Ala ($P = 0.049$) and Tyr ($P = 0.074$) clearances decreased with GLC, and Asp clearance increased ($P = 0.102$).

Mammary uptakes of all individual EAA increased by 22 (Arg) to 91% (Val) with infusion of EAA compared with SAL ($P < 0.001$; Table 7). The EAA infusion decreased uptakes of Asp ($P < 0.001$), Glu ($P = 0.002$), and Ser ($P = 0.035$) by 65, 34, and 30%, respectively, but did not affect uptakes of other NEAA. Infusion of GLC had no effect on the uptake of total EAA, BCAA, non-BCAA, or NEAA but tended to decrease mammary uptake of His, Trp, and Val by 20, 23, and 16%, respectively ($P < 0.1$) and increase the uptake of Ile, Phe, and Asp by 8, 8, and 64% ($P \leq 0.141$), respectively.

Insulin and Metabolites

Insulin concentration was unaffected by EAA ($P = 0.494$) but increased 53% ($P = 0.019$) with GLC infu-

sion (Table 8). Arterial plasma glucose concentration was not affected by EAA ($P = 0.681$) and increased 16% ($P < 0.001$) with GLC. Plasma BHB and acetate decreased with GLC infusion by 63 ($P = 0.008$) and 34% ($P = 0.012$), respectively. Arterial NEFA concentration increased 32% ($P = 0.004$) with EAA infusion, and decreased to SAL levels ($P < 0.001$) with GLC. Total long-chain fatty acid concentration tended to decrease when glucose was added to EAA infusions ($P = 0.123$). Plasma urea concentration during EAA infusion increased 2-fold compared with SAL ($P < 0.001$), whereas GLC had no effect.

Infusion of EAA had no effect on AV difference of any plasma metabolites (Table 9), but GLC infusion tended to decrease AV difference of acetate ($P = 0.122$) and NEFA ($P = 0.112$). Mammary gland clearance of NEFA tended to decrease 50% with EAA infusion ($P = 0.131$; Table 9). Compared with EAA, GLC tended to increase clearances of BHB 2.1-fold ($P = 0.148$) and TAG by 97% ($P = 0.144$). Mammary gland uptake of

plasma glucose tended to be 20% higher during GLC infusion ($P = 0.127$), but uptakes of all metabolites (Table 9) were not otherwise affected by EAA or GLC treatments.

Protein Abundance and Gene Expression

Abundance of BCKDH-K in skeletal muscle decreased with EAA ($P = 0.022$; Figure 1), and the abundance of BCAT1 was unaffected by EAA or GLC treatments. The expression of BCKDH mRNA was unaffected by treatment, but BCAT1 expression decreased when GLC was infused ($P = 0.040$; Figure 2).

DISCUSSION

AA Supply and Partitioning

The objective of this study was to determine whether glucose would stimulate milk protein yield when EAA

Table 5. Mammary gland arteriovenous differences (μM) of AA in lactating dairy cows ($n = 5$) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
Arg	30	37	46	39	38	5	0.061	0.551
His	12	19	22	13	15	3	0.016	0.028
Ile	29	52	58	43	56	6	0.001	0.310
Leu	46	80	88	65	84	9	0.002	0.236
Lys	40	71	86	57	75	8	<0.001	0.050
Met	11	16	20	14	15	3	0.025	0.135
Phe	19	32	40	30	35	4	0.001	0.332
Thr	25	39	46	33	37	6	0.035	0.268
Trp	5	7	10	5	6	2	0.051	0.073
Val	43	82	102	59	74	14	0.012	0.096
Ala	36	28	26	21	14	6	0.206	0.113
Asn	12	13	10	10	10	2	0.640	0.330
Asp	4	1	2	2	2	0.5	0.003	0.453
Gln	51	53	51	48	47	11	0.718	0.463
Glu	47	37	32	32	32	5	0.017	0.445
Gly	18	48	-6	23	13	18	0.661	0.873
Pro	16	14	12	11	10	3	0.187	0.328
Ser	29	22	20	21	20	4	0.088	0.786
Tyr	18	16	17	11	15	3	0.682	0.175
BCAA ⁴	118	214	248	167	214	29	0.004	0.147
EAA ⁵	260	435	518	358	433	49	0.001	0.093
Non-BCAA ⁶	141	221	271	191	220	23	0.001	0.076
NEAA ⁷	231	232	169	180	162	30	0.274	0.353
TAA ⁸	491	666	688	538	595	62	0.031	0.099

¹Data are least squares means from d 4 of each period.

²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).

³ P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.

⁴BCAA = branched-chain AA (Ile, Leu, and Val).

⁵EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

⁶Non-BCAA = EAA - BCAA.

⁷NEAA = Ala, Asn, Asp, Glu, Gln, Pro, Ser, and Tyr.

⁸TAA = EAA + NEAA.

were not limiting for mammary protein synthesis. Metabolizable protein supply from the diet was estimated using NRC (2001) to be 1,322 g/d on average and 2,170 and 2,432 g/d including the EAA infusates (844 and 1,126 g/d, respectively; Table 10). To the authors' knowledge, 1,126 g/d is the highest reported level of a complete EAA mixture to be infused into cows. Metcalf et al. (1996) infused 208 g/d of EAA into a jugular vein of cows fed a 14.5% CP diet and stimulated milk true protein yield by 143 g/d. When Doelman et al. (2015a,b) infused 563 g/d of exclusively EAA on an 11.2% CP diet, milk true protein yield increased by 165 and 145 g/d, respectively. Our daily infusion of 1126 g increased plasma EAA concentration 3.5-fold and stimulated milk true protein yield by 248 g/d.

Based on NRC (2001) calculations using basal diet intakes, the MP-allowable milk was 47% of the NE-allowable milk (Table 10) and the average DMI of the basal diet across all treatments supported a milk true

protein yield of 324 g/d (Table 11). During SAL infusion, the actual milk true protein yield of 820 g/d in comparison with the 332 g/d diet allowed milk protein yield suggests that this difference was supported by an additional 488 g/d of MP from spared catabolism and labile MP sources within the body, including muscle, splanchnic, and circulating protein and peptide pools (Table 11).

Milk true protein yield increased to 1,068 g/d in response to 1,126 g/d of EAA from 2EAA infusion, with the basal diet supporting 332 g/d of this protein yield (NRC, 2001; Table 11). Of the 736 g/d difference between diet-allowed protein yield and observed protein yield, 383 g/d were EAA that could have come entirely from the infusate, potentially mitigating the need for MP mobilization from labile pools. In fact, if the 383 g/d came entirely from the EAA infusate, then 743 g/d of infused EAA were available to replenish labile MP stores or be catabolized. Some of this excess

Table 6. Whole-mammary gland plasma flow (L/h) and clearances (k , L/h) of AA in lactating dairy cows ($n = 5$) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
Plasma flow	612	569	528	688	585	71	0.438	0.241
k								
Arg	551	352	282	500	273	88	0.046	0.448
His	1,133	140	117	100	80	110	<0.001	0.723
Ile	212	131	97	248	160	29	0.008	0.005
Leu	442	153	105	309	168	37	<0.001	0.004
Lys	1,114	523	381	652	346	111	<0.001	0.678
Met	1,067	153	106	115	75	42	<0.001	0.407
Phe	511	183	150	236	120	33	<0.001	0.708
Thr	243	141	114	76	75	20	<0.001	0.021
Trp	99	93	89	72	59	15	0.617	0.107
Val	173	87	64	105	71	13	<0.001	0.342
Ala	103	147	145	90	73	35	0.272	0.049
Asn	244	405	287	415	344	47	0.215	0.493
Asp	402	97	106	229	115	42	<0.001	0.102
Gln	227	366	293	486	296	87	0.416	0.469
Glu	367	218	150	236	196	42	<0.001	0.319
Gly	21	86	13	28	28	24	0.655	0.352
Pro	135	161	129	171	140	29	0.929	0.714
Ser	283	277	231	240	186	59	0.586	0.473
Tyr	424	352	214	172	187	81	0.026	0.074
BCAA ⁴	241	115	82	178	113	19	<0.001	0.009
EAA ⁵	327	147	110	180	122	18	<0.001	0.192
Non-BCAA ⁶	465	215	169	183	134	27	<0.001	0.241
NEAA ⁷	129	168	115	137	117	23	0.979	0.523
TAA ⁸	190	155	112	160	120	17	0.011	0.698

¹Data are least squares means from d 4 of each period.

²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).

³ P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.

⁴BCAA = branched-chain AA (Ile, Leu, and Val).

⁵EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.

⁶Non-BCAA = EAA – BCAA.

⁷NEAA = Ala, Asn, Asp, Glu, Gly, Gln, Pro, Ser, and Tyr.

⁸TAA = EAA + NEAA.

Table 7. Mammary gland uptakes (mmol/h) of AA in lactating dairy cows (n = 5) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
Arg	17.7	20.9	22.3	27.0	23.2	3.63	0.366	0.345
His	6.8	10.6	10.3	7.9	8.6	1.11	0.016	0.057
Ile	15.8	28.9	26.9	28.5	31.5	1.90	<0.001	0.125
Leu	26.2	44.4	40.8	43.0	47.1	2.55	<0.001	0.235
Lys	22.8	39.4	42.0	40.3	43.5	4.71	0.006	0.800
Met	6.2	9.1	9.1	8.4	8.2	0.86	0.010	0.302
Phe	10.9	17.7	19.0	20.0	19.6	0.99	<0.001	0.141
Thr	14.0	21.5	20.8	19.3	20.8	2.27	0.024	0.628
Trp	2.6	3.9	4.4	3.2	3.2	0.56	0.034	0.093
Val	24.0	46.3	45.5	35.8	41.1	4.37	0.001	0.095
Ala	19.3	16.0	13.9	13.1	9.1	3.73	0.272	0.257
Asn	6.4	7.4	4.8	7.1	5.8	0.96	0.417	0.612
Asp	2.1	0.6	0.8	1.5	0.8	0.24	<0.001	0.089
Gln	29.8	30.1	27.3	33.6	27.3	6.80	0.830	0.784
Glu	28.4	21.2	16.1	22.4	18.2	3.31	0.002	0.478
Gly	8.1	29.8	-0.5	11.3	6.8	9.48	0.999	0.518
Pro	8.6	7.7	5.3	7.3	5.6	1.27	0.093	0.959
Ser	15.9	12.0	10.3	14.4	11.1	1.89	0.035	0.379
Tyr	10.1	8.9	8.0	7.6	8.6	1.42	0.177	0.707
BCAA ⁴	66.1	119.7	113.2	107.3	119.8	7.14	<0.001	0.603
EAA ⁵	147.1	242.6	241.2	233.3	246.8	13.99	<0.001	0.869
Non-BCAA ⁶	81.0	123.0	128.0	126.0	127.1	9.57	0.001	0.899
NEAA ⁷	128.8	134.0	85.9	118.1	93.3	16.98	0.197	0.808
TAA ⁸	275.9	376.5	327.1	351.4	340.1	23.39	0.042	0.795

¹Data are least squares means from d 4 of each period.²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).³P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.⁴BCAA = branched-chain AA (Ile, Leu, and Val).⁵EAA = Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.⁶Non-BCAA = EAA - BCAA.⁷NEAA = Ala, Asn, Asp, Glu, Gly, Gln, Pro, Ser, and Tyr.⁸TAA = EAA + NEAA.

EAA was used to synthesize NEAA in the mammary gland because NEAA output in milk protein increased on 2EAA compared with SAL, even though mammary

NEAA uptake did not change. Between 2EAA and SAL, mammary uptake of EAA increased 311 g/d, which is 183 g/d more than the 128 g/d increase in

Table 8. Arterial plasma concentrations of metabolites and insulin in lactating dairy cows (n = 5) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
Glucose (mM)	2.87	2.67	2.90	3.03	3.45	0.09	0.681	<0.001
BHB (mM)	0.57	1.00	0.73	0.33	0.31	0.23	0.301	0.008
Acetate (mM)	1.78	1.80	1.48	1.05	1.12	0.22	0.387	0.012
Nonesterified fatty acids (μM)	131	171	176	117	130	12	0.004	<0.001
TAG (μM)	46	46	42	47	52	5	0.675	0.300
LCFA (μM)	270	310	303	259	286	21	0.195	0.123
Glycerol (μM)	37	22	28	23	19	6	0.210	0.508
Urea (mM)	0.62	1.11	1.48	1.06	1.28	0.15	<0.001	0.363
Insulin (μg/L)	0.74	0.90	0.89	1.39	1.35	0.22	0.494	0.019

¹Data are least squares means from d 4 of each period. TAG = triacylglycerol; LCFA = long-chain fatty acid.²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).³P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.

Table 9. Mammary gland arteriovenous (AV) differences, clearances (*k*), and uptakes of metabolites in lactating dairy cows (*n* = 5) receiving abomasal infusions of EAA and glucose (GLC) for 5 d¹

Item	Treatment ²					SEM	P-value ³	
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC		EAA	GLC
Glucose								
AV difference (mM)	0.60	0.58	0.72	0.56	0.81	0.09	0.360	0.698
<i>k</i> (L/h)	146	174	166	162	182	28	0.541	0.942
Uptake (mmol/h)	327.3	326.3	359.5	386.1	483.0	54.5	0.738	0.107
BHB								
AV difference (mM)	0.21	0.25	0.16	0.10	0.15	0.09	0.797	0.302
<i>k</i> (L/h)	460	326	144	387	603	190	0.239	0.148
Uptake (mmol/h)	116.3	152.3	89.2	68.4	92.0	58.3	0.884	0.417
Acetate								
AV difference (mM)	0.68	0.91	0.62	0.51	0.61	0.12	0.879	0.122
<i>k</i> (L/h)	418	765	441	691	709	136	0.430	0.402
Uptake (mmol/h)	354.5	494.2	309.2	376.4	356.7	63.8	0.927	0.592
Nonesterified fatty acids								
AV difference (μM)	-43	-31	-28	-55	-34	16	0.245	0.112
<i>k</i> (L/h)	-147	-79	-68	-219	91	53	0.131	0.050
Uptake (mmol/h)	-26.3	-17.6	-16.2	-37.9	-16.5	9.8	0.283	0.156
TAG								
AV difference (μM)	30	32	29	37	32	7	0.969	0.609
<i>k</i> (L/h)	1,820	1,422	1,308	4,097	1,274	845	0.662	0.144
Uptake (mmol/h)	17.4	18.3	13.8	26.4	18.6	4.2	0.668	0.151
LCFA								
AV difference (μM)	47	66	60	54	61	29	0.659	0.850
<i>k</i> (L/h)	116	169	126	216	208	79	0.813	0.397
Uptake (mmol/h)	26.0	37.4	25.2	41.2	39.2	16.1	0.888	0.569
Glycerol								
AV difference (μM)	14	0.6	7	-0.4	-1	5	0.169	0.373
<i>k</i> (L/h)	268	13	234	-22	-22	106	0.483	0.196
Uptake (mmol/h)	6.5	0.01	4.4	-0.3	-0.5	2.5	0.291	0.316

¹Data are least squares means from d 4 of each period.²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).³*P*_{EAA} = linear effect of EAA without GLC; *P*_{GLC} = effect of GLC.

EAA output in milk protein and is sufficient to account for the mammary NEAA deficit. This example of intramammary AA metabolism is in agreement with Doepel and Lapierre (2010), who reported increased mammary EAA uptake in support of both milk protein and NEAA synthesis within the gland.

Decreased mammary clearances for most EAA reflect the surplus of EAA supply for milk protein synthesis, consistent with their elevated plasma concentrations. Mammary plasma flow, which is a determinant of the arterial supply of EAA for mammary uptake, was not affected by EAA infusion. Thus, decreased clearances of EAA between SAL and EAA treatments were related to greater arterial and venous EAA concentrations, despite increased mammary uptake. This agrees with the 61% surplus of infused EAA not used for milk protein synthesis (Table 11), suggesting that mammary affinity for EAA is reduced when supply is abundant. Mammary clearance of AA and metabolites is part of an adaptive mechanism to match substrate supply with cellular needs of the gland for milk and component synthesis (Rius et al., 2010). The reduced mammary

clearance of EAA suggests mammary requirements for maximal protein synthesis were met and supports our hypothesis that surplus infused AA were available for catabolism.

It is interesting that DMI was not affected by EAA infusion, despite the significant increase in circulating EAA in plasma. Typically, intake of surplus AA reduces feed consumption (Gietzen, 1993), but our observations are in line with Weekes et al. (2006), who infused 1,104 g/d of a complete AA mixture and reported an increase in plasma AA concentration with no change in DMI.

That EAA infusion stimulated deposition of protein into labile pools and catabolism of AA is supported by the observation that plasma NEAA concentrations declined. Because NEAA were not included in the infusates, this decline indicates stimulation of NEAA utilization. The estimates of mammary NEAA clearance and uptake suggest that this stimulation did not occur at the mammary gland. However, elevated milk and plasma urea concentrations indicate that AA catabolism was indeed stimulated. Typically, when incomplete AA profiles are infused into cows or fed to nonruminants,

catabolism of all AA, including those not included in the supplement, increases (Cant et al., 2003). A portion of NEAA was likely catabolized in the liver for gluconeogenesis to support milk production (Vanhatalo et al., 2003b). Although we have no direct evidence that AA deposition into labile protein pools also increased, the elevation of plasma 3M-His indicates increased protein degradation in skeletal muscle during EAA infusion. The 3M-His concentration in saline-treated

Figure 1. Abundances of branched-chain α -keto acid dehydrogenase kinase (BCKDH-K) and branched-chain aminotransferase 1 (BCAT1) in skeletal muscle of lactating dairy cows ($n = 5$) after 5-d abomasal infusion of EAA and GLC. Values are presented as least squares means $\pm$ SE; SAL = saline; GLC = glucose; P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.

Figure 2. Skeletal muscle gene expression of branched-chain α -keto acid dehydrogenase (BCKDH) and branched-chain aminotransferase 1 (BCAT1) in lactating dairy cows ($n = 5$) after 5-d abomasal infusion of EAA and GLC. Values are presented as least squares means $\pm$ SE; SAL = saline; GLC = glucose; P_{EAA} = linear effect of EAA without GLC; P_{GLC} = effect of GLC.

cows was $17 \mu\text{M}$, which is similar to $14 \mu\text{M}$ reported for cows at 12 wk of lactation (Blum et al., 1985). Concentration of 3M-His increased 40% to $24 \mu\text{M}$ during EAA infusion. Appuhamy et al. (2011) reported plasma 3M-His levels of $10.4 \mu\text{M}$ for saline-treated cows that increased by 13% when jugular infusions of Met and Lys were supplemented with BCAA. Using labeled Leu, Bequette et al. (2002) found that protein synthesis and degradation rates in the hind limb of lactating goats

Table 10. Estimated MP and NE_L supplied from the basal diet at observed mean DMI and 5-d abomasal infusions of EAA and glucose (GLC) in lactating dairy cows (n = 5)¹

Item	Treatment ²				
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC
MP supplied (g/d)					
Diet	1,346	1,338	1,346	1,314	1,265
EAA portion from diet	693	689	693	677	654
Infusate	0	844	1,126	844	1,126
Total MP	1,346	2,182	2,472	2,158	2,391
Total EAA ³	693	1,533	1,819	1,521	1,780
BCAA	281	603	712	598	696
Non-BCAA	411	930	1,106	923	1,083
Mammary EAA uptake:MP supply	0.72	0.53	0.44	0.52	0.46
BCAA	0.71	0.60	0.48	0.54	0.52
Non-BCAA	0.73	0.48	0.42	0.50	0.43
Dietary MP-allowable milk (kg/d)	11.1	11.0	11.1	10.7	10.2
Intestinal glucose supply ⁴ (g/d)	1,609	1,606	1,609	2,582	2,525
NE _L supplied (MJ/d)					
Diet	113.0	113.0	113.4	110.9	107.9
Infusate	0	12.6	17.2	24.3	28.5
Total NE _L	113.0	125.6	130.6	135.2	136.4
Dietary NE-allowable milk (kg/d)	23.6	23.4	23.6	22.9	21.8
MP balance ⁵ (g/d)	148	819	1,094	759	979
NE balance ⁶ (MJ/d)	-6.3	-6.1	-1.4	-5.1	-6.4

¹Based on NRC (2001) calculations.²Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).³EAA supplied by diet + infused EAA.⁴Calculation uses resistant starch estimates from the CVB Table Ruminants (CVB, 2007) based on TMR ingredient composition.⁵Calculation includes MP from diet + EAA infusates.⁶Calculation includes energy from diet + EAA infusates.

both increased in response to infusion of a complete mix of AA, although synthesis was affected more, resulting in net AA gain. It is likely that a similar stimulation of protein synthesis and degradation in skeletal muscle

occurred during EAA infusion, producing a net gain of protein, which reconciles the rise in circulating 3M-His concentration with the higher MP balance and the lower plasma NEAA concentrations.

Table 11. Estimated EAA availability for mammary and extra-mammary protein synthesis in lactating dairy cows from the basal diet at observed mean DMI and 5-d abomasal EAA and glucose (GLC) infusions

Item	Treatment ¹				
	SAL	1.5EAA	2EAA	1.5+GLC	2+GLC
Mammary EAA uptake (g/d)	497	808	808	790	826
Milk true protein yield (g/d)	820	1,057	1,068	1,076	1,092
Diet-allowed milk protein yield ² (g/d)	332	329	332	322	307
Milk protein difference ³ (g/d)	488	728	736	754	785
Total EAA in milk protein ⁴ (g/d)	428	551	556	560	569
EAA in milk protein from diet ⁴ (g/d)	173	172	173	168	160
EAA difference (g/d)	255	379	383	392	409
Infused EAA (g/d)	0	844	1,126	844	1,126
Infused EAA not used for milk protein yield ⁵					
g/d	N/A	465	743	452	717
%	N/A	55	66	54	64

¹Infusion 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 of casein according to Metcalf et al. (1996), with or without the inclusion of 1 kg of glucose (1.5EAA, 2EAA, 1.5+GLC, and 2+GLC, respectively).²(MP-allowable milk × 0.03) × 1,000.³Milk true protein yield – diet-allowed milk protein yield.⁴AA composition estimated from Mephram (1987).⁵Infused EAA – EAA difference.

Despite similar energy balances between SAL and EAA-infused cows, plasma NEFA concentration significantly increased with EAA. The lack of a treatment effect on plasma concentrations of lipid metabolites, acetate, and BHB suggests that fat mobilization from adipose was not affected but that catabolism of AA may have spared NEFA oxidation. Additionally, the lack of response in NEFA, acetate, and BHB uptake; the decrease in clearance of NEFA; and the increase in AA uptake and higher MUN concentration suggest the milk fat response in this study was supported by intramammary AA catabolism. A similar response was observed in dairy cows abomasally infused with casein where milk fat yield and MUN increased without a change in lipid precursor uptake by the mammary glands (Vanhatalo et al., 2003a).

Glucose Effect on AA Partitioning

When glucose was added to EAA infusions, DMI decreased approximately 1 kg/d, dietary MP supply decreased from 1,341 to 1,290 g/d, and NE_L supply increased by 7.7 MJ/d (Table 10). Glucose did not stimulate milk protein production, leaving 54 and 64% of the 844 and 1,126 g/d of EAA infusate, respectively, that was not output into milk (Table 11).

Milk protein yield responses to provision of additional energy in the form of glucose, propionate, or acetate have been positive in some reports (Rulquin et al., 2004; Raggio et al., 2006a,b; Safayi and Nielsen, 2013), or nil in others (Vanhatalo et al., 2003b; Purdie et al., 2008; Curtis et al., 2014). When glucose was infused in this study, mammary uptakes of all AA groups were unaffected, including BCAA despite a decrease in arterial concentrations. The clearance of non-BCAA remained unchanged, in agreement with the level of milk protein synthesis during GLC infusion, but BCAA clearance increased suggesting their affinity for use by the gland changed with glucose supplementation. Mammary plasma flow was not affected by EAA or GLC infusions. Thus, it appears that when EAA are in abundant supply with or without glucose supplementation, mammary uptake of EAA is not affected by plasma concentrations or MPF after 5-d infusions, and is instead determined by AA-sequestering potential of mammary cells, which include processes of milk protein secretion and AA catabolism.

Whereas glucose had no effect on mammary utilization of AA groups, changes in milk urea and plasma AA concentrations suggest that glucose affected AA metabolism elsewhere. The decline in milk urea concentration indicates that AA catabolism was depressed during GLC infusion. Increased plasma concentration of the glucogenic AA Thr and Ser suggests that glu-

coneogenesis from AA was suppressed during glucose supplementation in agreement with others who have found the same effect of postruminal glucose supplementation (Lemosquet et al., 2004).

Of the EAA in plasma, only the concentrations of BCAA significantly decreased as a result of GLC infusion. Many others have found a suppressive effect of glucose or insulin on AA concentrations in lactating cows, with the BCAA being most dramatically reduced (Clark et al., 1977; Mackle et al., 2000; Curtis et al., 2014), and it has been suggested that partitioning of BCAA into skeletal muscle could account for their lower plasma concentrations (Clark et al., 1977; Hurtaud et al., 1998; Curtis et al., 2014). The BCAA were infused at 431 g/d on the 2EAA treatment, which elicited an increase in plasma BCAA concentration of 1,287 μM . Based on this rate:state relationship, the 401 μM decrease in BCAA due to glucose corresponds to a total BCAA efflux of 136 g/d. According to the BCAA content of bovine skeletal muscle (Early et al., 1990), 1.2 kg/d of muscle protein could theoretically be synthesized from 136 g/d BCAA. Whereas body protein loss from dairy cows between 14 d prepartum and 60 DIM ranges from 0.1 to 0.6 kg/d (Komaragiri and Erdman, 1997; Phillips et al., 2003; Chibisa et al., 2008), Botts et al. (1979) reported repletion of 0.4 kg/d of body protein when cows were re-fed a 22% CP diet after consuming 9% CP for 6 wk. Thus, 1.2 kg of net gain of muscle protein each day is double the highest reported rate of net loss or gain. However, whole-body protein synthesis based on Leu kinetics has been estimated at 3.7 kg/d when mid lactation cows were fed a high-MP diet (Lapierre et al. 2002). The absence of a glucose effect on plasma 3M-His concentration indicates that skeletal muscle protein degradation was unaffected. Plasma insulin concentration increased with GLC, and in mature ruminants the anti-proteolytic action of insulin on muscle protein has been well demonstrated (Lobley, 1998). Euglycemic insulin administration to lactating goats decreased whole body proteolysis in one study (Tesseraud et al., 1993) and had no effect on hind limb protein degradation in another (Bequette et al., 2002), although net protein gains were increased in both. Hyperaminoacidemia did not affect the protein degradation or accretion responses to insulin in either of these studies (Tesseraud et al., 1993; Bequette et al., 2002).

The problem with attributing the observed decrease in BCAA concentrations to sequestration in a protein pool is the absence of a glucose effect on the other EAA in circulation. The BCAA in particular appear to be subject to faster utilization. Catabolism of the BCAA differs from the other AA in that it occurs less in the liver and more in peripheral tissues (Lapierre et al.,

2002), particularly in muscle and fat (Goodwin et al., 1987; Bergen et al., 1988). This segregation of catabolic pathways may allow BCAA to be differentially regulated so their plasma concentration would only decline if peripheral catabolism were upregulated. Branched-chain AA catabolism is initiated by the sequential actions of BCAT and BCKDH, the latter of which is deactivated by BCKDH-K (Brosnan and Brosnan, 2006). Infusion of EAA decreased abundance of BCKDH-K in longissimus dorsi, which remained elevated with GLC and is consistent with lower plasma concentration of BCAA during GLC infusion. However, BCAT1 mRNA expression in muscle tended to increase with EAA and decreased with GLC. Although these effects were not detected at the BCAT1 protein level, the depression in mRNA expression does not support the possibility that BCAA catabolism was upregulated in skeletal muscle during GLC infusion. Similarly, when infusion of the glucogenic precursor propionate stimulated milk protein yield in cows and caused plasma BCAA concentrations to fall, whole-body oxidation of labeled Leu to CO₂ was not accelerated (Raggio et al., 2006b).

The decreases in acetate, BHB, and NEFA concentrations in plasma in response to GLC infusion indicate that adipose lipogenesis was stimulated, as expected from the insulin response (Eisemann and Huntington, 1994; Rigout et al., 2002; Lemosquet et al., 2009). Recent work demonstrates that plasma BCAA concentrations decrease as much as NEFA during glucose tolerance tests and that obesity and insulin resistance states reduce the magnitude of NEFA and BCAA responses to insulin equally (Shaham et al., 2008; Geidenstam et al., 2013). From flux of Val through BCKHD in explants, Herman et al. (2010) estimated that adipose tissue of mice could catabolize 950 nmol of BCAA/h, compared with 830 nmol/h in skeletal muscle. Branched-chain α -keto acids decarboxylated by BCKDH can serve as primers for de novo fatty acid synthesis by fatty acid synthase in adipose (Su et al., 2015), so effects of insulin on adipose lipogenesis would preferentially affect concentrations of BCAA in plasma in conjunction with other lipogenic precursors. In support of a role for adipose tissue in BCAA utilization, transplantation of adipose tissue from wild-type mice into BCAT2 knockout littermates caused plasma BCAA concentrations to fall (Herman et al., 2010). Although many studies report decreases in plasma BCAA with glucose or insulin infusion (Mackle et al., 2000; Bequette et al., 2001; Curtis et al., 2014), the only study of BCAA metabolism in ruminant adipose tissue of which we are aware showed that BCAA-C was incorporated into fatty acids by adipose tissue from adult sheep in vitro at 2.8% of the rate that acetate-C was used (Vernon et al., 1985). In our study, the absence of an effect of GLC on expression of

the enzymes of BCAA catabolism in muscle supports adipose tissue as the preferential fate of BCAA efflux during glucose supplementation.

CONCLUSIONS

This study demonstrates stimulation of milk protein synthesis in response to 5-d EAA and glucose supplementation in lactating dairy cows. The addition of glucose to EAA infusate did not stimulate greater milk protein yield, but did decrease MUN and plasma BCAA, while stimulating skeletal muscle accretion via increased insulin signaling. These results suggest that although milk protein yield is not stimulated further with postruminal energy supplementation when EAA are not limiting for milk protein synthesis, surplus AA are spared from gluconeogenesis and catabolism and may be directed toward extra-mammary tissues leading to improved N retention in the body and lower N loss into milk. Additionally, there may be a limit to the synthetic capacity of mammary cells after 5-d infusions if a surplus of milk protein precursor plus energy supply does not promote an unlimited response in protein yield. Furthermore, these results support the preferential use of BCAA in adipose metabolism in glucose-supplemented lactating cows when EAA are in abundant supply.

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