Effects of jugular infusions of isoleucine, leucine, methionine, threonine, and other amino acids on insulin and glucagon concentrations, mammalian target of rapamycin (mTOR) signaling, and lactational performance in goats

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ABSTRACT

The supply and profile of absorbed AA may affect milk protein synthesis through hormonal changes and mammalian target of rapamycin (mTOR) signaling pathways; and Ile, Leu, Met, and Thr (ILMT) are the 4 AA that have been reported to have the greatest effect on mammary mTOR signaling. The extent to which ILMT and the other remaining AA (RAA) differ in their effects on milk protein synthesis needs to be systematically investigated. In this study, 5 lactating goats, averaging 120 ± 10 d in milk, fitted with jugular vein and carotid artery catheters, were fasted for 24 h, followed by intravenous infusions of a mixture containing AA and glucose for 8 h in a 5×5 Latin square design. The AA mixtures were formulated according to the profile of casein. The amounts of AA infused were calculated based on supplies of AA when metabolizable protein (MP) was at requirement (MR). Treatments were an infusate containing glucose without AA (NTAA); an infusate containing $3 \times$ the MR of Ile, Leu, Met and Thr (3F0R); and infusates containing 3F0R plus 1, 2, or $3 \times$ MR of RAA (3F1R, 3F2R, and 3F3R, respectively) according to amounts provided when fed to meet MP requirements for maintenance and lactation for each goat. Milk, arterial blood, and mammary tissue samples were collected immediately after halting the infusion. Relative to NTAA, supplementation of ILMT tended to increase milk protein production and plasma glucose concentrations, and increased milk and lactose production, but had no effects on production or content of milk fat. Graded supplementation of RAA tended to quadratically affect production of milk and lactose. Arterial glucose and glucagon concentrations decreased linearly, and plasma insulin concentrations

decreased quadratically with increased RAA. Mammary p70-S6K1 phosphorylation was decreased by addition of ILMT compared with NTAA but increased linearly with increased RAA infusion. Furthermore, *EIF4EBP1* gene expression was much lower for 3F-treated goats than for the NTAA treatment. Both *MTOR* and *RPS6KB1* gene expressions were decreased quadratically with increased RAA supply. These results suggested that short-term milk protein yield tended to be increased by elevated ILMT availability, and this trend was not explained by variations in mammary mTOR signaling or pancreatic hormone secretions, whereas graded increase of RAA in combination with ILMT appeared to regulate the efficiency of conversion of glucose to lactose in a manner not involving milk protein production.

Key words: amino acids, insulin and glucagon, mTOR, milk protein

INTRODUCTION

A previous meta-analysis of 846 experimental diets determined that only 25% of dietary N was recovered in milk when feeding a grass-based diet of 17.8% CP (Hristov et al., 2004), but this efficiency increased to 30% under a grass-based diet with a well-balanced addition of His, Lys, Met, and Leu to a 15% CP diet (Haque et al., 2012). Postruminal infusions of select groups of AA or single AA both increased milk protein yields to varying degrees (Chamberlain and Yeo, 2003). These observations indicate that feeding a diet well balanced in absorbed AA profile can increase the efficiency of MP use for milk production in dairy cows and reduce the cost of dietary protein input (Haque et al., 2012).

Given the limitations in increasing dietary N digestibility, improving post-absorptive N efficiency without sacrificing milk yield is the key to increasing overall dietary N efficiency. This requires a better understanding of protein synthesis regulation in the mammary glands

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(Arriola Apelo et al., 2014a). The responses of milk protein synthesis to AA supply are complex, and substrate effects may not be the primary mechanism limiting milk protein synthesis during deficiency, a concept that is supported by studies observing no significant effects on uptake of deficient AA by mammary glands (Ying et al., 2013; Lin et al., 2014) or dramatic changes in milk protein yields (Bequette et al., 1996; Weekes and Cant, 2000).

It is generally accepted that 2 pathways are involved in the EAA supply-induced change in milk protein synthesis. One is the cellular mammalian target of rapamycin (**mTOR**) pathway, which can respond to circulating AA concentrations and modulate phosphorylation of downstream proteins that regulate the rate of translation initiation and elongation (Mahoney et al., 2009). The second pathway is mediated by hormonal state. Endocrine responses to total AA supply during an imbalance can override imperfections in the circulating AA profile to maintain milk protein yield at higher levels in dairy cows (Weekes et al., 2006). Insulin and glucagon are the 2 main hormones that control the balance between glucose storage and consumption. They have been reported to have significant effects on milk protein synthesis (Mackle et al., 2000; Bobe et al., 2009; Menzies et al., 2009).

It has been reported that Ile, Leu, Met, and Thr (**ILMT**) affect fractional synthesis rates in lactogenic mammary tissue slices, and variations in fractional synthesis rates can be partly explained by the phosphorylation of proteins in the mTOR signaling pathway (Appuhamy et al., 2012; Arriola Apelo et al., 2014a,b). Kuhara et al. (1991) found that the branched-chain AA (**BCAA**) tend to stimulate insulin concentrations and decrease glucagon secretions in sheep, and a diet deficient in Met has previously been shown to decrease plasma insulin concentrations in mice (Miller et al., 2005). Additionally, our previous study showed that Thr positively affected plasma insulin in lactating rats (Xu et al., 2018). The remaining AA (**RAA**) contained in casein have also been shown to affect milk protein yield. For example, His has been found to be limiting for milk production of dairy cows fed a grass silage and cereal grain-based diet (Vanhatalo et al., 1999; Korhonen et al., 2000), and Fraser et al. (1991) found that productive N (milk N + retained N) decreased by an estimated 91% when Lys was removed from an intragastrically infused EAA mixture in dairy cows. In addition, Clark et al. (1978) observed that Trp and Cys individually stimulated milk protein synthesis at the mammary secretory cell level. However, if ILMT and RAA differ in their effects on milk protein synthesis, the mechanisms mediating the responses need to be further investigated.

The objective of this work was to investigate the response of the mTOR signaling pathway and hormonal status to varying supplies of ILMT and RAA, ranging from deficiency to a large excess, and to evaluate the relative importance of these 2 pathways in mediating anabolic and catabolic responses to ILMT and RAA. The hypothesis was that mammary mTOR signaling and hormonal responses to ILMT and RAA differ, yielding differential milk protein synthesis responses.

MATERIALS AND METHODS

Animals and Diets

This study used 5 multiparous Laoshan dairy goats, averaging (mean $\pm$ SE) 50 ± 5 kg of BW and 120 ± 10 DIM. Surgery was performed after parturition under general anesthesia to elevate the right carotid artery to a subcutaneous position, and goats were allowed 1 mo for recovery before the start of the experiment. Thus, the goats were post-peak production at the start of the experiment. Goats were housed in individual stalls before and after each experimental period and fed a pelleted diet, which was formulated to provide 10.86 MJ of ME and 103 g of MP per kg of DM according to AFRC (1993; Table 1). Feed refusals were determined daily, and the amount of pelleted diet offered was adjusted to maintain a 5% refusal rate. Goats were milked twice daily at 0800 and 1800 h, except as noted below during the infusion. All animal handling and surgical

Table 1. Ingredient composition (% of DM) and nutrient levels of the pelleted diet fed to 5 mid-lactation multiparous Laoshan dairy goats

Item	Value
Ingredient	
Corn	14.42
Soybean meal	13.61
Wheat bran	4.86
Alfalfa hay	33.78
<i>Leymus chinensis</i>	28.85
Calcium carbonate	0.19
Calcium bicarbonate	3.73
Salt	0.38
Premix ¹	0.18
Nutrients	
ME, ² MJ/kg of DM	10.86
MP, ² g/kg of DM	103
CP, % of DM	16.41
NDF, % of DM	30.84
ADF, % of DM	18.89

¹Premix contained (per kg of DM): 100 kIU of vitamin A, 250 kIU of vitamin D₃, 2,400 mg of vitamin E, 2,000 mg of nicotinic acid, 2,000 mg of Fe, 3,000 mg of Mn, 3,000 mg of Cu, 14,000 mg of Zn, 100 mg of Se, 180 mg of I, and 40 mg of Co.

²Data were calculated according to AFRC (1993) models based on contents of EAA and RUP of the ingredients measured in our laboratory.

procedures were approved by the Welfare and Health Committee of Shandong Agricultural University.

Experimental Design and Procedure

The experimental design was a 5×5 Latin square with 5-d periods. Goats were moved into individual stalls and rested for 10 d between each experimental period. The goats were placed in individual metabolism cages 2 d before each experimental period, and indwelling catheters were placed in the left jugular vein and right carotid artery. After fasting for 24 h, the goats were assigned to 8-h intravenous infusions of an AA mixture plus glucose. The AA mixtures were formulated according to the profile of casein (Table 2), and the amounts of AA infused were calculated to match their MP requirements (**MR**) for maintenance plus lactation for each goat according to AFRC (1993), based on recorded milk yield over 3 successive days before each period. Treatments were infusion of mixtures containing glucose without AA (**NTAA**), glucose plus 4 AA (Ile, Leu, Met, and Thr) at $3 \times \text{MR}$ (**3F0R**), and **3F0R** plus 1, 2, or $3 \times \text{MR}$ of the other remaining AA (**3F1R**, **3F2R**, and **3F3R**, respectively).

The infusates were made as described by Guo et al. (2017). Briefly, the AA mixture and glucose for each goat was dissolved in saline 1 d before the infusion, adjusted to a pH of 7.4, and passed through a 0.22- μm sterile filter membrane, and the final volume was adjusted to 800 mL. The solutions were infused into the jugular vein catheter using a peristaltic pump (Type W0109-1, Baoding Longer Precision Pump Co. Ltd., Hebei, China) at a rate of 100 mL/h from 0800 to 1600 h. The amount of glucose in infused solutions was calculated to provide 3.6 mg of glucose per kg of BW per minute, which was the metabolic use rate observed in lactating goats (Annison and Linzell, 1964).

Sampling and Analysis

Goats were milked at 0 and 8 h relative to the start of the infusion. Milk from the second milking (milk secreted during the 8-h interval) was collected, weighed, and sampled for composition analysis using an infrared milk composition analyzer (Type 78110, Foss Analyt-ics, Hillerød, Denmark). Before each milking, 1 IU of oxytocin was intravenously injected to each goat, to allow for complete milk removal (Bequette et al., 1996).

From 1 to 8 h after the beginning of the infusion, blood samples of the last 3 h were collected from the carotid artery into heparinized tubes at hourly intervals, centrifuged at 4°C , $1,500 \times g$ (5424 centrifuge, Eppendorf, Hamburg, Germany) for 10 min to isolate plasma, and stored at -20°C for later analysis. Plasma

Table 2. Amounts of infused AA (g per 8 h) for each treatment¹

AA	Treatment ²				
	NTAA	3F0R	3F1R	3F2R	3F3R
Ile	0	5.36	5.35	5.28	5.26
Leu	0	10.34	10.31	10.18	10.14
Met	0	3.28	3.27	3.23	3.22
Thr	0	4.49	4.48	4.42	4.40
Arg	0	0	1.38	2.73	4.07
His	0	0	1.09	2.15	3.22
Lys	0	0	2.90	5.72	8.54
Phe	0	0	3.56	7.04	10.51
Trp	0	0	0.58	1.14	1.70
Val	0	0	2.41	4.77	7.12
Ala	0	0	1.09	2.15	3.22
Gly	0	0	0.69	1.36	2.03
Glu	0	0	3.85	7.60	11.35
Gln	0	0	3.79	7.48	11.18
Asn	0	0	1.31	2.60	3.88
Asp	0	0	2.64	5.22	7.79
Ser	0	0	1.95	3.86	5.76
Pro	0	0	3.22	6.35	9.49
Cys	0	0	0.23	0.45	0.67
Tyr ³	0	0	0	0	0

¹Data calculated according to the AA profile in casein (Bequette et al., 1996).

²NTAA = no total AA; 3F0R = $3 \times$ the amount of absorbed Ile, Leu, Met, and Thr present in the base diet at MP requirements; 3F1R, 3F2R, and 3F3R = 3F0R plus 1, 2, or $3 \times$ AA (excluding Ile, Leu, Met, and Thr) present in the base diet at MP requirements.

³Tyr was substituted by equal moles of Phe because of its low solubility.

glucose, urea, total protein, and albumin concentrations were measured by an automatic biochemical analyzer (Type 7020, Hitachi, Tokyo, Japan) with commercially available kits (Maccura Biotechnology Co. Ltd., Sichuan, China). Concentrations of individual AA in plasma were determined by liquid chromatography-tandem mass spectrometry (**LC-MS/MS**), using a Prominence LC-20AD XR binary pump (Shimadzu, Kyoto, Japan) coupled to a QTRAP 5500 hybrid triple quadrupole mass spectrometer (AB Sciex, Framingham, MA). Plasma insulin and glucagon concentrations were determined by ELISA using commercially available kits (Shanghai MLBIO Biotechnology Co. Ltd., Shanghai, China).

After the second milking, goats were injected subcutaneously with lidocaine hydrochloride, the skin was incised in the upper portion of the left-side mammary gland, and approximately 0.1 g of mammary tissue was collected with a 12-gauge, 16-cm core biopsy needle (MG1522 Bard Magnum reusable core biopsy instrument; Bard Medical Division, Covington, GA). The sample was rinsed in ice-cold saline solution and immediately frozen in liquid nitrogen for real-time PCR and Western blotting analysis. After sampling, the goats received an intramuscular injection of penicillin and streptomycin to reduce the likelihood of infection.

Real-Time PCR and Western Blotting

Total RNA was prepared and analyzed by real-time PCR as described by Xu et al. (2018). Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse-transcribed using a PrimeScript RT Reagent Kit with gDNA Eraser (catalog no. RR047A; Takara Bio-Company, Shiga, Japan). Real-time PCR was carried out using the SYBR Premix Ex Taq Kit (catalog no. RR420A; Takara Bio-Company), following the manufacturer's instructions. Gene expression of *MTOR*, *EIF4EBP1*, and *RPS6KB1* were calculated as $2^{-\Delta\Delta C_t}$. Expression of *ACTB* was used as an internal control. Primer sequences used for amplification are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-16102>). The process of Western blot analysis that we followed is described in Supplemental File 1 (<https://doi.org/10.3168/jds.2018-16102>), and results are shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2018-16102>).

Statistical Analysis

Statistical analyses were performed using the Mixed procedure of the SAS software, version 9.2 (SAS Institute Inc., Cary, NC), and the following model:

$$Y_{ijk} = \mu + \alpha_i + \beta_1 \times \text{RAA} + \beta_2 \times \text{RAA}^2 + P_j + \gamma_k + e_{ijk},$$

where Y_{ijk} is the dependent variable, μ = the overall mean, α_i = the fixed effect of the ILMT treatment, β_1 = the continuous linear effect of RAA dose, β_2 = the continuous quadratic effect of RAA dose, P_j = the fixed

effect of experimental period, γ_k = the random effect of goat, and e_{ijk} = random error. Both linear and quadratic effects of RAA were tested initially. When quadratic effects were nonsignificant, they were removed from the model. Plasma concentrations of metabolites, AA, and hormones were averaged for the last 3 hourly observations and were analyzed with the same model.

The relationships between circulating AA and insulin or glucagon concentrations were assessed by Pearson correlation test. To more fully evaluate how multiple AA affect insulin and glucagon responses, multiple linear regression with backward, stepwise elimination was used to relate AA concentrations to circulating insulin and glucagon concentrations. Multicollinearity among the variables was measured by the variance inflation factor (VIF). Variables were removed until all variables retained in the model were significant at $P < 0.10$ and had $VIF < 10$. Data were reported as least squares means, and differences were considered significant at $P < 0.05$, with tendencies at $P < 0.10$.

RESULTS

Milk Yield and Composition

Supplementation of ILMT increased milk and milk lactose yields ($P = 0.04$; Table 3), and tended to increase milk protein yield ($P = 0.08$) compared with the NTAA treatment. Graded increases of RAA in combination with ILMT tended to have a quadratic effect on milk production ($P = 0.08$), a linear effect on milk lactose concentration ($P = 0.09$), and a quadratic effect on milk lactose yield ($P = 0.04$). Milk fat-to-protein ratios tended to change quadratically with increased RAA supplementation ($P = 0.09$).

Table 3. Effects of different jugular AA infusion mixtures on lactational performance of 5 mid-lactation multiparous Laoshan dairy goats¹

Item	Treatment ²					P-value (ILMT ³)	P-value (RAA ⁴)	
	NTAA	3F0R	3F1R	3F2R	3F3R		Linear	Quadratic
Milk yield, g/8 h	109.5	151.0	184.9	174.6	119.9	0.04	0.32	0.08
Milk fat								
%	2.75	2.33	2.48	2.30	1.79	0.38	0.25	0.35
g/8 h	3.42	4.27	5.18	4.32	1.70	0.34	0.12	0.13
Milk protein								
%	2.97	2.80	2.62	2.60	2.74	0.26	0.66	0.16
g/8 h	3.35	4.31	5.12	4.83	3.44	0.08	0.31	0.12
Lactose								
%	4.55	4.64	4.83	4.94	4.97	0.66	0.09	0.60
g/8 h	4.89	6.81	9.06	8.80	6.03	0.04	0.06	0.04
Fat/protein	0.97	0.90	1.04	0.98	0.71	0.66	0.19	0.09

¹Data presented as least squares means (LSM).

²NTAA = no total AA; 3F0R = 3 × the amount of absorbed Ile, Leu, Met, and Thr present in the base diet at MP requirements; 3F1R, 3F2R, and 3F3R = 3F0R plus 1, 2, or 3 × AA (excluding Ile, Leu, Met, and Thr) present in the base diet at MP requirements.

³ILMT = Ile, Leu, Met, and Thr; P-value (ILMT) = NTAA vs. all 3F treatments.

⁴RAA = other remaining AA.

Arterial Free AA Concentrations

As expected, plasma Ile, Leu, Met, and Thr concentrations ($P < 0.001$), and the total of the 4 ($P < 0.0001$), increased with the 3F treatments compared with the NTAA treatment (Table 4). Additionally, circulating Ala ($P = 0.03$) and Glu ($P = 0.08$) concentrations followed the same pattern. Concentrations of plasma Phe ($P = 0.02$), Cys ($P = 0.03$), and Tyr ($P = 0.0029$) were lower for the 3F treatments than for NTAA. As expected, 10 of the 15 RAA as well as total RAA (**TRAA**) and total AA (**TAA**) had linear or quadratic increases in arterial concentrations with increased RAA ($P < 0.05$). However, the supply of RAA had no effects on plasma His, Cys, and ILMT concentrations ($P > 0.05$). Furthermore, supplementation of RAA linearly or quadratically decreased Phe, Trp, and Tyr concentrations ($P < 0.05$).

Plasma Metabolite and Hormone Concentrations

The effects of ILMT and RAA on plasma metabolite and hormone concentrations are presented in Table 5. Circulating glucose concentrations tended to be higher

($P = 0.09$) and urea concentrations were much higher ($P = 0.04$) for the 3F treatments than those for the NTAA treatment. Arterial glucose concentrations decreased linearly with increased RAA ($P = 0.02$), whereas plasma TP and urea concentrations were not affected by RAA supplementation ($P > 0.05$).

The 3F treatments had no influence on circulating insulin concentrations compared with the NTAA treatment ($P = 0.35$), but insulin concentrations decreased quadratically with increased RAA ($P = 0.02$). Arterial glucagon concentrations declined linearly with increased RAA ($P = 0.03$), and the plasma insulin-to-glucagon ratio (**IGR**) increased quadratically ($P = 0.02$) as RAA increased when combined with ILMT.

Correlations of Circulating Hormones to AA

Pearson correlation tests showed that plasma insulin concentrations were positively correlated with circulating concentrations of Val ($P < 0.01$), Glu ($P < 0.05$), Gln ($P < 0.01$), and Ala ($P < 0.05$), and plasma glucagon had significant positive associations with Ser ($P < 0.05$; Table 6). The AA most closely related to plasma IGR ($P < 0.05$) was Val. Multiple linear regression us-

Table 4. Effects of different jugular AA infusion mixtures on AA concentrations (μM) in the plasma of carotid artery of 5 mid-lactation multiparous Laoshan dairy goats¹

AA	Treatment ²					<i>P</i> -value (ILMT ³)	<i>P</i> -value (RAA ⁴)	
	NTAA	3F0R	3F1R	3F2R	3F3R		Linear	Quadratic
Ile	18.31	69.15	63.08	59.96	59.78	<0.0001	0.09	0.51
Leu	47.3	149.9	141.6	138.0	139.0	<0.0001	0.29	0.61
Met	7.07	25.13	24.69	23.87	22.68	0.0001	0.44	0.88
Thr	10.9	73.3	88.8	101.6	111.7	0.0005	0.01	0.82
Arg	70.7	85.1	115.0	156.2	208.8	0.56	0.0001	0.54
His	40.01	45.20	40.65	39.07	40.46	0.10	0.10	0.21
Lys	35.4	32.2	160.1	318.5	507.4	0.93	<0.0001	0.36
Phe	27.50	22.61	14.08	10.25	11.12	0.02	0.0003	0.0070
Trp	35.39	33.84	17.28	7.38	4.14	0.75	<0.0001	0.09
Val	36.2	37.3	42.8	70.0	118.9	0.88	0.52	0.001
Ala	120.2	180.8	210.8	300.8	450.7	0.03	0.99	0.01
Asn	8.65	14.65	23.91	38.13	57.30	0.29	<0.0001	0.26
Cys	2.98	2.31	2.32	2.22	2.01	0.03	0.33	0.61
Gln	31.5	78.2	92.5	176.1	328.9	0.16	0.59	0.02
Glu	69.7	91.4	110.4	152.8	218.6	0.08	0.59	0.02
Gly	196.2	247.7	466.5	650.0	798.1	0.67	0.0004	0.70
Pro	34.96	38.42	42.13	47.31	53.98	0.56	0.02	0.75
Ser	16.64	25.08	29.36	42.87	65.62	0.46	0.0054	0.31
Tyr	28.04	21.60	14.44	10.31	9.20	0.0029	0.0009	0.04
ILMT	76.1	309.5	301.4	306.9	326.0	<0.0001	0.68	0.57
RAA	646	903	1,307	1,937	2,793	0.36	<0.0001	0.30
TAA ⁵	718	1,209	1,602	2,241	3,126	0.11	<0.0001	0.29

¹Data presented as least squares means (LSM).

²NTAA = no total AA; 3F0R = 3 × the amount of absorbed Ile, Leu, Met, and Thr present in the base diet at MP requirements; 3F1R, 3F2R, and 3F3R = 3F0R plus 1, 2, or 3 × AA (excluding Ile, Leu, Met, and Thr) present in the base diet at MP requirements.

³ILMT = Ile, Leu, Met, and Thr; *P*-value (ILMT) = NTAA vs. all 3F treatments.

⁴RAA = other remaining AA.

⁵TAA = total AA.

Table 5. Effects of different jugular AA infusion mixtures on plasma metabolites and hormones of 5 mid-lactation multiparous Laoshan dairy goats¹

Item	Treatment ²					<i>P</i> -value (ILMT ³)	<i>P</i> -value (RAA ⁴)	
	NTAA	3F0R	3F1R	3F2R	3F3R		Linear	Quadratic
Glucose, mM	8.22	11.82	9.94	8.18	6.54	0.09	0.02	0.94
TP, ⁵ mg/mL	78.30	78.97	82.47	81.59	76.33	0.86	0.65	0.16
Urea, mM	13.89	17.53	17.37	17.97	19.33	0.04	0.33	0.55
Insulin, μ IU/mL	105.5	127.7	87.8	94.0	146.3	0.35	0.03	0.02
Glucagon, pg/mL	379.9	333.8	305.0	284.3	271.6	0.58	0.03	0.89
IGR ⁶	0.37	0.48	0.34	0.42	0.73	0.36	0.07	0.02

¹Data presented as least squares means (LSM).²NTAA = no total AA; 3F0R = 3 $\times$ the amount of absorbed Ile, Leu, Met, and Thr present in the base diet at MP requirements; 3F1R, 3F2R, and 3F3R = 3F0R plus 1, 2, or 3 $\times$ AA (excluding Ile, Leu, Met, and Thr) present in the base diet at MP requirements.³ILMT: Ile, Leu, Met, and Thr; *P*-value (ILMT) = NTAA versus all 3F treatments.⁴RAA = other remaining AA.⁵TP = total protein.⁶IGR = insulin-to-glucagon ratio.

ing backward elimination showed that plasma insulin concentrations were mainly correlated with circulating Val ($P = 0.0002$) and Ala ($P = 0.0074$) concentrations ($R^2 = 0.28$; Table 7). Each 2.56 μ mol/L increase in circulating Ser level ($P = 0.0075$, $R^2 = 0.12$) resulted in a 1 pg/mL increase in plasma glucagon concentration, and plasma Val level accounted for 9% of the variability in circulating IGR ($P = 0.03$).

Phosphorylation and Gene Expression of mTOR Pathway Proteins

The effects of treatments on phosphorylation of proteins in the mTOR signaling pathway are shown in Table 8. The 3F treatments significantly decreased p70-S6 kinase (**p70-S6K1**) phosphorylation compared with NTAA ($P = 0.0021$), whereas supplementing ILMT had no effects on mTOR ($P = 0.15$) or eukaryotic initiation factor 4E binding protein 1 (**4E-BP1**, $P = 0.11$) phosphorylation. However, increasing RAA linearly increased phosphorylation of p70-S6K1 ($P = 0.0052$).

Expression of *EIF4EBP1* decreased ($P = 0.04$), that of *RPS6KB1* tended to decrease ($P = 0.09$), and that of *MTOR* increased numerically ($P = 0.12$) for the 3F treatments compared with NTAA (Table 9). Additionally, expression of both *MTOR* and *RPS6KB1* decreased quadratically with increased RAA ($P = 0.0012$ and $P = 0.04$, respectively).

DISCUSSION

Plasma Insulin and Glucagon Concentrations

It is well established that AA can elicit positive and negative effects on insulin release in vitro and in vivo,

which mostly depend on AA type, duration of exposure, and concentrations (Newsholme and Krause, 2012). High insulin concentrations in the 3F3R group were consistent with the stimulatory effects of AA. Administered alone at physiological concentrations, AA do not modulate glucose-induced insulin release (GSIS), but supplementation in specific combinations at physiologi-

Table 6. Correlations among circulating AA and hormones in plasma of 5 mid-lactation multiparous Laoshan dairy goats receiving jugular infusions of different AA mixtures¹

AA	Insulin	Glucagon	IGR ²
Lys	0.22	0.15	0.06
Thr	0.13	0.06	0.04
Val	0.42**	0.07	0.29*
Met	-0.14	-0.05	-0.12
Ile	0.04	-0.10	0.06
Leu	0.06	-0.07	0.05
Phe	-0.01	0.15	-0.17
His	-0.17	0.04	-0.16
Arg	0.21	0.18	0.04
Trp	-0.18	0.03	-0.16
Ser	0.26	0.35*	-0.05
Glu	0.33*	0.04	0.24
Gly	0.13	0.07	0.07
Gln	0.36**	0.22	0.17
Ala	0.26*	0.05	0.2
Tyr	-0.09	-0.01	-0.08
Pro	-0.09	-0.02	-0.04
Asp	0.22	0.18	0.07
Cys	-0.14	-0.24	0.17
ILMT ³	0.07	-0.03	0.03
RAA ⁴	0.25	0.13	0.11
TAA ⁵	0.24	0.12	0.11

¹Asterisks indicate significant Pearson correlation coefficients (r): * $P < 0.05$; ** $P < 0.01$.²IGR = insulin-to-glucagon ratio.³ILMT = Ile, Leu, Met, and Thr.⁴RAA = other remaining AA.⁵TAA = total AA.

Table 7. Multiple regression analyses for relationships between circulating AA and hormones in plasma of 5 mid-lactation multiparous Laoshan dairy goats receiving jugular infusions of different AA mixtures

Dependent variable	Independent variable	Regression coefficient	P-value	R ²
Plasma	AA, $\mu\text{mol/L}$			
Insulin, $\mu\text{IU/mL}$	Val	1.47	0.0002	0.28
	Ala	-0.24	0.0074	
Glucagon, pg/mL	Ser	2.56	0.0075	0.12
IGR ¹	Val	0.0033	0.03	0.09

¹IGR = insulin-to-glucagon ratio.

cal concentrations or individually at elevated levels can promote GSIS (Newsholme et al., 2007; Newsholme and Krause, 2012). This suggests that multiple AA may have independent and additive effects where provision of a single AA at lower levels is not adequate to stimulate a detectable response. However, the AA profile resulting from RAA and ILMT supplementation may have provided a combination of positive and negative signals or some type of synergy resulting in the intermediate infusions of RAA, in turn resulting in lower insulin concentrations in 3F1R and 3F2R groups than those in the 3F0R and 3F3R groups. Previous work found that a synergistic effect was exerted by Arg and Leu, but not by Arg and Lys, in the stimulation of insulin release when these AA were administered intravenously (Floyd et al., 1970). Thus some component of the response appears to be additive, and another component responds synergistically to specific combinations of AA, in the ILMT and RAA treatments.

Arterial concentrations of glucagon declined linearly with increasing RAA supply, similar to research in rats where plasma glucagon concentrations were negatively correlated with circulating TAA concentrations (Xu et al., 2018). According to previous findings, divergent effects may take place depending on the type of AA. For instance, Arg, Ala, and Gln have been identified as potent stimulators of glucagon release (Pipeleers et al., 1985; Dumonteil et al., 2000), whereas Ile can inhibit α -cell secretion, and Leu was a positive stimulator at

physiological concentrations but inhibitory at elevated levels (Leclercq-Meyer et al., 1985). In addition, the concentrations of AA in plasma may also play an important role in glucagon secretion. Glucagon release stimulated by AA via an intracellular Ca influx, and concentrations of Ala and Glu in the micromolar range, contributed to the triggering of intracellular Ca oscillations, whereas these signals were inhibited with millimolar levels of these AA (Bode et al., 1999), which could partly account for the decline in glucagon concentration with increasing RAA. From the perspective of AA, the glucagon responses to the RAA supply in this trial were likely the result of a combination of stimulation and repression induced by AA, with the negative effects exceeding the positive.

Although bilaterally counterbalanced, plasma insulin and glucagon concentrations varied in the same direction initially with $1 \times$ RAA supplementation. Similar observations were reported in a previous study (Lemosquet et al., 2009). The quadratic effects of RAA on plasma IGR were mainly associated with the 3F3R treatment, indicating that the greatest concentrations of RAA have different effects on insulin and glucagon concentrations.

Circulating AA and Glucose Concentrations

As expected, arterial Ile, Leu, Met, and Thr concentrations were increased by supplementation of ILMT.

Table 8. Effects of different jugular AA infusion mixtures on phosphorylation [phosphorylation level (P)/total] of mammalian target of rapamycin (mTOR) pathway proteins in mammary gland tissue from 5 mid-lactation multiparous Laoshan dairy goats¹

Item	Treatment ²					P-value (ILMT ³)	P-value (RAA ⁴)	
	NTAA	3F0R	3F1R	3F2R	3F3R		Linear	Quadratic
P-mTOR/T-mTOR	1.43	1.01	1.26	1.29	1.08	0.15	0.66	0.29
P-4E-BP1/T-4E-BP1	1.25	0.96	1.18	1.23	1.12	0.11	0.25	0.20
P-p70-S6K1/T-p70-S6K1	1.54	0.79	1.12	1.28	1.29	0.0021	0.0052	0.17

¹Data presented as least squares means (LSM).

²NTAA = no total AA; 3F0R = $3 \times$ the amount of absorbed Ile, Leu, Met, and Thr present in the base diet at MP requirements; 3F1R, 3F2R, and 3F3R = 3F0R plus 1, 2, or $3 \times$ AA (excluding Ile, Leu, Met, and Thr) present in the base diet at MP requirements.

³ILMT = Ile, Leu, Met, and Thr; P-value (ILMT) = NTAA vs. all 3F treatments.

⁴RAA = other remaining AA.

Table 9. Effects of different jugular AA infusion mixtures on gene expression of mTOR pathway proteins in mammary gland tissue from 5 mid-lactation multiparous Laoshan dairy goats¹

Item	Treatment ²					<i>P</i> -value (ILMT ³)	<i>P</i> -value (RAA ⁴)	
	NTAA	3F0R	3F1R	3F2R	3F3R		Linear	Quadratic
<i>MTOR</i>	0.92	1.13	0.55	0.42	0.74	0.12	0.0005	0.0012
<i>EIF4EBP1</i>	1.10	0.86	0.83	0.83	0.85	0.04	0.64	0.57
<i>RPS6KB1</i>	0.77	0.45	0.15	0.09	0.27	0.09	0.44	0.04

¹Data presented as least squares means (LSM).

²NTAA = no total AA; 3F0R = 3 × the amount of absorbed Ile, Leu, Met, and Thr present in the base diet at MP requirements; 3F1R, 3F2R, and 3F3R = 3F0R plus 1, 2, or 3 × AA (excluding Ile, Leu, Met, and Thr) present in the base diet at MP requirements.

³ILMT = Ile, Leu, Met, and Thr; *P*-value (ILMT) = NTAA vs. all 3F treatments.

⁴RAA = other remaining AA.

Interestingly, plasma Ala and Glu levels also followed the same pattern. Circulating AA concentrations are determined by the balance of absorption, production (non-essential AA), anabolic use, and catabolic use. Isoleucine and Leu are deaminated in peripheral tissues, and Ala and Glu are the main products of nitrogen from the BCAA metabolism in muscle (Korhonen et al., 2002); thus the increase of Ala and Glu likely reflects additional synthesis in the peripheral tissues to transport the N from deaminated Ile and Leu back to the liver. Oxidation of Met across the portal-drained viscera (Lobley and Lapierre, 2003) and the oxidation of Thr in the pancreas (Le Floch et al., 1997) may also contribute to increased circulating Ala and Glu concentrations.

In contrast, circulating concentrations of Phe and Tyr were lower in 3F groups than among those in the NTAA group, which agreed with previous reports of Leu decreasing plasma concentrations of aromatic AA such as Phe (Harper et al., 1984), and Met infusions or excessive feeding levels decreasing plasma Phe concentration (Guinard and Rulquin, 1995; Varvikko et al., 1999). Uptake of BCAA by the tissue occurs through the L-system, which is also a carrier of Phe (Hyde et al., 2003). Thus, one possible explanation for the decrease in Phe concentrations in 3F treatments could be the competition or antagonism between these AA in transfer processes resulting in altered use of AA by the tissues. Plasma Phe and Tyr concentrations were likely influenced by the lack of Tyr in the infusates due to low solubility and potential changes in Phe degradation to Tyr. In addition, graded supplementation of RAA helped to reduce the Phe concentration and had no effects on His concentration, which can be partly explained by their metabolism in the liver. The degradative enzymes of His and Phe are restricted predominantly to the liver, and studies have found substantial hepatic removal of these 2 AA across the liver in dairy cows (Lobley and Lapierre, 2003). Such data suggest that the circulating concentration of a particular AA

results not only from its character in metabolism but also by the supply of other AA.

Evidence from previous studies suggests that the maximum contribution of AA to glucose synthesis varies from 5 to 30% (Lindsay, 1980; Reynolds et al., 1992; Lozano et al., 2000). In the current study, we found that circulating glucose concentrations tended to increase with addition of ILMT, which agreed with the previous report that addition of ruminally protected Met to the diet increased arterial concentrations of glucose (Berthiaume et al., 2001) and that abomasal infusion of Leu had no effects on plasma glucose concentrations (Huhtanen et al., 2002). Catabolism of Leu results in acetyl CoA, which cannot contribute net carbon to the tricarboxylic acid (TCA) cycle for use in gluconeogenesis, whereas Ile, Met, and Thr enter later in the TCA cycle, thus contributing net carbon that can be removed to create glucose. Therefore Ile, Met, and Thr, but not Leu, may provide extra carbon for glucose synthesis in the 3F treatments. However, graded supplementation of RAA linearly decreased circulating glucose, which may result from simulation of insulin secretion and a linear decrease in glucagon concentrations. It has been reported that a hyperinsulinemic, hypoglycemic state could be induced by protein meals (Hsu et al., 2001; de Lonlay et al., 2005). However, the biochemical mechanisms underlying this phenomenon require further investigation.

Mammary mTOR Pathway Signaling

Nutrients, especially AA, modulate the activity of several proteins involved in the control of mRNA translation. Activity of these signaling events is largely manifested through changes in phosphorylation, and a key pathway is the mTOR pathway (Proud, 2000). However, in the current work, goats receiving the 3F treatments showed no increases in mTOR or 4E-BP1 phosphorylation and even showed a reduction in p70-S6K1 phosphorylation compared with the NTAA treat-

ment. This observation was contrary to *in vitro* work (Sancak et al., 2010; Appuhamy et al., 2012), where Ile and Leu induced mTOR phosphorylation, but was consistent with a report of no effect of Met on p70-S6K1 phosphorylation and inhibition by Thr in murine and bovine mammary cells (Prizant and Barash, 2008). It is perhaps noteworthy that activation of the mTOR pathway is saturable with respect to AA (Arriola Apelo et al., 2014a), and thus the lack of a response could indicate oversupply at the base level. However, this seems unlikely for NTAA, as the goats should have been severely deficient in AA supply. It has been found in past studies that reducing the Lys-to-Ile ratio while maintaining an ideal ratio of Lys to Met and fixed ratios of other EAA in medium increased the mTOR phosphorylation of the mammary epithelial cell line, suggesting that the phosphorylation of mTOR may be affected by the AA profile (Dong et al., 2018). Thus, the ILMT-to-RAA ratio in the plasma of goats in this study may also partly account for the variability in mTOR phosphorylation of the mammary gland.

In a review of the literature, Avruch et al. (2006) noted that insulin can enhance Rheb-GTP charging, through the ability of activated Akt to inhibit the Rheb-GTPase-activating function of the tuberous sclerosis heterodimer, which enables the TOR complex 1 (TORC1) to achieve an active configuration. Glucagon has also been reported to stimulate phosphorylation of mTOR in rat hepatocytes (Mothe-Satney et al., 2004). Thus, the lack of an increase in mTOR phosphorylation corresponding to addition of ILMT and RAA may be due to the effects of low insulin concentrations in 3F1R and 3F2R treatments, as well as to the linear decreases in glucagon with increased RAA. The linear increases in p70-S6K1 phosphorylation with increasing RAA infusion suggested that RAA also independently stimulated downstream proteins in the mTOR signaling pathway. Collectively, the 4-AA addition showed no positive effects on mTOR signaling, which did not match what would be expected from the literature—that is, that these 4 AA all increased protein synthesis rates independently—suggesting that there are likely other factors affecting mTOR.

Milk Yield and Composition

Supplementation with ILMT tended to increase milk protein yield compared with the NTAA infusion. This observation was consistent with previous studies using mammary tissue (Arriola Apelo et al., 2014a), which found that increasing concentrations of Ile, Leu, Met, and Thr simultaneously increased casein synthesis curvilinearly. Considering that insulin and glucagon concentrations, as well as the phosphorylation status of

the mTOR signaling pathway, were not altered by the 3F0R treatment, the increased milk protein production shown in our study may be attributed to the substrate effect of ILMT. Graded supplementation of RAA had a near tendency for a positive quadratic effect on milk production, with a maximum near the 1× RAA level, which suggested that elevated RAA supply was initially stimulatory and subsequently inhibitory. Garlick (2004) observed that adverse effects were apparent when most (if not all) AA were consumed in amounts disproportionate to the normal diet composition in animals, with the greatest sensitivity to Met, Cys, and His.

In our study, milk lactose yield was increased by the addition of ILMT and changed quadratically with the increased RAA. Similarly, Galindo et al. (2015) found that abomasal infusion of free AA increased energy-corrected lactose yields in dairy cows. Given the role of α -lactalbumin as a subunit of lactose synthase, one would expect some link between lactose production and milk protein production. Other studies have found that Leu and Ile stimulate glucose uptake in an insulin-independent manner in C2C12 myotubes (Doi et al., 2003). Supplementation of ILMT may also potentiate glucose uptake by mammary glands, but the mechanism may be more due to increased protein synthesis than to actual transport activity. The major role lactose plays in terms of the osmotic potential of milk would seem to explain the common responses for milk and lactose yields in response to ILMT and RAA treatments.

CONCLUSIONS

Results of the present study showed that excessive postruminal supply of ILMT tended to increase short-term milk protein yield of mid-lactating goats in spite of reduced p70-S6K1 phosphorylation and unchanged insulin and glucagon concentrations, suggesting a mass-action substrate response. Graded increases of RAA in combination with ILMT resulted in increased efficiency of conversion of glucose to lactose in a manner not involving milk protein production, which may be due to the quadratically increased insulin secretion and linearly decreased glucagon secretion with elevated RAA. More knowledge of the regulation of pancreatic hormone secretions and mammary mTOR signaling by AA and the interactions among these 2 pathways and AA supply relative to the regulation of protein synthesis is required for these concepts to be incorporated into milk protein prediction models.

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