Effects of graded removal of lysine from an intravenously infused amino acid mixture on lactation performance and mammary amino acid metabolism in lactating goats

C. L. Guo,* Y. T. Li,* X. Y. Lin,* M. D. Hanigan,† Z. G. Yan,* Z. Y. Hu,* Q. L. Hou,* F. G. Jiang,* and Z. H. Wang*¹

*Ruminant Nutrition and Physiology Laboratory, College of Animal Science and Technology, Shandong Agricultural University, Taian 271018, P. R. China

†Department of Dairy Science, Virginia Tech, Blacksburg 24061

ABSTRACT

To investigate responses of milk protein synthesis and mammary AA metabolism to a graded decrease of postruminal Lys supply, 4 lactating goats fitted with jugular vein, mammary vein, and carotid artery catheters and transonic blood flow detectors on the external pudic artery were used in a 4 × 4 Latin square experiment. Goats were fasted for 24 h and then received a 9-h intravenous infusion of an AA mixture plus glucose. Milk yield was recorded and samples were taken in h 2 to 8 of the infusion period; a mammary biopsy was performed in the last hour. Treatments were graded decrease of lysine content in the infusate to 100 (complete), 60, 30, or 0% as in casein. Lysine-removed infusions linearly decreased milk yield, tended to decrease lactose yield, and tended to increase milk fat to protein ratio. Milk protein content and yield were linearly decreased by graded Lys deficiency. Mammary Lys uptake was concomitantly decreased, but linear regression analysis found no significant relationship between mammary Lys uptake and milk protein yield. Treatments had no effects on phosphorylation levels of the downstream proteins measured in the mammalian target or rapamycin pathway except for a tended quadratic effect on that of eukaryotic initiation factor 2, which was increased and then decreased by graded Lys deficiency. Removal of Lys from the infusate linearly increased circulating glucagon and glucose. Removal of Lys from the infusate linearly decreased arterial and venous concentrations of Lys. Treatments also had a significant quadratic effect on venous Lys, suggesting mechanisms to stabilize circulating Lys at a certain range. The 2 infusions partially removing Lys resulted in a similar 20% decrease, whereas the 0% Lys infusion resulted in an abrupt 70% decrease in mammary Lys uptake compared with that

of the full-AA mixture infusion. Consistent with the abrupt decrease, mammary Lys uptake-to-output ratio decreased from 2.2 to 0.92, suggesting catabolism of Lys in the mammary gland could be completely prevented when the animal faced severe Lys deficiency. Mammary blood flow was linearly increased, consistent with the linearly increased circulating nitric oxide by graded Lys deficiency, indicating mechanisms to ensure the priority of the mammary gland in acquiring AA for milk protein synthesis. Infusions with Lys removed increased mammary clearance rate of Lys numerically by 2 to 3 fold. In conclusion, the decreased milk protein yield by graded Lys deficiency was mainly a result of the varied physiological status, as indicated by the elevated circulating glucagon and glucose, rather than a result of the decreased mammary Lys uptake or depressed signals in the mTOR pathway. Mechanisms of Lys deficiency to promote glucagon secretion and mammary blood flow and glucagon to depress milk protein synthesis need to be clarified by future studies.

Key words: goat, lactation, lysine, milk protein

INTRODUCTION

Based on present knowledge of AA nutrition, postabsorptive efficiency of N use for milk or tissue proteins is determined by the amount of MP supplied relative to animal needs, the profile of absorbed EAA, and the rate of protein synthesis. Postabsorptive N efficiency would then be maximized if we could match an animal's MP needs exactly with a diet well balanced in absorptive EAA profile. By maximal utilization of the strategy, Baker (1996) demonstrated that postabsorptive N efficiency in pigs could be increased to 85%, which is in agreement with the theoretical efficiency of the mammary gland to convert an ideal EAA profile into milk protein proposed by AFRC (1993).

The ideal EAA profile and requirements for EAA are at present evaluated according to the theory of limiting AA, which predicates that protein synthesis is limited

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¹Corresponding author: zhwang@sdau.edu.cn

by the most deficient EAA relative to requirement (Mitchell and Block, 1946). The predication is obviously violated by observations that suggest the supply of individual EAA has independent and additive effects on protein synthesis (Hanigan et al., 2000), which implies that the theory may be an oversimplification and is inappropriate to predicate the responses of milk protein synthesis to EAA supply (Arriola Apelo et al., 2014a).

The relationship of EAA supply with protein synthesis is much more complex than what the limiting AA theory describes. Though it cannot be excluded, mass action may not be the main mechanism for the supply of individual EAA to affect protein synthesis. It has long been recognized that the deficiency of an AA must be severe to have any effect on the concentrations of intracellular aminoacylated transfer RNA, the actual substrate for protein synthesis (Rogers, 1976; Flaim et al., 1982a,b). In respect to the mammary glands, they seem to have the ability to modify AA extraction efficiencies to meet a preset need of AA utilization (Mackle et al., 2000). For instance, removal of individual EAA (Arg, His, Lys, or Met) from an intravenously infused AA mixture had no significant effects on their respective mammary uptake in lactating goats restrictively fed a basal diet meeting only maintenance MP requirements (Ying et al., 2013). The liver modulates AA supply for peripheral tissues, including the mammary glands, but largely in response to recycling of the excess not removed by peripheral tissues (Arriola-Apelo et al. 2014a). The endocrine system also plays a role in mediating the effects of EAA supply on protein synthesis. Insulin (Bequette et al., 2001; Lemay et al., 2007; Bionaz and Loores, 2011), prolactin, glucocorticoid (Doppler et al., 1989), and hormones in the somatotrophic axis (Cant et al., 1999) have all been shown to affect milk protein synthesis. However, these effects may not be all direct as the supply of some EAA affect the secretion of insulin (Davis et al., 2003; Bolster et al., 2004; Xiao et al., 2014), glucagon (Tovar et al., 2002), IGF-I (Wheelhouse et al., 1999; Noguchi, 2000; Stubbs et al., 2002), and parathyroid hormone (Conigrave et al., 2004). Supply of individual EAA may also affect protein synthesis through the mechanistic target of rapamycin complex 1 (**mTORC1**), which is an important intracellular signaling pathway that integrates environmental and intracellular signals to regulate cell growth and proliferation (Kim et al., 2013). A recent in vitro study using mammary tissue slices from lactating cows demonstrated that Ile, Leu, Met, and Thr affected casein synthesis through the mTORC1 pathway (Arriola Apelo et al., 2014b,c); the respective supply of these 4 EAA may also affect milk protein synthesis through mTORC1 pathway.

The relative importance of individual mechanisms mediating the effects of EAA supply on milk protein synthesis has not been fully investigated. The aim of the present study was to observe the effects of a graded deficiency of Lys on milk production, mammary AA metabolism, circulating hormone levels, and phosphorylation status of selected agents in the mTORC1 pathway in lactating goats.

MATERIALS AND METHODS

Animals and Diet

Four multiparous Laoshan dairy goats averaging 110 ± 10 DIM and 45 ± 5 kg of BW were used. Surgery was performed about 1 mo before the start of the experiment to elevate the right carotid artery to a subcutaneous level and fit a blood flow detector (6 mm, Transonic Systems Inc., Ithaca, NY) to the right external pudic artery. Goats were housed in individual stalls before and after each experimental period, and fed and milked twice daily at 0800 and 1800 h. The diet was pelleted and predicted to provide 49.44 g of MP/kg of DM and 10.86 MJ of ME/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 allowed free access to water throughout the experiment. All animal-handling and surgical procedures were approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University.

Experimental Design and Procedure

The experimental design was a 4×4 Latin square. Treatments were intravenous infusion of an AA mixture containing 100 (complete), 60, 30, or 0% of the Lys present in casein. Temporal catheters were introduced into the left side jugular vein, right side carotid artery, and right side mammary vein of each goat 2 d before each experimental period, and goats were moved into individual cages. After fasting for 24 h, goats received a 9-h infusion of an AA mixture and glucose. Feeding was restored after completion of each infusion. Goats were moved into individual stalls and rested for 10 d between each experimental period. Similar starvation-refeeding procedures have been used in lactating goats (Chaiyabutr et al., 1983) and lactating cows (Toerien et al., 2010). No carryover effects on milk production were observed in those experiments.

The complete AA mixture was formulated according to the profile of casein (Table 2). Lysine was substituted by equal moles of glutamate to achieve the Lys deficient infusates. The infusion rate was set to match the MP

requirement of each goat according to AFRC (1993) based on milk yield recorded for 3 successive days before the infusion. The amount of total AA infused ranged from 4.6 to 6.5 g/h. The amount of glucose infused was calculated to provide 3.6 mg of glucose/kg of BW per minute, which was the observed metabolic use rate in lactating goat measured using isotope tracer technology (Annison and Linzell, 1964).

Infused solutions were made fresh on the day before the infusion. The appropriate amount of AA mixture and glucose for each goat was dissolved in saline, adjusted to a pH of 7.4 using HCl and NaOH, passed through a 0.22- μ m sterile filter membrane, and kept at 4°C until use. The final volume of each solution was 900 mL. On the day of infusion, solutions were warmed to 37°C in a water bath and infused into the jugular vein catheter using a peristaltic pump (Type W0109-1, Baoding Longer Precision Pump Co. Ltd., Hebei, China)

Table 1. Ingredients, nutrient, and EAA compositions of the pelleted diet

Item	Value
Ingredient, % of DM	
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	49.44
CP, ³ %	16.41
NDF, ³ %	30.84
ADF, ³ %	18.89
EAA, ⁴ g/kg of DM	
Lys	10.31
Met	2.69
His	4.32
Arg	10.88
Thr	7.02
Val	9.04
Ile	10.85
Leu	11.48
Phe	9.59
Trp	0.61
Lys/MP, %	20.85
Met/MP, %	5.44

¹Contains per kilogram of premix: 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.

²Calculated data.

³Laboratory determined data.

⁴Calculated data from a laboratory determined data set of AA composition of feed ingredients.

Table 2. Amino acid profile of the complete AA mixture¹

Item	Content, g/100 g
EAA	
Lys	7.49
Met	2.82
Leu	8.89
Ile	4.61
Val	6.24
Thr	3.86
Phe	4.75
His	2.82
Arg	3.57
NEAA	
Trp	1.49
Ala	2.82
Gly	1.78
Glu	9.95
Gln	9.80
Asn	3.40
Asp	6.83
Ser	5.05
Pro	8.32
Cys	0.59
Tyr	4.90

¹Adopted from Bequette et al. (1996).

at a rate of 100 mL/h. Infusion was started at 0700 h and stopped at 1600 h.

Sampling and Analysis

Goats were milked twice in each experimental period at 1 and 8 h after the start of the infusion. Milk collected at the second milking (milk secreted during the 7 h after the first milking) was collected, weighed, recorded, and sampled for later analysis. Prior to each milking, 1 IU of oxytocin was intravenously injected into each goat to allow for complete milk removal (Bequette et al., 1996). Milk samples were divided into 2 subsamples: one for AA determination after hydrolysis using an AA analyzer (Type 835-50, Hitachi, Tokyo, Japan), and the other for milk composition analysis using an infrared milk composition analyzer (Type 78110, FOSS, Hillerød, Denmark).

Five milliliters of blood was simultaneously collected from the carotid artery and mammary vein into heparinized evacuated tubes at hourly intervals from 1 to 8 h after the start of the infusion. Animals were kept in standing position during blood sampling. A total of 7 samples were taken from each blood vessel and were pooled together and kept at -20°C for later analysis of free AA concentration using an AA analyzer (Type 835-50, Hitachi). Plasma was prepared from an aliquot of each blood sample taken from the mammary vein and used for latter analysis of metabolites and hormones. Plasma urea nitrogen and glucose were determined by an automatic biochemical analyzer (Type 7020, Hita-

chi), total protein and nitric oxide were determined by commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and IGF-I, insulin, glucagon, and growth hormone (**GH**) were determined by RIA using commercial assay kits (Tianjin Jiuding Biochemical Engineering Co. Ltd., Tianjin, China). Mammary blood flow during each infusion period was measured using a transit time ultrasonic flow system (Type MC6PSS -LS -WCS10 -GC, Transonic Systems Inc.) calibrated according to the manufacturer's instructions.

Biopsies were taken from the upper portion of the left side mammary gland following the second milking (between h 8 and 9 of the infusion) during each period. Mammary biopsies were performed using a 12-gauge, 16-cm MG1522 Bard Magnum reusable core biopsy instrument (Bard Medical Division, Covington, GA). About 0.1 g of mammary tissue was sampled after subcutaneous injection of lidocaine hydrochloride and skin incision at the sampling position. Mammary tissue was immediately rinsed in ice-cold saline, frozen, and stored in liquid nitrogen for latter analysis of phosphorylation of ribosomal protein S6 kinase beta-1 (p70S6k), eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), and eukaryotic translation initiation factor 2 α (eIF2 α) by Western blotting.

Calculation and Statistics

Mammary clearance rates ($K_{\text{clearance}}$; Hanigan et al., 1998) were calculated to assess tissue affinity for blood metabolites:

$$K_{\text{clearance}}, \text{ L/time} = [(CA - CV)/CV] \times BF,$$

where CA = arterial concentration of the measured AA, CV = venous concentration of the measured AA,

and BF = mammary blood flow. Mammary uptake ($\mu\text{mol/h}$) of individual AA was calculated by multiplying arteriovenous concentration differences with mammary blood flow times 2 to express on a whole-tissue basis. Mammary uptake-to-milk output ratios (**U:O**) for individual AA were calculated as mammary uptake of the AA divided by the amount secreted in milk during the 7 h between milkings. Relative recovery of each infused EAA to milk protein was calculated by dividing the amount of individual EAA secreted into milk protein by the amount of that EAA infused. The recovery of Lys in the 0% Lys infusion treatment was incalculable, as no Lys was infused.

Data were subjected to mixed-effect regression analysis by SAS (version 9.0, SAS Institute Inc., Cary, NC). The statistic model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk},$$

where μ = overall mean, α_i = class effect of experimental period, β_j = continuous effect of lysine dose, γ_k = random effect of animal, and e_{ijk} = random error. Both linear and quadratic effects of Lys dose were tested initially. When quadratic effects were nonsignificant, they were removed from the model. Significance was declared at $P \leq 0.05$ and tendency was declared at $P \leq 0.1$.

RESULTS

Milk Yield and Composition

Removing Lys from the infusate linearly decreased milk yield, milk protein content, and milk protein yield ($P < 0.05$; Table 3). Lactose yield was decreased and milk fat to protein ratio was increased linearly to the declared tendency level ($P < 0.1$). Milk fat was

Table 3. Effects of graded removal of Lys from the AA mixture infused into jugular vein on lactation performance of lactating goats

Item	Content of Lys in the complete mixture				SE	P-value	
	100%	60%	30%	0%		Linear	Quadratic
Milk yield, g/7 h	408.30	363.75	314.00	344.25	0.317	0.045	0.194
Milk fat							
%	5.16	5.23	5.07	5.81	0.006	0.405	0.471
g/7 h	20.76	19.26	15.12	18.11	0.020	0.102	0.243
Milk protein							
%	4.37	4.13	3.96	3.73	0.001	0.003	0.837
g/7 h	17.37	14.74	12.28	12.49	0.013	0.004	0.277
Lactose							
%	4.46	4.56	4.54	4.56	0.002	0.448	0.650
g/7 h	18.62	16.57	14.32	15.75	0.017	0.081	0.266
Fat/protein	1.18	1.28	1.31	1.57	0.002	0.061	0.451

unaffected by treatments except for a near tendency decrease in milk fat yield by Lys removed infusions.

Arterial Free AA Concentrations

Graded removal of Lys from the infusate linearly decreased arterial concentration of Lys ($P < 0.05$; Table 4). The decrease was sharp and we noted a near tendency for a quadratic effect ($P = 0.132$) with the 60% and 30% Lys treatments, which harvested a similar arterial concentration of Lys; the concentration was about one-third of that of the complete mixture infusion but was more than 2 times of that of the 0% Lys treatment. Apart from Lys, arterial concentrations of Thr, Gly, and Tyr were also linearly decreased ($P < 0.05$) and the concentrations of Arg and Pro tended to be linearly decreased ($P < 0.1$) with the 0% Lys treatment. Treatments tended to affect arterial concentration of Thr quadratically ($P < 0.1$), with the concentration in the 30% Lys treatment higher than those in the 30 and 0% Lys treatments. Arterial concentrations of all the other measured AA and total AA (the sum of the entire measured AA) were unaffected but numerically decreased by Lys removed from the infusions.

Venous AA Concentration

Venous concentration of Lys decreased linearly and quadratically with decreased content of Lys in the infusate ($P < 0.05$; Table 5). The decrease was as sharp as that in arterial plasma; infusing the 60 or 30% Lys infusate decreased venous Lys to 27%, and infusing the

0% Lys infusate decreased venous Lys to 14% of that of the complete mixture infusion. For those AA that were significantly decreased (Thr and Gly) or tended to be decreased (Tyr, Arg, and Pro) in arterial plasma by decreased Lys infusions, their decreases in venous plasma were not significant but were to a near tendency level; however, Tyr decreased to the declared tendency level. Venous concentrations of all the other measured AA and total AA were unaffected but were numerically decreased by the 0% Lys treatment except for that of venous Val and Met.

Mammary Clearance Rates of AA

Graded removal of Lys from the infusate had no significant effects on mammary clearance rates of the AA measured ($P > 0.05$; Table 6). Numerically, mammary clearance rate of Lys increased by 3.1, 2.6, and 2.2 fold, respectively, for the 60, 30, and 0% Lys treatment compared with that of complete mixture infusion, but the differences were not significant due to the large variation in measurements.

Mammary Uptakes of AA

Graded removal of Lys from the infusate linearly decreased mammary uptake of Lys ($P < 0.05$; Table 7). The decrease was numerically slight until all the Lys was removed from the infusate when more than 70% of mammary uptake of Lys was cut down, which underscores the priority of milk protein synthesis in acquiring AA and the buffering capacity of endogenous

Table 4. Effects of graded removal of Lys from the AA mixture infused into the jugular vein on AA concentrations in the plasma of carotid artery of lactating goats

Item, μM	Percentage of Lys in complete AA mixture				SE	<i>P</i> -value	
	100%	60%	30%	0%		Linear	Quadratic
EAA							
Lys	320.14	109.62	113.42	46.32	0.551	0.002	0.132
Thr	188.13	61.26	105.32	87.63	1.292	0.040	0.093
Val	240.30	185.88	223.44	187.67	0.450	0.415	0.768
Met	33.72	26.12	28.34	21.71	0.094	0.303	0.967
Ile	83.38	49.45	72.73	42.25	0.234	0.217	0.901
Leu	130.31	81.41	112.39	65.11	0.374	0.208	0.965
Phe	85.50	46.67	70.02	46.20	0.196	0.162	0.604
His	69.10	39.29	56.06	39.26	0.169	0.207	0.615
Arg	167.97	91.49	134.15	71.55	0.338	0.052	0.788
NEAA							
Ser	84.02	42.33	64.88	53.48	0.196	0.273	0.304
Glu	361.8	193.2	289.7	182.8	0.982	0.192	0.676
Gly	572.3	285.5	469.6	341.7	1.499	0.030	0.473
Ala	181.32	159.92	166.71	146.24	0.492	0.556	0.999
Cys	58.51	23.20	63.39	19.03	0.190	0.231	0.866
Tyr	87.69	75.30	76.08	74.97	0.050	0.043	0.159
Pro	291.63	156.70	203.74	139.78	0.602	0.064	0.474
Total AA	2,975.32	1,645.98	2,291.97	1,586.94	6.448	0.121	0.541

Table 5. Effects of graded removal of Lys from the AA mixture infused into jugular vein on AA concentrations in the plasma of mammary vein in lactating goats

Item, μM	Percentage of Lys in complete AA mixture				SE	<i>P</i> -value	
	100%	60%	30%	0%		Linear	Quadratic
EAA							
Lys	271.59	74.09	74.09	36.83	0.012	0.004	0.035
Thr	139.89	54.54	87.29	74.08	0.340	0.148	0.158
Val	191.90	148.87	193.40	157.67	0.448	0.677	0.867
Met	21.76	20.30	25.23	16.41	0.095	0.746	0.658
Ile	60.86	40.40	51.11	29.29	0.168	0.162	0.956
Leu	94.35	66.89	79.58	47.90	0.269	0.188	0.911
Phe	66.42	43.24	60.30	39.30	0.154	0.225	0.898
His	59.76	34.32	43.79	32.71	0.160	0.193	0.582
Arg	127.16	82.39	113.16	55.66	0.319	0.115	0.819
NEAA							
Ser	69.81	35.24	58.38	45.33	0.178	0.366	0.407
Glu	316.10	169.10	232.40	148.00	0.886	0.155	0.660
Gly	497.00	272.10	407.40	307.30	1.209	0.275	0.492
Ala	154.72	127.22	152.36	125.56	0.536	0.722	0.974
Cys	49.96	28.29	62.91	18.08	0.165	0.294	0.460
Tyr	80.36	72.54	71.51	70.07	0.048	0.072	0.465
Pro	246.63	140.22	202.74	110.15	0.708	0.165	0.893
Total AA	2,448.26	1,426.20	1,924.72	1,330.08	5.902	0.164	0.647

Lys sources in mitigating short-term Lys deficiency. Removal of Lys from the infused mixture more than halved mammary uptake of Thr, but the decreases were statistically not significant ($P = 0.173$). Treatments had no significant effects on mammary uptakes of the other AA measured.

U:O of AA

Graded removal of Lys from the infusate decreased mammary U:O of Lys to a near significant level ($P = 0.059$; Table 8). Mammary uptake of Lys was 2 times (2.20~2.30) more than its output from milk unless all

Table 6. Effects of graded removal of Lys from the AA mixture infused into jugular vein on mammary clearance rates ($K_{\text{clearance}}$) of AA in lactating goats¹

Item, L/7 h	Percentage of Lys in complete AA mixture				SE	<i>P</i> -value	
	100%	60%	30%	0%		Linear	Quadratic
EAA							
Lys	17.58	53.79	46.38	37.95	0.213	0.407	0.181
Thr	32.06	30.82	26.46	43.49	0.219	0.711	0.604
Val	26.75	31.58	17.42	24.93	0.291	0.623	0.321
Met	81.14	42.31	19.96	69.35	0.476	0.649	0.240
Ile	37.15	62.49	70.04	70.50	0.328	0.335	0.679
Leu	41.50	60.70	42.84	52.42	0.391	0.891	0.856
Phe	29.80	20.25	20.93	35.18	0.210	0.854	0.473
His	16.45	39.64	62.32	45.04	0.260	0.264	0.562
Arg	30.55	33.11	27.74	56.10	0.204	0.341	0.419
NEAA							
Ser	20.58	27.19	19.22	46.59	0.174	0.270	0.452
Glu	15.08	30.40	31.51	41.02	0.201	0.261	0.897
Gly	19.90	14.64	14.38	47.43	0.221	0.321	0.250
Ala	21.08	46.80	13.23	27.33	0.225	0.899	0.653
Cys	17.55	20.42	0.73	3.28	0.121	0.168	0.839
Tyr	8.75	6.59	6.07	9.17	0.065	0.999	0.625
Pro	21.81	59.93	11.39	101.93	0.372	0.161	0.407
Total AA	22.48	32.61	21.92	40.10	0.145	0.402	0.753

¹ $K_{\text{clearance}}$ was calculated as $\text{BF} \times (\text{CA} - \text{CV})/\text{CV}$, according to Hanigan et al. (1998), where BF = mammary blood flow, CA = arterial AA concentration, and CV = venous AA concentration.

Table 7. Effects of graded removal of Lys from the AA mixture infused into jugular vein on mammary AA uptakes in lactating goats

Item, $\mu\text{mol/h}$	Percentage of Lys in complete AA mixture				SE	P-value	
	100%	60%	30%	0%		Linear	Quadratic
EAA							
Lys	1,302.2	1,034.2	1,044.6	383.7	2.583	0.013	0.278
Thr	1373.8	202.5	550.7	554.4	4.721	0.173	0.105
Val	1,345.1	1,537.3	986.6	1262.9	3.175	0.490	0.995
Met	341.4	195.5	108.4	246.4	1.196	0.332	0.156
Ile	643.3	303.5	720.5	523.5	2.580	0.995	0.637
Leu	1,010.4	475.9	1,059.3	697.7	4.013	0.748	0.712
Phe	587.2	131.2	319.3	277.3	2.072	0.262	0.192
His	238.2	180.9	479.3	270.1	2.072	0.585	0.753
Arg	1,099.0	328.6	707.5	647.6	4.205	0.441	0.263
NEAA							
Ser	377.7	230.2	190.4	335.5	1.219	0.621	0.122
Glu	1,109.3	869.3	1,889.0	1,399.5	6.391	0.410	0.938
Gly	2,399	443	1,768	1,567	16.697	0.783	0.480
Ala	712.9	1268.8	471.4	847.5	6.328	0.894	0.779
Cys	237.8	38.0	21.8	38.8	3.015	0.679	0.254
Tyr	186.5	128.9	134.5	192.0	1.337	0.993	0.594
Pro	1,374.3	837.3	297.3	1,275.5	7.588	0.696	0.228
Total AA	1,843.6	1,479.9	1,067.9	1,316.9	4.520	0.206	0.472

Lys was removed from the infusate, in which case it decreased abruptly to 0.92. Treatments tended to affect the U:O of Ser quadratically ($P < 0.1$). The U:O decreased and then increased with decreased content of Lys in the infusate. Treatments had no significant effects on mammary U:O of any of the other AA measured.

Venous Metabolites, Hormones, and Mammary Blood Flow

Graded removal of Lys from the infusate linearly increased the concentration of venous nitric oxide and mammary blood flow (**MBF**; $P < 0.05$; Table 9). Venous glucose and glucagon were linearly increased

Table 8. Effects of graded removal of Lys from the AA mixture infused into jugular vein on mammary uptake-to-output ratio (U:O) of AA in lactating goats

Item	Percentage of Lys in complete AA mixture				SE	P-value	
	100%	60%	30%	0%		Linear	Quadratic
EAA							
Lys	2.30	2.22	2.20	0.92	0.006	0.059	0.128
Thr	3.62	0.59	1.68	1.84	0.013	0.288	0.105
Val	1.36	1.54	1.09	1.66	0.005	0.792	0.669
Met	1.54	0.98	0.58	0.95	0.005	0.166	0.204
Ile	2.15	1.17	2.60	2.16	0.008	0.661	0.574
Leu	1.47	0.80	1.72	1.32	0.006	0.870	0.687
Phe	1.97	0.78	1.14	1.16	0.008	0.446	0.146
His	1.39	1.13	2.78	2.09	0.013	0.400	0.946
Arg	5.31	2.89	2.72	4.34	0.034	0.884	0.320
NEAA							
Ser	1.09	0.69	0.64	1.15	0.0001	0.109	0.096
Glu	0.69	0.55	1.27	1.23	0.005	0.202	0.768
Gly	12.50	1.76	10.49	9.16	0.099	0.916	0.512
Ala	9.16	4.73	1.63	3.42	0.024	0.894	0.765
Cys	0.83	-1.45	0.16	0.15	0.014	0.850	0.275
Tyr	0.71	0.59	0.49	0.86	0.006	0.874	0.586
Pro	1.65	0.95	0.25	1.81	0.011	0.902	0.200
Total AA	1.68	1.60	1.09	1.53	0.005	0.596	0.622

Table 9. Effects of graded removal of Lys from the AA mixture infused into jugular vein on mammary blood flow (MBF) and concentrations of metabolites and hormones in the plasma of mammary vein in lactating goats

Item	Percentage of Lys in complete AA mixture				SE	P-value	
	100%	60%	30%	0%		Linear	Quadratic
Urea N, mmol/L	4.38	4.62	4.51	4.66	0.002	0.488	0.588
Glucose, mmol/L	4.20	4.75	5.12	5.42	0.004	0.008	0.820
Total protein, mg/mL	74.43	70.12	70.03	66.25	0.025	0.016	0.986
IGF-1, ng/mL	94.56	87.51	84.95	82.90	0.095	0.256	0.798
Growth hormone, ng/mL	0.70	0.72	0.53	0.75	0.002	0.299	0.679
Insulin, μ IU/mL	48.38	51.29	58.79	51.83	0.036	0.153	0.112
Glucagon, pg/mL	137.12	157.91	169.92	170.86	0.092	0.005	0.260
Nitric oxide, μ mol/L	7.24	9.54	16.94	21.22	0.058	0.035	0.681
MBF, L/7 h	105.60	130.03	123.56	149.29	0.138	0.025	0.904

and venous total protein was linearly decreased with decreased content of Lys in the infusate ($P < 0.05$). Treatments had no significant effect on venous urea nitrogen and GH concentrations. Removing Lys from the infusate numerically decreased venous concentration of IGF-1 ($P = 0.256$) and numerically increased the concentration of venous insulin ($P = 0.153$).

Phosphorylation of Downstream Proteins in the Mammalian Target of Rapamycin Pathway

Treatments tended to affect the phosphorylation state of eIF2 quadratically ($P < 0.1$, Table 10). The ratio of phosphorylated to total eIF2 was at first increased and then decreased with increased removal of Lys from the infusate. Treatments had no significant effects on the phosphorylation of 4EBP1 and p70S6k. But both the linear and quadratic effects on phosphorylation of p70S6k, which decreased and then increased with increased Lys removal, were near the declared tendency.

Relative Recovery of Intravenously Infused AA in Milk Protein

Supposing intravenously infused AA was the only exogenous AA supplied to the animals during each infusion period, relative recovery of individual AA in milk protein was then calculated by dividing the

amount of the specific AA recovered in milk protein by the amount of the AA infused during each of the 7-h milk collection periods. As no Lys was infused in the 100% removal treatment, relative recovery of Lys for that treatment was indeterminate. Graded removal of Lys from the infusate increased the relative recovery of Lys quadratically ($P < 0.05$; Table 11), indicating that the increase was accelerated with intensified Lys deficiency. For the other EAA measured and total AA, their respective relative recovery either decreased to the declared tendency (Arg, His, Leu, Val, and total AA; $P < 0.1$) or was only numerically decreased (Phe, Ile, Thr) by increased removal of Lys from the infusate.

DISCUSSION

Milk Yield and Composition

Variation in postruminal Lys supply may affect milk protein synthesis through mass action, changed hormonal status, changed phosphorylation in the mammalian target of rapamycin (**mTOR**) pathway, or all of them, as mentioned above. To investigate which is the main pathway, one needs to be sure of significantly changed milk protein yield by experimental treatments. Postruminal Lys supply could be easily changed by postruminal Lys infusion, but the extent of Lys shortage in such experiments was limited by Lys supply from

Table 10. Effects of graded removal of Lys from the AA mixture infused into jugular vein on phosphorylation level (phosphorylated/total AA $\times$ 100) of downstream proteins in the mammalian target of rapamycin signaling pathway of mammary gland tissue from lactating goats

Item ¹	Percentage of Lys in complete AA mixture				SE	P-value	
	100%	60%	30%	0%		Linear	Quadratic
P-4EBP1/4EBP1	74.87	67.13	75.01	70.79	0.045	0.710	0.540
P-p70S6k/p70S6k	40.58	21.31	24.84	25.78	0.083	0.143	0.109
P-eIF2/eIF2	52.69	84.61	57.69	45.72	0.006	0.147	0.096

¹p70S6k = ribosomal protein S6 kinase beta-1; 4EBP1 = eukaryotic translation initiation factor 4E-binding protein 1; eIF2 α = eukaryotic translation initiation factor 2 α .

the basal diets and there was little chance to have a significant change in milk protein yield, as indicated by previous studies. For example, in the 5 abomasal Lys infusion trials performed by Schwab et al. (1976), milk protein yield was significantly increased only in one of them. Both Guinard and Rulquin (1994) and Varvikko et al. (1999) failed to promote milk protein yield by postruminal Lys infusion in lactating cows. Out of the 7 experiments investigating milk protein response to varied postruminal Lys supply since 2000, 6 observed no significant effects (Misciatteilli et al., 2003; Wang et al., 2010; Lee et al., 2012a,b; Paz et al., 2013; Paz and Kononoff, 2014) and the other reported a lower milk protein yield for the low MP diet supplemented with Lys compared with that of the MP-adequate diet (Lee et al., 2015). It was easier to elevate milk protein yield by co-supplementation of Lys and Met (Schwab et al., 1992a,b), especially when supplemented to low-protein diets (Appuhamy et al., 2011a; Lee et al., 2012b); still, the effect was not consistently observed across previous studies (Mullins et al., 2013). Such experimental procedures are also inappropriate if one only want to investigate the effects of individual EAA on milk protein synthesis. To avoid the limitation of basal diets on extent of individual AA deficiency, Fraser et al. (1991) investigated the effect of postruminal supply of individual EAA on milk protein yield by subtracting approximately 45% of each EAA from the full mixture of EAA infused into cows maintained by total intragastric infusion of nutrients. Removal of Lys caused the greatest depression in milk protein yield, and the productive N value (milk N + retained N) was estimated to decrease by 91%; this phenomenon was not observed in an experiment with lactating goats. Removal of Lys from an abomasally infused AA mixture only numerically decreased milk protein yield (Ying et al., 2013). Of note, these goats were restricted-fed a pelleted diet

to fulfill maintenance MP requirement rather than maintained by total intragastric infusion of nutrients.

To confirm an observed response in milk protein yield, animals were starved for 24 h before the start of an intravenous infusion of glucose and AA mixture to provide the required energy and protein in the present study. The time length of feed deprivation was based on a time previously reported to reversibly decrease milk yield in lactating goats (Linzell et al., 1967). Graded removal of Lys from the infusate linearly decreased milk protein content and yield. Considering the concomitant linear decrease in mammary Lys uptake (Table 7), it seemed reasonable to interpret the result as a mass action of Lys supply. However, the interpretation was questioned by the fact that mammary Lys uptake was only sharply decreased when all Lys was removed from the infusate (Table 7) and no significant linear regression was found between milk protein yield and mammary Lys uptake ($y = 0.004x + 10.236$, $R^2 = 0.487$, $P = 0.302$). It has been suggested that milk production in the mammary glands appear to operate at a predetermined rate set by external communications of milk withdrawal rate, physiological state, and overall nutritional status (Cant et al., 2003). As both nutritional and physiological statuses were changed by Lys removed from the infusions in the present study, as evidenced by the linearly changed blood glucose, glucagon, total protein, and nitric oxide, the decreased milk protein may be a result of decreased mammary activity rather than the mass action of Lys supply. Lactose is the major osmotic substance in milk that is responsible for drawing water into milk. Therefore, the linearly decreased milk yield with graded Lys removal in the present study could be reasonably interpreted as a result of the tendency for decreases in lactose yield. The linearly decreased milk protein content concomitant with the unaffected milk fat content explained the tendency for

Table 11. Effects of graded removal of Lys from a jugular vein-infused AA mixture on the apparent recovery of infused individual AA in milk protein in lactating goats

Item	Percentage of Lys in complete AA mixture				SE	P-value	
	100%	60%	30%	0%		Linear	Quadratic
Lys	21.64	32.01	59.03	—	0.002	0.436	0.012
Met	24.11	21.45	21.98	22.97	0.030	0.724	0.440
Arg	15.17	12.90	11.72	12.77	0.013	0.072	0.123
His	18.36	16.42	15.32	16.09	0.013	0.086	0.230
Phe	20.35	18.06	16.76	18.41	0.017	0.214	0.166
Leu	18.82	16.18	15.23	16.26	0.015	0.098	0.129
Ile	17.73	13.92	13.30	14.54	0.013	0.326	0.151
Thr	23.16	20.78	19.41	20.45	0.018	0.137	0.274
Val	38.00	32.57	29.20	31.61	0.034	0.068	0.182
Total AA	25.43	22.30	20.72	22.05	0.019	0.092	0.178

an increase in milk fat-to-protein ratio observed in the present study.

Circulating Concentrations and Mammary Metabolism of AA

Decreasing postruminal supply of an EAA will generally decrease circulating concentration of that specific EAA, as revealed by previous studies. Plasma Lys concentration decreased by 11.8% when postruminal Lys supply decreased from 118 to 104% of requirements in lactating cows (Paz et al., 2013). Removing Lys from an AA mixture abomasally infused into lactating goats more than halved the arterial concentration of Lys (Ying et al., 2013), and removing Met decreased by one-fourth the arterial concentration of Met (Lin et al., 2014). Removing about 40% of postruminal His supply resulted in an extremely low arterial His concentration (8 vs 73 μM ; Bequette et al., 2000). Consistent with previous studies, arterial and venous concentrations of Lys decreased linearly by the graded Lys deficiency in the present study. Arterial and venous Lys concentrations in the 2 partially removed Lys infusions were numerically similar, which were about 34 and 27%, respectively, of the full-AA mixture infusion. Both arterial and venous Lys decreased further to 14% of those of full-AA mixture infusion by removing all Lys from the infusate. The quadratic effect was significant for venous Lys but was not significant for arterial Lys, though its *P*-value was near the declared tendency. Whether it implied homeostatic regulation of circulating Lys in lactating goats or not was unclear and remained an open question for further investigation. Apart from arterial Arg, arterial and venous concentrations of the other measured EAA were unaffected by treatments, which supported the independent effects of individual EAA on protein synthesis suggested by Hanigan et al. (2000). Arginine is an intermediate metabolite in ureogenesis. The linearly decreased arterial Arg by graded Lys deficiency could be explained by elevated systemic AA catabolism, as indicated by the decreased apparent recovery of infused total AA in milk protein (Table 11).

An increase in MBF seems a general effect of individual EAA deficiency (Cant et al., 2003). Graded removal of Lys from the infusate increased MBF linearly in the present study. The increase was consistent with the linearly increased venous NO, which is synthesized from Arg and has been proposed as one of the local vasodilatory compounds (Laughlin and Korzick, 2001). Apart from MBF, we found evidence that mammary affinities to AA also responded to varied supplies of AA. Abomasal infusion of an EAA mixture decreased mammary clearance rate by 61% in lactating cows (Nichols et al., 2016). The ^{15}N -Lys decay curve analysis

in the plasma revealed a faster mammary extraction rate of Lys for the MP-deficient diets (Lee et al., 2015). High-starch diets increased mammary clearance rate of Lys concomitant with an increased milk protein yield (Cantalapiedra-Hijar et al., 2015). The modeling work by Hanigan et al. (2000) showed that the Michaelis-Menten representation of mammary net uptake of EAA reduced prediction errors compared with a mass action representation; this implies that the tissue may resist changes in uptake driven only by supply through a reduction in apparent affinity and, conversely, increases in apparent affinity when protein synthesis is stimulated. With a graded Lys deficiency, mammary clearance rate of Lys was increased by 3.1, 2.6, and 2.2 times, respectively, to that of the full-AA mixture infusion by the 60, 30, and 0% Lys treatments, but the increases were statistically not significant. This may be because of the relatively large variations in mammary clearance measurements, and replicate animals should be used in future studies to yield more powerful statistics.

Variation in MBF and mammary affinity compensate for part of the varied supply of individual EAA; thus, changes in mammary uptake of EAA with respect to supply are heavily buffered, often resulting in no significant change. It has been reported that removal of Lys from the abomasally infused AA mixture decreased its mammary uptake in lactating cows (Lapierre et al., 2009); this result was not observed in lactating goats (Ying et al., 2013). Glucose infusion decreased arterial concentrations of branched-chain AA by 29%, but increased their mammary clearance rates by 48% and their mammary uptakes were unchanged (Nichols et al., 2016). Graded removal of Lys linearly decreased mammary Lys uptake in the present study; the decrease was small and was only about 20% of that of full mixture infusion until 70% of Lys was removed from the infusate. Mammary Lys uptake then decreased abruptly by more than 70% by the 0% Lys infusion, which may indicate failure of the mechanisms to stabilize mammary Lys uptake, perhaps due to exceeding the potential mitigation range. In conjunction with the abrupt drop, mammary Lys U:O decreased from ~2.2 to 0.92, which apparently maximized efficiency of use within the tissue to further mitigate the decrease. This result suggests that mammary catabolism of Lys can be completely prevented under severe deficiency conditions, indicating that catabolism is not a true requirement but simply a reflection of tissue operation within the range of Lys supply normally provided.

Postruminal Lys Supply and Milk Protein Synthesis

The correlation between the linearly decreased mammary Lys uptake and milk protein yield was not

significant as mentioned above. Whereas similar mammary Lys uptakes in the 60 and 30% Lys treatments decreased to a respective of 15 and 29% milk protein yield compared with that of the full-AA mixture infusion, a 63% abrupt decrease in mammary Lys uptake by the 0% Lys treatment failed to drop milk protein yield further compared with that of the 30% Lys treatment. It is therefore illogical to interpret the decreased milk protein yield in the present study as a result of decreased supply of Lys as precursors for milk protein synthesis. Physiological and overall nutrition status of animals were changed by graded Lys deficiency, as evidenced by the varied circulating metabolites and hormones, which may decrease mammary activity or set point for milk protein synthesis, as suggested by Cant et al. (2003); however, the underlying details are unclear at present. We observed linearly increased circulating glucagon by graded removal of Lys from the infusate in the present study. Glucagon promotes hepatic extraction of circulating AA for gluconeogenesis (Brockman et al., 1975), and hence decreases the amount of AA for protein synthesis. It has been observed that infusion of glucagon decreased milk protein yield in lactating cows (Hippen et al., 1999; She et al., 1999). Although it is a hormone defending against low circulating glucose, glucagon secretion seems more sensitive to the supply of protein rather than glucose. Hypoglycemia was only a weak stimulus to glucagon secretion compared with effective stimulation of an AA mixture in isolated rat pancreas (Pagliara et al., 1974). High-protein diets promoted glucagon secretion in rats (Eisenstein et al., 1979), and 17 of the 20 individual AA intravenously infused into dogs caused substantial increase in plasma glucagon (Rocha et al., 1972). Peritoneal injection of Arg, Ala, or Leu promoted glucagon secretion in rats (Ishizuka et al., 2012). To our knowledge, no report has investigated the effects of imbalanced AA supply or individual EAA subtraction on glucagon secretion. Whether the increased plasma glucagon in the present study implied a direct stimulation or an indirect effect of Lys deficiency (or imbalanced AA supply) on glucagon secretion remains a question for further investigation. Increases in plasma glucose could be explained as a result of promoted gluconeogenesis by increased glucagon secretion, which in turn promoted insulin secretion. The milk protein-depressing effect of glucagon may therefore partly counteracted by insulin, a well-established stimulus of milk protein synthesis (Bequette et al., 2001; Lemay et al., 2007; Bionaz and Loo, 2011).

Phosphorylation status of the measured downstream proteins in the mTOR signaling pathway were not significantly changed by removal of Lys from the infused AA mixture except for a tended quadratic effect on that

of eIF2, which was increased at first by Lys removed infusion and then decreased with increased Lys removal. Appuhamy et al. (2011b) did not observe significant effects of Lys on mTOR signaling in either bovine mammary epithelial cell line T cells or lactogenic mammary tissue slices. The combined observations suggest that most, if not all, of the observed decline in milk protein synthesis associated with Lys deficiency was mediated by a substrate limitation and little, if any, by the mTOR pathway. With decreased postprandial Lys supply, more metabolizable Lys was allocated to milk protein synthesis. The apparent marginal efficiency of metabolizable Lys use was a maximum of 60%, which is still less than the 67% efficiency assumed in the NRC (2001) and prior models. However, this marginal efficiency was only achieved in association with significant reductions in milk protein yield.

CONCLUSIONS

Graded removal of Lys from the intravenously infused AA mixture linearly decreased milk protein yield. The decrease was not a result of the decreased mammary Lys uptake, as the correlation between them was not significant. The mTOR pathway also did not play an important part, if any, in depressing milk protein synthesis. The observed decline in milk protein yield was therefore mainly an effect of the varied physiological status, as indicated by the elevated circulating glucagon and glucose; however, details of the process remain open for further investigation. There were several mechanisms to mitigate Lys deficiency, including the linearly increased MBF and the completely prevented catabolism of Lys in the mammary gland when the animals faced severe Lys deficiency. Details of these mechanisms need to be clarified by future studies.

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