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Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids

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ABSTRACT

The objective of this study was to evaluate local molecular adaptations proposed to regulate protein synthesis in the mammary glands. It was hypothesized that AA and energy-yielding substrates independently regulate AA metabolism and protein synthesis in mammary glands by a combination of systemic and local mechanisms. Six primiparous mid-lactation Holstein cows with ruminal cannulas were randomly assigned to 4 treatment sequences in a replicated incomplete 4 × 4 Latin square design experiment. Treatments were abomasal infusions of casein and starch in a 2 × 2 factorial arrangement. All animals received the same basal diet (17.6% crude protein and 6.61 MJ of net energy for lactation/kg of DM) throughout the study. Cows were restricted to 70% of ad libitum intake and abomasally infused for 36 h with water, casein (0.86 kg/d), starch (2 kg/d), or a combination (2 kg/d starch + 0.86 kg/d casein) using peristaltic pumps. Milk yields and composition were assessed throughout the study. Arterial and venous plasma samples were collected every 20 min during the last 8 h of infusion to assess mammary uptake. Mammary biopsy samples were collected at the end of each infusion and assessed for the phosphorylation state of selected intracellular signaling molecules that regulate protein synthesis. Animals infused with casein had increased arterial concentrations of AA, increased mammary extraction of AA from plasma, either no change or a trend for reduced mammary AA clearance rates, and no change in milk protein yield. Animals infused with starch had increased milk and milk protein yields, increased mammary plasma flow, reduced arterial concentrations of AA, and increased mammary clearance rates and net uptake of some AA. Infusions of starch increased plasma concentrations of glucose, insulin, and insulin-like growth factor-I. Starch infusions increased phosphorylation of ribosomal protein S6 and endothelial nitric oxide synthase, consistent with changes in milk protein yields and plasma flow, respectively. Phosphorylation of the mammalian target of rapamycin was increased in response to starch only when casein was also infused. Thus, cell signaling molecules involved in the regulation of protein synthesis differentially responded to these nutritional stimuli. The hypothesized independent effects of casein and starch on animal metabolism and cell signaling were not observed, presumably because of the lack of a milk protein response to infused casein.

Key words: amino acid, cell signaling, mammary gland

INTRODUCTION

Stimulation of milk protein synthesis at a given supply of AA is a potential strategy to reduce AA catabolism. If AA supply remains constant while use for milk protein synthesis is increased, reductions in arterial concentrations of AA should lead to reduced AA catabolism by the splanchnic tissues (Hanigan et al., 1998a). Conversely, increased AA removal by the mammary glands (**MG**) in the absence of greater protein synthesis would likely increase AA degradation within the tissue (Bequette et al., 1996a,b). Experiments to increase intracellular concentrations of AA resulted in increased oxidation of AA (Bequette et al., 1996a; Raggio et al., 2006). Reduced concentrations of circulating AA triggered a greater proportional mammary capture, although there was some loss of production (Bequette et al., 2000; Raggio et al., 2006). The increased AA capture occurs through stimulation of mammary AA clearance rate. For example, limiting arterial concentrations of His from 73 to 8 μM elicited a 43-fold increase in its clearance rate by the MG (Bequette et al., 2000). This is likely an adaptive mechanism used to

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Table 1. Ingredient composition of the basal diet during the experiment offered to lactating dairy cows infused with starch and casein in the abomasum

Item	% of DM
Corn silage	40.00
Mix grass + legume silage	5.00
Soybean hulls	21.66
Corn grain, ground, dry	14.50
Soybean meal, solvent (48% CP)	12.40
Protected soybean meal	4.78
Calcium carbonate	0.30
Calcium phosphate (di-)	0.20
Sodium bicarbonate	0.20
Sodium selenate	0.04
Salt	0.40
Vitamins and trace minerals[1]	0.01

[1]Vitamin A, D, E, magnesium oxide, manganous oxide, zinc oxide, hydrated sulfate, cobalt carbonate, ferrous sulfate, and copper sulfate.

maintain milk protein output in the face of shortages in one or more AA. Similar adaptations in hepatic tissue have not been reported (Hanigan et al., 1998b, 2004). Therefore, limiting the supply of AA to the animal by low-protein diets should shift AA partitioning toward milk production and increase overall N efficiency. The challenge is to achieve this while avoiding reductions in milk protein output.

In general, increasing the intake of nonstructural carbohydrate, particularly corn starch, increases milk and milk protein yield and N efficiency in lactating cows (Broderick, 2003; Rius et al., 2010). Increasing energy supply to the MG was suggested as a plausible nutritional intervention to stimulate mammary protein synthesis and milk protein production (Hanigan et al., 1998a). For example, increased duodenal glucose supply in cows fed isonitrogenous diets caused hyperglycemia and stimulated insulin secretion and milk protein yield (Rulquin et al., 2004). Similar results were observed when insulin and glucose were infused systemically (Mackle et al., 2000), suggesting that the effect is entirely postabsorptive. Therefore, energy supply either directly or via insulin should interact with AA supply in determining milk protein synthesis and overall N efficiency.

Insulin and AA have been reported to regulate initiation of protein synthesis independently in muscle tissue of growing animals (Davis et al., 1991, 2001) by activation of the mammalian target of rapamycin (**mTOR**) signaling cascade (Escobar et al., 2006). Activity of mTOR signaling proteins in mammary cells increased with greater AA availability, and this response was enhanced by lactogenic hormones (Burgos et al., 2010). Increased mTOR activity corresponds with greater AA utilization for protein synthesis in growing animals; however, this molecular mechanism is not fully understood in the MG of lactating cows.

Greater milk and milk protein production in lactating cows fed high-starch diets compared with those fed low-starch diets has been reported (Rius et al., 2010). This response in production was observed even though dietary protein was lower than recommended (15.2% CP, DM basis). However, mammary metabolism and molecular mechanisms were not evaluated. The objectives of the work reported herein were to determine the effects of starch and protein intake on MG blood flow, AA capture, intracellular signaling, and protein synthesis. It was hypothesized that AA and glucogenic substrate could alter intracellular signaling and protein synthesis independently.

MATERIALS AND METHODS

Animals and Housing

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech. Six primiparous Holstein cows averaging 612 kg of BW and 160 DIM were fitted with a ruminal cannula 55 d before commencement of the study. Animals were housed in a freestall barn with constant access to water and feed. A common TMR (Table 1) was mixed at 0800 h and offered daily during 14-d periods. Feed was offered ad libitum the first 12 d and restricted to 70% of ad libitum intake on an individual basis for the last 48 h of each period. Diets were formulated to meet all NRC (2001) recommendations for a mid-lactation cow weighing 650 kg, consuming 22 kg of TMR, and producing 40 kg/d of milk with 3.0% CP and 3.5% fat. Final diets contained 45% forage and 55% concentrate on a DM basis. The DM content of the forages was monitored weekly and used to adjust the mix to maintain constant DM proportions in the final diet. Samples of forages and concentrates were taken daily during the last week of each period, pooled, and submitted to Dairyland Laboratories (Arcadia, WI) for analyses of nutrients.

Treatments

The effect of 2 levels of abomasal starch and casein infusions were tested in a 2 × 2 factorial arrangement using a replicated Latin square design with 2 squares and 4 periods. One square was complete and 4 cows were used. The second square was incomplete and 2 cows were used. Cows were randomly assigned to treatment sequence within square. The study consisted of four 14-d periods. The first 10 d of each period were allotted for diet acclimation of the animals and washout of the previous treatment. On d 11, cows were moved to a metabolism unit with individual tie stalls, and

abomasal infusion lines were placed manually via the ruminal cannula and checked daily to ensure proper placement. There were no problems with lines becoming dislodged after the initial placement. When cows were in the metabolism unit, rectal temperatures were taken twice daily and cows were observed for signs of illness. Milk samples were checked at each milking for signs of mastitis. On d 13 and 14, the TMR offered was restricted to 70% of the observed ad libitum intake over the previous 10 d for each animal. During the time that intake was restricted, the TMR was subdivided into 8 equal proportions delivered at 0300, 0600, 0900, 1200, 1500, 1800, 2100, and 2400 h on d 13 and 0300, 0600, 0900, 1200, and 1500 h on d 14. At 0400 h on d 13, abomasal infusions were started and consisted of 1) water; 2) starch (2 kg/d); 3) casein (0.86 kg/d); or 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein). Starch and casein were purchased from National Starch and Chemistry Company (Bridgewater, NJ) and International Ingredient Corporation (St. Louis, MO), respectively. Infusates were delivered in a total of 95 kg of water/d using peristaltic pumps (Harvard Apparatus Co. Inc., Millis, MA) set to deliver approximately 0.066 kg/min. Suspensions were maintained by continuous stirring.

Milking and Milk Samples

Animals were milked at 0800 and 2000 h daily from d 1 to 13 of each period. On d 14, cows were milked every hour from 0800 to 1600 h (the last 8 h of the infusion) and samples were collected. An aliquot of milk was submitted for analyses of fat, true protein, lactose, total solids, and somatic cells (AOAC, 1997) by infrared analyses (DHIA, Blacksburg, VA; Foss 4000 Combi North America, Eden Prairie, MN). The MUN analyses were conducted using the Berthelot procedure (ChemSpec 150 Analyzer; Bentley Instruments, Chaska, MN).

Surgeries and Catheter Maintenance

Two weeks before the onset of the study, a permanent indwelling catheter was introduced approximately 40 cm into the intercostal artery (for catheter details, see Mackle et al., 2000). During the study, 4 catheters failed and replacement catheters were inserted into the contralateral intercostal artery. On d 11, one indwelling catheter was inserted into the jugular vein and another catheter was inserted into the subcutaneous abdominal vein. The venous catheters were removed on d 14 of each period after completion of sampling. Arterial catheters were flushed weekly with a 0.9% NaCl solution containing 400 IU of heparin (Baxter, Deerfield, IL). A second solution containing 20 IU of heparin was used to flush the catheters during the sampling sessions.

Blood Collection, Hormone, and Metabolite Concentrations

During the last 8 h of each infusion period, arterial and venous samples (~7 mL) were collected simultaneously every 20 min into heparinized syringes. They were subsequently transferred to heparinized tubes to be analyzed for glucose concentrations. Blood was harvested while cows were standing to ensure representative sampling from the abdominal vein. Plasma was prepared by centrifugation (2,000 × *g* for 10 min), pooled by hour within cow, and stored at −20°C until analysis. Samples were subsequently analyzed for glucose, insulin, IGF-I, and AA. Glucose concentrations were determined using an enzymatic method (according to manufacturer procedures; Beckman Coulter Inc., Fullerton, CA). The intraassay CV was <3%. Insulin and IGF-I were determined by double-antibody RIA as described by Daniels et al. (2008). For the IGF-I assay, acid-ethanol extraction of binding proteins preceded the RIA. Intra- and interassay CV were 2.1 and 1.3%, respectively, for IGF-I, and 3.2 and 2.4%, respectively, for the insulin concentrations. Equal hourly blood aliquots were pooled by period and cow and analyzed for hematocrit. Equal hourly plasma aliquots were pooled by period and cow and analyzed for NEFA, triglyceride (**TG**), and IGF-I-binding proteins (**IGFBP**)-3, 4, and 5. Concentrations of NEFA (Wako Chemicals, Richmond, VA) and TG were determined using an enzymatic method (Beckman Coulter Inc.). The intraassay CV was less than 1% for each metabolite. Ligand blotting was used to determine the relative abundance of IGFBP-3, 4, and 5 as described by Daniels et al. (2008). Gels were electrophoresed at the same time to minimize gel-to-gel variation. Proteins were electrotransferred to a nitrocellulose membrane incubated with ^{125}I-IGF-I (1×10^6 cpm/mL) overnight, washed in Tris-buffered saline, and visualized by autoradiography (Amersham, Piscataway, NJ) for 24 h at −80°C. The relative abundance of binding proteins was determined by scanning densitometry (Un-Scan It v6.1, Orem, UT). Free AA were determined by isotope dilution using a GC-MS (Focus-PolarisQ GC-MS, Thermo Electron Corp., Waltham, MA) according to the methods of El-Kadi et al. (2004) and Calder et al. (1999).

Mammary Biopsy

Mammary glands were prepared for biopsy and tissue was collected at the end of the infusion periods from right and left rear MG as described by Harvatine

and Bauman (2006). Briefly, mammary biopsies were collected using a biopsy tool (Magnum Core Biopsy System, Bard, Covington, GA) fitted with a 12-gauge needle. One portion of the collected tissue (~0.1 g) was immediately snap-frozen in liquid N and stored at −80°C for later analysis of AA. A second portion was processed for Western blot analysis. Clots resulting from the biopsies were removed by hand over the next 2 to 3 d. All biopsied quarters were cultured for pathogens subsequent to the biopsy and found to remain clear of infection.

Cell Signaling Analysis

Tissue samples from mammary biopsies (0.1 g) were processed for Western immunoblotting analysis as described previously (Escobar et al., 2006). Briefly, tissue was mixed 1:7 (wt/vol) with homogenization buffer containing a mix of protease and phosphatase inhibitors (Sigma Chemical Co., St. Louis, MO), homogenized for 40 s (Power Gen 1000, Fisher Scientific, Waltham, MA), and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was diluted in Laemmli SDS sample buffer, boiled for 5 min, and stored at −80°C until protein immunoblot analyses. Proteins were electrophoretically separated in polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA), blocked with 5% nonfat dry milk in a Tris-base Tween buffer (10 m*M* Tris base, 150 m*M* NaCl, and 1% Tween 20), and probed with various antibodies recognizing the signaling proteins of interest including endothelial nitric oxide synthase (**eNOS**), protein kinase B (**AKT**), ribosomal protein S6 (**rpS6**), and mTOR as described previously (Escobar et al., 2006). All antibodies were procured from Cell Signaling Technology (Danvers, MA) and included anti-phospho-eNOS (Ser^{1177}), anti-α tubulin, anti-phospho-AKT (Ser^{473}), anti-total AKT, anti-phospho-mTOR (Ser^{2448}), anti-total mTOR, anti-phospho-rpS6 ($Ser^{235/236}$ and $Ser^{240/244}$), and anti-total rpS6. Membranes for eNOS determination were probed for anti-phospho-eNOS, and then stripped and reprobed to determine α tubulin. The antibody recognizing total eNOS did not cross-react with the bovine molecule, thus necessitating the use of the α tubulin antibody as a loading control. The remaining membranes were probed initially for the phosphorylated initiation factors, and then stripped and reprobed to determine the abundance of the total form of these initiation factors. Blots were developed using an enhanced chemiluminescence kit (ECL Plus, Amersham, Piscataway, NJ) and visualized using enhanced chemiluminescence film and a medical film processor (SRX 101A Konica Minolta, Wayne, NJ). The films were scanned and bands quantified by densitometry as described above. Band intensities were used to calculate the ratio of phosphorylated to total protein (or tubulin in the case of eNOS), which is indicative of the phosphorylation state of that particular protein.

Measurements and Calculations

Mammary plasma flow (**MPF**, L/h) was calculated according to the Fick principle using plasma and milk Met, Thr, Lys, and Phe plus Tyr flux with an allowance for 3.5% contribution from blood-derived proteins (Thivierge et al., 2002) and assuming quantitative conversion to milk protein:

$$MPF = (AA_m \times 0.965)/AV_{AA} \times (1 - \text{hematocrit}),$$

where AA_m represented each milk AA (μmol/h) and AV_{AA} represented the arterio-venous (**AV**) concentration differences (μmol/L) for each essential AA (**EAA**). Milk AA composition was calculated from Hanigan et al. (2004). The independent estimates of flow provided by each AA were averaged to derive a final flow for each time point and animal.

Clearance rates (*k*, L/h) of the MG were calculated from the model of Hanigan et al. (1998b) as applied in Bequette et al. (2000):

$$k_i = MPF \times AV/[V_i],$$

where $[V_i]$ represented venous plasma concentrations of the *i*th AA (μmol/L). The advantage of this form over the use of extraction efficiency is that the clearance rate accommodates changes in plasma flow. Clearance is the net uptake of the metabolite relative to its venous concentrations. Net uptake (μmol/h) of metabolites across the MG was calculated as

$$Uptake_i = MPF \times AV_i.$$

Statistical Analysis

The main effects of starch and casein and the interaction between starch and casein were tested as summarized in Table 2. Statistical computations were performed using the Proc Mixed procedure of SAS (2001, version 3.01; SAS Institute Inc., Cary, NC). Cow was defined as a random effect, and period and treatment were defined as fixed effects for the analyses of hormones, NEFA, TG, and Western blotting. The same model was used for analyses of milk yield, milk composition, and milk component yields, plasma AA and glucose, plasma flow, and mammary calculations with added consideration for the effects of repeated measures in time. The autoregressive covariance struc-

Table 2. Definition of the effects for the statistical model

Effect	Type	df	ddfm[1]
Starch (S)[2]	Fixed	1	8
Casein (CN)[2]	Fixed	1	8
CN × S	Fixed	1	8
Period[2]	Fixed	3	8
Cow[2]	Random	5	
Error	Random	8	
		19	
Hour (H)[2]	Fixed	8	128
S × H	Fixed	8	128
CN × H	Fixed	8	128
CN × S × H	Fixed	8	128
Residual	Random	128	
Total		179	

[1]ddfm = denominator degrees of freedom for *F*-test.
[2]The effects of starch, casein, period, cow, and hour.

ture was used based on goodness of fit as indicated by Akaike's information criterion, Bayesian information criterion, and Akaike's information criterion corrected. Unless otherwise stated, significance was declared at $P \leq 0.05$.

RESULTS

Intake, Milk Production, and Milk Components

The chemical composition of dietary ingredients is presented in Table 3, and animal performance data are presented in Table 4. Intake of TMR (not considering infusates) was greater in animals infused with starch relative to cows infused with casein ($P = 0.01$), primarily because of a greater intake for cows infused only with starch (casein × starch interaction, $P = 0.01$).

Milk production and composition are presented in Table 4. Infusions of starch increased milk yield ($P = 0.02$); however, there were no evident effects of casein infusion on milk yield, and there was no interaction between casein and starch. However, there was an interaction between casein and starch for milk protein yield with an increase in response to casein in the presence of starch ($P = 0.02$). Relative to starch, infusions of casein increased milk protein percentage ($P = 0.03$); however, there was a negative response to casein in the presence of starch that reduced milk protein percentage (casein × starch interaction, $P = 0.04$). Milk urea N was reduced by infusion of starch ($P = 0.01$) and increased by the infusion of casein ($P = 0.01$). Lactose yield was greater in cows treated with starch ($P = 0.01$). Neither starch nor casein had an effect on milk fat yield, but there was an interaction, with a positive response to casein in the presence of starch ($P = 0.03$). There was an increase in SNF yield in cows infused with starch ($P = 0.03$) and a casein × starch interaction ($P = 0.03$) with a negative response to casein in the absent of starch and a positive response in the presence of starch.

Insulin, IGF-I, and IGFBP

Insulin, IGF-I, and IGFBP data are presented in Table 5. Cows that received starch infusions had increased insulin and IGF-I concentrations ($P = 0.01$). There was a trend for an insulin interaction between casein and starch, with a negative response to casein in the presence of starch ($P = 0.08$). There was an interaction between casein and starch for relative abundance of IGFBP-4 and 5, with a negative response to casein in the presence of starch ($P = 0.05$). There was a trend for an interaction of casein and starch on IGFBP-3 relative abundance with a negative response to casein in the presence of starch ($P = 0.06$).

Metabolite Kinetics and Plasma Flow

Arterial plasma concentrations of energy-yielding metabolites, AV differences, clearance rates, and net uptake are presented in Table 6. Plasma flow was increased in cows infused with starch ($P = 0.01$) as were arterial plasma concentrations of glucose ($P = 0.01$),

Table 3. Chemical composition of the feed ingredients of the basal diet (% DM basis)

Item	Haylage	Corn silage	Forage mix	Concentrate
DM	55.7	27.8	30.4	91.5
NDF	43.2	43.3	43.9	31.2
ADF	34.2	26.1	26.3	21.0
CP	21.6	7.2	9.7	23.5
Fat	3.3	4.1	3.8	2.9
Lignin	7.1	2.1	2.6	1.9
Starch	0.5	30.6	23.6	17.8
Sugar	2.6	1.0	2.2	5.7
Minerals				
Ash	11.1	3.1	4.8	6.4
Calcium	1.0	0.2	0.6	0.8
Phosphorous	0.4	0.2	0.3	0.4
Magnesium	0.3	0.2	0.3	0.3

Table 4. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on total DMI, milk yield, and milk composition

	Experimental treatment[1]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
DMI + infusate,[2] kg/d	13.0	14.7	17.9	16.9	0.8	0.01	0.4	0.01
DMI,[3] kg/d	13.0	13.6	15.6	13.6	0.8	0.01	0.2	0.01
Milk yield, kg/h	0.98	0.83	1.09	1.20	0.10	0.02	0.8	0.2
Protein yield, g/h	31.4	25.0	30.2	37.5	4.2	0.05	0.8	0.02
Lactose yield, g/h	45.5	35.3	44.6	56.5	7.0	0.01	0.8	0.1
Fat yield, g/h	48.0	34.9	42.6	50.0	4.7	0.3	0.4	0.03
SNF yield, g/h	85.0	68.7	84.0	104.0	12.7	0.03	0.8	0.03
MUN, mg/dL	12.0	14.7	9.2	13.1	0.5	0.01	0.01	0.2
Protein, %	3.05	3.29	3.15	3.18	0.11	0.9	0.03	0.04
Lactose, %	4.35	4.41	4.59	4.62	0.12	0.01	0.3	0.7
Fat, %	4.68	4.40	4.25	3.73	0.30	0.05	0.1	0.6
SNF, %	8.32	8.68	8.66	8.68	0.12	0.01	0.01	0.01

[1]Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

[2]TMR intake including infusate.

[3]Voluntary TMR intake without infusate.

but there was a casein × starch interaction for glucose, with the starch response greater in the absence of casein ($P = 0.01$). Arterio-venous concentration differences of glucose increased in response to casein infusion in the absence of starch but decreased in response to casein in the presence of starch ($P = 0.02$). Net uptake of glucose was increased by infusion of starch ($P = 0.01$). These changes in uptake were likely caused, at least partially, by changes in mammary glucose clearance rate, which increased in response to casein in the absence of starch, but declined in response to casein in the presence of starch ($P = 0.01$).

Arterial NEFA concentrations were lower in cows infused with casein ($P = 0.05$) but were unaffected by starch. Net uptake of NEFA by MG was reduced in cows infused with starch ($P = 0.01$) but increased in cows infused with casein (trend, $P = 0.07$).

Arterial concentrations of AA are presented in Table 7. Casein infusions increased plasma concentrations of all EAA ($P = 0.02$) except Phe. Casein infusions increased plasma concentrations of Cys, Pro, Ser, and Tyr ($P = 0.02$) but reduced arterial concentrations of Gln ($P = 0.01$). Starch infusions reduced arterial concentrations of Ile, His, Leu, Lys, Phe, and Val ($P = 0.05$) and tended to reduce Met concentrations ($P = 0.07$). Infusions of starch reduced arterial concentrations of the nonessential AA Asp, Gln, Glu, and Pro ($P = 0.01$) and tended to reduce Trp and Ala plasma concentrations ($P = 0.08$). However, starch infusions increased Gly concentrations ($P = 0.01$). There was a casein × starch interaction for Leu and Trp with a positive response to casein in the absence of starch ($P = 0.01$). There was also a casein × starch interaction for arterial concentrations of Ala, Cys, and Gln ($P = 0.01$) with reduced Cys concentrations and increased Ala and Gln concentrations in response to casein in the presence of starch.

Arterio-venous concentration differences of AA are presented in Table 8. Infusions of casein increased AV for Ile, Leu, Lys, Thr, Phe, Glu, Gln, Gly, Pro, and

Table 5. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on the arterial concentration of insulin, IGF-I, and IGF-I binding proteins (IGF-BP)

	Experimental treatment[1]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
Hormone								
Insulin, ng/mL	0.78	0.85	1.29	1.0	0.18	0.01	0.8	0.08
IGF-I, ng/mL	352	376	399	405	51	0.01	0.3	0.5
IGF binding proteins[2]								
IGF-BP3	326	350	356	261	30	0.3	0.2	0.06
IGF-BP4	247	349	277	246	33	0.2	0.3	0.05
IGF-BP5	171	170	183	127	15	0.2	0.04	0.05

[1]Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

[2]Arbitrary density units.

Table 6. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on plasma flow, arterial concentration, arterio-venous (AV) difference, net uptake, and clearance rates of energy-yielding metabolites

	Experimental treatment[1]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
Plasma flow, L/h	419	324	547	505	75	0.01	0.3	0.6
Arterial concentrations								
Glucose, mmol/L	4.1	4.3	4.6	4.3	0.16	0.01	0.5	0.01
NEFA, mEq/L	0.24	0.17	0.14	0.16	0.03	0.4	0.05	0.1
Triglycerides, mg/dL	9.0	9.1	8.8	8.4	0.7	0.6	0.9	0.8
AV difference								
Glucose, mmol/L	0.49	0.78	0.63	0.51	0.11	0.4	0.3	0.02
NEFA, mEq/L	0.001	0.002	−0.031	−0.009	0.007	0.01	0.1	0.1
Triglycerides, mg/dL	3.7	3.5	3.0	3.1	1.1	0.5	0.9	0.8
Net uptake[2]								
Glucose, mmol/h	201	252	409	330	88	0.01	0.8	0.2
NEFA, mEq/h	−0.24	4.37	−20.7	−5.5	5.0	0.01	0.07	0.3
Triglycerides, mg/h	1,693	1,642	1,618	1,510	565	0.8	0.8	0.9
Clearance rate[3]								
Glucose, L/h	70	135	122	82	20	0.9	0.5	0.01

[1]Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

[2]Net uptake of metabolites across the mammary glands was calculated as $MPF \times AV_{met}$, where MPF is the mammary plasma flow (L/h) and AV_{met} represents metabolite differences between arterial and venous concentrations.

[3]$k_i = MPF \times AV_{met}/[V_i]$ (from Hanigan et al., 1998b), where k_i is the clearance rate and $[V_i]$ represents venous plasma concentrations of glucose (mmol/L).

Ser ($P = 0.04$), but reduced AV for Ala and Cys ($P = 0.04$). Infusions of starch increased AV for Ala, Glu, and Cys ($P = 0.05$). There was a reduction in AV for Phe in cows infused with starch ($P = 0.01$). There was a trend to reduce the AV of Leu by starch infusion ($P = 0.07$). Arterio-venous differences of Met, His, Thr, and Gln were affected by the interaction between casein and starch, with an increase in response to casein in the absence of starch and a reduction in response to casein in the presence of starch ($P = 0.03$). There was a trend

Table 7. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on arterial concentrations (μM) of AA

	Experimental treatment[1]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
Essential AA								
Arg	76	98	72	99	7	0.8	0.01	0.6
Ile	82	131	64	109	8	0.01	0.01	0.6
His	30	39	27	36	3	0.05	0.01	0.9
Leu	116	205	85	150	6	0.01	0.01	0.01
Lys	63	96	52	83	5	0.01	0.01	0.7
Met	22	27	19	27	2	0.07	0.01	0.2
Phe	89	94	64	77	13	0.04	0.2	0.5
Thr	59	85	57	82	6	0.7	0.01	0.6
Trp	95	99	99	100	1	0.08	0.02	0.01
Val	190	350	140	272	18	0.01	0.01	0.2
Total essential AA	753	1,147	622	962	80	0.01	0.01	0.5
Branched-chain AA	375	683	294	533	26	0.01	0.01	0.1
Nonessential AA								
Ala	160	143	148	170	12	0.07	0.5	0.01
Asp	7	7	5	6	1	0.01	0.1	0.07
Cys	16	17	19	15	1	0.5	0.02	0.01
Gln	92	33	32	71	11	0.01	0.01	0.01
Glu	30	32	25	28	2	0.01	0.1	0.8
Gly	191	192	222	216	10	0.01	0.7	0.6
Pro	57	136	42	122	8	0.01	0.01	0.9
Ser	64	84	62	83	4	0.6	0.01	0.8
Tyr	37	58	32	57	3	0.1	0.01	0.3
Total nonessential AA	693	682	639	761	25	0.5	0.02	0.1

[1]Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

Table 8. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on arterio-venous difference (μM) of AA[1]

	Experimental treatment[2]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
Essential AA								
Arg	15	24	22	28	9	0.4	0.3	0.1
Ile	24	32	21	35	3	0.8	0.01	0.3
His	1	6	5	3	2	0.3	0.1	0.03
Leu	36	56	30	48	4	0.07	0.01	0.8
Lys	25	35	25	36	3	0.9	0.01	0.09
Met	6	9	10	8	1	0.1	0.05	0.01
Phe	38	84	21	53	37	0.01	0.01	0.2
Thr	10	23	18	17	3	0.1	0.01	0.01
Trp	12	12	13	13	2	0.5	0.9	0.9
Val	30	25	23	26	7	0.6	0.8	0.5
Total essential AA	121	242	174	169	35	0.6	0.02	0.01
Branched-chain AA	80	128	83	111	15	0.6	0.01	0.41
Nonessential AA								
Ala	12	1	23	2	7	0.05	0.01	0.1
Asp	2	3	2	2	1	0.6	0.7	0.5
Cys	1	1	8	3	2	0.01	0.04	0.03
Gln	3	3	−3	12	3	0.5	0.01	0.01
Glu	14	19	19	26	2	0.01	0.01	0.6
Gly	−14	−13	−25	3	7	0.7	0.04	0.06
Pro	2	16	4	10	3	0.6	0.01	0.1
Ser	8	18	9	15	4	0.5	0.01	0.5
Tyr	9	9	11	11	2	0.4	0.94	0.9
Total nonessential AA	1	37	36	53	15	0.06	0.05	0.4

[1]Positive values indicate plasma AA removal; negative values indicate AA released from the mammary glands.

[2]Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

to increase AV of Lys and Gly in response to casein with or without starch ($P = 0.09$). Arterio-venous differences of Cys did not change in response to casein in the absence of starch but were reduced in the presence of starch ($P = 0.03$).

Clearance rates of plasma AA from the MG are presented in Table 9. Clearance rates of Arg, Ile, Leu, Lys, Phe, Trp, Ala, Glu, and Tyr were increased during starch infusions ($P = 0.03$). There was a trend to increase the clearance rate of Asp by starch infusion ($P = 0.07$). The clearance rates of Ile, Ala, and Cys were reduced, whereas that of Gln was increased by the infusion of casein ($P = 0.05$). Clearance rates of His, Met, Phe, Thr, Ala, and Tyr were affected by the casein × starch interaction with a positive response to casein in the absence of starch and a negative response in the presence of starch ($P = 0.05$). There was a similar trend for a casein × starch interaction in the clearance rate of Trp ($P = 0.08$). The net uptake of AA by the mammary gland is presented in Table 10. Infusions of starch increased the net uptake of Arg, Ile, Lys, Phe, Trp, Ala, Asp, Cys, Gln, Glu, Ser, and Tyr ($P = 0.05$). There was a trend for starch infusions to increase Val uptake ($P = 0.08$). Casein infusions reduced net uptake of Ala and Cys ($P = 0.05$), whereas casein tended to increase uptake of His and Gln ($P = 0.09$). Casein infusions increased net uptake of Pro, Ser, and Tyr ($P = 0.04$). Net uptake of His, Met, Thr, and Ala were affected by the casein and starch interaction with greater uptake in response to casein in the absence of starch and a reduction in the presence of starch ($P = 0.05$). Net uptake of Trp showed a trend for the casein × starch interaction, with greater uptake in response to casein in the absence of starch and a reduction in the presence of starch ($P = 0.08$).

Cell Signaling

The ratio of phosphorylated to total abundance of mTOR, AKT, and rpS6 in the MG are presented in Figure 1. Infusions of starch increased the phosphorylation ratio of rpS6 ($P = 0.03$). However, infusion of casein failed to increase the phosphorylation ratio of mTOR. There was an interaction between starch and casein for phosphorylation state of mTOR with increased phosphorylation in response to casein when starch was present and a decline in response to casein in the absence of starch ($P = 0.05$). There was a trend for the interaction of casein and starch on the phosphorylation ratio for AKT ($P = 0.14$) with an increase in

Table 9. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on clearance rates[1] (L/h) of AA by the mammary glands

	Experimental treatment[2]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
Essential AA								
Arg	10	112	241	228	67	0.01	0.4	0.3
Ile	179	99	265	193	43	0.03	0.05	0.8
His	−34	125	127	36	41	0.2	0.3	0.01
Leu	153	153	337	225	50	0.01	0.7	0.9
Lys	220	270	469	359	77	0.01	0.6	0.1
Met	178	304	350	159	115	0.8	0.6	0.02
Phe	33	128	611	266	124	0.01	0.2	0.05
Thr	87	265	199	117	35	0.6	0.2	0.01
Trp	−2.1	0.5	2.2	1.1	1.5	0.02	0.6	0.08
Val	76	38	75	77	19	0.2	0.9	0.2
Nonessential AA								
Ala	2.0	22	86	7.8	18	0.01	0.04	0.01
Asp	151	247	518	376	118	0.07	0.8	0.3
Cys	14	−83	69	−68	40	0.3	0.01	0.6
Gln	−94	22	−120	−13	58	0.1	0.01	0.8
Glu	66	713	1,108	1,227	393	0.02	0.2	0.4
Gly	−44	−28	−31	−10	31	0.4	0.3	0.9
Pro	45	60	52	34	18	0.6	0.9	0.3
Ser	105	98	85	130	41	0.8	0.5	0.4
Tyr	28	178	293	180	56	0.02	0.7	0.03

[1] $k_i = MPF \times AV_{met}/[V_i]$ (from Hanigan et al., 1998b), where k_i is the clearance rate, $[V_i]$ represents venous plasma concentrations of the ith AA (μmol/L), and MPF is the mammary plasma flow (L/h). Positive values indicate uptake and negative values indicate output by the mammary glands.

[2] Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

Table 10. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on AA net uptake (M/h) in the mammary glands[1]

	Experimental treatment[2]					*P*-value		
Item	Water	CN	S	S+CN	SEM	S	CN	CN × S
Essential AA								
Arg	3.4	4.4	10	14	3.4	0.01	0.4	0.7
Ile	8.9	8.5	11	18	2.5	0.01	0.1	0.1
His	0.3	3.3	1.3	0.3	1.0	0.5	0.09	0.01
Leu	15	16	16	22	5.0	0.3	0.4	0.5
Lys	9.3	11	13	18	2.6	0.03	0.1	0.6
Met	1.6	3.7	3.4	1.3	1.0	0.6	0.9	0.01
Phe	3.1	4.6	20	15	5.7	0.04	0.4	0.3
Thr	3.3	11	10	5.6	2.5	0.5	0.6	0.01
Trp	0.2	1.5	2.2	1.1	1.0	0.02	0.6	0.08
Val	6.7	10	13	19	4.3	0.08	0.2	0.7
Nonessential AA								
Ala	0.8	1.7	14	1.5	3.5	0.02	0.01	0.01
Asp	0.7	0.7	1.3	1.0	0.4	0.05	0.7	0.9
Cys	0.8	0.1	2.4	0.5	0.7	0.03	0.01	0.9
Gln	−1.1	1.3	−3.6	0.2	1.3	0.01	0.06	0.7
Glu	7.5	8.5	10	14	3.2	0.05	0.2	0.4
Gly	−5.1	−1.7	−8.4	3.9	4.0	0.4	0.2	0.9
Pro	0.5	8.4	2.3	6.6	1.4	0.9	0.01	0.3
Ser	0.1	5.6	5.2	7.7	2.0	0.04	0.04	0.5
Tyr	0.1	5.1	6.4	7.3	1.5	0.01	0.05	0.2

[1] Net uptake of AA across the mammary glands was calculated as $MPF \times AV_{AA}$, where AV_{AA} represents AA differences between arterial and venous concentrations and MPF is the mammary plasma flow (L/h). Positive values indicate net removal and negative values indicate net release from the mammary glands.

[2] Treatments: 1) water (control); 2) casein (0.86 kg/d); 3) starch (2 kg/d); and 4) the combination of starch plus casein (2 kg/d of starch + 0.86 kg/d of casein).

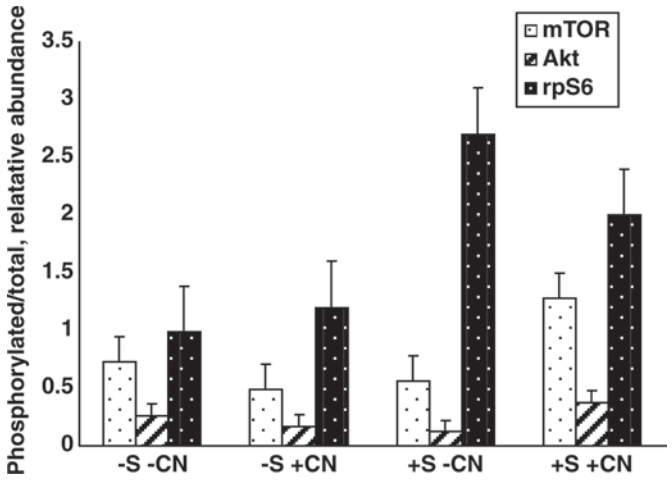

Figure 1. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on protein initiation factor activities in the mammary glands. Bars represent LSM (±SE) of phosphorylated to total relative abundance ratios of arbitrary density units. There was an interaction of CN and S on mammalian target of rapamycin (mTOR; $P = 0.05$), with the response to CN in the absence of S negative and the response to CN in the presence of S positive. Cows infused with S increased the ratio of phosphorylated to total relative abundance of ribosomal protein S6 (rpS6; $P = 0.03$). Akt = protein kinase B.

response to casein in the presence of starch. Infusions of starch tended ($P = 0.06$) to increase the ratio of phosphorylated eNOS to tubulin (Figure 2).

DISCUSSION

A critical limitation to current MP and AA requirement systems is the assumption that a given level of milk protein output requires a fixed proportion of metabolizable AA, despite observations of large variations in postabsorptive conversion efficiencies (Hanigan et al., 1998a; Cant et al., 2002; Hanigan, 2005). Variable efficiency is consistent with the biological mechanisms regulating N metabolism. Approximately 50% of cardiac output, and therefore systemic AA, flows through the splanchnic tissues (Davis et al., 1988; Huntington et al., 1990), and thus AA not removed from blood for milk protein synthesis on a given transit through the udder are subsequently subject to clearance by the splanchnic tissues (Hanigan, 2005). If mammary uptake of AA is stimulated, recycling and use by other tissues will decline if AA supply is fixed; this is consistent with the model of postabsorptive AA metabolism presented by Hanigan et al. (1998a).

The observed increase in milk protein output (Table 4) when cows were infused with starch probably resulted from increased insulin and IGF-I concentrations (Table 5). It has been previously demonstrated that hyperinsulinemia stimulates milk protein synthesis whether occurring in response to infused insulin (McGuire et al., 1995; Bequette et al., 2001) or when caused by elevated glucose concentrations (Rulquin et al., 2004). Increased insulin and IGF-I concentrations are consistent with increased rpS6 phosphorylation (Davis et al., 2001; Proud, 2007). Ribosomal protein S6 is thought to stimulate ribosomal biogenesis through preferential stimulation of initiation rates of mRNA characterized by a 5′ tract of oligopyrimidines, although this role has been questioned (Proud, 2007). Its phosphorylation has been reported to occur via the AKT (O'Connor et al., 2003) and mTOR pathway (Proud, 2007; Figure 3). However, in the current study, phosphorylation of mTOR increased only when both casein and starch were infused, and there was no evidence for increased AKT phosphorylation in response to the elevated insulin and IGF-I concentrations during starch infusions. Phosphorylation of rpS6 and mTOR was found to be highly correlated using an in vitro bovine mammary cell model (Appuhamy et al., 2009). It is possible that an IGF-I–induced rpS6 phosphorylation occurs via the mitogen-activated protein kinase or the AMP kinase pathways (see Proud, 2007), which acts independently of AKT. This, however, does not explain the lack of a phosphorylation response in mTOR when animals were infused with starch in the absence of casein infusions. Phosphorylation of rpS6 can also occur via RSK (ribosomal S6 kinase; Roux et al., 2007), which may explain the difference found between mTOR and rpS6 phosphorylation. Additionally, the downstream target of mTOR, p70S6K1, has been reported to inhibit insulin actions via phosphorylation of IRS-1 (insulin receptor substrate 1; Tremblay et al.,

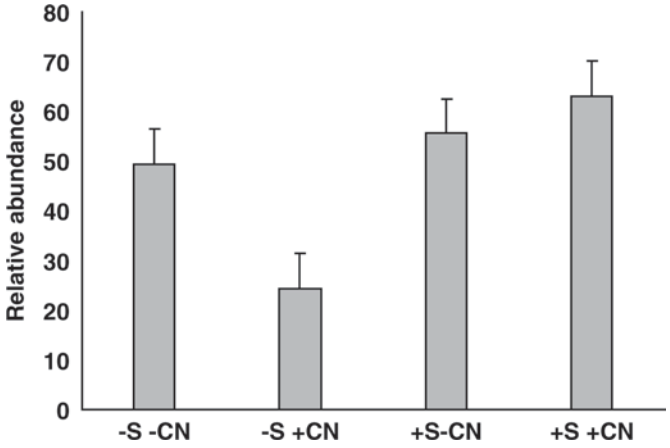

Figure 2. Effect of 2 levels of starch (S) and casein (CN) infused in the abomasum of intake-restricted lactating dairy cows on activity of endothelial nitric oxide synthase (eNOS) to α tubulin ratio in the mammary glands. Bars represent LSM (±SE) of phosphorylated eNOS to α tubulin relative ratios of arbitrary density units. Starch tended to increase the ratio of phosphorylated eNOS to α tubulin relative abundance ($P = 0.06$).

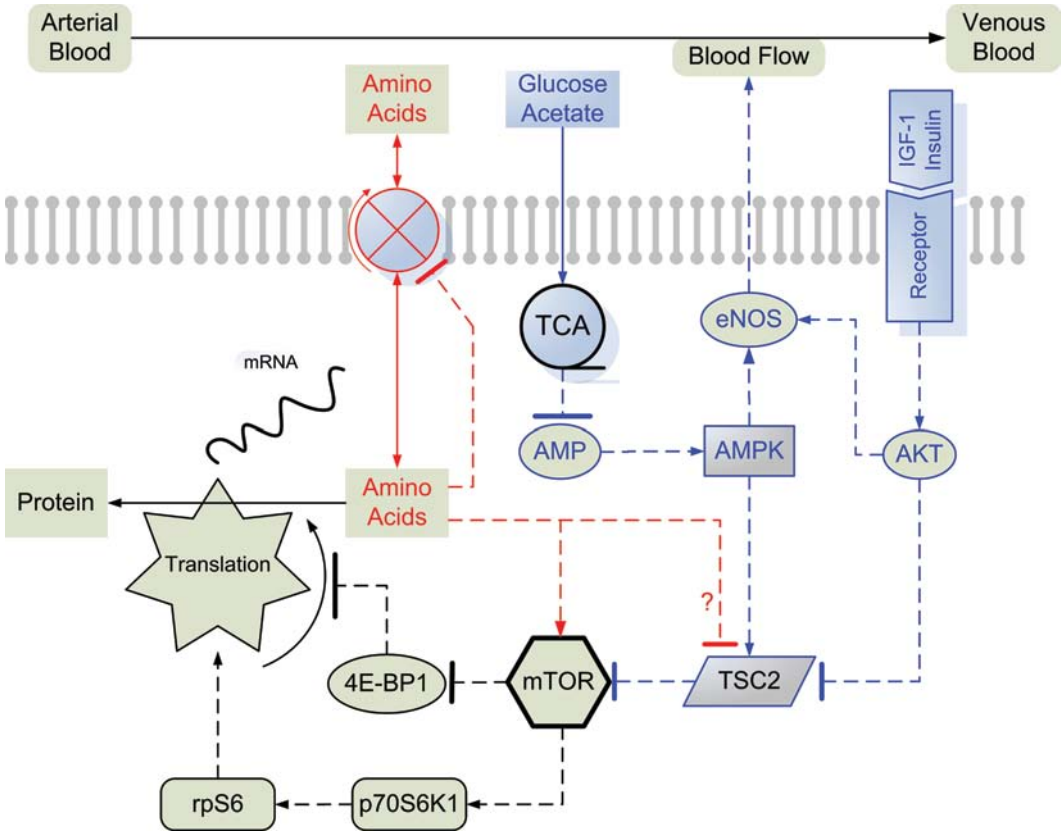

Figure 3. A partial schematic representation of the signaling pathways thought to regulate protein synthesis and their purported interactions based on the literature. TCA represents the tri-carboxylic acid cycle; → represent fluxes, – –> represents activation, and – –| represents inhibition. Color version available in the online PDF.

2007), which may explain the lack of both an AKT and an mTOR response. Finally, recent work has identified an alternative mTOR phosphorylation site that purportedly responds to AKT and AA and controls mTOR activity (Acosta-Jaquez et al., 2009). It is possible that the phosphorylation of the alternative site is not highly correlated to phosphorylation of Ser^{2448}, resulting in the disparate signaling pattern. However, we have previously observed independent effects of insulin and AA on Ser^{2448} in bovine mammary cells in vitro (Bell et al., 2009) and have found phosphorylation of Ser^{2448} to be highly correlated with rates of protein synthesis in mammary tissue slices from lactating cows (Appuhamy et al., 2009).

The lack of milk protein yield and mTOR phosphorylation responses to casein infusions are also surprising. Burgos et al. (2010) reported that AA availability in mammary epithelia increased protein synthesis by 50% and regulated signaling activity, which was enhanced by adding a combination of hydrocortisone, insulin, and prolactin to the medium. Escobar et al. (2006) reported that Leu activated the mTOR cascade and protein accretion in the muscle of growing piglets. The supply of AA is a key signal that activates mTOR signaling (Bolster et al., 2004); however, in the experiment reported here, increasing the mammary supply of AA did not stimulate the mTOR cascade or protein synthesis. It may be that the change in AA concentrations achieved with a 30% feed restriction and casein infusion for 36 h was not adequate to detect differences in mTOR activity and protein synthesis. Escobar et al. (2006) fasted their piglets overnight and infused with large doses of individual AA, which created 3-fold or greater changes in blood concentrations compared with responses of 1-fold or less in the study reported herein. Burgos et al. (2010) increased AA availability 4 times the arterial concentrations determined in well-fed lactating cows reported by Mackle et al. (2000).

Essential AA required by mammary cells to support increased milk protein output during starch infusions were acquired through a combination of increased mammary clearance rates for certain AA (Table 9) and increased MPF (Table 6). The combination resulted in increased net uptake (Table 10) of AA despite reduced

arterial concentrations (Table 6) during starch infusions. Increased mammary clearance rates of AA are part of a local adaptive mechanism to support protein synthesis, wherein clearance rate is matched to cellular needs (Moore et al., 1977; Bequette et al., 2000; Raggio et al., 2006) and is predicted by the model of Hanigan et al. (2000).

The decline in arterial AA concentrations during starch infusions might be a result of a decrease in AA catabolism. The reduction of AA catabolism is evidenced by the observed decline in MUN concentrations (Table 4). These results are consistent with the initial hypothesis regarding the ability to regulate and reduce catabolic losses of AA and improve N efficiency. If other tissues are catabolizing AA by mass action (Hanigan et al., 1998a), any decline in AA concentrations in blood would lead to a decline in catabolic flux, an increase in postabsorptive and animal efficiencies, and reduced N loss to the environment.

The increase in MPF may have resulted from increased insulin or IGF-I concentrations. Both have been reported to increase blood flow to the MG via AKT-dependent phosphorylation of eNOS resulting in greater nitric oxide production (Lacasse et al., 1996; Zeng and Quon, 1996; Iantorno et al., 2007). Although nitric oxide was not measured in the present study, there was a trend for increased phosphorylation and thus activation of eNOS ($P = 0.06$) in association with starch infusions. Such changes are consistent with the observed increases in MPF. However, as for mTOR, no changes in AKT phosphorylation were observed, and thus it is unclear what mechanism is responsible for the changes in eNOS phosphorylation.

Increased MPF is not consistent with the observations of Cant et al. (2002), in which increased local glucose supply (517 mM/h) resulted in increased arterial concentration (6.37 mM) but a decreased MPF. However, the increase in arterial glucose concentrations, in the study reported here, was much less than the increase elicited by the local glucose infusions used in the work of Cant et al. (2002), perhaps allowing the effects of insulin and IGF-I to predominate. Rigout et al. (2002) reported similar responses in MPF when varying glucose supply, suggesting that the observations reported herein are valid.

The variable efficiency in N use resulting from the effect of energy supply and hormonal signals to the MG are not captured in the current NRC (2001) model, which contributes to the overall poor relative efficiency of dairy cows in converting dietary N to milk N (Bequette et al., 2003) when fed to NRC requirements.

CONCLUSIONS

Provision of starch during dietary restriction can stimulate the MG to synthesize more milk protein. Ribosomal protein S6 appears to mediate this effect, although phosphorylation of AKT and mTOR did not mirror that of rpS6. Endothelial nitric oxide synthase may be, at least in part, responsible for a greater MPF during starch infusions. The increase in milk protein output was supported by an increase in MG affinity for some EAA. These actions resulted in decreased arterial AA concentrations and reduced AA catabolism. Collectively, this information can be used to improve the representation of protein synthesis regulation in metabolic models and lead to more accurate predictions of AA requirements and improved N efficiency.

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