J. Dairy Sci. 97:430–438
http://dx.doi.org/10.3168/jds.2013-7252

Decline in mammary translational capacity during intravenous glucose infusion into lactating dairy cows

R. V. Curtis,*[1] J. J. M. Kim,* D. L. Bajramaj,* J. Doelman,† V. R. Osborne,* and J. P. Cant*
*Department of Animal and Poultry Science, University of Guelph, Ontario, N1G 2W1, Canada
†Nutreco Canada AgResearch, Guelph, Ontario, N1G 4T2, Canada

ABSTRACT

The objective of this study was to determine effects of glucose on milk protein yield and mammary mammalian target of rapamycin (mTOR) activity in dairy cattle in early lactation. Eight multiparous cows at 73 ± 8 d in milk were randomly assigned to 2 treatments in a crossover design for two 6-d periods. Treatments were jugular infusion of either saline (Sal) or 896 g/d glucose (Glc). All cows were fed a total mixed ration with 42% neutral detergent fiber, had free access to water, and were milked twice a day. Within each period, blood samples were taken (d 5) and mammary tissue was collected by biopsy (d 6) from each hindquarter for Western blot analysis. In addition to Sal and Glc treatments, on d 6, rapamycin dissolved in 50% dimethyl sulfoxide was administered via the teat canals into the left quarters, with a control solution administered into the right quarters. Rapamycin had no effect on milk protein yields or phosphorylation state of mTOR signaling proteins. Infusions of Glc significantly increased milk yield but only tended to increase milk protein yields. Milk fat tended to be decreased in cows infused with Glc, whereas lactose yields were significantly increased. Glucose infusion did not increase plasma glucose levels, but insulin and nonessential AA concentrations increased by 21 and 16%, respectively, branched-chain AA concentrations decreased 24%, and essential AA concentrations tended to decrease by 14%. Infusion of Glc significantly decreased abundances of both phosphorylated and total ribosomal S6 kinase 1 (S6K1) in mammary tissue by 27 and 11%, respectively. Abundance of phosphorylated eukaryotic initiation factor 4E-binding protein 1 (4EBP1) decreased significantly by 25%, whereas total 4EBP1 exhibited a tendency to decrease by 16%. We conclude that the mTOR signaling pathway is not the only regulator of milk protein synthesis. Decreases in essential AA concentrations in plasma suggest that protein synthesis was stimulated in nonmammary tissues of the body, presumably skeletal muscle.

Key words: glucose infusion, dairy cow, mammary protein synthesis, mammalian target of rapamycin (mTOR)

INTRODUCTION

Milk protein synthesis in the lactating dairy cow is greatly influenced by 2 major nutritional sources, protein and energy, with recent research attention being heavily focused on energy. Many studies have found that glucose infusion in dairy cows increases milk protein yield (Rulquin et al., 2004; Al-Trad et al., 2009; Toerien et al., 2010), whereas others have shown glucose to have no effect on milk protein yield (Hurtaud et al., 1998; Cant et al., 2002; Lemosquet et al., 2009). As a result of these inconsistencies, the mechanism behind how glucose carries out its effects on milk protein synthesis remains unknown.

Glucose infusion does not increase essential AA (**EAA**) concentrations in circulation but Raggio et al. (2006) found that mammary uptake of nonessential AA (**NEAA**) was increased during propionate infusion. Rulquin et al. (2004) suggested that glucose directs AA to the mammary glands by stimulating mammary blood flow. This hyperemia could be mediated by insulin, which stimulates mammary blood flow and milk protein yield in cows (Mackle et al., 2000). However, elevating mammary blood flow with vasodilators does not increase milk protein yield (Lacasse and Prosser, 2003); therefore, blood flow itself cannot explain the effect of glucose or insulin on milk protein yield. If, instead, glucose or insulin stimulated milk protein synthesis, the associated ATP expenditure would be expected to increase mammary blood flow according to a metabolic control hypothesis (Cant et al., 2003). Toerien et al. (2010) found that glucose infusion into fasted cows caused activation of mammary eukaryotic initiation factor 2 (**eIF2**) by dephosphorylation of its α subunit; eIF2 is an important controller of the rate of initiation of mRNA translation (Proud, 2005). Rius et al. (2010) found that the mammalian target of ra-

Received July 12, 2013.
Accepted October 8, 2013.
[1]Corresponding author: rcurtis@uoguelph.ca

pamycin (**mTOR**) pathway of translational regulation was activated in mammary glands of cows infused abomasally with starch. Both insulin and intracellular energy charge are known activators of the mTOR pathway (Wullschleger et al., 2006) and have been shown to activate mTOR in mammary epithelial cells in culture (Appuhamy et al., 2011; Burgos et al., 2013).

We hypothesized that signaling to the translation apparatus in mammary tissue is responsible for the milk protein response to glucose. Therefore, the objective of the present study was to determine effects of glucose on milk protein yield and mammary mTOR activity in dairy cattle in early lactation. Intramammary infusions of the mTOR inhibitor rapamycin were given to test whether mTOR is involved in the effects of glucose on milk protein synthesis.

MATERIALS AND METHODS

Animals and Housing

The Animal Care Committee at the University of Guelph approved all experimental procedures in this study. Eight multiparous Holstein cows began the experiment at 73 ± 8 DIM and 674 ± 84.7 kg of BW. Cows were housed in a tiestall barn at the Ponsonby Livestock Research Station (Ponsonby, ON, Canada) and had free access to feed and water throughout the study. A high-forage diet (Table 1) containing 42% NDF was formulated for 32 kg/d of ME-allowable and MP-allowable milk according to the Cornell Net Carbohydrate and Protein System (CNCPS v6.1; AMTS LLC, Cortland, NY). Feed offered and refused was recorded throughout the study for determination of daily ad libitum feed intakes of individual cows. Feed samples were collected daily and pooled weekly over the 5-wk study and submitted for nutrient composition analysis by wet chemistry at a commercial laboratory (Agri-Food Labs, Guelph, ON, Canada). Orts from individual cows were sampled daily and pooled weekly. Dry matter contents of feed and orts samples were determined using a forced-air oven at 60°C.

Treatments

Cows were randomly assigned to a 6-d continuous infusion into the jugular vein of either a physiological saline (**Sal**) treatment or 896.2 ± 12.9 g/d of glucose (**Glc**) treatment via peristaltic pump. After a 7-d rest period between infusion periods, cows were switched to the opposite treatment. One day before each infusion period, cows were weighed and fitted with long-term catheters (14-gauge, 20 cm; MILA International Inc., Erlinger, KY) in the left jugular vein. Ceftiofur (Excede; Zoetis Canada, Kirkland, QC, Canada) was administered after catheter insertion as a precautionary measure to prevent infection. Glucose was completely dissolved in 3 L of 0.9% NaCl saline and both infusates were sterilized via autoclave.

To test the role of mTOR signaling in effects of glucose on milk protein synthesis, 10 mg of rapamycin dissolved in 150 mL of 50% dimethyl sulfoxide (**DMSO**) solution was rapidly infused, following the 1530 and 0500 h milkings on d 5 and 6, respectively, of each period, into the 2 left mammary glands of each cow via the teat canals. As a control, 150 mL of 50% DMSO was infused into each of the right mammary glands.

Milking and Milk Sampling

During infusion periods, cows were milked twice daily at 0500 and 1530 h using a bucket milker modified to collect milk from the 2 udder halves separately. Milk yields from each udder half were recorded daily and samples were collected and analyzed for fat, lactose and protein by spectroscopy at the Laboratory Services Division, University of Guelph (Guelph, ON, Canada).

Blood Sampling and Metabolite and Hormone Concentrations

Blood samples were collected from the tail vein on d 5 of each period after the morning milking and on d

Table 1. Ingredient and chemical composition (% of DM unless otherwise noted) of the experimental TMR (DM basis) fed to lactating dairy cattle (n = 8) infused i.v. with physiological saline or glucose for 6 d

Item	Value
Ingredient composition	
Alfalfa silage	29.4
Corn silage	24.6
Soybean hulls, ground	11.1
Mixed grains, chopped	7.8
Tripro soy plus[1]	7.3
Corn grain, ground fine	6.9
Straw	5.0
Corn distillers	2.1
Wheat shorts	1.0
Soybean meal	0.9
Canola meal	0.9
Vitamin and minerals	2.9
Chemical composition	
CP	15.1
Soluble CP (% of CP)	35.6
NDICP[2] (% of CP)	32.4
NDF	42.1
ADF	29.1
Lignin	5.7
NFC	37.8
Starch (% of NFC)	40.2
Ether extract	3.1
NE_L (Mcal/kg)	1.4

[1]West Central (Ralston, IA).
[2]Neutral detergent-insoluble CP.

6 before mammary biopsies. Tubes were placed on ice until centrifuged at 2,000 × *g* for 10 min and plasma was transferred to polypropylene tubes to be stored at −20°C. Plasma was analyzed as described by Weekes et al. (2006) for glucose (kit no. GAGO-20; Sigma-Aldrich, St. Louis, MO), insulin (kit no. 90060; Crystal Chem Inc., Downers Grove, IL), BHBA, NEFA (kit no. 999-34691; Wako Chemicals, Neuss, Germany), and triacylglycerol (kit no. TR0100; Sigma-Aldrich). Acetate (kit no. K-ACETRM; Megazyme International, Bray, Ireland) was analyzed following the manufacturer's protocol. Plasma AA concentrations were analyzed using ultra-performance liquid chromatography in conjunction with Empower Chromatography Data Software (Waters Corporation, Milford, MA) according to the protocol described by Boogers et al. (2008).

Mammary Biopsies

On d 6 of each infusion period, following the morning milking, mammary samples were collected via biopsy (Farr et al., 1996) from rapamycin- and DMSO-infused hindquarters after sedation with 0.5 mL of xylazine i.v. and 5 mL of lidocaine injected subcutaneously at the biopsy site. Ketoprofen (3 mg/kg of BW) was administered intramuscularly. Biopsy samples (~0.5 g) were immediately rinsed with saline, snap-frozen in liquid N_2, and stored at −80°C for further analysis.

Cell Signaling Analysis

Approximately 500 mg of mammary tissue was homogenized with 1 mL of lysis buffer (1% Triton X-100, 0.1% SDS, 50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (Thermo Scientific, Nepean, ON, Canada) for 10 s before inversion for 1 h at 4°C. Lysates were centrifuged at 13,000 × *g* for 15 min at 4°C, supernatants extracted, and stored at −20°C. The DNA concentration was measured using the Qubit 2.0 Fluorometer (Life Technologies Inc., Burlington, ON, Canada) with the Qubit dsDNA BR Assay.

Mammary supernatant samples were diluted in lysis buffer and sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.125 *M* Tris HCl, and 0.004% bromophenol blue) and boiled at 95°C for 5 min. Samples (30 μg of total protein), along with BLUeye Prestained Protein Ladder (Froggabio, Toronto, ON, Canada), were separated by 10% SDS-PAGE at 120 V for approximately 90 min. Proteins were electrotransferred (Bio-Rad Laboratories Inc., Mississauga, ON, Canada) onto polyvinylidene difluoride membranes (Millipore, Mississauga, ON, Canada) at 100 V for 60 min. Membranes were incubated in blocking buffer for 1 h at room temperature, followed by three 5-min washes in Tris-buffered saline-Tween (**TBST**) buffer. Membranes were incubated for 1 h at room temperature with rabbit monoclonal antibodies to phospho-S6K1 (Thr389, 1:800; #ab2571, Abcam, Cambridge, MA), phospho-4EBP1 (Thr37/46,1:1,000; #9459, Cell Signaling, Danvers, MA), or eIF4E (1:1,000, #9742, Cell Signaling). All antibodies were diluted using 1% milk TBST buffer. Membranes were washed with TBST buffer and incubated at room temperature for 1 h with horseradish peroxidase-linked anti-rabbit IgG (1:2,000; #7074, Cell Signaling). Membranes were washed and proteins were developed by autoradiography using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Membranes first probed with the phosphorylated protein were immediately stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) according to the manufacturer's protocol and re-probed with antibodies against the corresponding nonphosphorylated proteins S6K1 (1:800; #ab9366, Abcam) or 4EBP1 (1:1,000; #9452, Cell Signaling). Finally, images from radiographic film (VWR International, Pittsburgh, PA) were scanned and the integrated density was determined by using ImageJ software (http://rbs.info.nih.gov/ij). Densities were normalized to the DNA content of homogenates, and phosphorylated protein densities were normalized to those of the corresponding nonphosphorylated protein.

Statistical Analysis

Cow performance was averaged for the 3 d of each period before intramammary infusions. Cow performance and plasma metabolite observations (Y_{ijk}) were subjected to ANOVA using PROC MIXED (SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ijk} = \mu + cow_i + per_j + trt_k + \varepsilon_{ijk},$$

where μ = overall mean, cow_i = random effect of cow (i = 1 to 8), per_j = fixed effect of period (j = 1 or 2), trt_k = fixed effect of treatment (k = 1 or 2), and ε_{ijk} = experimental error. For milk composition and Western blot results after intramammary infusions, the treatment effect was split into intravenous, intramammary, and intravenous × intramammary effects. Yields of milk from control and rapamycin-infused udder halves were not equal before intramammary infusion, so ratios of post- to preinfusion milk yield and composition were calculated and subjected to ANOVA to determine effects of rapamycin. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.15$.

Table 2. Dry matter intake, milk yield, and milk composition of lactating dairy cattle (n = 8) infused i.v. with Sal or Glc for 6 d[1]

	Treatment[2]			
Item	Sal	Glc	SEM	*P*-value
DMI (kg/d)	22.9	22.1	0.72	0.14
Yield				
Milk (kg/d)	37.3	39.5	1.13	0.006
Fat (g/d)	1,666	1,610	71.4	0.12
Protein (g/d)	1,223	1,257	48.9	0.15
Lactose (g/d)	1,803	1,945	56.6	0.001
Percentage				
Fat	4.44	4.07	0.17	<0.001
Protein	3.27	3.19	0.09	0.003
Lactose	4.82	4.89	0.02	<0.001

[1]Data for statistical analysis were taken from the last 3 d of each period.
[2]Sal = infusion of physiological saline; Glc = infusion of 896.2 ± 12.9 g/d of glucose.

RESULTS

Lactational Performance

Dry matter intake did not differ between Glc and Sal treatments (Table 2). Cows infused with Glc produced 2.2 kg/d more milk than those infused with Sal ($P = 0.006$). Milk fat and protein yields of cows on the Glc treatment tended to decrease by 56 g/d ($P = 0.12$) and to increase by 34 g/d ($P = 0.15$), respectively, whereas daily lactose yield was 142 g/d higher in cows infused with Glc ($P = 0.001$). Fat and protein percentages decreased by 0.37 ($P < 0.001$) and 0.08 ($P = 0.003$) percentage units on the Glc treatment, respectively. The Glc-infused cows had, on average, lactose contents 0.07 percentage units higher ($P < 0.001$) compared with those infused with Sal.

Milk yield of rapamycin-infused udder halves was 100% of the yield of the previous 2 d from that half, whereas yield from DMSO-infused halves was 80% of the previous yield. The difference between rapamycin and DMSO treatments was significant ($P = 0.02$). We observed no effect of intramammary rapamycin on milk fat, protein, or lactose percentages ($P > 0.27$).

Plasma Constituents

Glucose treatment did not have a significant effect on plasma glucose (Table 3); however, it did increase insulin concentrations by 21% compared with Sal ($P = 0.004$). Infusions of Glc tended to decrease plasma NEFA concentrations by 16% ($P = 0.09$) relative to Sal, and tended to increase circulating triacylglycerol concentrations by 8% ($P = 0.06$). We found no significant effect on plasma BHBA or acetate concentrations.

The Glc treatment led to a 16% increase of NEAA concentrations in plasma (Table 4; $P = 0.03$) while having a tendency to decrease EAA by 14% ($P = 0.12$). Concentrations of Gln ($P = 0.006$) and Gly ($P = 0.01$) both increased in cows on Glc treatments, by 25 and 28%, respectively. Cows receiving the Glc treatment had 24% lower ($P = 0.044$) concentrations of branched-chain amino acids (**BCAA**) relative to those receiving Sal. Specifically, Glc decreased Val ($P = 0.03$) by 23%, while having the tendency to decrease Leu by 25% ($P = 0.05$) and Ile by 24% ($P = 0.07$). Total circulating AA concentrations did not differ between the 2 treatments.

Table 3. Plasma concentrations of insulin and metabolites in lactating dairy cattle (n = 8) infused i.v. with Sal or Glc for 6 d

	Treatment[1]			
Item	Sal	Glc	SEM	*P*-value
Glucose (m*M*)	3.11	3.26	0.06	0.12
Insulin (ng/mL)	1.23	1.49	1.00	0.004
BHBA (m*M*)	0.71	0.67	0.08	0.75
NEFA (μ*M*)	118	99	6.48	0.09
Acetate (m*M*)	1.37	1.39	0.08	0.88
Triacylglycerol (μ*M*)	99.0	107.0	5.77	0.06

[1]Sal = infusion of physiological saline; Glc = infusion of 896.2 ± 12.9 g/d of glucose.

Translational Proteins

Treatments did not differ in abundance of total eukaryotic initiation factor 4E (eIF4E; data not shown). We observed decreases in phosphorylated and total S6K1 abundances of 27% ($P = 0.05$) and 11% ($P = 0.005$), respectively, in mammary tissue of cows on the Glc treatment (Figure 1). However, Glc failed to have an effect on phosphorylated S6K1 normalized to S6K1 abundance in mammary tissue. The Glc treatment led to a 25% decrease in phosphorylation of mammary 4EBP1 (Figure 2; $P = 0.009$) and a tendency to decrease total 4EBP1 by 16% ($P = 0.12$). Phosphorylated 4EBP1 normalized to total 4EBP1 tended to be 16% lower in mammary tissue of cows that received Glc ($P = 0.07$). Rapamycin treatment tended to increase total S6K1 abundance in mammary tissue ($P = 0.06$) by 7%. We observed an interaction between intravenous and intramammary infusions ($P = 0.001$), where rapamycin decreased abundance of total S6K1 on Sal treatment and increased it on the Glc treatment. However, the interaction was opposite for total 4EBP1 ($P = 0.08$), where rapamycin increased abundance on the Sal treatment and decreased it on Glc.

DISCUSSION

Contrary to significant increases in milk protein yield in response to infusion of glucose or glucogenic substrates (Rulquin et al., 2004; Raggio et al., 2006; Rius et al., 2010; Toerien et al., 2010), we found only a tendency for an increased milk protein yield. In agreement with our study, Huhtanen et al. (2002) reported only a tendency for increased milk protein yield with abomasal infusion of 250 g/d of Glc for 14 d. Others reported no significant change in protein yield with Glc (Oldick et al., 1997; Hurtaud et al., 1998; Cant et al., 2002). It is interesting to note that cows infused with Glc had greater milk production efficiency as a result of a tendency for lower DMI and significantly higher milk production than cows on the Sal treatment. This efficiency difference can be explained by the lactose synthesis response to Glc infusion, causing increased water secretion into milk and thus, increased milk yields. This lactose response was also responsible for the decreases in milk fat and protein percentages on the Glc treatments, which Cant et al. (2002) reported in their study.

The increase in lactose production accounted for approximately 17% of the 896 g/d of infused glucose. It is

Table 4. Plasma concentrations of amino acids (μM) in lactating dairy cattle (n = 8) infused i.v. with Sal or Glc for 6 d

	Treatment[1]			
AA	Sal	Glc	SEM	*P*-value
His	32	35	2.0	0.32
Asn	61	66	4.6	0.43
Ser	56	67	7.58	0.24
Gln	503	674	28.89	0.006
Arg	83	70	5.81	0.17
Gly	184	255	20.98	0.01
Glu	95	96	3.54	0.80
Thr	87	102	6.40	0.14
Ala	288	282	20.03	0.76
Pro	84	83	4.58	0.99
Lys	71	60	5.44	0.20
Tyr	57	51	3.84	0.33
Met	19	19	1.33	0.91
Val	276	214	18.27	0.03
Ile	126	96	9.98	0.07
Leu	159	119	13.86	0.05
Phe	48	39	3.22	0.10
BCAA[2]	561	429	41.74	0.04
EAA[3]	900	774	57.89	0.12
NEAA[4]	1,332	1,589	64.23	0.03
TAA[5]	2,232	2,343	85	0.38

[1]Sal = infusion of physiological saline; Glc = infusion of 896.2 ± 12.9 g/d of glucose.

[2]BCAA = branched-chain amino acids (Val, Ile, and Leu).

[3]EAA = essential amino acids.

[4]NEAA = nonessential amino acids (Ser, Gln, Arg, Gly, Asp, Glu, Ala, Pro, Asn, and Tyr).

[5]TAA = EAA + NEAA.

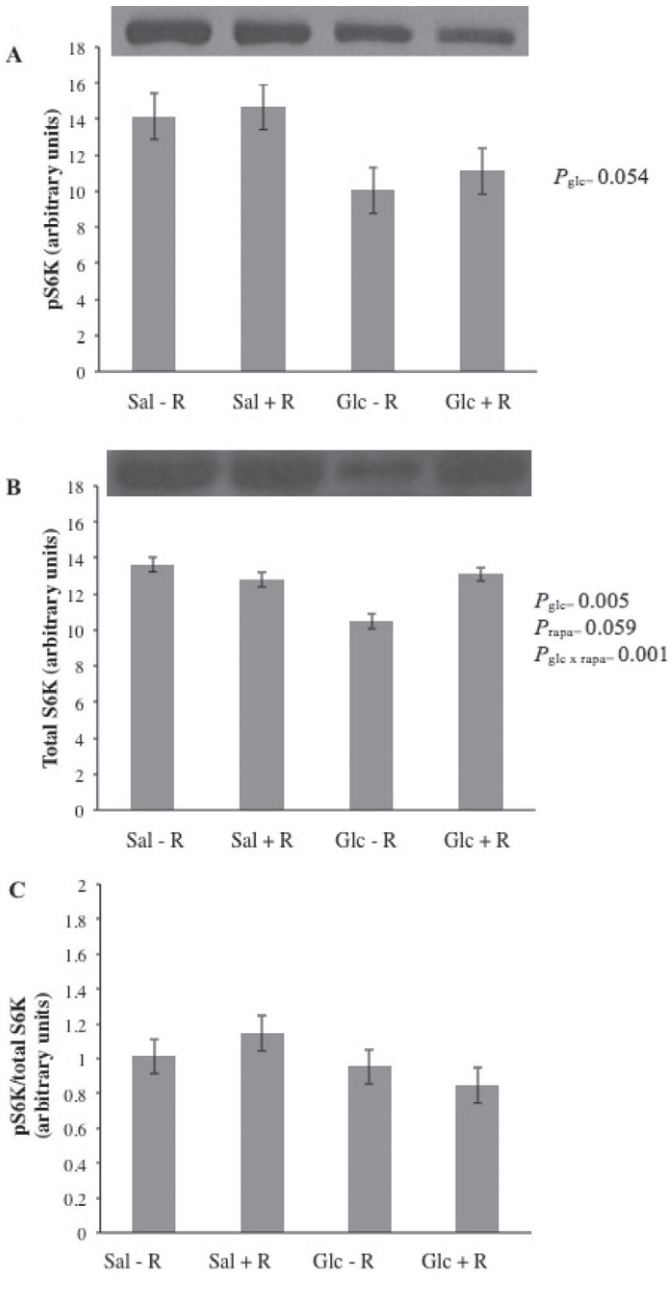

Figure 1. Abundances of (A) phosphorylated ribosomal S6 kinase 1 (pS6K1), (B) total S6K1 normalized per unit of DNA, and (C) pS6K1 normalized to total S6K1 in mammary tissue of lactating dairy cattle (n = 8) infused i.v. with glucose (Glc) or saline (Sal), with and without intramammary rapamycin (R) treatment. Values are mean densities and P_{glc}, P_{rapa}, and $P_{glc \times rapa}$ represent *P*-values for Glc, rapamycin, and the interaction between Glc and rapamycin, respectively.

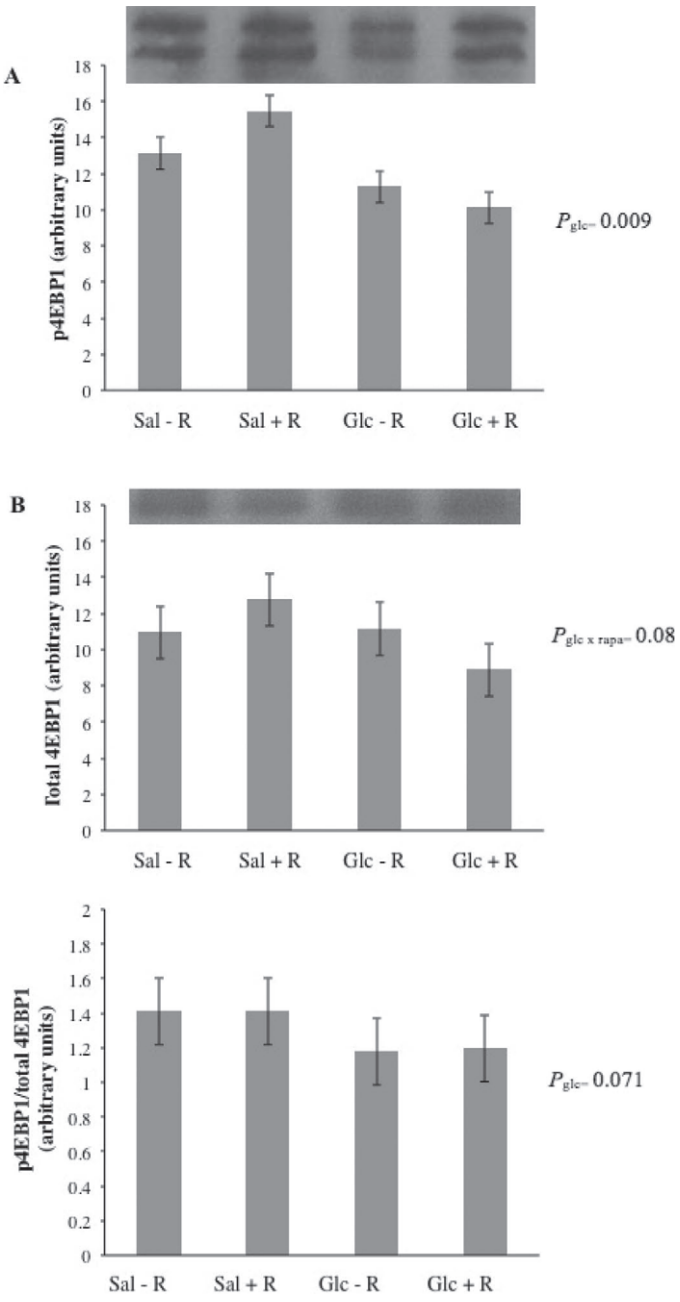

Figure 2. Abundances of (A) phosphorylated eukaryotic initiation factor 4E-binding protein 1 (p4EBP1), (B) total 4EBP1 normalized per unit DNA, and (C) p4EBP1 normalized to total 4EBP1 in mammary tissue of lactating dairy cattle (n = 8) infused with glucose (Glc) or saline (Sal), with and without rapamycin (R) treatment. Values are mean densities and P_{glc}, P_{rapa}, and $P_{glc \times rapa}$ represent *P*-values for Glc, rapamycin, and the interaction between Glc and rapamycin, respectively.

possible that some of the remaining 83% of infused glucose spared gluconeogenesis in the liver, because cows infused with Glc had higher plasma NEAA concentrations than those on Sal treatments. Nonessential AA are consumed in the liver for gluconeogenesis, resulting in elevated levels of NEAA in the blood when gluconeogenesis is suppressed. This conclusion is in agreement with that of Lemosquet et al. (2004), who also found an increase in glucogenic NEAA concentrations when Glc or propionic acid was infused into lactating dairy cattle.

Plasma BCAA concentrations were decreased in cows receiving Glc treatments, in accordance with previous experiments (Raggio et al., 2006; Rius et al., 2010; Toerien et al., 2010). However, these previous studies found an increase in milk protein yield, whereas ours did not. A decrease in plasma BCAA concentrations can be indicative of muscle protein synthesis because these EAA account for a disproportionately large percentage of the AA profile of skeletal muscle protein (Anthony et al., 2001). Lower BCAA levels in blood, in conjunction with unaffected milk protein yields, suggest that protein synthesis was stimulated in muscle rather than the mammary glands with infusion of Glc.

Effects of glucose on mammary and muscle protein synthesis could be mediated by insulin. Plasma insulin concentration was elevated 21% with Glc, which is similar to increases observed during previous glucose infusions (Hurtaud et al., 2000; Lemosquet et al., 2004; Rulquin et al., 2004). Insulin clamp studies in dairy animals increase plasma insulin 3 to 4 times over basal concentrations (Mackle et al., 2000; Bequette et al., 2001). Accompanying these elevated insulin levels is a reduction of circulating BCAA (Mackle et al., 2000; Bequette et al., 2001), as well as a higher efficiency of BCAA extraction by skeletal muscle for protein synthesis. Insulin clamps caused higher EAA extraction efficiency by the mammary glands (Bequette et al., 2001) as well as increased mammary blood flow to support protein synthesis (Mackle et al., 2000; Bequette et al., 2001). Because increasing glucose via infusions causes blood insulin to increase simultaneously, glucose effects on milk protein yield may, in part, be explained by insulin and its influence on protein synthesis in mammary and muscle tissue.

Although the milk protein yield response to Glc appears to be associated with changes in mammary blood flow (Cant et al., 2002; Rulquin et al., 2004), the flow response cannot be causing protein yields to increase because increasing flow by vasodilation does not affect milk protein synthesis or secretion (Lacasse and Prosser, 2003). The mTOR signaling pathway, which is responsible for translational regulation of protein synthesis in many tissues, is activated by both insulin and intracellular ATP levels in mammary epithelial cells in culture (Appuhamy et al., 2011; Burgos et al., 2013). Glucose infusion can affect insulin concentration, as in our experiment, and mammary energy charge. Although 9 h of glucose infusion into fasted cows did not affect mammary mTOR activity (Toerien et al., 2010), Rius et al. (2010) found that abomasal infusion of starch for 36 h led to activation of mammary S6, a substrate of the mTOR substrate S6K1. We found that Glc tended to decrease the phosphorylation state of 4EBP1 and had no effect on S6K1 phosphorylation, indicating that mTOR was not activated and was potentially depressed. Furthermore, total and phosphorylated S6K1 abundances were decreased by Glc, suggesting that translational capacity was reduced.

We were unable to use rapamycin to probe the involvement of mammary mTOR in the milk protein response to glucose because intramammary rapamycin infusion failed to inhibit mTOR signaling, as evident from the lack of decrease in milk protein yield and small, inconsistent S6K1/4EBP1 phosphorylation responses. It is plausible that the route of administration was ineffective in delivery of rapamycin into the glands and across mammary epithelial cell membranes. The 150-mL volume of solution infused into the mammary glands may not have reached cells that comprised the tissue collected by biopsy. Although the 10-mg dose of rapamycin is sufficient for inhibition of mTOR in an animal with a BW of 13 kg (Suryawan et al., 2008), greater than the weight of a bovine mammary gland, rapid washout of the dose into the venous circulation may necessitate much higher doses to maintain a prolonged inhibition of mammary mTOR.

Our finding that abundance of mTOR signaling proteins was depressed highlights a novel aspect of long-term translational control. Changes in the phosphorylation state of these proteins occurs rapidly, within minutes, and has been shown to be important in short-term upregulation of protein synthesis in tissues after a meal (Davis et al., 2000), for example. Long-term effects of a change in nutritional status, such as after 6 d of glucose infusion, may be expected to be mediated not just through phosphorylation state of these regulatory proteins but also through transcriptional regulation of their abundance. In support of this contention, Sciascia et al. (2013) found that after a 6-d growth hormone treatment of lactating cows, abundances of S6K1 and several other mTOR signaling compounds were elevated in mammary tissue. Transcriptional regulation of S6K1 is poorly understood. Estrogen stimulates mammary S6K1 expression (Maruani et al., 2012) but, to our knowledge, the panel of transcription factors binding upstream of the S6K1 gene has not been identified. Amino acids, particularly Leu, are potent activators of mammary mTOR signaling in vitro (Burgos et al., 2010; Appuhamy et al., 2012), so the decrease in plasma EAA concentrations, particularly BCAA, could account for the reduction in phosphorylated S6K1 and 4EBP1 abundances.

If mTOR, particularly through the key signaling proteins 4EBP1 and S6K1, was solely responsible for regulating milk protein synthesis, a decrease in milk protein yield would have been expected as a consequence of mTOR downregulation. However, we found no such decline in milk protein yield. We conclude that

mechanisms other than mTOR signaling can influence milk protein yields. The integrated stress response network involving eIF2 phosphorylation and the insulin signaling pathway impinging on eIF2B activity are 2 alternative translational control mechanisms (Proud, 2005). Transcriptional regulation of the milk protein genes is also a potential mechanism for milk protein synthesis control.

CONCLUSIONS

In the present study, our objective to stimulate a milk protein response to Glc infusion into dairy cattle was not met. We propose that skeletal muscle was more sensitive than mammary tissue to Glc treatment in this study. The decreased concentrations of BCAA in plasma, in conjunction with decreased phosphorylation state of 4EBP1, decreased abundance of S6K1 in mammary tissue, and the lack of a milk protein response, collectively support the proposition that glucose directs amino acids into skeletal muscle, rather than into mammary glands for milk protein synthesis. In addition, the maintenance of milk protein yield while mTOR signaling was depressed indicates that the mTOR pathway is not the only regulator of milk protein synthesis.

ACKNOWLEDGMENTS

The authors gratefully thank Laura Wright and staff at the Ponsonby Dairy Research Station (Ponsonby, ON, Canada) for their technical help and animal care during the experiment. The authors also acknowledge the financial support of Dairy Farmers of Canada (Mississauga, ON, Canada), Agriculture and Agri-Food Canada (Ottawa, ON, Canada), and Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA; Guelph, ON, Canada). R. Curtis was the recipient of an OMAFRA HQP Scholarship.

REFERENCES

Al-Trad, B., K. Reisberg, T. Wittek, G. B. Penner, A. Alkaassem, G. Gabel, M. Furll, and J. R. Aschenbach. 2009. Increasing intravenous infusions of glucose improve body condition but not lactation performance in midlactation dairy cows. J. Dairy Sci. 92:5645–5658.

Anthony, J. C., T. G. Anthony, S. R. Kimball, and L. S. Jefferson. 2001. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. J. Nutr. 131:856S–860S.

Appuhamy, J. A. D. R. N., A. L. Bell, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2011. Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. J. Nutr. 141:1209–1215.

Appuhamy, J. A. D. R. N., N. A. Knoebel, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. J. Nutr. 142:484–491.

Bequette, B. J., C. E. Kyle, L. A. Crompton, V. Buchan, and M. D. Hanigan. 2001. Insulin regulates milk production and mammary gland and hind-leg amino acid fluxes and blood flow. J. Dairy Sci. 84:241–255.

Boogers, I., W. Plugge, Y. Q. Stokkermans, and A. L. L. Duchateau. 2008. Ultra-performance liquid chromatographic analysis of amino acids in protein hydrolysates using an automated pre-column derivatisation method. J. Chromatogr. A 1189:406–409.

Burgos, S. A., M. Dai, and J. P. Cant. 2010. Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. J. Dairy Sci. 93:153–161.

Burgos, S. A., J. J. M. Kim, M. Dai, and J. P. Cant. 2013. Energy depletion of bovine mammary epithelial cells activates AMPK and suppresses protein synthesis through inhibition of mTORC1 signaling. Horm. Metab. Res. 45:183–189.

Cant, J. P., R. Berthiaume, H. Lapierre, P. H. Luimes, B. W. McBride, and D. Pacheco. 2003. Responses of the bovine mammary glands to absorptive supply of single amino acids. Can. J. Anim. Sci. 83:341–355.

Cant, J. P., D. R. Trout, F. Qiao, and N. G. Purdie. 2002. Milk synthetic response of the bovine mammary gland to an increase in the local concentration of arterial glucose. J. Dairy Sci. 85:494–503.

Davis, T. A., H. V. Nguyen, A. Suryawan, J. A. Bush, L. S. Jefferson, and S. R. Kimball. 2000. Developmental changes in the feeding-induced stimulation of translation initiation in muscle of neonatal pigs. Am. J. Physiol. Endocrinol. Metab. 279:E1226–E1234.

Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. J. Dairy Sci. 79:543–549.

Huhtanen, P., A. Vanhatalo, and T. Varvikko. 2002. Effects of abomasal infusions of histidine, glucose, and leucine on milk production and plasma metabolites of dairy cows fed grass silage diets. J. Dairy Sci. 85:204–216.

Hurtaud, C., S. Lemosquet, and H. Rulquin. 2000. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. J. Dairy Sci. 83:2952–2962.

Hurtaud, C., H. Rulquin, and R. Verite. 1998. Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on corn silage. J. Dairy Sci. 81:3239–3247.

Lacasse, P., and C. G. Prosser. 2003. Mammary blood flow does not limit milk yield in lactating goats. J. Dairy Sci. 86:2094–2097.

Lemosquet, S., G. Raggio, G. E. Lobley, H. Rulquin, J. Guinard-Flament, and H. Lapierre. 2009. Whole-body glucose metabolism and mammary energetic nutrient metabolism in lactating dairy cows receiving digestive infusions of casein and propionic acid. J. Dairy Sci. 92:6068–6082.

Lemosquet, S., S. Rigout, A. Bach, H. Rulquin, and J. W. Blum. 2004. Glucose metabolism in lactating cows in response to isoenergetic infusions of propionic acid or duodenal glucose. J. Dairy Sci. 87:1767–1777.

Mackle, T. R., D. A. Dwyer, K. L. Ingvartsen, P. Y. Chouinard, D. A. Ross, and D. E. Bauman. 2000. Effects of insulin and postruminal supply of protein on use of amino acids by the mammary gland for milk protein synthesis. J. Dairy Sci. 83:93–105.

Maruani, D. M., T. N. Spiegel, E. N. Harris, A. S. Shachter, H. A. Unger, S. Herrero-Gonzalez, and M. K. Holz. 2012. Estrogenic regulation of S6K1 expression creates a positive regulatory loop in control of breast cancer cell proliferation. Oncogene 31:5073–5080.

Oldick, B. S., C. R. Staples, W. W. Thatcher, and P. Gyawu. 1997. Abomasal infusion of glucose and fat—Effect on digestion, production, and ovarian and uterine functions of cows. J. Dairy Sci. 80:1315–1328.

Proud, C. G. 2005. eIF2 and the control of cell physiology. Semin. Cell Dev. Biol. 16:3–12.

Raggio, G., S. Lemosquet, G. E. Lobley, H. Rulquin, and H. Lapierre. 2006. Effect of casein and propionate supply on mammary protein metabolism in lactating dairy cows. J. Dairy Sci. 89:4340–4351.

Rius, A. G., J. A. D. R. N. Appuhamy, J. Cyriac, D. Kirovski, O. Becvar, J. Escobar, M. L. McGilliard, B. J. Bequette, R. M. Akers, and M. D. Hanigan. 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. J. Dairy Sci. 93:3114–3127.

Rulquin, H., S. Rigout, S. Lemosquet, and A. Bach. 2004. Infusion of glucose directs circulating amino acids to the mammary gland in well-fed dairy cows. J. Dairy Sci. 87:340–349.

Sciascia, Q., D. Pacheco, and S. A. McCoard. 2013. Increased milk protein synthesis in response to exogenous growth hormone is associated with changes in mechanistic (mammalian) target of rapamycin (mTOR) C1-dependant and independent cell signaling. J. Dairy Sci. 96:2327–2338.

Suryawan, A., A. S. Jeyapalan, R. A. Orellana, F. A. Wilson, H. V. Nguyen, and T. A. Davis. 2008. Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. Am. J. Physiol. Endocrinol. Metab. 295:E868–E875.

Toerien, C. A., D. R. Trout, and J. P. Cant. 2010. Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal S6 kinase 1. J. Nutr. 140:285–292.

Weekes, T. L., P. H. Luimes, and J. P. Cant. 2006. Responses to amino acids imbalances and deficiencies in lactating dairy cows. J. Dairy Sci. 89:2177–2187.

Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. Cell 124:471–484.