Mammary mTOR Activity during Intravenous Glucose Infusions into Lactating Dairy Cows

by

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ABSTRACT

MAMMARY mTOR ACTIVITY DURING INTRAVENOUS GLUCOSE INFUSIONS INTO LACTATING DAIRY COWS

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The effects of glucose on milk protein yield and mammary mTOR activity were studied in eight multiparous early lactation Holstein cows fed a TMR with 40% NDF content. Treatments were jugular infusions of either physiological saline (SAL) or approximately 900 g/d glucose (GLC) in a cross over design for two 6 d periods during which blood, milk and mammary tissue were sampled. GLC infusion increased plasma insulin but did not significantly increase plasma glucose and only tended to increase milk protein yields. NEAA and BCAA plasma concentrations significantly increased and decreased, respectively, with GLC treatments. Furthermore, reduction of the phosphorylation state of mTOR signalling protein 4EBP1, as well as reduction of total and phosphorylated S6K1 abundances resulted from the infusion of GLC. The expected increase in milk protein synthesis due to glucose infusion was not observed, presumably because protein synthesis was stimulated in muscle tissue rather than the mammary glands.

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To my dad, I dedicate my thesis to you. You always knew I would be doing something special with my life and this research is exactly that.

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LIST OF ABBREVIATIONS

4EBP1	eukaryotic initiation factor (eIF) 4E-binding protein 1
AA	amino acid(s)
AKT/PKB	protein kinase B
AMPK	5’ adenosine monophosphate activated kinase
ATP	adenosine triphosphate
BCAA	branch chain amino acids
BHBA	beta hydroxybutyrate
BW	body weight
DIM	days in milk
DM	dry matter
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EAA	essential amino acids
eIF/2/2B/2α/4E/4A/4G/4F	eukaryotic initiation factor/2/2B/2α/4E/4A/4G/4F
GCN2	general control non-repressed 2 protein kinase
GDP/GTP	guanosine diphosphate/triphosphate
GLC	glucose
HRI	heme-regulated inhibitor kinase
ISR	integrated stress response network
mRNA	messenger ribonucleic acid
mTOR (C1/2)	mammalian target of rapamycin (complex 1/2)
NADPH	nicotinamide adenine dinucleotide phosphate

NDF	neutral detergent fibre
NEAA	non-essential amino acids
NSC	non-structural carbohydrates
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PI3K	phosphatidylinositol-3-kinase
PKR	protein kinase R
S6K/1	ribosomal S6 kinase 1
SAL	saline
SCC	somatic cell count
TAA	total amino acids
TAG	triacylglycerol
TMR	total mixed ration
tRNA	transfer ribonucleic acid
VFA	volatile fatty acids

CHAPTER 1

LITERATURE REVIEW

General Introduction

The mammary glands of a dairy cow produce and secrete a remarkable amount of milk protein during lactation. Many years of research have shown that milk protein yields in lactating dairy cattle are influenced by supplemental glucose when supplied to cows by either feeding a high-concentrate diet, postruminal infusion of glucose, propionate or starch, as well as by euglycemic infusion of insulin. Although greatly studied, the mechanism by which glucose manipulates milk protein yield remains unknown. Discovering the mechanism would enhance our understanding of mammary gland dynamics; particularly how nutrients impact milk protein yield in the mammary gland. More importantly, this discovery would allow dairy nutritionists and feed formulators to better utilize feed ingredients, particularly protein, for managing milk protein yield. As a result of improved feed ingredient utilization in ration formulation, dairy producers' feed costs could potentially be reduced along with a reduction in environmental impact of dairy farming in Canada. The objective of this study was to investigate potential mechanisms responsible for the milk protein synthesis response to postruminal glucose.

Energy Metabolism In Dairy Cows For Protein Synthesis

The main substrates of digestion in the rumen of dairy cattle are structural carbohydrates of plants, non-structural carbohydrates (NSC) and nitrogen-containing compounds. NSC, such as sugars and starch, are of particular interest in relation to protein synthesis in the mammary gland. Compared to structural carbohydrates, NSC are much more rapidly hydrolysed to mono and disaccharides by microbial amylases upon entry into the rumen (Figure 1). Rumen microbes

consume these mono and disaccharides to yield adenosine triphosphate (ATP) for cell function, volatile fatty acids (VFA) as waste products and cell components, including microbial protein. The 3-carbon VFA, propionate, is absorbed into the blood stream across the rumen wall to be converted into glucose via gluconeogenesis in the liver. A large portion of glucose made in the liver is used by the mammary glands for lactose synthesis, but is also used in the mammary glands to yield nicotinamide adenine dinucleotide phosphate (NADPH) for fat synthesis, as well as energy in the form of ATP. The 2-carbon VFA, acetate, is taken out of blood by the mammary glands to provide the majority of ATP for milk synthesis. Microbial protein passes from the rumen into the lower gut where it is broken down into individual amino acids (AA). These AA for use by the cow, along with other AA obtained from dietary protein that escapes rumen degradation, enter the blood stream and are taken up by the mammary gland for protein synthesis. It is the combination of ATP from VFA and AA from microbial and feed proteins that supply substrate for milk protein synthesis.

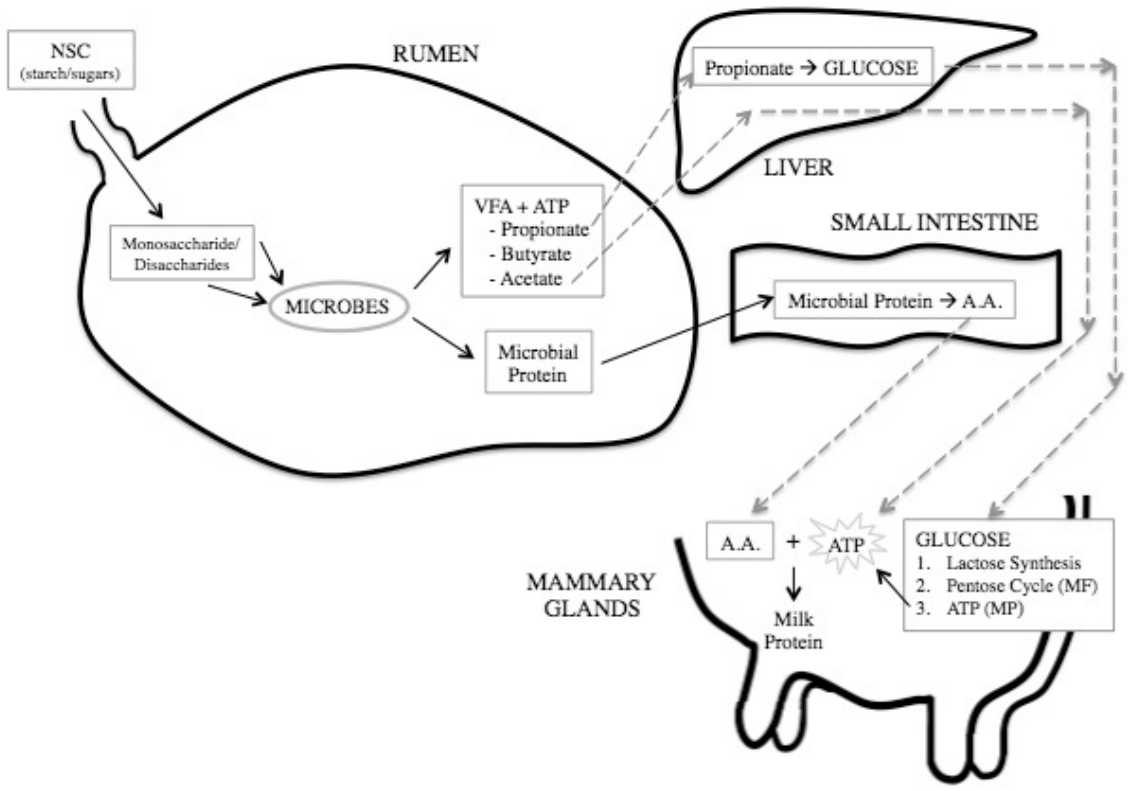

Figure 1. General overview of non-structural carbohydrate (NSC) digestion in the dairy cow with regard to milk protein synthesis.

Milk Protein Synthesis Response to Glucose

There has been extensive research conducted over the last few decades regarding dietary glucose utilization by the lactating dairy cow. Glucose has been shown to have the ability to stimulate milk protein synthesis when supplied to the cow in different ways. Multiple theories have been presented which help to suggest how this response is occurring.

The main explanation of how milk protein yield is regulated in response to dietary changes has to do with AA supply to the mammary gland, specifically essential AA (EAA). The mammary gland is a major net user of EAA, for the purpose of building milk proteins as well as synthesizing non-essential AA (NEAA). Certain EAA can be limiting factors for the synthesis of protein in the mammary glands. Lys and Met are largely considered first-limiting AA as increased post-ruminal supply of either AA can increase milk protein yield (Bequette et al., 1998; Crompton et al., 2002). Other studies have also found His to be stimulatory to production of protein within the mammary glands (Vanhatalo et al., 1999a).

The simplest method researchers have used to increase glucose supply to dairy cows is to increase the dietary proportion of energy concentrates containing NSC. Increasing the concentrate content in the diet of lactating cows has shown to increase milk protein percent by 5-15% (Macleod et al., 1983; Sahn et al., 2002). Moreover, similar findings were revealed in pastured dairy goats fed varying concentrate supplementation, where goats fed higher concentrate levels produced, on average, up to 26% more milk protein (Min et al., 2005). This milk protein stimulation by increased concentrate in the diet could be attributed to an increased ruminal microbial protein outflow, supplying more EAA to the mammary gland for milk protein synthesis, as demonstrated in a study by Rode et al. (1985). However, a study found no significant difference in microbial nitrogen output when comparing cows that were fed a control

versus starch diet and a urea versus urea + starch diet (Cameron et al., 1991). Therefore, increased microbial protein flow to the mammary gland does not always explain the milk protein stimulation by concentrate feeding. Abomasal infusion of glucose on a low protein diet marginally increase milk protein synthesis, which would favour the argument of increases in protein synthesis.

Postruminal glucose infusions, either directly into the abomasum or administered into circulation, have elicited an increase in milk protein yield (Wilson et al., 1967; Vik-Mo et al., 1974; Raggio et al., 2006a; Toerien et al., 2009; Rius et al., 2009). Numerous studies have not found statistically significant effects, but only tendencies for glucose to stimulate milk protein yield (Hurtaud et al., 2000; Huhtanen et al., 2002; Rigout et al., 2003; Vanhatalo et al., 2003 Rulquin et al., 2004). Moreover, some studies have reported glucose to have no effect on milk protein yield when infused into cows (Clark et al., 1977; Frobish and Davis, 1977; Chalmers et al., 1980; Oldick et al., 1997; Hurtaud et al., 1998; Cant et al., 2002).

The response to glucose is interesting because postruminal infusion does not influence the absorptive AA supply, and thus the limiting EAA explanation for milk protein stimulation does not hold. In fact, concentrations of many EAA, especially the branch chain AA (BCAA) Leu, Ile and Val, are decreased in circulation during glucose infusion due to uptake by the mammary gland (; Lemosquet et al., 2004). Despite such decreases, mammary uptakes of EAA are often increased (Rulquin et al., 2004; Raggio et al., 2006b), which may be related to increases in mammary blood flow (Rulquin et al., 2004; Raggio et al., 2006b; Rius et al., 2010). Mammary uptake of glucose also typically increases with glucose infusion (Rigout et al., 2002b), supplying more energy to the mammary glands for protein synthesis. However, Lemosquet et al. (2009) found no change in glucose uptake during infusion of propionic acid, yet still observed an

increase in milk protein yield, suggesting that glucose uptake itself does not increase mammary protein synthesis.

When discussing effects of glucose on mammary protein synthesis, it is essential to also consider insulin and its physiological effects in the body. Insulin is a peptide hormone produced by β cells in the pancreas that is responsible for the homeostatic regulation of blood glucose concentration. After consuming a meal, especially one high in carbohydrates in the monogastric, glucose levels in the blood spike, causing insulin to be rapidly released into the bloodstream. Insulin signals to cells to take up glucose, thereby lowering blood glucose levels back to normal. In the non-lactating animal, insulin stimulates glycolysis and ATP production from glucose in muscle and adipose cells, lipogenesis in liver and adipose tissue, and protein synthesis in skeletal muscle (Frayn, 2009). The role of insulin in the lactating animal is extended to stimulation of the mammary gland where insulin has been shown to have large effects on mammary glucose uptake and lipogenesis in lactating rats (Burnol et al., 1988; Oller do Nascimento et al., 1989), but less of an effect on glucose utilization in lactating dairy cows (Mackle et al., 2000). Several days of euglycemic insulin infusion into lactating ruminants increases milk protein yields by 10 to 25%, despite decreases in circulating concentrations of EAA, particularly BCAA (Mackle et al., 2000; Bequette et al., 2001), reminiscent of the responses to glucose. The decreased AA concentrations are likely due to insulin stimulation of protein synthesis in various tissues of the body including skeletal muscle and the mammary glands (Bequette et al., 2001). Insulin infusions have increased plasma insulin concentrations approximately 4-fold (Mackle et al., 2000; Bequette et al., 2001), while glucose infusions typically increase plasma insulin by only 15-22% (Lemosquet et al., 2004; Toerien et al., 2009). Nevertheless, the evidence suggests that mammary gland protein

synthesis is under the influence of the insulin-signaling cascade and the milk protein response to glucose may be mediated by insulin.

As a result of the anabolic effects of insulin, not only does glucose concentration in blood decrease, but EAA, acetate, fatty acids and ketone bodies are all decreased in blood (Brockman and Laarveld, 1985; Eisemann and Huntington, 1994; Mackle et al., 2000). These blood metabolite changes can be expected to influence blood flow rate to the mammary glands. Cant et al. (2003) describe an energy balance hypothesis stating that mammary blood flow is locally regulated to match ATP expenditure with ATP utilization. For instance, if protein synthesis is high, yet plasma concentrations of energy metabolites are low, then a hyperemic response would take place in order to match energy supply with demands within the glands. The energy balance hypothesis explains the observed increase in mammary blood flow during euglycemic insulin infusion as a consequence of the drop in energy metabolite concentrations (Cant et al., 2003). Similarly, glucose infusion for 14 d caused plasma concentrations of acetate, ketone bodies, and fatty acids to decrease and mammary blood flow increased (Rigout et al., 2002). After only 10 h of glucose infusion, which did not affect plasma concentrations of energy metabolites besides glucose, mammary blood flow was depressed, consistent with the energy balance hypothesis, and milk protein yield was not affected. Rulquin et al. (2004) suggested that glucose directs AA to the mammary glands by stimulating mammary blood flow. However, elevating mammary blood flow with vasodilators does not increase milk protein yield (Lacasse and Prosser, 2003) so blood flow itself cannot explain the effect of glucose or insulin on milk protein yield.

Findings in the literature support the hypothesis that some or all of the effect of glucose on milk protein yield is mediated by insulin, and that insulin could be directly stimulating protein synthesis within the mammary glands themselves. In tissues of growing animals, insulin

stimulates protein synthesis through an important protein kinase called mammalian target of rapamycin (mTOR). It is possible that mammary mTOR is involved in the stimulation of milk protein production by postruminal glucose.

Regulation of Protein Synthesis via the mTOR Pathway

mTOR is a highly conserved signaling protein across eukaryotes that plays a critical role in regulating the rate of cell growth, metabolism and proliferation by sensing a variety of nutritional and hormonal stimuli (Wullschleger et al., 2006). mTOR functions as a serine/threonine (Ser/Thr) kinase and is a member of the phosphatidylinositol-3-kinase (PI3K)-related kinase family (Holz and Blenis, 2005). mTOR is considered to be the principal regulator of protein synthesis, and because protein synthesis is an energy demanding activity, cells have evolved a highly sensitive mechanism to control protein translation rates (Tee and Blenis, 2005). mTOR regulates protein synthesis through its downstream effectors, which consequently control translation efficiency within cells. These essential targets include the eukaryotic initiation factor 4E-binding factor (4EBP1), the eukaryotic initiation factor 4E (eIF4E) and the ribosomal S6 kinase 1 (S6K1) (Tee and Blenis, 2005).

Eukaryotic initiation factors (eIF) help to regulate messenger ribonucleic acid (mRNA) translation by controlling initiation of translation, a multi-step process. A control point for translation initiation is formation of the eIF4F complex, which is responsible for recruiting ribosomal subunits to the mRNA for cap-dependent translation. This complex is comprised of three polypeptides, those being eIF4A, eIF4E and eIF4G. In order for the eIF4F complex to become functional, a series of interactions between the subunits and mRNA must occur. An important control point of this cascade involves the eIF4E subunit and 4EBP1. When in its

unphosphorylated state, 4EBP1 interacts with eIF4E at the eIF4G-binding site, thereby blocking eIF4G attachment and formation of the eIF4F complex. This ultimately inhibits 5'-cap-dependent mRNA translation, slowing protein synthesis (Tee and Blenis, 2005). Alternatively, when 4EBP1 is phosphorylated by mTOR, it possesses less affinity for eIF4E allowing eIF4G to bind to initiate mRNA translation into protein (Gingras et al., 1999).

It is still poorly defined how exactly S6K1 stimulates protein synthesis. It was once thought that phosphorylation of ribosomal protein S6 by mTOR-activated S6K1 led to accelerated translation of mRNAs coding for ribosomal proteins and translation factors (Meyuhas, 2000). However, studies with mutant S6 constructs established that S6 is not required for mTOR to stimulate synthesis of translational proteins (Magnuson et al., 2012). Now it is known that S6K1 phosphorylates many participants in global protein synthesis, including those that facilitate mRNA splicing and unwinding, ribosome translocation, and protein folding (Magnuson et al., 2012).

Figure 2 shows the mTOR signaling pathway and the steps potentially modified in mammary glands during glucose infusion. Insulin, through the PI3K/Akt signalling pathway, activates mTOR. Glucose taken up into mammary cells may be catabolized to generate ATP, which stimulates mTOR through inhibition of the adenosine monophosphate (AMP)-activated kinase AMPK. AA, particularly Leu, are also mTOR activators, and Leu concentrations are typically lowered during glucose infusion. Thus, both positive and negative regulation of mTOR may be expected.

Mammary glands of lactating cows expressed a lower association of eIF4E with 4EBP1 compared to non-lactating cows (Toerien and Cant, 2007). S6K1 abundances were also elevated in lactating mammary tissue, implying S6K1 plays a role in regulation of mammary protein

synthesis (Toerien and Cant, 2007). A study by the same group found there to be no significant change in phosphorylated S6K1 when fasted cows were infused with glucose for 9 h (Toerien et al., 2009). However, abomasal infusions of starch for 36 hours caused an increase in the phosphorylation state of ribosomal protein S6, in addition to increased protein yields (Rius et al., 2010). Ribosomal protein S6 is phosphorylated by active S6K1, suggesting mTOR is in fact involved in the protein synthesis response to glucose. No other studies of mammary mTOR have been conducted to further support or dismiss these findings.

The mTOR protein is associated with two distinct multiprotein complexes termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). It is only mTORC1 that is sensitive to stress and nutrients, as well as an mTOR inhibitor known as rapamycin (Wullschleger et al., 2006). Rapamycin, sold under the trade names rapamune or sirolimus, is a bacterial macrolide drug originally discovered to be an anti-fungal agent (Chen and Fang, 2002). It is a potent inhibitor of the mTOR pathway, disrupting the cell cycle and more specifically, protein synthesis within the cell. mTOR pathway inhibition by rapamycin is accomplished by impeding its ability to form the mTORC1 complex, which is necessary for downstream signaling to important target proteins such as 4EBP1 and S6K1. It is thought that rapamycin interacts with the cellular receptor protein FKBP12, which prevents important regulatory proteins from binding (Tee and Blenis, 2005). Rapamycin has been used in vivo and in vitro to inhibit protein synthesis via the mTOR pathway (Hosoi et al., 1999; Bodine et al., 2001); however, it has not been used in the mammary glands of lactating dairy cows.

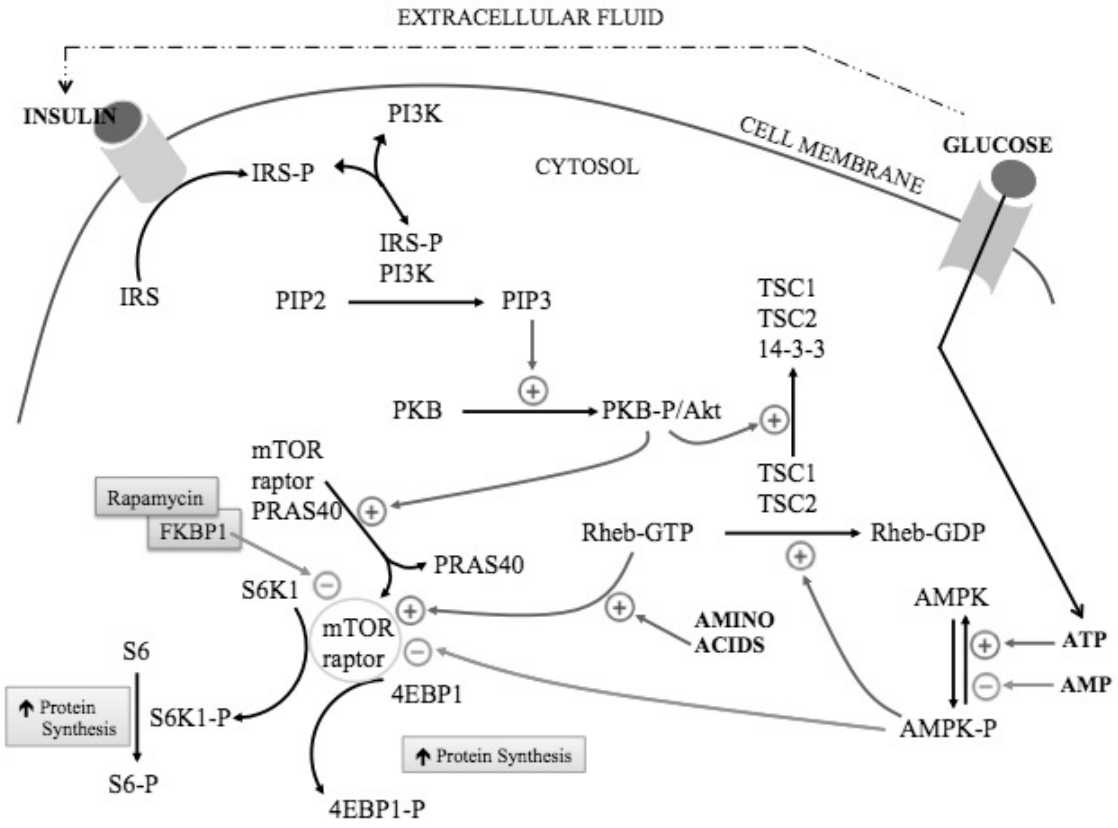

Figure 2. The mammalian target of rapamycin (mTOR) signaling pathway and its signaling proteins, such as S6K1 and 4EBP1, involved in the regulation of protein synthesis in mammalian cells.

Regulation of Protein Synthesis via ISR Network

In addition to the mTOR signaling pathway, another mechanism plays a role in regulation of global protein synthesis. The integrated stress response (ISR) network is responsible for a decrease in protein synthesis by slowing down initiation of mRNA translation via eIF2 and its exchange factor called eIF2B. eIF2 is regulated by a mechanism involving both phosphorylation and guanine nucleotide exchange. eIF2B acts as the guanine nucleotide exchange factor as it activates eIF2 by exchanging guanosine diphosphate (GDP) with guanosine triphosphate (GTP). The active eIF2-GTP complex allows for methionyl-tRNA to bind to the small ribosomal subunit, which is necessary for the initiation of mRNA translation (Proud, 2005). If eIF2 becomes phosphorylated by one of its regulatory kinases, PERK, GCN2, PKR, or HRI, protein synthesis slows due to inhibition of eIF2B is by phosphorylated eIF2 (Figure 3). Mammary eIF2 phosphorylation was measured in a study conducted by Toerien et al. (2010) and the glucose treatment alone caused a 62% decrease in eIF2 phosphorylation. This result suggests that the ISR network was responsible for the 27% increase in milk protein yield that resulted from the glucose infusion. No other studies have tested whether the ISR network is indeed the mechanism by which glucose exerts its effects on protein synthesis.

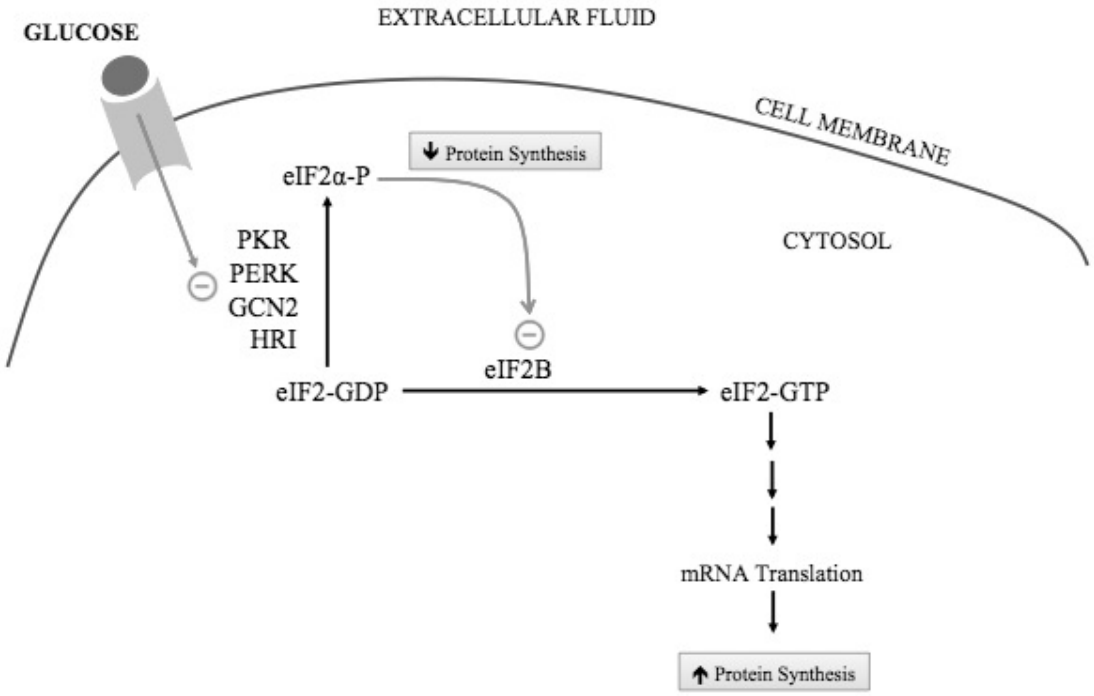

Figure 3. The integrated stress response (ISR) network and its eukaryotic initiation factors, eIF2 and eIF2B, responsible for regulating protein synthesis in mammalian cells.

Study Objectives

Previous literature has presented inconsistent results and multiple theories on the mechanism behind the milk protein yield response to supplemental glucose in dairy cows. Further understanding of mammary gland nutrient utilization for protein synthesis is of importance for nutritionists and dairy farmers to enhance feed ingredient utilization for management of milk protein yields, and reduce feed costs. We hypothesized that signalling to the translation apparatus in mammary tissue is responsible for the milk protein response to glucose. Therefore, the purpose of this study was to determine effects of glucose on milk protein yield and mammary mTOR activity in early lactation dairy cows. Intramammary infusions of rapamycin were also given to inhibit the mTOR signaling pathway and test if mTOR is involved in the effects of glucose on milk protein synthesis.

CHAPTER 2

DECLINE IN MAMMARY TRANSLATIONAL CAPACITY DURING INTRAVENOUS GLUCOSE INFUSION INTO LACTATING DAIRY COWS

ABSTRACT

The objective of this study was to determine effects of glucose on milk protein yield and mTOR activity in dairy cattle in early lactation. Eight multiparous cows at 73 $\pm$ 8 DIM were randomly assigned to two 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 TMR with 42% neutral detergent fibre (NDF), 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% DMSO solution was administered via the teat canals into the right quarters with a control solution in the left quarters. Rapamycin had no effect on milk protein yields or phosphorylation state of mTOR signalling proteins. GLC infusions significantly increased milk yield but only tended to increase milk protein yields (P = 0.15). Milk fat tended (P = 0.12) to be decreased in cows infused with GLC while lactose yields were significantly increased. GLC infusion did not increase plasma glucose levels, but insulin and NEAA concentrations increased by 21 and 16%, respectively, BCAA concentrations decreased 24%, and there was a tendency (P = 0.12) for EAA concentrations to decrease by 14%. GLC infusion significantly decreased both phosphorylated and total S6K1 abundances in mammary tissue by 27% and 11%, respectively. Abundance of phosphorylated 4EBP1 significantly decreased by 25%, whereas total 4EBP1 exhibited a tendency (P = 0.12) to decrease by 16%. It is concluded

that GLC failed to increase milk protein yield because of a decline in activity of mTOR-regulated initiation factors. Decreases in EAA concentrations in plasma suggest that protein synthesis was stimulated in non-mammary tissues of the body, presumably skeletal muscle.

Key words: glucose, dairy cow, mammary protein synthesis, S6K1, 4EBP1

INTRODUCTION

Milk protein synthesis in the lactating dairy cow is greatly influenced by two major nutritional sources, protein and energy, with recent research attention heavily focused on energy. Glucose has the ability to stimulate milk protein synthesis; however, results remain conflicting in the literature. 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., 2009), 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 effects milk protein synthesis is still unknown.

Glucose infusion does not increase EAA concentrations in circulation but Raggio et al. (2006b) found that mammary uptake of 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. (2009) found that glucose infusion into fasted cows caused activation of mammary 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 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 signalling 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 if 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 $\pm$ 8 DIM and 674 $\pm$ 84.7 kg body weight (BW). Cows were housed in a tie stall barn at the Ponsonby Livestock Research Station 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 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-week study and submitted for nutrient composition analysis by wet chemistry at a commercial laboratory (Agri-Food Labs, Guelph, Ont.). Orts from individual cows were sampled daily and pooled on a weekly basis. Dry matter contents of feed and orts samples were determined using a forced-air oven at 60°C for 24 h.

Table 1. Ingredient and chemical composition of the experimental TMR (DM basis) fed to lactating dairy cattle (n=8) infused i.v. with SAL or GLC for 6 d.

Component	% of DM
Ingredient composition	
Alfalfa silage	29.4
Corn silage	24.6
Soybean hulls, ground	11.1
Mixed grains, chopped	7.8
Tripro soy plus	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
CP	15.1
Soluble CP (% of CP)	35.6
ND-insoluble CP (% 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

Treatments

Cows were randomly assigned to a 6 d continuous infusion into the jugular vein of equal amounts 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 opposite treatments. One d prior to 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) was administered after catheter insertion as a precautionary measure to prevent infection. GLC was completely dissolved in 3 L 0.9% saline and both infusates were sterilized via autoclave.

In order to test whether mTOR signaling is involved in the effects of glucose on milk protein synthesis, 10 mg rapamycin was dissolved in 150 ml dimethyl sulfoxide (DMSO) diluted to 50% with distilled water. The rapamycin infusate was rapidly infused into the two left mammary glands of each cow via the teat canals, following the 1530 and 0500 h milkings on days 5 and 6, respectively, of each period. One hundred and fifty ml of 50% DMSO was infused into each of the right mammary glands as a control.

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 left and right halves separately. Milk yields from each udder half were recorded daily and samples were collected and analysed for fat, lactose and protein by spectroscopy at the Laboratory Services Division, University of Guelph (Guelph, ON).

Blood Sampling, Metabolite and Hormone Concentrations

Blood samples were collected from the coccygeal vessels on d 5 of each period after AM milking and on d 6 prior to mammary biopsies. Tubes were placed on ice and immediately centrifuged at 2000 g for 10 min and plasma was transferred to polypropylene tubes to be stored at -20°C. Plasma was analysed as described by Weekes et al. (2006) for glucose (kit no. GAGO-20; Sigma-Aldrich, St. Louis, USA;), insulin (kit no. 90060; Crystal Chem Inc., Downers Drive, USA), β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA; kit no. 999-34691; Wako Chemicals, Neuss, Germany) and triacylglycerol (TAG; kit no. TR0100; Sigma-Aldrich, St. Louis, USA). Acetate (kit no. K-ACETRM; Megazyme International, Bray, Ireland) was analysed following the manufacture's protocol. Plasma AA concentrations were analysed using Ultra Performance Liquid Chromatography in conjunction with Empower Chromatography Data Software (Waters Corporation, Milford, USA) 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 according to the procedure of Farr et al. (1996) from rapamycin and DMSO-infused hindquarters after sedation with 0.5 ml xylazine i.v. and 5 ml lidocaine injected subcutaneously at the biopsy site. Ketoprofen (3 mg/kg body weight) 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 lysis buffer (1% Triton X-100, 0.1% SDS, 50mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (Thermo Scientific, Nepean, ON) for 10 s prior to inversion for 1 h at 4°C. Lysates were centrifuged at 13,000 x g for 15 min at 4°C, supernatants extracted and stored at -20°C. DNA concentration was measured using the Qubit 2.0 Fluorometer (Life Technologies Inc, Burlington, ON) with the Qubit dsDNA BR Assay.

Mammary supernatant samples were diluted in lysis buffer and sample buffer (4% SDS, 20% glycerol, 10% beta-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, CA), were separated by 10% SDS-PAGE at 120 V for approximately 90 min. Proteins were electrotransferred (Bio-Rad Laboratories Inc., Mississauga, CA) onto polyvinylidene difluoride membranes (Millipore, Mississauga, ON) at 100 V for 60 min. Membranes were then incubated in blocking buffer for 1 h at room temperature, followed by three 5-min washes in TBST buffer. Membranes were incubated for 1 h at room temperature with rabbit monoclonal antibodies to either phospho-S6K1 (Thr389; 1:800, Abcam, #ab2571), phospho-4EBP1 (Thr37/46; 1:1000, Cell Signaling, #9459) or eIF4E (1:1000, Cell Signaling, #9742). All antibodies were diluted using 1% milk TBST buffer. Membranes were washed with TBST buffer and incubated at room temperature for 1 h with HRP-linked anti-rabbit IgG (1:2000; Cell Signaling, #7074). Membranes were washed and proteins were developed by autoradiography using Enhanced chemiluminescence (Amersham, Arlington Heights, USA). 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 non-phosphorylated proteins S6K1 (1:800, Abcam, Cambridge, USA, #ab9366) or 4EBP1 (1:1000, Cell Signaling, #9452). Finally, images from radiographic film (VWR International, Pennsylvania, USA) were scanned and the integrated density was determined by ImageJ software (http://rbs.info.nih.gov/ij). Densities were normalized to DNA content of homogenates and phosphorylated protein densities were normalized to those of the corresponding non-phosphorylated protein.

Statistical Analysis

Cow performance was averaged for the 3 d of each period prior to intramammary infusions. Cow performance and plasma metabolite observations (Y_{ijk}) were subjected to ANOVA using PROC MIXED of SAS (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. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.15$.

RESULTS

Lactational Performance

There was no difference in DMI 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 = 0.122) and increase by 34 g/d (P = 0.149), 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 percentage units (P = 0.003) on the GLC treatment, respectively. GLC-infused cows had, on average, 0.07 percentage-units higher (P < 0.001) lactose contents compared to those infused with SAL.

Milk yield of rapamycin-infused udder halves was 100% of the previous 2-day's yield from that half, while yield from DMSO-infused halves was 80% of the previous yield. The difference between rapamycin and DMSO was significant (P = 0.023). There was no effect of intramammary rapamycin on milk fat, protein or lactose percentages (P > 0.271).

Plasma Constituents

GLC treatment did not have a significant effect on plasma glucose (Table 3); however, it did increase insulin concentrations by 21% compared to SAL (*P* = 0.004). GLC infusions tended to decrease plasma NEFA concentrations by 16% (*P* = 0.091) relative to SAL, and tended to increase circulating TAG concentrations by 8% (*P* = 0.065). There was no significant effect on plasma BHBA or acetate concentrations between treatments.

GLC caused a 16% increase of NEAA concentrations in plasma (Table 4; *P* = 0.032) while having a tendency to decrease EAA by 14% (*P* = 0.124). Gln (*P* = 0.006) and Gly (*P* =

0.011) concentrations 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 BCAA relative to those receiving SAL. Specifically, GLC decreased Val (P = 0.029) by 23%, while having the tendency to decrease Leu by 25% (P = 0.054) and Ile by 24% (P = 0.067). Total circulating amino acid (TAA) concentrations did not differ between the two treatments.

Table 2. DMI, milk yield and milk composition of lactating dairy cattle (n=8) infused i.v. with SAL or GLC for 6 d[1].

	Treatments[1]			
Item	SAL	GLC	SEM	*P*
DMI, kg/d	22.9	22.1	0.72	0.144
Yield				
Milk, kg/d	37.3	39.5	1.13	0.006
Fat, g/d	1666	1610	71.4	0.122
Protein, g/d	1223	1257	48.9	0.149
Lactose, g/d	1803	1945	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 were taken from the last 3 d of each period for statistical analysis

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

	Treatments			
Item	SAL	GLC	SEM	*P*
Glucose, m*M*	3.11	3.26	0.06	0.124
Insulin, ng/ml	1.23	1.49	1.00	0.004
BHB, m*M*	0.71	0.67	0.08	0.753
NEFA, µ*M*	118	99	6.48	0.091
Acetate, m*M*	1.37	1.39	0.08	0.879
TAG, µ*M*	99.0	107.0	5.77	0.065

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

	Treatments[1]			
A.A.	SAL	GLC	SEM	*P*
His	32	35	2.0	0.322
Asn	61	66	4.6	0.430
Ser	56	67	7.58	0.237
Gln	503	674	28.89	0.006
Arg	83	70	5.81	0.174
Gly	184	255	20.98	0.011
Glu	95	96	3.54	0.796
Thr	87	102	6.40	0.143
Ala	288	282	20.03	0.763
Pro	84	83	4.58	0.996
Lys	71	60	5.44	0.196
Tyr	57	51	3.84	0.328
Met	19	19	1.33	0.908
Val	276	214	18.27	0.029
Ile	126	96	9.98	0.067
Leu	159	119	13.86	0.054
Phe	48	39	3.22	0.099
BCAA[1]	561	429	41.74	0.044
EAA	900	774	57.89	0.124

NEAA[2]	1332	1589	64.23	0.032
TAA[3]	2232	2343	85	0.381

[1]BCAA = Branch chain amino acids (Val, Ile and Leu)

[2]NEAA = Non-essential amino acids (Ser, Gln, Arg, Gly, Asp, Glu, Ala, Pro, Asn and Tyr)

[3]TAA = EAA + NEAA

Translational Proteins

Treatments did not differ in abundance of total eIF4E (data not shown). There were 27 (P = 0.054) and 11% (P = 0.005) decreases in phosphorylated and total S6K1 abundances, respectively, in mammary tissue of cows on the GLC treatment (Figure 4). However, GLC failed to have an effect on phosphorylated S6K1 normalized to S6K1 abundance in mammary tissue. GLC treatment led to a decrease in phosphorylation of mammary 4EBP1 by 25% (Figure 5; P = 0.009) and a tendency to decrease total 4EBP1 by 16% (P = 0.121). Phosphorylated 4EBP1 normalized to total 4EBP1 tended to be 16% lower in mammary tissue of cows that received GLC (P = 0.071). Rapamycin treatment tended to increase total S6K1 abundance in mammary tissue (P = 0.059) by 7%. There was an interaction between intravenous and intramammary infusions (P = 0.001) where rapamycin decreased abundance of total S6K1 on SAL treatment and increased it on GLC treatment. However, the interaction was opposite for total 4EBP1 (P = 0.080) where rapamycin increased abundance on SAL treatment and decreased it on GLC.

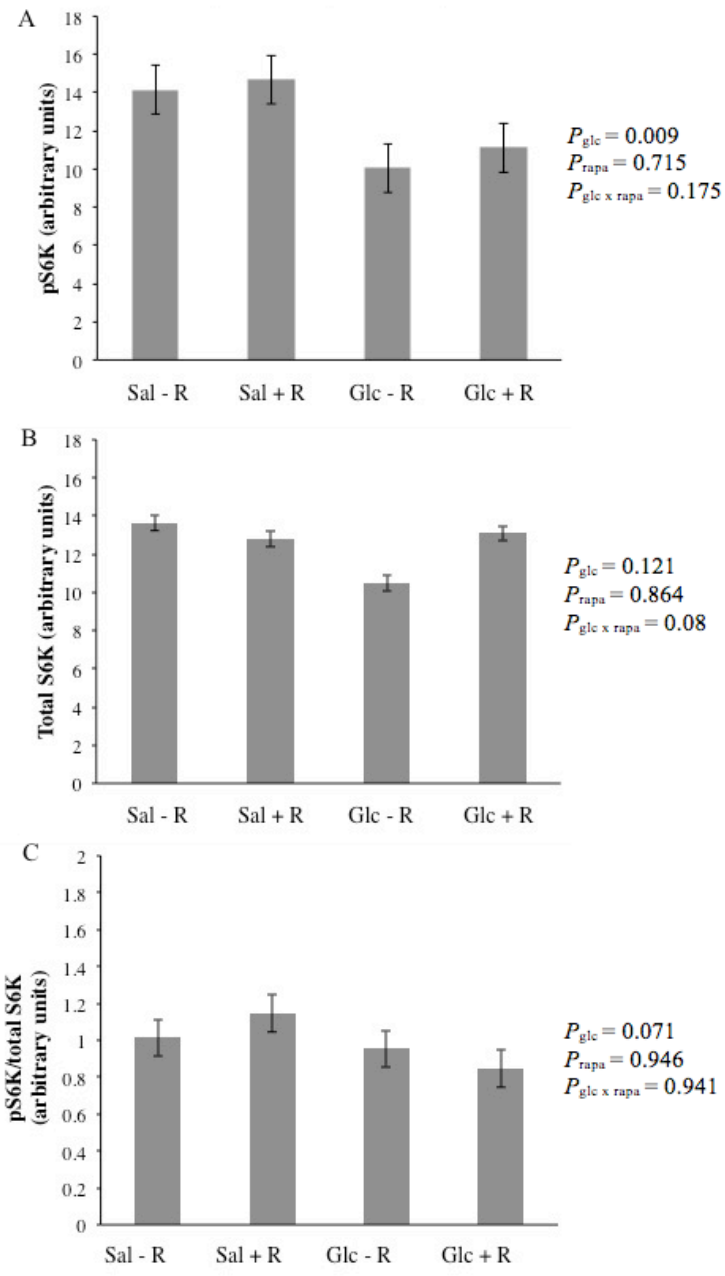

Figure 4. Abundances of phosphorylated S6K1 (A) and total S6K1 (B) normalized per unit DNA and pS6K1 normalized to total S6K1 (C) in mammary tissue of lactating dairy cattle infused i.v. with GLC or SAL, with and without intramammary rapamycin treatment. Values are mean densities and P_{glc}, P_{rapa} and $P_{glc\ x\ rapa}$ represent *P* values for GLC, rapamycin, and the interaction between GLC and rapamycin, respectively.

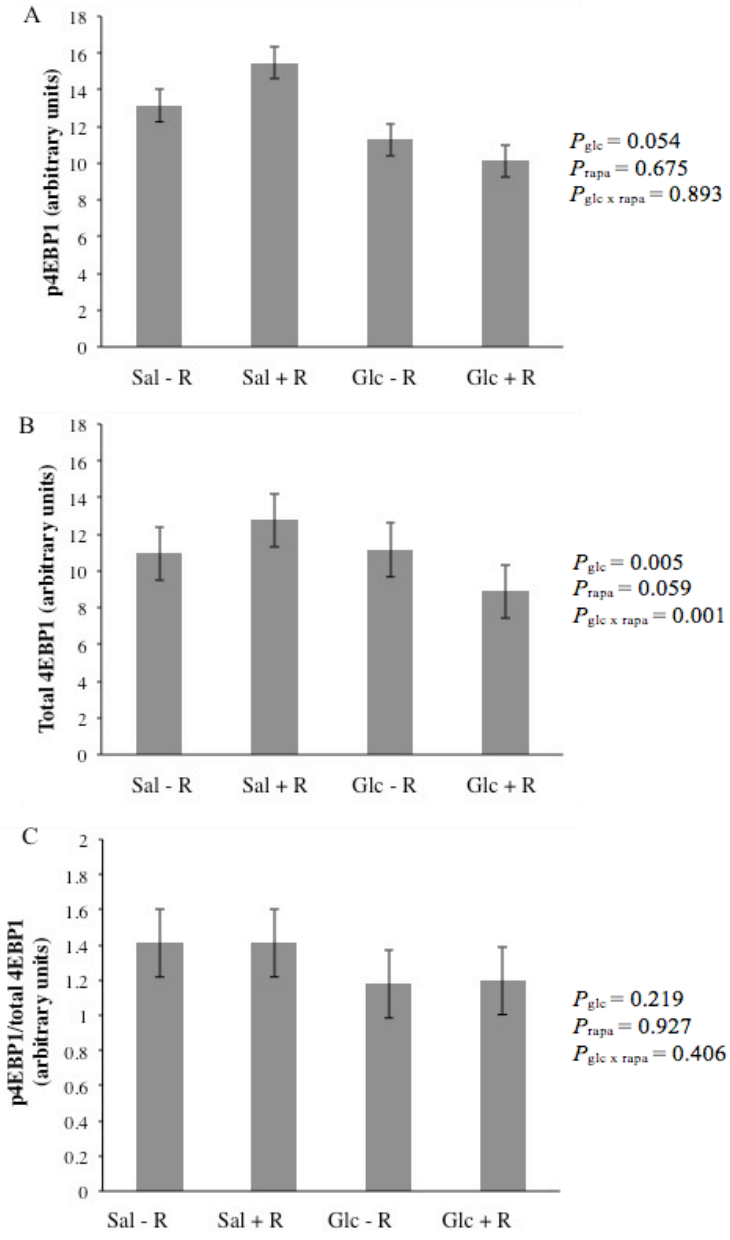

Figure 5. Abundances of phosphorylated 4EBP1 (A) and total 4EBP1 (B) normalized per unit DNA and p4EBP1 normalized to total 4EBP1 (C) in mammary tissue of lactating dairy cattle infused with GLC or SAL, with and without rapamycin treatment. Values are mean densities and P_{glc}, P_{rapa} and $P_{glc \times rapa}$ represent *P* values for GLC, rapamycin, and the interaction between GLC and rapamycin, respectively.

DISCUSSION

Contrary to studies showing significant increases in milk protein yield in response to infusion of glucose or glucogenic substrates (Raggio et a., 2006; Rulquin et al., 2004; Toerien et al., 2009; Rius 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 GLC for 14 d. Others reported no significant change in protein yield with GLC (Hurtaud et al., 1998; Cant et al., 2002; Rigout et al., 2003). It is interesting to point out 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 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. NEAA 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 Lemosquet et al. (2004), who also found an increase in glucogenic NEAA concentrations when GLC or propionic acid were infused into lactating dairy cattle.

Plasma BCAA concentrations were decreased in cows receiving GLC treatments, as seen with previous experiments (Raggio et al.,2006a; Toerien et al., 2009; Rius 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 as these EAA account for a disproportionately large percentage of the amino acid profile of skeletal muscle protein (Anthony et al., 2001). Lower BCAA levels in blood in conjunction with unaffected milk protein yields, as demonstrated in the present study as well as others (Hurtaud et al., 1998; Clark et al., 1977), suggest that protein synthesis was stimulated in the 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 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 an increased mammary blood flow to support protein synthesis (Mackle et al., 2000; Bequette et al., 2001). Since increasing glucose via infusions causes blood insulin to rise 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.

The increase in mammary blood flow during euglycemic insulin infusion was attributed by Cant et al. (2003) to a decrease in circulating acetate and BHBA concentrations due to the anabolic effects of insulin in non-mammary tissues. If the mammary glands control their own rate of blood flow to match ATP expenditure with supply, the loss of energy substrate from

plasma would induce a hyperemic response. Within 10 h of doubling the mammary arterial glucose concentration by close arterial infusion, mammary blood flow had dropped (Cant et al., 2002), consistent with the energy balance hypothesis. On the other hand, GLC infusion for 14 d caused plasma concentrations of acetate, BHBA, NEFA and TAG to decline and mammary blood flow increased (Rigout et al., 2002), which is again consistent with the energy balance hypothesis. In the 10-h study, when mammary blood flow dropped, milk protein yield did not increase, while in the 14-d study when mammary blood flow increased, milk protein yield also increased. In the current study, after 6 d of GLC, concentrations of energy metabolites in plasma were not significantly depressed. The energy balance hypothesis, according to the simulation model of Cant et al. (2003), predicts no change in mammary blood flow from the effect of treatment on plasma metabolite concentrations and, like Cant et al. (2002), there was no significant effect of GLC on milk protein yield.

Although the milk protein yield response to GLC appears to be associated with changes in mammary blood flow, 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 energy charge 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 the 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. The decrease in abundance of a key regulator of mRNA translation, along with depressed mTOR activity, could explain why there was no milk protein yield response to GLC. However, the reason for reduced mTOR signaling remains unclear. 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. AA, particularly Leu, are potent activators of mammary mTOR in vitro (Burgos et al., 2010; Appuhamy et al., 2012) so the decrease in plasma EAA concentrations, particularly the BCAA, could account for the reduction in phosphorylated S6K1 and 4EBP1 abundances.

Intramammary rapamycin infusion failed to inhibit mTOR signaling, as evident from the lack of effect on 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. While the 10-mg dose of rapamycin is sufficient for inhibition of mTOR in an animal of 13 kg bodyweight (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.

CONCLUSION

In the present study, our objective to stimulate a milk protein response to GLC infusion into dairy cattle was not seen. However, new insight on how protein synthesis is influenced by supplemental glucose was gained. 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 and reduced S6K1 abundance in mammary tissue, and the lack of a milk protein response, collectively support the proposition that glucose directed AA into skeletal muscle, rather than into mammary glands for milk protein synthesis.

CHAPTER 3

GENERAL DISCUSSION

Protein is an expensive feed ingredient in the rations of dairy cows. Unfortunately, the efficiency of conversion of dietary protein into milk is low in dairy cows, less than 25%. The typical TMR currently fed to cows in Canada contains between 17 and 18% crude protein (CP) of dietary dry matter. Marginal efficiencies of protein capture in milk fall to around 5% at these high protein intakes, meaning that 95% of excess protein fed is lost to the environment in manure nitrogen. A reduction of 2% of dietary CP content would decrease nitrogen emissions from Ontario dairy farms by 25 000 tonnes per year. This is equivalent to 60 million dollars per year in feed costs paid for by dairy producers. However, the challenge lies in a compromise in milk production when CP levels in dairy rations are reduced. Therefore, it would be beneficial both environmentally and economically, to determine a method of lowering CP in the ration of lactating dairy cows without compromising lactational performance.

Dietary carbohydrates, including starch and sugars, provide dairy cows with glucose, which is converted into energy substrates and microbial protein by microbes present in the rumen. These products from microbial digestion of glucose, among other sources, are used to synthesize protein in the mammary glands. Glucose has the ability to stimulate milk protein yield in dairy cows; however there still remains conflicting evidence in the literature. Many studies have shown glucose to significantly increase milk protein (Rulquin et al., 2004; Al-Trad et al., 2009; Toerien et al., 2009), yet others have reported no change (Cant et al., 2002; Lemosquet et al., 2009, chapter 2). Researchers are beginning to investigate the mechanism behind how glucose elicits its effect on milk protein synthesis. It is suggested that glucose may interact with the mTOR and ISR regulatory networks to influence milk protein yield (Toerien et al., 2009;

Rius et al., 2010, Chapter 3), but it has not been confirmed to date. In this study, we found that glucose had no effect on protein synthesis in the mammary glands. Considering this result, along with blood and milk composition results, we suggested that absorbed AA were directed to the muscle instead of the mammary glands. Shedding light on the mechanism behind how glucose affects milk protein synthesis is crucial to the development of lower protein dairy rations that would not compromise lactation performance.

There have been many mathematical models developed over the last few decades describing the fate of nutritional components, including carbohydrates, once they enter the rumen (France et al., 1982; Dijkstra et al., 1992; Weisbjerg et al., 1998). Current dairy ration formulating programs, such as AMTS (Agricultural Modeling and Training Systems, LLC), use these nutrition models to formulate diets that meet nutrient requirements of a cow for optimal production capacity. Modeling nutrient utilization after absorption into the bloodstream for processes such as protein synthesis in muscle and mammary glands have also been theoretically described, but have yet to be implemented in feed formulation software.

Revealing the mechanism behind the effect of glucose on milk protein yield, and using this knowledge to further improve mammary protein synthesis models for industry application, is of importance to Canadian dairy farming. With the use of feed formulation software, lower protein rations could be formulated by substituting a percentage of dietary protein with carbohydrates. These test diets could then be fed to lactating dairy cows in commercial herds to test whether or not N waste can be reduced without compromising production efficiency. Besides reducing feed costs for dairy producers, this research demonstrates a great deal of potential to reduce the environmental impact of dairy farming in Canada, while still maintaining production performance of dairy herds.

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