Nitrogen Efficiency and Regulation of Protein Synthesis in Lactating Dairy Cows

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Dissertation submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for degree of

Doctor of Philosophy

in

Animal Science, Dairy

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April 28th , 2009

Blacksburg VA

Key words: amino acids, energy, mammary gland

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ABSTRACT

Dairy herds are major contributors to N pollution because 70% of the N intake is lost to the environment and 30% or less is retained in milk protein. Plasma amino acids (AA) that are not used for protein synthesis in mammary glands (MG) are catabolized in post splanchnic tissues (liver plus gastrointestinal tract, pancreas, spleen, portal system, and associated adipose tissue) and two thirds of the net supply of essential AA (EAA) are cleared in splanchnic tissues. Thus, increasing AA capture in MG would be expected to reduce AA catabolism and thereby increase efficiency of AA utilization. The objectives of the work presented in this dissertation were to test the effect of energy and N intake on cell regulatory mechanisms, nutrient kinetics, milk, milk protein yield, and N efficiency in dairy cows.

The aim of the first study was to test whether metabolizable protein (MP) and dietary energy exerted independent effects on milk protein synthesis and postabsorptive N efficiency. Forty mid-lactation cows (32 multiparous Holstein and 8 primiparous Holstein x Jersey cross-breds) were used in a complete randomized design with a 2 x 2 factorial arrangement of diets. Cows were assigned to one of four dietary treatments: high-energy, high-protein (HE/HP); high-energy, low-protein (HE/LP); low-energy, high-protein (LE/HP); and low-energy, low-protein (LE/LP). Energy concentrations were 1.55 (HE/HP and HE/LP) or 1.44 (LE/HP and LE/LP) Mcal NE_L/kg DM according to the NRC model. Changes in predicted MP were achieved by feeding diets with 6.6 (HE/HP and LE/HP) or 4.6% (HE/LP and LE/LP) ruminally undegradable protein (DM basis).

Ruminally degradable protein was held constant at 10.1% of DM. All cows were fed HE/HP diet from day 1 to 21 followed by the respective treatments from day 22 to 43 (n=10). Milk protein yield was reduced as dietary energy was reduced. There were no interactions between dietary energy and protein for either milk or protein yield. Milk urea N was significantly affected by energy and protein with an interaction (HE/HP=17.2, HE/LP=12.2, LE/HP=21.0, LE/LP=12.2 mg/dl). Nitrogen efficiency was affected by energy and protein supplies with no interaction and ranged from a low of 31% (LE/HP) to a high of 43% (HE/LP). Although energy and protein independently affected milk and protein yield the tissue and cellular mechanisms that regulate milk production were not studied.

The second experiment studied cellular mechanisms in MG that contributed to the regulation of protein synthesis in the presence of energy or protein supply. We hypothesized that metabolism of AA in the MG is controlled by systemic and local tissue adaptations and when combined with altered mammary cell function controlled milk protein yield. Six primiparous mid-lactation Holstein cows with rumen cannulas were randomly assigned to abomasal infusions of casein and starch using a 2 x 2 factorial arrangement. The design was a replicated incomplete 4 x 4 Latin-square. All animals received the same basal diet (17.6% CP and 1.58 Mcal NE_L/kg DM) throughout the study. Cows were restricted to 70% of ad libitum intake and infused abomasally for 36 h with water, starch (2 kg/d), casein (0.86 kg/d), or the combination (2 kg/d starch + 0.86 kg/d casein) using peristaltic pumps. Milk weights, milk samples, and arterial and venous blood samples were collected during the last 8 h of infusions. Mammary biopsy samples were collected and tissue protein prepared to evaluate cell signaling. Animals infused with casein had increased arterial concentrations of NEAA and EAA, as well as

net uptake and clearance; however, milk protein yield did not increase. Animals infused with starch however, exhibited reduced arterial concentrations of NEAA and EAA but increased clearance and net uptake of most AA. Additionally, infusions of starch increased circulating concentration of insulin, IGF-I, and glucose as well as the rate of mammary plasma flow. Abomasal infusions of starch activated mammary activity of ribosomal protein S6 irrespective of other treatments. However, mammary tissue mTOR increased activity in response to casein only when starch was present during the infusions. These results suggest that cell signaling activation responded to different nutritional stimuli. Milk and protein yield increased in animals infused with starch. Therefore, MG positively responded to energy supply and engaged local and intracellular regulatory mechanisms to achieve that response. Understanding these adaptations could be beneficial in the development of mathematical representations for nutrients utilization in lactating animals. These two studies supported our hypotheses that regulatory mechanism are activated during limiting supply of AA to sustain protein synthesis in MG. The accuracy of mathematical models for lactating animals would increase if effects of energy on AA metabolism and cell signaling related to protein synthesis were included in the representation of milk protein synthesis.

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AKNOWLEDGEMENTS

I would like to extend sincere thanks to my committee members for the support and guidance on my research over these years at VT. To my major advisor, Dr. Mark Hanigan that his leadership, constructive criticism, and encouragement to keep me going at all times of the PhD journey. Thank you for always having time not only for me but for all of us to review results, discussions, and thoughts. Thank you for your patience and support in organizing ideas and putting them on paper. I will take that with me as extra gifts from my times at VT. I am sure they will become handy in the years to come. I am thankful to the rest of my committee members Drs. McGilliard, Escobar, Corl, and Akers for their support, knowledge, guidance and collaboration in this dissertation.

The invaluable hard work and support from the VT dairy complex farm crew, graduate and undergraduate students are gratefully acknowledged. Thank you all to the Dairy Science faculty and staff for their great contribution to this wonderful department in the prestigious institute of Virginia Tech. I thank present and past members of the Dairy Science Department family for being part of this great department. Finally I would like to express my gratitude to my family and special thanks to Naiara for her love, confidence, care, and endless support. Thanks to the Cooperative State Research, Education and Extension Service, the Virginia Agricultural Council, Land O' Lakes/Purina Feed, Inc., and the general departmental funding provided by the Virginia State Dairymen's Association for the financial support to conduct these studies

Chapter 1:

Introduction

Excess of N from dairy operations represents an environmental hazard in the US (NRC, 2003). Ruminants generally consume large amounts of N but less than 30% is typically retained in marketable products (Tamminga, 1992). The efficiency of N utilization in synthesis and secretion of milk in dairy cows is equally poor i.e. only 20 to 30% is captured in milk protein (Bequette et al., 1998). Urea is excreted in urine and the N released can lead to pollution of air, soil, and surface water. However, it is believed possible to reduce N output to the environment and increase N retention in farm animals (Jonker et al., 2002).

The metabolizable protein (MP) efficiency of utilization in pigs ranges from 74% to 80% (Chung and Baker, 1992) but dairy cows typically convert less than 37% of the MP into milk protein (Rius et al., unpublished data). Thus, it might be possible to narrow the gap between simple-stomached and ruminant animals and increase MP and overall N efficiencies in the lactating cow. Reducing CP content from 18.7 to 14.8% (DM basis), which reduces N losses, is a possible mechanism to increase N efficiency without compromising milk production (Ipharraguerre and Clark, 2005). Reducing urinary N losses would increase overall efficiency in lactating cattle (Hanigan, 2005). The majority of N losses occur in splanchnic tissues. Portal-drained viscera (PDV) catabolized an average of 30% and the liver 20% of the EAA that are absorbed from the gut lumen (MacRae et al., 1997; Wray-Cahen et al., 1997). The majority of catabolized AA are drawn from arterial supply (MacRae et al., 1997), therefore, recycling of AA back to these tissues in arterial blood is a key determinant in setting catabolic rate. Increasing the

supply of AA to these tissues resulted in increased catabolism with the extra AA carbon funneled into gluconeogenesis and N into ureagenesis (Wray-Cahen et al., 1997). If utilization of AA for milk protein synthesis could be stimulated the proportion of AA recycled to the splanchnic tissues where they are subjected to catabolism would be reduced thereby improving animal efficiency (Hanigan, 2005).

Stimulation of milk protein synthesis at a given supply of AA is a potential strategy to reduce recycling of AA to splanchnic tissues, and if AA supply remains constant, reductions of arterial concentrations of AA should be observed (Hanigan et al., 1998a). If true this mechanism could lead to even further progress by increasing use of AA for protein synthesis and reducing AA supply to the peripheral tissues. Indeed, reduced circulating AA concentrations triggered greater proportional mammary capture which partially sustained protein synthesis (Bequette et al., 2000; Raggio et al., 2006). Conversely, increased mammary AA removal in the absence of greater protein synthesis would likely increase AA degradation (Bequette et al., 1996a; Bequette et al., 1996b). Greater intracellular concentrations of AA resulted in increased oxidation to a large extent (Bequette et al., 1996a; Raggio et al., 2006). Thus, increasing protein synthesis should be associated with greater AA removal, reduced arterial AA concentrations and less AA degradation.

Increasing dietary energy content at a given CP level may stimulate mammary protein synthesis and milk protein production (Hanigan et al., 1998a). Increasing duodenal glucose supply in cows fed isonitrogenous diets stimulated insulin secretion and milk protein yield (Rulquin et al., 2004). Greater insulin concentration and glucose supply to the animal increased mammary capture of AA to sustain greater protein

synthesis (Mackle et al., 2000; Rulquin et al., 2004). The latter could compensate for reductions in arterial AA concentrations if low protein diets are fed and protein synthesis is stimulated in lactating animals.

Insulin stimulated initiation of protein synthesis at the molecular level in growing animals (Davis et al., 2001) and the mTOR signaling cascade was up-regulated during protein synthesis in muscle tissue (Escobar et al., 2006). Increased mTOR activity was associated with greater AA usage for protein synthesis in growing animals, however this molecular mechanism has not been evaluated in MG of lactating cows (Escobar et al., 2006). Increases in protein synthesis are independently regulated by both nutrient intake and insulin (Davis et al., 2001; Davis et al., 1991). Therefore feeding high energy diets to stimulate insulin release should stimulate mammary milk protein synthesis even when dietary CP is low. Mammary glands should increase their affinity for EAA and capture of AA if arterial concentrations are reduced by feeding low protein diets. Thus high energy diets may stimulate the mammary secretory cell to maintain milk protein synthesis and yield by increasing mTOR activity even when low protein diets are fed.

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Impact of Nitrogen in the Dairy Farm and the Environment

Soybean meal is a major source of crude protein (CP) and widely used in North American dairy rations. The Dec 2008-USDA national price received by farmers averaged $11 per bushel, an all-time high for soybeans. Thus, an increase in the efficiency of nitrogen (N) utilization should maintain milk production with less N consumed. This would benefit the dairy industry by reducing feeding costs.

In addition, concentrated animal operations, particularly dairy farms, are a significant source of pollution through exports of nitrate to land and water and ammonia (NH_3) to the atmosphere via urine and feces. The NRC (2003) indicates that NH_3 from animal farm operations contributes to the deterioration of air quality at regional, national, and global levels. Aerial ammonia concentrations up to 50 ppm resulted in 51% greater bacterial load in the lungs of growing piglets as compared with control piglets. High levels of NH_3 impaired growth and feed conversion in growing piglets (Drummond et al., 1980). Therefore, lower NH_3 emissions should benefit animal health and production.

Livestock are estimated to be responsible for 80% of the United Sates NH_3 emissions and cattle are the largest contributor (Battye, 1994). For example, N content in urine and feces equaled more than 70% of the N intake (Tamminga, 1992). Therefore strategies to lessen the negative aspects of dairy farms should include nutritional interventions to both increase N efficiency and reduce N pollution.

Nitrogen Retention in Cattle

Nitrogen efficiency varies from 20 to 30% in lactating cattle in field conditions (Moorby and Theobald, 1999). Investigations conducted in the United States to study N balance

on dairy farms showed that cows retained only 12 to 36% of the dietary N in milk products whereas most of the remaining 64 to 88% was released into the environment (Castillo et al., 2000; Spears et al., 2003). Therefore, increasing N efficiency is a promising opportunity to both increase N retention in marketable products and reduce N pollution arising from dairy operations (Jonker et al., 2002; Rotz et al., 1999).

Nitrogen efficiency increased from 25 to 33% when lactating cows were fed 14.8% CP as compared to cows fed 18.7% CP (DM basis; Ipharraguerre and Clark, 2005). Typically N efficiency is poor in lactating animals that are fed high forage diets with low levels of energy compared to cows that are fed medium or high grain content diets (Broderick, 2003; Castillo et al., 2001). Thus, overall N efficiency increased when cows were fed low forage combined with low N and high concentrate diets.

Milk and protein yields increased when high levels of corn were substituted for barley in diets of lactating cows (Castillo et al., 2001). However, high corn diets with limited digestion of starch in the rumen can lead to fermentation in the hind gut with reduced or no benefit to the animal (Orskov, 1986). Conversely, rapidly fermented grains can cause reductions in ruminal pH which can impair rumen function, fiber digestion, N flow, production, and N efficiency. Reducing ruminal pH below 5.6 for long intervals increased the likelihood of subacute acidosis and caused health problems including laminitis, ruminal abscesses, and liver abscesses (Stone, 2004). Thus high starch diets could negatively impact the N efficiency and performance of the lactating cow if too much starch is fermented in the rumen. Dietary corn grain with low ruminal degradability is more likely than high ruminal degradability sources such as wheat and barley to increase N retention and efficiency without the negative health effects.

Deleterious effects observed with high levels of starch in the diet must be prevented to successfully use these feedstuff to improve N efficiency.

Nitrogen Recommendations for Dairy Cattle

Crude protein requirements for ruminants include sources of N which are available to ruminal microorganisms and N available for the host animal. The latter is provided by both microbial protein and dietary protein escaping ruminal fermentation. Maximizing microbial growth improves digestibility, productivity, and N utilization in the lactating cow (Allen, 2000). Therefore, sufficient ruminal NH_3 concentrations are necessary for maintenance of normal microbial function. Diets with as low as 14.7% CP (DM basis) provided sufficient levels of NH_3 to maintain milk production (Cyriac et al., 2008). However, increasing dietary CP to more than 19% impaired microbial growth (Maeng et al., 1976). Indeed diets with 17% CP content or more lowered production and reproductive activity in lactating cows (Butler, 1998). However, reductions of dietary CP below 14.2% could impair bacteria growth and diminish milk production in dairy cows (Ipharraguerre and Clark, 2005).

Dietary protein that escaped rumen fermentation is partially digested and absorbed in the small intestine. Manipulations of dietary CP to change AA composition and to increase dietary AA flow to the small intestine did not show benefits to lactating cows (Santos et al., 1998). Thus varying AA composition and supply does not seem to offer a plausible mechanism to increase N efficiency. However, methionine is thought to be the first limiting AA in dairy cows fed typical North American rations (Leonardi et al., 2003). Therefore the addition of ruminally protected methionine to diets could

ameliorate any such deficiencies. However, supplementing dairy rations with protected methionine failed to increase milk and protein production, and N efficiency when cows were fed either 16.1 or 18.8% CP. Presumably, the response to Met supplementations should be observed in animals fed diets with low CP percentage (e.g. 14.0% CP; Leonardi et al., 2003). Interestingly, the authors concluded that reducing dietary CP from 18.8 to 16.1% was an effective approach to reduce N losses and increase N efficiency (Leonardi et al., 2003).

Pigs can be fed low CP diets (e.g. 12.0%) if diets are supplemented with limiting EAA hence increasing N efficiency (Chung and Baker, 1992), but in ruminants it is difficult to establish an order of limiting AA. Nonetheless as with swine, AA requirements for lactating cows could be met feeding low protein diets. Thus this approach would be a feasible platform to maintain production, reduce AA catabolism, and increase N efficiency in lactating cows.

Greater Energy Supply Improves N efficiency

Castillo et al., (2000) indicated that milk protein yield was highly correlated with both N and energy intake. The variation in milk protein yield explained by energy intake was greater than the proportion explained by the variation in N supply. In fact, increasing energy intake increased milk protein yield in a non linear manner (Broderick, 2003; Hanigan et al., 1998a). Part of the positive response may be associated with greater microbial growth in the rumen (Castillo et al., 2001). Readily fermentable carbohydrates such as those found in barley, provide energy to sustain microbial growth during dietary N reductions which in turn increases N efficiency (Castillo et al., 2001). However,

propionate infused in the rumen and starch infused in the duodenum stimulated milk and milk protein synthesis (Raggio et al., 2006; Rulquin et al., 2004) when neither would be expected to elicit a change in microbial flow. Thus N efficiency and milk protein production can be manipulated by altering propionate production or the supply of post-ruminal carbohydrates and this effect is not related to changes in microbial yields.

Infusion of glucose in the duodenum was associated with increased mammary blood flow (MBF), mammary EAA uptake, and insulin release (Rulquin et al., 2004). Nocek and Tamminga (1991). Reynolds et al., (1994) speculated that postruminal starch digestion increased glucose absorption and promoted milk protein yield by sparing AA utilization in the splanchnic tissues allowing more AA to reach the MG. Collectively, dietary carbohydrates with different fermentation patterns activate ruminal and post-ruminal metabolism to maintain production even when cows were fed low protein diets. Thus high levels of dietary energy would increase N efficiency while maintaining productivity.

Nitrogen Metabolism in Splanchnic Tissues

Splanchnic tissues (liver plus gastrointestinal tract, pancreas, spleen, portal system, and associated adipose tissue) are responsible for digestion, absorption, and intermediary metabolism of AA (Reynolds et al., 1991). In lactating cows, the largest losses of dietary N occurred in the splanchnic tissues (Hanigan et al., 2004). Casein delivered to the duodenum resulted in greater absorption and concentration of AA but also correspondingly greater removal of AA and catabolism by splanchnic tissues (Hanigan et al., 2004).

Liver AA affinity is very low with only 0 to 10% of AA in blood being removed in a single pass (Hanigan, 2005). Thus most absorbed AA reach arterial blood as evidenced by increased arterial AA concentrations associated with casein infusion (Guinard and Rulquin, 1994). However approximately 50% of the cardiac output, and thus systemic AA, flows back to the splanchnic tissues. Therefore AA not used for productive purposes or catabolized by peripheral tissues will be recycled to the splanchnic tissues (Hanigan, 2005). Even though fractional removal is low in the splanchnic tissues, this constant recycling results in large catabolic removal with 60 % of the daily supply of postabsorptive AA removed by these tissues (Hanigan, 2005; Hanigan et al., 1998b). This ensures that almost all of the absorbed AA are presented for use (e.g. protein synthesis) and only those not used by peripheral tissues are cleared from the system. Therefore increasing MG removal of AA would reduce recycling and improve AA utilization in the peripheral tissues.

High starch diets can trigger insulin release which increases AA capture in milk protein (Mackle et al., 2000). Part of the effects of energy status are mediated by increased blood supply to MG (Bequette et al., 2002). Both, greater systemic insulin and local blood flow in MG were associated with greater AA capture and protein synthesis even under dietary CP restrictions. These mechanisms offer the opportunity to reduce AA catabolism in splanchnic tissues (Hanigan et al., 2004).

Mammary Glands Maintain Protein Synthesis When Amino Acids are Limiting

The udder can locally regulate AA supply and extraction to maintain milk synthesis even when concentrations of EAA are low (Bequette et al., 2000). Mammary tissue regulated

EAA extraction as observed in lactating goat with limiting arterial supply of His. When arterial concentrations of His were extremely low (8 µM), MG responded with a 43-fold increase in the capacity to remove plasma His (Bequette et al., 2000).

The mammary cell synthesizes nitric oxide (NO) from Arg which diffuses from capillaries in the vicinity of the alveoli. Increased concentration of NO acts to relax the smooth-muscle arterioles and increased MBF (Lacasse et al., 1996). However, this mechanism has not been shown to mediate increases of MBF during shortages of His or EAA.

Administration of leucine increased protein synthesis in muscle of growing animals (Escobar et al., 2006). Although, greater protein intake linearly increased the circulating concentrations of Leu and MG oxidation from 4.7 to 13.6% with no increase in milk protein output while reduced arterial concentration of Leu reduced MG oxidation by 33% (Bequette et al., 1996b). Inducing an increased MP flow also increased oxidation of Leu by 259% in MG (Raggio et al., 2006). Clearly, reduced AA supply triggered mammary mechanisms to maintain production, while, greater MP supply increased AA catabolism in MG. Possible mechanisms engaged to maintain production include greater EAA uptake, and diminished AA oxidation in MG. Each of these would improve N efficiency by improving AA capture in protein and reducing catabolism in mammary tissue.

Mammary Glands Adapt to Energy Supply

Mammary glands have the capacity to regulate nutrient supply and uptake to sustain milk production (Delamaire and Guinard-Flament, 2006). Guinard et al., (1994) concluded

that greater milk yield was associated with greater plasma concentrations of acetate, glucose, beta-hydroxybutyrate (BHBA), and nonesterified fatty acids (NEFA) by 3, 18, 22, and 91% respectively. Hormones (e.g. insulin and IGF-I) and locally produced metabolites regulate mammary metabolic activity which in turn regulates MBF and milk production (Mackle et al., 2000).

Elevations in insulin increased milk and milk protein yield by increasing extraction of EAA particularly branched chain AA (BCAA; Mackle et al., 2000). The concentrations of circulating plasma EAA were reduced by 33% and BCAA by 41% during insulin infusion and milk protein yield was increased by 15% (Mackle et al., 2000). Restriction of energy and glucogenic factors limited extractions of AA and synthesis of milk. However, intravenous infusion of glucose increased the AA extraction when compared to either intravenous or postruminal infusion of AA (Schei et al., 2007). Moreover, greater insulin concentration, which is associated with hyperglycemia, increased MBF and reduced Leu oxidation in the udder of lactating animals (Bequette et al., 2002).

Insulin and IGF-I stimulate NO production via phosphorylation and cellular activation of PI-3 kinase which leads to greater blood flow (Zeng and Quon, 1996). Nitric oxide was proposed to be an endocrine regulator that stimulated insulin secretion in the pancreas (Schmidt et al., 1992). These hormonal responses suggest that carbohydrates supplied post ruminally may trigger hormonal release and local adaptation in MG.

Collectively, adaptations at the MG include greater MBF and AA extraction and reductions in AA oxidation. Digestion of carbohydrates in the duodenum may increase

glycemia and insulin release which can increase mammary metabolism. Therefore under these circumstances, reduction of mammary AA catabolism, greater AA capture to maintain milk protein synthesis, and greater N efficiency are expected.

Glucose and Amino Acids Increased Protein Synthesis at Cellular Level

Increased dietary energy, which increases milk production, triggers hormonal changes including elevations in insulin, somatotropin, and IGF-I. However, there is no conclusive demonstration of the complete mechanism of action of these hormones on milk and protein synthesis. Cellular signaling cascades have been shown to mediate and integrate signals arising from changes in AA and hormonal concentrations. These signaling proteins act to modify rates of protein synthesis (Bolster et al., 2004).

The mammalian target of rapamycin (mTOR) signaling cascade regulates protein synthesis in growing muscle in part through its ability to phosphorylate and control the activity of the translational regulators, p70 S6 kinase 1 (p70 S6K1), ribosomal protein S6 (rpS6), and eukaryotic initiation translation factor 4E binding protein 1 (4E-BP1; see figure 1-1). This pathway has been explored in single-stomach animals and cell culture. In turn, mTOR signaling is controlled through its phosphorylation state which is mediated by upstream kinases including AKT also known as Protein kinase B. AKT is activated by insulin, IGF-I and AMP-activated protein kinase (AMPK; Bolster et al., 2004), the latter being regulated by energy intake and energy status of the cell. Additionally, mTOR phosphorylation and activity is particularly sensitive to Leu concentrations (Escobar et al., 2006). However, the mTOR cascade has not been explored in MG of lactating cows.

Protein kinase B also phosphorylates endothelial nitric oxide synthase (eNOS), which catalyzes the synthesis of NO and controls local blood flow (Boo and Jo, 2003; Zeng and Quon, 1996). NO production from endothelial cells is promoted by a number of factors. eNOS can be activated by shear stress, acetylcholine, growth factors such as IGF-I, vascular endothelial growth factor, histamine, and estrogen. However, the activity of eNOS is largely mediated by at least 5 specific phosphorylation sites (Boo and Jo, 2003). Protein kinase B has shown to regulate activity of eNOS and inhibitors of PI3K (upstream regulator of AKT) failed to phosphorylate eNOS during stimulations with IGF-I (Boo and Jo, 2003). Thus if the energy status of the mammary cell and growth factor concentrations increase activating protein kinase B, it could in turn activate eNOS, NO production, and increase local BF. The latter could contribute to maintenance of mammary AA supply when arterial concentrations of AA are low. Reducing dietary CP was proposed as a means to reduce AA recycling, increase mammary AA capture while maintaining protein synthesis and increase overall N efficiency. However, limiting delivery of AA could also down regulate mTOR activity and negatively affect protein synthesis (Bolster et al., 2004).

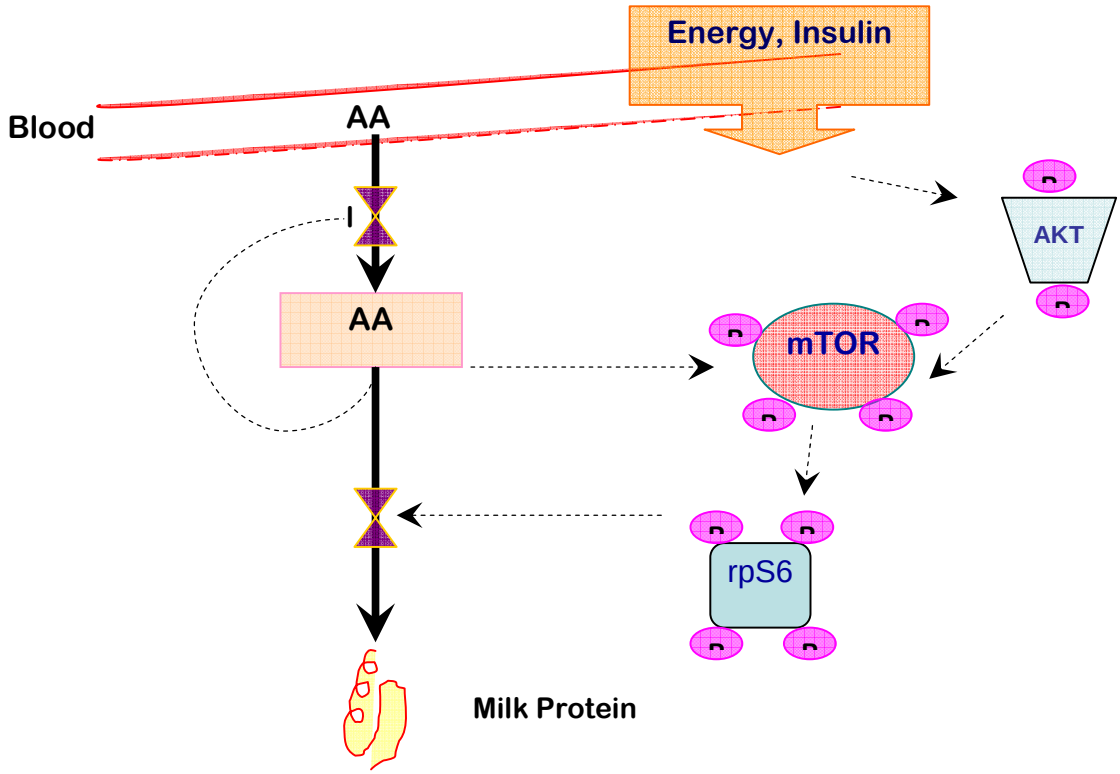
It is likely that similar signaling cascades exist in bovine MG including mTOR, eNOS and intermediary signaling proteins. Given the role of mTOR in integration of signals arising from AA supply, energy status of the cell, and hormonal signals it may be possible to manipulate mTOR to achieve greater efficiency of AA use for milk protein synthesis. Specifically, increased energy supply and the associated increase in insulin and IGF-I concentrations may be able to maintain mTOR signaling activity during a loss of signaling from AA associated with a reduction in protein supply. If true, this would

maintain mammary AA capture and use for milk protein synthesis at the expense of splanchnic catabolism of AA. Such a mechanism would allow increased N efficiency, reduced N intake, and reduced released of N to the environment.

We hypothesized that milk and protein yield could be maintained and N efficiency improved by reducing MP in combination with high energy diets as compared to a low energy low protein diets. Our first study was conducted to test our hypotheses at the animal level. The first objective of this study was create an MP deficiency and assess N efficiency when dietary energy was varied. The second objective was to test the NRC (2001) model predictions of energy and protein requirements for lactating dairy cows.

The second study was conducted to test our hypothesis at the animal, tissue, and cellular levels. We hypothesized that AA and glucogenic substrate support protein synthesis independently. We reasoned that the efficiency of protein synthesis in mammary tissue is associated with local adaptive mechanisms at the cellular level including greater activity of the protein translation factors consistent with the mechanisms described in muscle tissue of growing animals. The objectives of this study were to test if MG can regulate blood flow, AA uptake, and protein synthesis when AA supply was reduced and energy status increased.

Figure 1-1. Proposed aggregated representation of the mTOR signaling cascade in the mammary secretory cell. This figure partially represents initiation of the protein synthesis translation process. Some of the mTOR (mammalian target of rapamycin) signaling cascade is illustrated. Energy status of the cell and insulin up regulate the mTOR cascade. Phsophorylation (P) of AKT (protein kinases B) activates and phosphorylates mTOR which phosphorylates rpS6 (ribosomal protein S6) which promotes initiation of protein synthesis. Additionally, amino acids (AA) activates mTOR which promotes initiation of protein synthesis. Also, increased cellular concentrations of AA inhibit the AA uptake.



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Chapter 3

Interactions of Energy and Predicted Metabolizable Protein in Determining Nitrogen Efficiency in the Lactating Dairy Cow

ABSTRACT

Lactating cows are relatively inefficient in converting dietary N to milk N as compared to the efficiency of N use for growth in simple-stomached animals. The majority of productive N losses occur in the postabsorptive system. The aim of the study was to test whether predicted metabolizable protein (MP) and dietary energy exerted independent effects on milk protein synthesis and postabsorptive N efficiency. If true, postabsorptive N efficiency could be expected to be greater when animals are fed high energy diets. Forty mid-lactation cows (32 multiparous Holstein and 8 primiparous Holstein x Jersey cross-breds) were used in a complete randomized design with a 2 x 2 factorial arrangement of diets. Cows were assigned to one of four dietary treatments: high-energy, high-protein (HE/HP); high-energy, low-protein (HE/LP); low-energy, high-protein (LE/HP); and low-energy, low-protein (LE/LP). Energy concentrations were 1.55 (HE/HP and HE/LP) or 1.44 (LE/HP and LE/LP) Mcal NE_L/kg DM. Changes in predicted MP were achieved by feeding diets with 6.6 (HE/HP and LE/HP) or 4.6% (HE/LP and LE/LP) ruminally undegradable protein (DM basis). Ruminally degradable protein was held constant at 10.1% of DM. All cows were fed HE/HP diet from day 1 to 21 followed by the respective treatments from day 22 to 43 (n=10). Milk protein yield was reduced as dietary energy was reduced. Milk yield followed a similar pattern as milk protein yield. There was a trend for decreased milk yield as CP was reduced. There were

no interactions between dietary energy and protein for either milk or protein yield. Plasma amino acid concentrations were not affected by treatment. Milk urea N was affected by energy and protein with a significant interaction (HE/HP=17.2, HE/LP=12.2, LE/HP=21.0, LE/LP=12.2 mg/dl). Nitrogen efficiency calculated from predicted MP supply was affected by energy and protein supplies with no apparent interaction and ranged from a low of 31% (LE/HP) to a high of 43% (HE/LP). The NRC model would predict N efficiency more accurately if a representation of the effects of energy on N efficiency were included in the postabsorptive system.

(Key words: metabolizable protein, energy, nitrogen efficiency, cow)

INTRODUCTION

The lactating dairy cow has a relatively low efficiency of converting dietary N to milk N when fed to NRC requirements (N output in milk/N intake; Bequette et al., 1998; Castillo et al., 2000), and efficiency decreases as dietary CP content increases (Broderick, 2003; Ipharraguerre and Clark, 2005). An improvement in N efficiency would lead to increased N capture and reduced N losses in the lactating cow (Jonker et al., 2002). The majority of the N losses occur in the postabsorptive tissues (e.g. gut, liver), and the magnitude of the losses are variable (Hanigan, 2005). The NRC (2001) assumes fixed conversion partial efficiencies in calculating N requirements for postabsorptive processes in the lactating cow. The efficiency of use of MP for lactation is assumed to be a constant 0.67 meaning that each gram of milk protein output requires 1.5 g of metabolizable protein (**MP**). Similarly the efficiency of MP use is assumed constant for maintenance functions. The model thus predicts that an increase in milk protein output in response to increased

dietary energy can only occur if additional MP is provided to support the increase in milk protein output for a given animal.

A meta analysis demonstrated that energy supply affects the capacity for milk protein synthesis, but it did not suggest that the efficiency of conversion of dietary protein to milk protein was affected by energy supply when cows were fed at or below N requirements (Hanigan et al.1998). It is possible that the effects of energy on milk protein synthesis are caused entirely by changes in microbial growth and outflow from the rumen. However, Castillo et al., (2001) reported that the efficiency of N utilization was improved and N excretion reduced when cows were fed diets low in degradable starch which is not consistent with the hypothesis of a microbial protein response. Ruminal infusions of propionate were also observed to increase milk protein percentage and yield (Raggio et al., 2006), and postruminal infusions of starch stimulated milk protein output (Reynolds et al., 2001). These observations all support a role for energy supply in regulating postabsorptive N utilization and efficiency independent of the effects of metabolizable protein supply.

Rulquin et al., (2004) concluded that lactating cows fed isonitrogenous, isoenergetic diets and duodenally infused with glucose increased arterial flux and mammary uptake of essential amino acids (**EAA**) which led to increased milk protein output. While Lapierre et al., (2006) concluded that the splanchnic tissues (liver and portal-drained viscera) were the primary cause of poor efficiency of postabsorptive AA use for milk protein, the relatively low affinity for amino acids (**AA**) exhibited by these tissues does not support that hypothesis. First pass removal has been shown to be low, and thus these tissues are not preventing AA from reaching the mammary gland (**MG**).

The problem lies with recycling of AA not used by mammary in a given pass. The splanchnic tissues receive 50% of the cardiac output (Davis et al., 1988). Therefore, a large proportion of AA not used by the MG are returned to the splanchnic tissues (Hanigan, 2005). This constant recycling of unused AA to splanchnic tissues results in a 60% loss of the postabsorptive AA supply on a daily basis (Hanigan, 2005). Thus, increased mammary uptake and use for protein synthesis would reduce recycling of AA to the splanchnic tissues and improve efficiency of AA use by the MG, i.e. excessive splanchnic catabolism is an effect rather than a cause.

Certainly the MG can be very efficient at extracting AA when they are needed. A dietary deficiency of a single EAA triggered a 43-fold increase in transport activity in the MG for that single EAA plus a 33% increase in blood flow reaching the MG (Bequette et al., 2000). Thus it is clear that the MG is able to alter EAA transport activity which will increase in response to either decreased supply of EAA or increased use of EAA for protein output (i.e. increased milk protein synthesis). This results in variable postabsortive transfer efficiencies from the gut to milk protein. Therefore, if energy supply can stimulate milk protein output, the MG should respond by increasing AA removal from blood resulting in reduced recycling of AA to other tissues and improved efficiency.

We hypothesized that milk protein yield could be maintained and N efficiency improved when reduced dietary MP (elicited by changes in RUP) was combined with high dietary energy as opposed to the single limiting nutrient paradigm assumed by the NRC (2001) model. This can only be true if energy and protein exert independent, additive effects on milk production. The first objective of this study was to assess the

efficiency of N utilization under predicted conditions of MP deficiency when dietary energy density was varied. A second objective was to test the NRC (2001) model predictions of energy and protein requirements for the lactating dairy cow.

MATERIAL AND METHODS

Animals and Diets

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech. Thirty-two multiparous Holstein and 8 primiparous Jersey × Holstein cross-bred cows (186 ± 89 DIM) were selected from the Virginia Tech dairy herd. Cows were housed in a free-stall barn at the Virginia Tech dairy complex and individually fed using a Calan door system (American Calan, Inc., Northwood, NH). Animals were milked twice daily at 0230 and 1500 h. Milk weights and BW were recorded automatically at each milking. Body condition scores (**BCS**) were recorded for each cow at d 21 and 43 according to the methods of Wildman (1982). Animals were balanced (stratified) in four groups based on DIM, parity, breed, body weight (**BW**), milk production, and randomly assigned to one of the four treatments. The study was a complete randomized design with a 2 x 2 factorial arrangement of treatments. Dietary treatments were 2 levels of energy (high and low) and 2 levels of MP (high and low) indicated as following: high-energy, high-protein (**HE/HP**); high-energy, low- protein (**HE/LP**); low-energy, high- protein (**LE/HP**); and low-energy, low- protein (**LE/LP**; Table 1). Diets were formulated to meet NRC (2001) recommendations for RDP, minerals, and vitamins of a mid-lactation dairy cow weighing 635 kg (BCS = 3.0) and producing 36.3 kg milk/d containing 3.5% fat and 3.0% protein and consuming 22.9 kg/d

DM. Final diets contained either 50% forage and 50% concentrate (LE/HP and LE/LP) or 39% forage and 61% concentrate (HE/HP and HE/LP) on a DM basis (Table 1). Ruminally undegradable protein was manipulated while holding RDP constant through the use of varying amounts of soybean meal and ruminally protected soybean meal (HiVap®). The latter was manufactured by Land O' Lakes/Purina Feed (Gainesville, GA) using an extrusion based process (Patent No. 5683739) resulting in conformational and chemical changes in the protein that confer ruminal stability. The resulting product was previously found to contain 51.6% CP which was 15% soluble, 85% insoluble, and the insoluble portion had a ruminal degradation rate of 0.02%/h (Cyriac et al., 2008). Tallow was included in two treatments to maintain constant energy concentrations as dietary protein was manipulated and to compensate for the fat present in the protected soybean meal. The percentages of forages and concentrate were adjusted weekly on an as fed basis to reflect changes in the DM content of the forages and concentrates. Diets were mixed at 0800 and fed once daily as a total mixed ration (TMR). Feed offered and refused was recorded daily. Feed offered each day was adjusted to achieve between 5 and 10% refusals. The HE/HP diet was fed from d 1 through 21 (the covariate period) followed by the respective experimental diets from d 22 to 43 (the treatment period).

Sample Collection and Analyses

Samples of forages, concentrates, and orts were collected twice weekly to assess DM content of the ingredients and refusals. Feed samples were dried overnight at 105ºC for DM content (Mechanical Convection Oven, Freas 645, Thermo Electron Corporation, Waltham, MA). During collection weeks, samples of ingredients were obtained daily and

stored frozen at −20ºC for later chemical analyses. Daily subsamples were dried at 55ºC for 48 h and ground through a Wiley mill (1-mm screen; Arthur H. Thomas, Philadelphia, PA). Subsamples were combined by treatment and period on an equal weight basis, and submitted to Cumberland Valley Analytical Service Inc., (Hagerstown, MD) for analysis of ether extract, Kjeldahl N (AOAC, 1990) starch, sugars, lignin, ADF, NDF (Van Soest et al., 1991) and Ca, P, Mg, and K by inductively coupled plasma spectrometry.

Milk samples were collected from 6 consecutive milkings during d 19 through 21 for covariate period analysis and d 41 through 43 for treatment period analysis (wk 3 and 6). Individual milk samples were analyzed for fat, true protein, lactose, total solids, and somatic cells by infrared analyses (DHIA, Blacksburg, VA; AOAC (1997); Foss North America, Eden Prairie, MN). The MUN analyses were conducted using the Berthelot procedure (ChemSpec 150 Analyzer; Bentley Instruments, Chaska, MN). Daily milk composition was calculated from the weighted AM and PM observations.

Two blood samples were collected from the coccygeal vessel on d 21 and 43 into vacuutainer tubes containing either sodium heparin or sodium floride plus EDTA and placed immediately on ice (10 or 5 ml; Becton Dickinson and Co., Frankin Lakes, NJ). Plasma was harvested by centrifugation at 1,300 x *g* for 20 min at 4ºC within 2 h of blood collection. Plasma was stored at -20ºC until analyses were conducted for AA, glucose, acetate, lactate and beta-hydroxybutyrate. Free AA were determined by isotope dilution using a gas chromatograph-mass spectrometer (GC-MS; Focus-PolarisQ GC-MS, Thermo Electron Corporation; Waltham, MA) according to the methods of El-Kadi et al., (2006) and Calder et al., (1999).

Proton nuclear magnetic resonance spectroscopy (**NMR**) was conducted on the NaF treated plasma samples to assess glucose, acetate, lactate and beta-hydroxybutyrate concentrations (Beckonert et al., 2007). Briefly, 0.5 mL of plasma was deproteinized by addition of an equal volume of acetonitrile. Samples were vortexed and centrifuged at 14,000 *g* for 20 min at 4ºC. The supernatant was removed and freeze dried (Savant SpeedVac® SC 110, GMI Inc.; Ramsey MN). Dried samples were re-suspended in 0.5 mL of phosphate buffered (100 mM) D_2O (Sigma Aldrich, St. Louis, MO) with 0.5% Na azide and 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as an internal standard (0.45 mM final concentration). Proton NMR (Varian 400; Palo Alto, CA) was performed and the spectra were baseline adjusted, phase corrected, and the peak areas integrated using the Know-It-All Informatics System (Bio-Rad; Hercules, CA). Validation of this procedure using samples from a previous study indicated the peak areas were linearly correlated with observed concentrations. However, apparent recoveries were not 100% when comparing the NMR determined concentrations with enzymatically determined concentrations. Although the technique clearly detects relative differences among samples with reasonable precision, we are not confident the method can accurately determine concentrations. Thus metabolite values were presented as relative peak areas with respect to the DSS peak area.

Statistical Analysis

Data were tested for normality using the univariate procedure of SAS (SAS Institute, 2004). Nutrient and DM intake, BW, milk yield, and milk composition data were analyzed as repeated measures in time (days) using the Mixed procedure of SAS (SAS

Institute, 2004) to test for differences in the treatment means. Excepting milk composition, data from the last 7 d of each period were analyzed to allow for complete diet adaptation. Milk composition data from d 19, 20, 21, 41, 42, and 43 were subjected to analyses. Body condition score and blood metabolites were analyzed using the Mixed procedure without the effect of time. The statistical model used is described in Table 2. Interactions between main effects were removed form the model if they were not significant. The autoregressive covariance structure was used for each variable analyzed. This structure was chosen based on AIC and AICC fit statistics and the expected relationship among days. The smallest value for AIC and AICC information criteria was used to identify the appropriate covariance structure. Observations from d 15 to 21 were used for covariate analysis of BW, DMI, and milk yield. Milk composition data on d 19, 20, and 21 were used for covariate analysis of milk component percentages and yield. The covariate was removed from the model if it was not significant. Observations from the covariate period were averaged and included as a covariate adjustment in the model. Unless otherwise stated, significance was declared at $P < 0.05$. All results are reported as least square means (LSM).

NRC Model Analysis

Predictions of NE_L and MP by the NRC model (2001) were assessed for accuracy using the observed values for production and N efficiency. Model predictions were compared to the LSM values from the statistical analysis of DMI, milk yield, milk composition, and BW for each dietary treatment. Diet composition was set to values listed in Table 3 and observed DMI was used to set the feeding rate of each ingredient. The nutrient content of

each grain mix was then calculated using tabular values and compared to the observed chemical composition of the grain mix in Table 3. Adjustment was needed because the tabular values of CP, ADF, and NDF differed from the observed values obtained by chemical analysis. Because ruminally protected SBM and SBM were the major contributors of CP in the grain mixes, the tabular CP content of these two ingredients was increased to bring the average CP of the grain mixes in line with the predicted values (from 52.2 to 65% of DM for ruminally protected SBM and from 53.8 to 54.9% of DM for SBM). The large adjustment required for the ruminally protected SBM to bring observed and predicted grain mix CP contents in line with observed values likely reflects mixing or formulation errors at the plant given that it would be highly improbable that a ruminally protected SBM ingredient could have such high CP content. It is also possible the chemical analyses of the grain mixes were in error, but reanalyses confirmed the original observations. The ADF and NDF content of cotton seed hulls and soybean hulls were modified in a similar manner to reflect the observed chemical analysis of the grain mixes. The tabular ADF content for cotton seed hulls was reduced from 65.0 to 55.0% DM, and the tabular value for soybean hulls was increased from 44.6 to 47.0% DM. The tabular value for NDF in soybean hulls was increased from 60.3 to 70.0% DM.

RESULTS

Chemical Composition of the Diets and Feed Ingredients

The nutrient requirements as predicted by NRC (2001) are shown in Table 1. The observed nutrient concentrations of dietary components are listed in Table 3. The CP, ADF, NDF, and NFC contents of the experimental diets differed from the formulated

values due to nutrient variation in the ingredients as compared to tabular values used for formulation and possibly variation in formulation as noted above. The predicted energy content of the treatments was slightly lower than expected probably because of the greater ADF and NDF contents in the concentrate mixes (Table 4), but the differences among treatments was maintained. As intended, the CP difference between the HP and LP treatments was greater than 3.0 percentage units (Table 4).

Animal Performance

Least squares means for DMI, BW, BCS, milk yield, and milk composition are presented in Table 5. Dry matter intake was not affected by treatments. As anticipated, CP intake was greater for cows consuming the HE/HP and LE/HP diets than for those consuming the HE/LP and LE/LP diets (4.70 and 4.68 versus 3.72 and 3.64 kg/d respectively). Reduced dietary energy decreased milk production ($P < 0.003$), and there was a trend for decreased production in association with inadequate dietary CP ($P < 0.08$). There was no apparent interaction of energy and CP effect on milk production. Lactose concentration and lactose yield decreased in association with feeding the low energy diets ($P < 0.01$ and 0.01 respectively). Cows that received high energy diets (HE/HP and HE/LP) produced more milk protein ($P < 0.001$; 1.13 and 1.03 kg/d respectively) than those fed low energy diets (LE/HP and LE/LP; 0.91 and 0.87 kg/d respectively). Milk protein percentage was not affected by dietary energy, but there was a trend for a CP effect to increased protein percentage ($P < 0.10$). Energy and CP both affected milk fat percentage ($P < 0.004$ and 0.01 respectively), and there was no interaction between treatments. However, milk fat yield was not different among treatments. Non-fat milk solids percent and yield were

reduced ($P < 0.001$ and 0.01 respectively) when dietary energy was inadequate (LE/HP and LE/LP). Milk urea N was reduced in cows fed low CP diets (HE/LP and LE/LP; 12.2 and 12.0 mg/dl respectively) as compared to cows fed high CP diets (HE/HP and LE/HP; 17.2 and 21.0 mg/dl) but at low CP, provision of addition energy did not result in a reduction in MUN concentrations. The interaction between energy and CP was significant for MUN ($P < 0.005$). Somatic cell counts were reduced in cows fed high energy diets ($P < 0.02$). Reduced CP intake was associated with reduced ending BW ($P < 0.04$). Body condition scores were not significantly affected by energy or protein and there was no interaction.

NRC Model Analysis

Observed DMI was greater than that used in formulating the diets, resulting in a nutrient supply greater than expected (Table 4). After adjusting for observed intakes, ingredient composition, and milk performance the NRC (2001) model overpredicted allowable milk yields for the HE/HP, LE/HP, and LE/LP diets and slightly underpredicted yields for cows fed the HE/LP diets. The relative predicted responses to energy on the high CP diets were close to observed with the model predicting a 3.9 kg/d response and an observed response of 5.4 kg/d. However, the relative responses to CP were greatly overpredicted with predicted responses of 9.0 (HE/HP - HE/LP) and 8.2 kg/d (LE/HP - LE/LP) for the high and low energy diets, respectively, and observed responses of 2.8 and 4.7 kg/d, respectively.

Amino Acids and Blood Metabolites

Although DMI, CP, and milk protein yield were affected by treatments, neither concentrations of plasma free NEAA nor EAA changed across treatments (Table 6). There was an effect of energy on plasma beta-hydroxybutyrate concentrations ($P < 0.001$), and an interaction between energy and CP on blood glucose concentrations ($P < 0.01$; Table 7).

Nitrogen Utilization

Nitrogen supply was reduced for low protein diets (Table 8). Cows that received the HE/HP and LE/HP diets had greater predicted urinary N excretion (292 and 338 g/d, respectively) relative to those that received the HE/LP and LE/LP treatments (216 and 202 g/d, respectively) and there was an interaction ($P < 0.01$). High CP diets increased predicted urinary N excretion but especially so at low dietary energy. There was an effect of energy on milk N output ($P < 0.002$) with cows fed high energy diets increasing N output in milk relative to those animals fed low energy diets. Consistent with our hypothesis, postabsorptive N efficiency improved as energy increased, with the greatest efficiency observed for cows fed high energy diets ($P < 0.001$; HE/HP and HE/LP; 37.1 and 43.0% respectively) relative to cows fed low energy diets (LE/HP=31.0 and LE/LP=38.5%). Likewise, cows fed low CP diets had greater N efficiency compared with cows fed high CP diets ($P < 0.001$).

DISCUSSION

Lactation Response

Cows fed the low CP diet were apparently protein deficient based on the trend for a milk yield response to increased CP supply ($P < 0.08$) and reduced ending BW ($P < 0.04$). Based on the single-limiting nutrient paradigm used in the NRC, these protein deficient animals should not have been able to respond to increased energy supply, yet they clearly did. The lack of a significant interaction between energy and CP for both milk and milk protein yields supports this conclusion. Therefore, the effects of energy and CP were additive and independent. Therefore, greater efficiency of N use in the postabsorptive system can be expected when animals are fed a high energy diet as compared to a low energy diet. The independent effects of energy and CP are consistent with observations at the tissue level wherein increased rates of MG protein synthesis resulted in increased removal of AA from blood and reduced recycling of AA to and catabolism by the splanchnic bed and other peripheral tissues (Bequette et al., 2000; Bequette et al., 2001; Hanigan et al., 2000; Hanigan, 2005). Our observations are also consistent with the observations of Raggio and coworkers (2006) who concluded that propionate infusions promoted milk protein concentration and yield by increasing AA uptake by the MG. The observed improvement in N efficiency in the current study from 38.5% for the LE/LP diet to 43.0% for the HE/LP diet (Table 8) are also consistent with this conclusion. Finally, the N efficiency results are consistent with those of Broderick (2003) if one assumes the responses in the prior work resulted from changes in RUP rather than RDP as concluded by the authors. The lack of an interaction between energy and protein supply was also consistent with the observations of Broderick (2003) and supportive of a postabsorptive mechanism given that dietary RDP concentrations exceed NRC requirements in both studies and were controlled in the current work.

Milk urea nitrogen was affected by both energy and CP supply, and there was a significant interaction. Provision of additional protein in the diet when dietary energy was low resulted in the greatest MUN concentrations. The HE/HP diet had intermediate MUN concentrations consistent with increased use of CP for milk protein. Low dietary CP resulted in comparably low MUN regardless of dietary energy. It was anticipated that the HE/LP diet would result in the lowest MUN concentration given the comparable N intakes and significant effects of energy on milk protein output and N efficiency (Table 8).

Concentrations of MUN were greater than those reported by other authors (Broderick, 2003; Ipharraguerre and Clark, 2005). Kohn and coworkers (2004) compared different methods to determine MUN and concluded that the Bentley, CL 10, Foss 6000, and Skalar instruments explained more than 98% of the variance that was due to farm-to-farm variance. Machine accuracy and precision in DHIA laboratories is checked monthly against known standards (CL10 method) and results from a split-sample test indicated the machine was properly calibrated and reporting accurately.

Plasma beta-hydroxybutyrate concentrations were affected by dietary energy content. The large changes in ruminally fermentable carbohydrate content may have contributed to this, however endogenous production from partial oxidation of fatty acids in the liver was likely stimulated by the dramatic drop in energy metabolite supply to the animal which would be expected to elicit a change in fatty acid mobilization from adipose. The liver would also be expected to release acetate under these conditions (Hanigan et al., 2004) which may have been adequate to offset any reductions in acetate supply associated with lower diet digestibility. Low milk and lactose yield would be

likely to reflect for reductions in mammary glucose uptake. Thus reduced mammary removal would have caused increased glycemia.

Nitrogen Utilization

Predicted postabsorptive N efficiency was affected by dietary energy and CP independently with the greatest efficiency observed for cows fed the HE/LP diet (43.0%) and lesser efficiency for HE/HP and LE/HP diets (37.1 and 31.0% respectively). These results are in agreement with those presented by Broderick (2003) that reported an increase in N efficiency as CP in the diet was decreased and energy density increased and no significant interaction between CP and energy. Reducing N intake by approximately 100 g resulted in less than a 16 g reduction in milk N output while changing energy density in the diet changed milk N output by approximately 30 g/d. The present study is consistent with observations of Castillo et al., (2000) who reported that reductions in N intake from 750 to 400 g/d had a greater impact on N partitioning to urinary output (R^2=0.62) than to milk protein output (R^2=0.14). These findings suggest that postabsorptive N efficiency in lactating cows is variable and independently affected by N and energy supply. Feeding diets with less RUP than recommended by the NRC (2001) in combination with high concentrations of dietary energy may be a useful strategy to improve the N capture in milk protein.

NRC analysis

The partitioning of absorbed AA is represented by a fixed set of transfer coefficients in the NRC (2001) model. Further, this model assumes that a single nutrient limits animal

performance. Thus if CP is the first limiting nutrient as appears to be the case herein for the HE/LP and LE/LP diets (Table 5), then the model predicts that the animals cannot respond to provision of any other nutrient. However, milk production increased by 6.6 kg/d and milk protein output by 160 g/d when additional energy was provided with fixed dietary CP concentrations (LE/LP versus HE/LP) resulting in a 4.5% unit increase in the efficiency of MP use for milk production. These observations are consistent with those of Bequette et al. (2000) where the efficiency of transfer of AA into milk protein in the mammary gland was found to be variable. Not only did the model fail to predict the response to energy in a protein limiting situation, but it also overpredicted responses to CP in general. The model predicted a 9 kg/d milk response to provision of additional CP to the HE/LP diet and 8.2 kg of response for the LE/LP diet, but the cows exhibited a 2.8 kg/d response for the HE/LP diet and a 4.7 kg/d response for the LE/LP diet, i.e. the observed response was at best 50% of the predicted response. These observations are consistent with the independent effects of energy and N supply, and variable N capture efficiencies observed by Hanigan et al. (1998). As this variable efficiency is not captured in the NRC (2001) model, an improved representation of postabsorptive partitioning of N should result in a model that more accurately predicts N requirements. Additionally, the new representation would result in improved feeding programs and reduced N release to the environment.

CONCLUSIONS

The effects of postabsorptive energy and CP supplies on milk and protein yield were independent and greater energy or CP was able to stimulate production. The efficiency

of N utilization was maximum when feeding the combination of high energy and low CP in the diet. Therefore, the single limiting nutrient concept used in the NRC should be replaced with a multi-nutrient representation that accommodates at least energy and CP supply to better predict milk protein output and N efficiency.

ACKNOWLEDGEMENTS

This material is based upon work supported by the Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture, under Project No. NC-1009. The authors acknowledge funding provided by the Virginia Ag Council and Land O' Lakes/Purina Feed, Inc. in support of this study, general departmental funding provided by the Virginia State Dairymen's Association, and the Virginia Tech Dairy Complex crew for help in conducting the study. The contributions of J. Cyriac, A. Elgin, G. Moyer, H. Weeks, N. Ianni, and A. Cornman are gratefully acknowledged. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture, the Virginia State Dairymen's Association, or Land O' Lakes/Purina Feed, Inc.

Table 1. Predicted nutrient requirements as determined from NRC (2001) on % of DM basis.

	Treatments			
Energy	High	High	Low	Low
Protein	High	Low	High	Low
Corn silage	34.97	34.89	45.86	45.82
Mixed grass + legume silage	4.81	4.80	4.80	4.80
Cottonseed hulls	-	-	20.53	17.89
Soybean hulls	25.70	31.62	10.70	19.55
Corn grain, ground, dry	22.07	22.03	-	-
Tallow	-	1.18	-	1.13
Urea	1.16	1.24	0.74	0.79
Soybean meal, solvent (48% CP)	3.06	2.53	9.17	8.25
Protected soybean meal[1]	6.56	-	6.55	-
Calcium carbonate	0.30	0.30	0.30	0.30
Calcium phosphate (Di-)[2]	0.35	0.57	0.48	0.63
Sodium bicarbonate	0.20	0.20	0.20	0.20
Salt	0.24	0.24	0.24	0.22
Trace mineral and vitamin mix[3]	0.10	0.10	0.10	0.10

1HiVap®, Land O' Lakes / Purina Feed, Gainesville, GA.

[2]Contained 22% Ca and 19.3% P.

[3]Land O' Lakes/Purina Feed, Gainesville, GA; formulated to provide (per kg of dietary DM) 25×10^5 IU of vitamin A, 400,000 IU of vitamin D, and 10×10^5 IU of vitamin E, 0.1 mg of Co, 12 mg of Cu, 0.7 mg of I, 60 mg of Fe, 48 mg of Mn, 48 mg of Zn, 0.3 mg of Se.

Table 2. Definition of effects for the statistical model.

Effect	Type	df[2]	ddfm[2]
Energy (E) [1]	Fixed	1	31
Crude Protein (P) [1]	Fixed	1	31
Breed (B) [1]	Fixed	1	31
ExB	Fixed	1	31
PxB	Fixed	1	31
ExP	Fixed	1	31
ExPxB	Fixed	1	31
b[1]	Fixed	1	31
Cow (ExPxB)	Random	31	
Day (D) [1]	Fixed	6	192
ExD	Fixed	6	192
PxD	Fixed	6	192
ExPxD	Fixed	6	192
BxD	Fixed	6	192
ExBxD	Fixed	6	192
PxBxD	Fixed	6	192
ExPxBxD	Fixed	6	192
Residual	Random	192	
Total		279	

[1]The energy effect (high or low E), the crude protein effect (high or low P), the breed effect (Holstein or crossbreed), the effect of the regression analysis of the covariate b for the variable of interest, and the effect of day (1 through 7).
[2]df = degrees of freedom, ddfm = balanced denominator degrees of freedom for F-test

Table 3. Analyzed chemical composition of the feed ingredients used in the experimental diets (% DM basis).

	Ingredients					
	Corn silage	Haylage	Conc. mix A	Conc. Mix B	Conc. mix C	Conc. mix D
Item						
DM, % of feed	28.5	50.9	90.3	89.5	89.8	90.2
NDF, % of DM	43.7	48.8	36.0	41.5	50.1	54.6
ADF, % of DM	26.3	38.0	22.5	27.2	36.1	40.9
CP, % of DM	8.9	17.6	24.2	20.0	30.6	21.8
Fat, % of DM	3.6	2.5	2.8	3.7	2.1	4.0
NFC, % of DM	42.1	22.1	34.1	32.2	11.9	14.6
Lignin, % of DM	3.0	7.4	1.8	1.8	6.0	5.5
Starch, % of DM	30.0	1.2	28.5	24.3	4.0	2.8
Sugar, % of DM	1.2	4.3	3.7	3.9	5.1	3.6
Ash, % of DM	3.1	10.8	5.6	5.8	8.6	8.6
Calcium, % of DM	0.17	0.86	0.61	0.83	0.98	0.97
Phosphorous, % of DM	0.20	0.29	0.33	0.47	0.49	0.50
Magnesium, % of DM	0.15	0.23	0.19	0.20	0.27	0.25
Potassium, % of DM	1.04	3.01	1.07	0.94	1.50	1.23
Sulfur, % of DM	0.13	0.26	0.19	0.13	0.25	0.19
Sodium, % of DM	0.002	0.027	0.479	0.497	0.849	0.663

Table 4. Observed composition of experimental diets and predicted nutrient requirements as determined from the NRC(2001)[1].

	Treatments			
Energy	High	High	Low	Low
Protein	High	Low	High	Low
DM, %	49.6	47.8	46.0	44.8
CP, % of DM	18.7	15.2	19.1	15.5
NDF, % of DM	38.7	42.1	48.9	51.8
ADF, % of DM	24.5	26.8	31.2	33.4
RDP, % of DM	11.5	10.5	11.2	10.3
RUP, % of DM	7.2	4.7	7.9	5.2
NFC, % of DM	35.6	34.9	25.3	25.0
Crude fat, % of DM	3.3	4.4	2.9	4.0
NE_L Mcal/kg	1.54	1.53	1.45	1.45
RDP required, g/d	2463	2453	2355	2141
RDP supplied, g/d	2907	2639	2866	2364
RDP balance, g/d	444	186	511	223
RUP required, g/d	1333	1265	1203	1075
RUP supplied, g/d	1826	1176	2026	1203
RUP balance, g/d	493	-89	823	128
MP required, g/d	2574	2424	2395	2089
MP balanced, g/d	412	-68	679	97
MP supplied, g/d	2986	2356	3074	2186
MP allowable milk, kg/d	43.5	32.0	45.6	28.9
NE_L allowable milk, kg/d	41.0	39.6	37.1	30.3

[1]Values predicted using actual DMI, milk yield, and components for each treatment. NRC (2001) ingredients composition was modified to reflect the actual chemical values for CP, ADF, and NDF or, in the case of the grain mixes, the composition that would be required to achieve the observed values.

Table 5. Least square means of intake, milk production body weight and body condition score for cows fed varying amounts of energy and protein.

	Experimental Diets							
Energy	High	High	Low	Low		Effect ($P <$)		
Protein	High	Low	High	Low	SEM	Energy	MP	E*MP
Intake, kg/d								
DM	24.8	24.4	24.9	23.2	0.8	0.50	0.21	0.39
CP	4.70	3.72	4.68	3.64	0.13	0.001	0.001	0.84
NDF	9.53	10.27	12.23	12.00	0.18	0.001	0.25	0.02
ADF	6.03	6.53	7.80	7.73	0.10	0.001	0.12	0.04
NE_L	38.8	37.8	35.3	33.9	1.50	0.01	0.39	0.90
Milk Production								
Milk yield, kg/d	36.1	33.3	30.7	26.0	2.0	0.003	0.08	0.64
Lactose, kg/d	1.71	1.69	1.49	1.28	0.12	0.01	0.32	0.43
True protein, kg/d	1.13	1.03	0.91	0.87	0.05	0.001	0.16	0.57
Fat, kg/d	1.14	1.26	1.13	1.18	0.09	0.61	0.32	0.72
MSNF, kg/d[1]	3.13	3.04	2.72	2.41	0.20	0.01	0.29	0.58
Lactose, %	4.97	4.94	4.76	4.79	0.03	0.001	0.95	0.34
True protein, %	3.24	3.12	3.19	3.16	0.04	0.90	0.10	0.29
Fat, %	3.46	3.75	3.82	4.30	0.15	0.004	0.01	0.54
MSNF, %	9.12	8.98	8.85	8.88	0.04	0.001	0.24	0.07
MUN, mg/dl	17.2	12.2	21.0	12.0	0.81	0.01	0.001	0.005
SCC, 1000 cells/ml	32.79	23.57	132.8	161.35	56.03	0.02	0.85	0.71
BW, kg	642	635	650	640	4.0	0.13	0.04	0.80
BCS	3.09	3.11	2.90	2.97	0.08	0.24	0.35	0.54

[1]Milk solid non-fat

Table 6. Concentrations of free AA in plasma of cows fed varying amounts of energy and protein.

	Experimental Diets							
Energy	High	High	Low	Low		Effect ($P <$)		
Protein	High	Low	High	Low	SEM	Energy	MP	E*MP
	---------- μM ----------							
Total Essential AA	907	924	913	997	53	0.47	0.36	0.53
Arg	109	118	100	116	14	0.71	0.38	0.81
Ile	100	100	104	100	6	0.79	0.72	0.72
Leu	150	145	150	221	29	0.19	0.26	0.19
Lys	82	85	93	91	7	0.26	0.92	0.75
Met	10	17	13	14	2	0.92	0.23	0.27
Phe	96	56	67	62	18	0.56	0.24	0.37
Thr	85	93	86	92	7	0.99	0.35	0.91
Trp	40	44	47	46	3	0.13	0.68	0.44
Val	207	236	211	221	16	0.73	0.27	0.57
Total Nonessential AA	623	622	605	635	42	0.95	0.74	0.72
Ala	184	199	196	187	10	0.99	0.80	0.25
Asp	14	15	15	14	1	0.80	0.50	0.34
Glu	52	50	46	45	3	0.18	0.69	0.92
Gly	262	239	246	252	34	0.96	0.79	0.67
Pro	82	90	85	93	4	0.64	0.09	0.98
Ser	89	97	99	100	7	0.40	0.60	0.66
Tyr	50	52	46	50	3	0.49	0.46	0.87

Table 7. Relative amount of plasma metabolites with regard to DSS[1] concentration (100%) as analyzed by NMR method in cows fed the experimental diets.

	Experimental Diets							
Energy	High	High	Low	Low		Effect ($P <$)		
Protein	High	Low	High	Low	SEM	Energy	MP	E*MP
Metabolite								
Acetate	59.9	70.4	75.1	91.2	14.0	0.18	0.32	0.83
Beta-hydroxybutyrate	18.9	16.3	31.0	30.1	3.2	0.001	0.56	0.78
Lactate	75.3	89.4	70.5	91.7	21.1	0.95	0.40	0.86
Glucose	26.2	23.1	23.2	27.4	1.4	0.62	0.70	0.01

[1]The DSS peak area (0.45 mM) was set to 100% and the other metabolite peak areas were expressed as a percentage of the DSS peak area.

Table 8. Nitrogen utilization in cows fed varying amounts of energy and protein.

	Experimental Diets							
Energy	High	High	Low	Low		Effect ($P <$)		
Protein	High	Low	High	Low	SEM	Energy	MP	E*MP
N supplied in MP, g/d	470	372	468	364	14	0.71	0.001	0.84
Milk protein N, g/d	176	160	143	136	8	0.002	0.19	0.63
Predicted Urinary N[1], g/d	292	216	338	202	11	0.20	0.001	0.01
N efficiency[2] %	37.1	43.0	31.0	38.5	1.4	0.001	0.001	0.53

[1]Estimated urine N output = 0.026 × MUN (mg/dl) × BW (kg)(Kauffman and St-Pierre, 2001).
[2]N efficiency (%) = 100×Milk N (g/d) / N supplied in MP (g/d).

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Chapter 4

Interactions of Energy and Amino Acids in Determining the Regulation of Protein Synthesis in Mammary Glands of Lactating Dairy Cows

ABSTRACT

The objective of this study was to test local and molecular adaptations regulating protein synthesis in the mammary gland. We hypothesized that mammary glands can regulate AA metabolism via local and cellular adaptations to maintain milk protein yield when AA is limiting production. Six primiparous mid-lactation Holstein cows with ruminal cannulas were randomly assigned to casein and starch infused abomasally in a 2 x 2 factorial arrangement. The design was a replicated incomplete 4 x 4 Latin-square. All animals received the same basal diet (17.6% CP and 1.58 Mcal NE_L/kg DM) throughout the study. Cows were restricted to 70% of ad libitum intake and infused for 36 h with water, casein (0.86 kg/d), starch (2 kg/d), and the combination (2 kg/d starch + 0.86 kg/d casein) using peristaltic pumps. Milk yields and composition was assessed throughout the study. Arterial and venous blood samples were collected during the last 8 h of infusions. Mammary biopsy samples were collected at the end of each infusion and assessed for cell signaling phosphorylation state. Animals infused with casein had increased arterial concentrations of AA, increased mammary extraction of AA from plasma and no change or a trend to reduce mammary affinity for AA, however milk protein yield did not increase. Animals infused with starch experienced reduced arterial concentrations of AA but increased affinity and net uptake of some AA. Additionally, infusions of starch increased glucose concentrations, insulin, IGF-I, mammary plasma

flow, and activated the ribosomal protein S6. However, mTOR activity increased in response to casein only when starch was present. Thus, cell signaling activation responded to different nutritional stimuli. Milk and protein yield increased in animals infused with starch. Therefore, starch infusions increased metabolism of mammary glands and engaged local and intracellular regulatory mechanisms to stimulate milk protein synthesis. These metabolic and cellular adaptations should be included in current models to improve the accuracy of prediction of nutrient utilization in lactating cows.

(**Key words**: amino acids, cell signaling, mammary gland)

INTRODUCTION

The efficiency of N utilization is relatively poor in lactating cows, and reducing CP content from 19.1 to 15.2% (DM basis), which reduces N losses, is a possible tool to increase N efficiency although it compromises milk production (Rius et al unpublished data). Lactating cows fed diets with 18.3% CP had reduced N efficiency as compared to cows fed 15.4% CP diets (27.7 vs 35.5 %) with no loss in milk or milk protein yield (Cyriac et al., 2008).

The majority of N losses occur in the postabsorptive system (e.g. splanchnic tissues; Hanigan, 2005). Under normal feeding conditions, less than a third of posthepatic AA supplied to mammary glands (MG) are used for milk protein synthesis (Bequette et al., 1998). The majority of circulating AA are drawn from arterial supply and catabolized in the splanchnic tissues due to poor capture at the peripheral tissues (Hanigan, 2005; MacRae et al., 1997). Therefore, recycling of AA back to the splanchnic

tissues in arterial blood is a key determinant of catabolic rate. If utilization of AA for milk protein synthesis could be stimulated, the proportion of AA recycled to the splanchnic tissues would be reduced thereby improving animal efficiency (Hanigan, 2005).

Stimulation of milk protein synthesis at a given supply of AA is a potential strategy to reduce recycling of AA to splanchnic tissues. If AA supply remains constant while use for milk protein synthesis is increased, reductions in arterial concentrations of AA should be observed (Hanigan et al., 1998a). If true this should lead to greater use of AA for protein synthesis and reduced AA catabolism by the splanchnic tissues. Conversely, increased mammary AA removal in the absence of greater protein synthesis would likely increase AA degradation within the tissue (Bequette et al., 1996a; Bequette et al., 1996b). Greater intracellular concentrations of AA resulted in increased oxidation to a large extent (Bequette et al., 1996a; Raggio et al., 2006).

Reduced concentrations of circulating AA triggered greater proportional mammary capture which partially sustained protein synthesis (Bequette et al., 2000; Raggio et al., 2006). Limiting arterial concentrations of His from 73 to 8 µM elicited an increase of 43-fold in the transport activity for removal of His by the MG (Bequette et al., 2000).

Therefore limiting the supply of AA to MG should trigger local adaptations to increase affinity and removal of EAA thereby partially mitigating losses in rates of milk protein synthesis.

Increasing energy intake increased milk and milk protein yield and N efficiency (Broderick, 2003; Rius et al., unpublished). Increasing dietary energy content at a given

CP level may stimulate mammary protein synthesis and milk protein production (Hanigan et al., 1998a). Increasing duodenal energy supply in cows fed isonitrogenous diets stimulated insulin secretion and milk protein yield (Rulquin et al., 2004). Greater insulin concentration and glucose supply to the animal increased mammary capture of AA to sustain greater protein synthesis (Mackle et al., 2000; Rulquin et al., 2004). The latter could compensate for reductions in arterial AA concentrations if low-protein high-starch diets are fed and protein synthesis is stimulated in lactating animals.

Insulin stimulated initiation of protein synthesis at the molecular level in growing animals (Davis et al., 2001) and the mTOR signaling cascade was up-regulated during protein synthesis in muscle tissue (Escobar et al., 2006). Increased mTOR activity was associated with greater AA utilization for protein synthesis in growing animals, however this molecular mechanism has not been evaluated in MG of lactating cows (Escobar et al., 2006). Increases in protein synthesis are independently up regulated by either greater AA intake or greater insulin concentrations (Davis et al., 2001; Davis et al., 1991). Therefore feeding high energy diets to stimulate insulin release can maintain milk protein synthesis even when dietary CP is low.

Mammary glands should increase their affinity for EAA and capture of AA if arterial concentrations are reduced by feeding low protein diets (Bequette et al., 2000). High starch diets stimulate mammary secretory cells which can maintain milk protein synthesis and yield by increasing mTOR activity even when low protein diets are fed. Collectively the reduction in AA supply and the stimulus of protein synthesis could be the platform to maintain production and to increase N efficiency at the MG.

The objectives of this study were to test if MG can regulate blood flow, AA capture, and protein synthesis when AA supply was reduced and energy status increased. We hypothesized that AA and glucogenic substrate could support protein synthesis independently. The efficiency of protein synthesis could be associated with local adaptive mechanisms at the cellular level including greater activity of the protein translation factors as it is described in muscle tissue of growing animals.

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 (612 kg of BW) were fitted with a ruminal cannula 55 d before commencement of the study. Animals were housed in a free stall barn with constant access to water and feed. A common TMR was mixed 0800 daily and offered ad libitum for the first 12 d of each period and restricted to 70% of ad libitum intake on an individual basis for the last 36 h of each period. Diets were formulated to meet NRC (2001) recommendations with low protein content 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 two levels of abomasal starch and casein infusions were tested in a 2 x 2 factorial arrangement. Cows were assigned to one of two, 4 x 4, incomplete Latin squares. Starch and casein were purchased from National Starch and Chemistry Company (Bridgewater, NJ) and International Ingredient Corporation (St Louis, MO), respectively. The study consisted in 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 day 11, cows were moved to a metabolism unit with individual tie stalls and abomasal infusion lines were placed via the ruminal cannula. 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 treatment infusions, the TMR offered was subdivided into equal 3 h proportions which were delivered at 0300, 0600, 0900, 1200, 1500, 1800, 2100, and 2400 on d 13 and 0300, 0600, 0900, 1200, and 0300 h on d 14. At 0400 on d 13, abomasal infusions were started and consisted of: 1) control tap water; 2) starch (2 kg/d); 3) casein (0.86 kg/d); 4) and the combination of starch plus casein (2 kg/d of starch + 0.86 of casein kg/d). Infusates were delivered in a total of 95 kg of tap water/d. Suspensions were maintained by continuous stirring. Peristaltic pumps (Harvard apparatus Co., Inc. Millis Mass) were set at a ~66 ml/min to deliver equal volumes of the infusates into the abomasum.

Milking and Milk Samples

Animals were milked at 0800 and 2000 daily from d 1 to 13 on each period. On d 14 cows were milked out every hour from 0800 to 1600 and samples were collected (during

the last 8 h of the infusion on each period). An aliquot was collected and submitted for analyses of fat, true protein, lactose, total solids, and somatic cells by infrared analyses (DHIA, Blacksburg, VA; AOAC, 1990; Foss 4000 Combi North America, Eden Prairie, MN). The MUN analyses were conducted using the Berthelot procedure (ChemSpec 150 Analyzer; Bentley Instruments, Chaska, MN).

Mammary Biopsy

Mammary glands were prepared for biopsy and tissue was collected at the end of the infusion periods according to 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 aliquot of collected tissue (~0.1 g) was immediately snap frozen in liquid N and stored at -80 °C for later analysis. A second aliquot was processed for western blot analysis.

Catheters Surgery and Maintenance

Two weeks before the onset of the study a permanent indwelling catheter was introduced ~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 day 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 day 14 at the end of each infusion period. Arterial catheters were flushed weekly with a 0.9% NaCl solution containing 400 IU of heparin (Baxter; Deerfield, IL) prior to initiation of the study and between sapling periods. 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 syringes treated with sodium heparin. Plasma was prepared by centrifugation (2000 x *g* for 10 min), pooled by hour and cow, and stored at -20 °C until analysis. Blood was harvested while cows were standing to insure representative sampling from the abdominal vein. Samples were subsequently subjected to glucose, insulin, insulin-like growth factor I (IGF-I), and AA analysis. Glucose concentrations were determined using an enzymatic method (according to manufacture procedures; Beckman Coulter Inc.; Fullerton, CA). The intra-assay 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 inter-assay CV were 2.1 and 1.3% for the IGF-I, and 3.2 and 2.4% respectively for the insulin concentrations. Equal hourly plasma aliquots were pooled by period and cow, and analyzed for hematocrit, nonesterified fatty acids (NEFA), triglyceride (TG), and IGFI-binding proteins 3, 4, and 5 (IGFBP-3, 4, and 5). Nonesterified fatty acids (Wako Chemicals; Richmond, VA) and TG concentrations were determined using an enzymatic method (Beckman Coulter Inc.; Fullerton, CA). The intra-assay CV were less than 1% for each metabolite. Western ligand blotting was used to determine relative abundance of IGFBP 3, 4, and 5 as described by Daniels et al (2008). Two 12% gels were electrophoresed at the same time to minimize gel to gel

variation. Briefly, proteins were electrotransferred to a nitrocellulose membranes' incubated with ^{125}I-IGF-I (1 x 10^6 cpm/mL) overnight, washed in tris-buffered saline and visualized by autoradiography (Amersham; Piscataway, NJ) for 24 h at –80 °C. Relative abundance of binding proteins were determined by scanning densitometry (Un-Scan It v6.1; Orem, UT). Free AA was determined by isotope dilution using a gas chromatograph-mass spectrometer (Focus-PolarisQ GC-MS, Thermo Electron Corporation; Waltham, MA) according to the methods of El-Kadi et al., (2004) and Calder et al., (1999).

Cell Signaling Analysis

Tissue samples from mammary biopsies (0.1 g) was processed for Western blot analysis (Escobar et al., 2006). Briefly, the tissue was mixed 1:7 with homogenization buffer containing a mix of protease and phosphatase inhibitors (Sigma Chemical Company; St. Louis, MO), homogenized (Power Gen 1000; Fisher Scientific, Waltham, MA), and centrifuged at 10,000 x *g* for 10 min at 4°C. The supernatant was diluted in Laemmli sodium dodecyl sulfate 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 PVDF membrane (Bio-Rad, Hercules, CA), blocked with 5% of nonfat dry milk on tris-base tween buffer (10 mM Tris-base, 150 mM NaCl, and 1% tween 20), and probed with various antibodies made against the signaling proteins of interest as previously described (Escobar et al., 2006). Antibodies against the phosporylated forms of each of these initiation factors were procured from Cell Signaling Technology and included anti-phospho-AKT (Ser^{473}), anti-phospho-mTOR (Ser^{2448}),

anti-phospho-rpS6 ($Ser^{235/236}$ and $Ser^{240/244}$). Membranes were probed initially for the phosphorylated initiation factors then stripped, and re-probed to determine the total form of initiation factors. Total- AKT1, mTOR and ribosomal protein S6 (rpS6) were quantified using antibodies from Cell Signaling Technology (Danvers, MA). Blots were developed using an enhanced chemiluminescense kit (ECL Plus, Amersham; Piscataway, NJ), visualized using ECL film and a medical film processor (SRX 101A Konica Minolta; Wayne, NJ). The films were scanned and bands quantified by densitometry as described above.

Measurements and Calculations

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

$MPF = ([AA_m] \times 0.965) / (AV_{AA}) \times (1 - hematocrite)$

where $[AA_m]$ represented milk concentrations of AA in µmol/h and (AV_{AA}) represented arterio-venous concentration differences for each EAA in µmol/L. The Fick principle assumes that milk output = arterio-venous plasma concentration differences (AV) of AA x mammary plasma flow. Essential AA listed above were used assuming negligible degradation in the MG. Milk AA composition was calculated from Hanigan et. al. (2004).

Clearance or transport activity (*k*, L/h) of AA and metabolites by MG were calculated from the model of Hanigan et al. (1998b) as applied in Bequette et al (2000):

$k_i = ([A_i] \text{ x } MPF / [V_i]) - MPF$

where $[A_i]$ and $[V_i]$ represented arterial and venous plasma concentrations of the i^{th} AA (µmol/L) and MPF (L/h). The value of *k* represents the transport activity of AA or metabolites in the MG (L/h) or the ability of the gland to clear AA or metabolites from blood. The advantage of this form over the use of an extraction efficiency is that the transport activity accommodates changes in plasma flow. Net Uptake or extraction of AA or metabolites across the MG were calculated as:

Uptake = $(MPF) \text{ x } (AV_{AA})$

Uptake is expressed in µmol/h [(L/h) x (µmol/L)]

Statistical Analysis

The main effects of starch and casein and the interaction between starch x casein were tested (Table 3). Statistical computations were performed using the Proc Mixed procedure of SAS (2001; 3.01, SAS Institute Inc., Cary, NC). Cow was defined as random effect whereas periods and treatments 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, AA, glucose, plasma flow, and mammary calculations as repeated measures in time using the MIXED procedure of SAS to test for differences. The autoregressive covariance structure (AR1) was used based on goodness of fit as indicated by BIC and ACIC. Unless otherwise stated, significance was declared at $P < 0.05$.

RESULTS

The ingredient composition of the basal diet is presented in Table 1 and the chemical composition of the ingredients are presented in Table 2.

Milk production, Milk Components, and Intake

Intake of TMR DM did not change among cows however, total DMI (TMR + infusate) increased in animals that received starch and casein treatments ($P < 0.03$; Table 4). Milk production, milk solid percentages and yields are presented in Table 4. Infusion of starch resulted in increased milk yield ($P < 0.02$) however, there were no observed effects of casein infusion on milk yield. There was no interaction between casein and starch for milk yield. However, there was an interaction between casein and starch for milk protein yield with a positive response to casein in the presence of starch ($P < 0.02$). The infusion 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 x starch interaction, $P < 0.04$). Milk urea N was reduced by infusion of starch ($P < 0.01$; 11.1 mg/dl), and increased by the infusion of casein ($P < 0.01$; 13.9 mg/dl). Lactose yield was greater in cows treated with starch ($P < 0.01$). There was neither an effect of starch nor casein on milk fat yield, but there was an interaction with a positive response to casein in the presence of starch that increased fat yield (casein x starch interaction, $P < 0.03$; 42.6 vs. 50.0 g/h). There was a positive effect on SNF yield in cows infused with starch ($P < 0.03$; 84.5 g/h) and there was an interaction with a positive response to casein in the presence of starch (casein x starch interaction, $P < 0.03$; 104.0 g/h).

Insulin, IGF-I, and IGF Binding Proteins

Insulin, IGF-I, and IGFBP data are presented in Table 5. As expected, cows that received starch infusions had increased insulin and IGF-I concentrations ($P < 0.01$). There was a trend for an interaction between casein and starch with a negative response to casein in the presence of starch that reduced insulin concentration ($P < 0.08$). There was an interaction of casein and starch for IGF-BP4 and 5 abundance with a negative response to casein in the presence of starch ($P < 0.05$). There was a trend for the interaction of casein and starch with a negative response to casein in the presence of starch on IGF-BP3 abundance ($P < 0.06$).

Metabolite Kinetics and Plasma Flow

Arterial plasma concentrations of energy yielding metabolites, AV, transport activity, and net uptake are shown in Table 6. Plasma flow was increased in cows infused with starch ($P < 0.05$). Arterial plasma concentrations of glucose were increased in cows infused with starch ($P < 0.01$) and there was a casein x starch interaction ($P < 0.01$). There was a positive effect of casein in the absence of starch that increased glycemia however, there was a negative effect of casein in the presence of starch that reduced glycemia. Arterio-venous concentration differences and net uptake of glucose increased in response to casein infusion in the absence of starch but, AV and net uptake of glucose decreased in response to casein in the presence of starch (casein x starch interaction, $P < 0.02$). These changes in uptake were caused by changes in mammary transport activity which increased in response to casein in the absence of starch however, the transport activity of glucose was reduced in response to casein in the presence of starch (casein x starch interaction, $P < 0.01$). There was a trend for increased transport activity of glucose in

cows infused with starch ($P < 0.08$). Arterial NEFA concentrations were greater in cows infused with casein relative to cows infused with starch ($P < 0.05$, 0.20 vs. 0.15 mEq/L). Arterio-venous concentration differences of NEFA was reduced during starch infusions ($P < 0.01$; -0.02 mEq/L). Net uptake of NEFA were reduced in cows infused with starch ($P < 0.01$; -21.0 mEq/L) but, increased in cows infused with casein ($P < 0.01$; 5.0 mEq/L). Likewise, transport activity of NEFA dramatically declined in cows infused with starch ($P < 0.01$; -153.2 mEq/L). However, there was a trend for increased NEFA transport activity ($P < 0.07$; 13.2 mEq/L) in cows infused with casein alone.

Amino Acids Kinetics

Arterial concentrations of AA are presented in Table 7. Casein infusions increased plasma concentrations of all EAA except Phe ($P < 0.02$). Casein infusions increased plasma Cys, Pro, Ser, and Tyr ($P < 0.02$). Infusions of casein reduced arterial concentrations of Gln ($P < 0.01$). Infusions of starch reduced arterial concentrations of Ile, His, Leu, Lys, Phe, and Val ($P < 0.05$). Moreover, there was a trend for reduced Met concentrations in cows infused with starch ($P < 0.07$). Infusions of starch reduced arterial concentrations of the NEAA Asp, Gln, Glu, and Pro ($P < 0.01$) and there was a trend for a reduction in Ala ($P < 0.07$). However, starch infusions increased Gly concentrations ($P < 0.01$). Leu and Trp were affected by the casein and starch interaction with a positive response to casein in the absence of starch ($P < 0.01$). There was a casein x starch interaction for arterial concentrations of Ala, Cys, and Gln ($P < 0.01$) and the effect of casein in the presence of starch reduced Cys arterial concentrations. However, Ala and Gln concentrations increased in response to casein in the presence of starch.

Arterio-venous concentration differences of AA are presented inTable 8. Casein infusion increased AV for Arg, Ile, Leu, Lys, Thr, and Val ($P < 0.03$) and Cys, Gln, Gly, Pro, and Ser ($P < 0.02$). However, Ala AV were reduced in cows infused with casein ($P < 0.01$). Infusions of starch increased AV for Met, Thr, Ala and Glu ($P < 0.05$), and there was a trend for Phe ($P < 0.06$). There was a trend for a reduction in AV difference for Leu in cows infused with starch ($P < 0.08$). Arterio-venous concentration differences of Cys, His, Phe, and Thr 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 ($P < 0.03$).

Transport activity of AA are shown in Table 9. Clearance of Arg, Leu, Lys, Met, Phe, and Trp were increased during starch infusions ($P < 0.05$). Clearance of His and Thr were affected by the casein x starch interaction and the effect of casein in the absence of starch increased clearance however, casein reduced clearance of His and Thr in the presence of starch ($P < 0.05$). Similarly there was trend by the casein x starch interaction for clearance of Lys, Met, and Trp and the effect of casein in the absence of starch increased clearance however, casein reduced clearance of Lys, Met, and Trp in the presence of starch ($P < 0.07$). Conversely, there was a significant casein x starch interaction for the clearance of Val and the effect of casein in the absence of starch reduced clearance however, the effect of casein in the presence of starch increased clearance the of Val ($P < 0.05$). Clearance of Ala, Asp, and Tyr were increased by starch infusions ($P < 0.05$). However, the clearance of Pro was reduced during infusion of starch ($P < 0.03$). The clearance of Gln was increased by the infusion of casein however, the clearance of Tyr was reduced by the infusion of casein ($P < 0.05$).

The net uptake of AA in the mammary gland is shown in Table 10. The infusion of starch increased the net uptake of Arg, Phe, and Trp ($P < 0.05$). Net uptake of His, Met, and Thr were affected by casein x starch interaction with a greater uptake in response to casein in the absence of starch however, a net uptake reduction when casein was infused in the presence of starch ($P < 0.04$). The infusion of starch increased the net uptake of Ala, Asp, and Glu ($P < 0.05$). Casein infusions reduced net uptake of Ala ($P < 0.02$). However, casein infusions increased uptake of Gly ($P < 0.04$). Net uptake of Ala, Cys, and Tyr were affected by casein x starch interaction with a greater uptake in response to casein infusions in the absence of starch however, the uptake of Ala, Cys, and Tyr was reduced in response to casein in the presence of starch ($P < 0.02$)

Cell Signaling

The ratio of phosphorylated to total abundance of cell signals factors mTOR, AKT, and rpS6 in the mammary gland are presented in Figure 1. The phosphorylated ratio of mTOR increased in response to casein when starch was present and declined in response to casein in the absence of starch (casein x starch, interaction $P < 0.05$). There was a trend for the interaction of casein x starch on the phosphorylation ratio for AKT ($P < 0.14$) with an increase in response to casein in the presence of starch. Infusions of starch increased the phosphorylation ratio of rpS6 ($P < 0.03$). However, infusion of casein failed to increase phosphorylation ratio of mTOR.

DISCUSSION

The study described in this paper was designed to test the effect of infusions of starch, casein and the interaction of the main effects on systemic, tissue, and cellular adaptations to regulate protein synthesis in MG of lactating cows. The mTOR cell signaling cascade was evaluated in mammary tissue and the role of selected cell signaling mediators in regulating mammary protein synthesis was determined

The presence and activity of these selected cell signaling factors were identified in the mammary tissue and the activity of these factors demonstrated to change when treatments were imposed. In our study, starch infusion increased the activity of rpS6 factor. Infusion of casein in the presence of starch increased mTOR activity (Figure 1). This likely produced an increase in rpS6 which increased AA capture in milk protein (Table 3). In contrast, this cell signaling cascade did not respond to casein infusions in our study. Escobar et al (2006) reported that Leu activated the mTOR cascade and protein accretion in muscle of growing piglets. The supply of AA is a key signal that activates mTOR signaling (Bolster et al., 2004), however, in our experiment increasing mammary supply of AA did not stimulate the mTOR cascade.

The positive effect of starch on cell signaling activity could have been partially mediated by greater concentrations of insulin and IGF-I in agreement with O'Connor et al., (2003). They proposed that AA and insulin independently increased protein synthesis and activity of this cell signaling cascade in muscle tissue (O'Connor et al., 2003). However in our study, casein infusions did not stimulate the mTOR cascade. The identification and the evaluation of the activity of the mTOR cascade in bovine mammary tissue presents a potential regulatory mechanism to allow manipulation of protein synthesis through nutritional and hormonal interventions. However, the precise

molecular adaptation and change in the mTOR cascade remains uncertain in the present study.

Release of insulin and IFG-I increased blood flow, activity of endothelial nitric oxide synthase (eNOS), and nitric oxide production (NO; Iantorno et al., 2007; Zeng and Quon, 1996). Nitric oxide that was produced in mammary tissues increased MBF (Lacasse et al., 1996). mTOR, which is activated by insulin, and eNOS partially share a cell signaling mechanism that activates NO production (Iantorno et al., 2007). Although NO was not measured in the present study, phosphorylation and activity of eNOS by insulin and IFG-I secretion provides a plausible molecular mechanism to explain increased MPF even when glucose arterial concentration increased (Cant et al., 2002).

Net uptake and affinity of AA was increased in animals infused with starch in the present study. This is part of a local adaptive mechanism orchestrated to support protein synthesis in MG (Bequette et al., 2000). Furthermore, AA captured to sustain proteins synthesis should improve the efficiency of AA utilization by MG. In our study the average efficiency of extraction of EAA increased more than 50% in cows infused with starch however, intracellular AA were not used exclusively for milk protein synthesis. Perhaps catabolism of AA contributed to intracellular uses of AA in MG (Raggio et al., 2006). Reductions of EAA supply to MG did not impair milk protein yield in animals infused with starch. These finding agree with results reported by Bequette et al., (2000) where lactating goats with limiting supply of His dramatically reduced concentration of circulating His but increased transport activity by 43-fold in MG. Similarly, Raggio et al., (2006) found that cows infused with propionate in the rumen increased AA net uptake to support milk protein synthesis. In the present study net uptake and clearance of

glucose responded positively to casein in the absence of starch but the response to casein declined in the presence of starch. Thus transport activity was up-regulated to maintain glucose extraction for metabolic functions in MG.

As expected, measured increases in MUN during casein infusion suggested that AA derived from casein in the absence of adequate energy supply were oxidized rather than used for protein synthesis in MG (Raggio et al., 2006). The infusion of starch had a positive effect on milk yield and protein yield which agrees with results from Rulquin et al., (2004).

Collectively, animals that were infused with starch expressed increases in AA affinity, MPF, and net uptake to support nutrient partitioning to MG. The rise in insulin concentration probably activated the mTOR signaling cascade that channeled AA removed from blood supply through protein synthesis. It is plausible that these local and cellular adaptations regulated at least partially, milk protein synthesis during lactation. However, the long term effect on cell signaling activity and local adaptive mechanisms need to be addressed to fully understand responses to protein and energy supply and regulation of protein synthesis in MG.

CONCLUSIONS

The efficiency of protein synthesis could be associated with molecular adaptive mechanisms including greater activity of the protein translation factors mTOR and rpS6. The objectives of this study were to determine if MG can regulate blood flow, AA capture, and protein synthesis when AA supply was reduced and energy status increased.

Indeed, local adaptations occurred and protein yield was maintained. We hypothesized that AA and glucogenic substrate could support protein synthesis independently but only starch increased protein synthesis. These findings provide valuable information to better understand dynamic of AA and nutrients in lactating animals. Collectively, this information can be used to empower mechanistic models to predict milk protein production. The identification of key molecular signaling cascade involved in protein synthesis could be included in mathematical models to increase accuracy in predicting AA capture and protein synthesis regulation in lactating animals.

ACKNOWLEDGEMENTS

This material is based upon work supported by the Cooperative State Research, Education and Extension Service, U.S. Department of Agriculture. The authors acknowledge general departmental funding provided by the Virginia State Dairymen's Association, and the Virginia Tech Dairy Complex crew for help in conducting the study. The dedicated and expert support from C. Parsons and Dr. Akers in the analysis of insulin and IGF-I are gratefully acknowledged. The contributions of H. Weeks, J. Bross, A. Bell, A. Kopanko, T. Emmons, B. Slayers, M. Herbert, D. Flugel, L. Kelly , S. Hylton, A. Sabat, and J. McFadden are gratefully acknowledged. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture or the Virginia State Dairymen's Association.

Table 1. Ingredient composition of the basal diet.

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 Min[1]	0.01

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

Table 2. 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 3. Definition of the effects for the statistical model

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

[1]The effects of S, CN, period, cow, and H.
[2]df = degrees of freedom, ddfm = balanced denominator degrees of freedom for F-test.

Table 4. Effect of starch (S) and casein (CN) infusion in the abomasum of dairy cows on total dry matter intake, milk yield, and milk composition.

	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
DMI, kg/d[1]	13.0	14.7	17.9	16.9	0.8	0.01	0.4	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]Voluntary total mixed ration intake plus infusate

Table 5 Effect of starch (S) and casein (CN) infusion in the abomasum of dairy cows on arterial insulin, IGF-I, and IGF-I binding proteins.

	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
Hormones								
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								
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

Table 6. Plasma flow, arterial concentration, arterio-venous difference, net uptake, and transport activity of energy metabolites in dairy cows abomasally infused with starch (S) and casein (CN).

	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
Plasma Flow, L/h	190	366	522	439	100	0.05	0.6	0.2
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								
Glucose, mmol/h	63	301	447	104	86	0.5	0.5	0.01
NEFA, mEq/h	-3.6	13.6	-31.4	-11.0	6.0	0.01	0.01	0.7
Triglycerides, mg/h	182.9	203.5	243.4	197.1	83.2	0.06	0.8	0.6
Transport Activity[1]								
Glucose, L/h	0.9	4.0	6.8	2.5	1.3	0.08	0.6	0.01
NEFA, L/h	-30.8	4.6	-223.9	-82.5	44.0	0.01	0.07	0.2
Triglycerides, L/h	38.1	22.8	51.9	49.3	18.6	0.3	0.6	0.7

[1] $Ki = ([Ai] \times MPF/[Vi]) - MPF$ (Obtained from Hanigan et al., 1998b)
Where *Ki* is the transport activity of glucose, [*Ai*] and [*Vi*] represented arterial and venous plasma concentrations of glucose (mmol/L) and MBF is the mammary plasma flow (L/h).

Table 7. Arterial concentration of amino acids in lactating dairy cows abomasally infused with starch (S) and casein (CN).

	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
Essential AA	---------- *μM* ----------							
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
EAA	769	1028	518	843	80	0.01	0.01	0.5
BCAA	386	681	293	530	30	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
NEAA	564	654	564	704	23	0.01	0.1	0.1

Table 8. Arterio-venous difference of amino acids in lactating dairy cows abomasally infused with starch (S) and casein (CN).

	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
Essential AA	----------	*μM*	----------					
Arg	8	29	17	24	8	0.7	0.03	0.3
Ile	19	30	23	29	4	0.6	0.01	0.5
His	1	6	5	3	2	0.3	0.1	0.03
Leu	35	55	33	38	7	0.08	0.03	0.1
Lys	22	38	25	37	4	0.7	0.01	0.4
Met	4	8	9	10	2	0.03	0.2	0.5
Phe	1	27	37	19	11	0.06	0.8	0.01
Thr	4	22	18	18	3	0.05	0.01	0.01
Trp	10	10	12	12	2	0.2	0.9	0.7
Val	26	37	18	34	7	0.3	0.03	0.6
EAA	121	242	174	169	35	0.6	0.02	0.01
BCAA	80	128	83	111	15	0.6	0.01	0.4
Nonessential AA								
Ala	8	1	20	4	5	0.03	0.01	0.4
Asp	2	3	3	2	1	0.8	0.7	0.3
Cys	1	2	6	1	1	0.01	0.03	0.01
Gln	-6	4	-4	1	3	0.7	0.01	0.2
Glu	16	18	21	24	2	0.04	0.3	0.8
Gly	-27	-13	-23	2	6	0.1	0.01	0.3
Pro	1	17	6	11	5	0.8	0.01	0.1
Ser	10	19	9	14	4	0.2	0.02	0.5
Tyr	8	12	10	10	2	0.8	0.2	0.2
NEAA	1	37	36	53	15	0.06	0.05	0.4

Table 9. Effect of starch (S) and casein (CN) abomsally infused on lactating dairy cows on transport activity[1] of amino acids in the mammary gland.

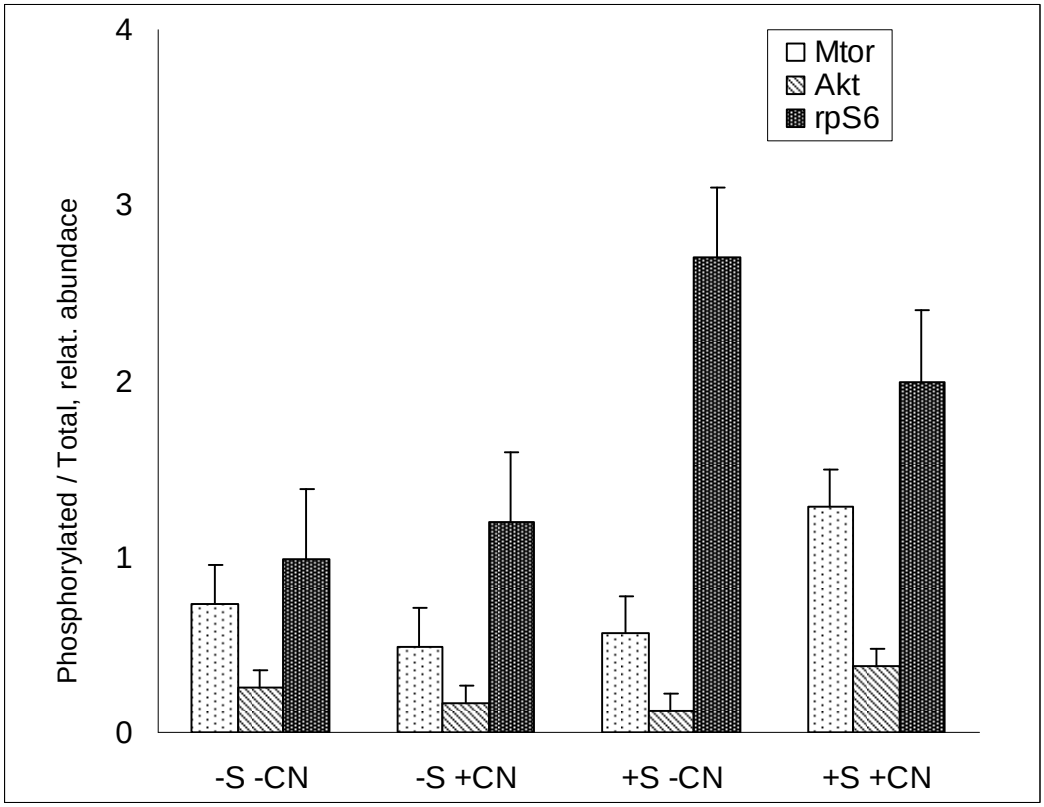
	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
Essential AA	---------- *L/h* ----------							
Arg	24	80	266	135	63	0.03	0.5	0.1
Ile	185	119	226	176	49	0.2	0.2	0.8
His	6.1	123	136	44	54	0.5	0.7	0.02
Leu	62	80	232	246	46	0.01	0.7	0.9
Lys	138	209	423	255	71	0.01	0.4	0.06
Met	77	154	304	146	58	0.04	0.4	0.07
Phe	65	55	509	277	137	0.04	0.4	0.4
Thr	89	97	171	63	23	0.3	0.08	0.05
Trp	-43	-0.8	28	13	13	0.01	0.3	0.06
Val	75	32	59	98	19	0.2	0.9	0.05
Nonessential AA								
Ala	2.8	6.0	60	15	15	0.02	0.1	0.1
Asp	107	150	389	249	89	0.05	0.6	0.3
Cys	19	19	34	15	17	0.7	0.5	0.5
Gln	-43	29	-46	-9.8	40	0.3	0.05	0.4
Glu	306	193	629	390	313	0.7	0.5	0.5
Gly	-49	-43	-43	-42	37	0.7	0.8	0.8
Pro	68	70	53	6.0	14	0.03	0.1	0.1
Ser	57	57	61	106	37	0.3	0.4	0.4
Tyr	100	54	285	106	42	0.02	0.03	0.1

[1]$Ki = ([Ai] \times MBF/[Vi]) - MBF$ (Obtained from Hanigan et al., 1998b)
Where *Ki* is the transport activity, [*Ai*] and [*Vi*] represented arterial and venous plasma concentrations of the i^{th} AA (µmol/L) and MBF is the mammary plasma flow (L/h).

Table 10. Effect of starch (S) and casein (CN) abomasally infused in lactating dairy cows on amino acids net uptake in the mammary gland.

	Experimental Treatments							
Starch	-	-	+	+		Effect (P<)		
Casein	-	+	-	+	SEM	S	CN	CN*S
Essential AA	---------- *mmol/h* ----------							
Arg	-0.3	3.9	13	11	3.1	0.01	0.7	0.2
Ile	8.5	10	11	14	3.0	0.3	0.4	0.8
His	0.3	4.6	2.0	0.4	1.3	0.2	0.2	0.02
Leu	16	18	18	21	6.3	0.6	0.6	0.9
Lys	9.7	10	12	11	3.5	0.6	0.9	0.8
Met	0.7	2.5	3.2	0.6	0.8	0.6	0.6	0.03
Phe	1.7	1.7	18	6.4	4.4	0.02	0.4	0.1
Thr	5.3	12	9.4	5.1	2.2	0.5	0.6	0.04
Trp	-3.7	0.3	2.0	1.2	1.3	0.05	0.3	0.1
Val	12	8.9	12	14	3.6	0.4	0.8	0.5
Nonessential AA								
Ala	-1.5	0.1	12	1.0	2.7	0.01	0.05	0.02
Asp	0.1	-0.1	1.3	0.6	0.3	0.01	0.2	0.4
Cys	-0.1	0.6	0.8	0.2	0.2	0.2	0.8	0.02
Gln	0.1	1.5	-2.2	0.2	1.3	0.1	0.09	0.6
Glu	5.5	5.5	11	12	2.6	0.02	0.7	0.7
Gly	-5.5	-1.9	-9.8	2.4	4.2	0.9	0.04	0.2
Pro	3.1	6.4	2.6	3.0	1.4	0.2	0.2	0.3
Ser	5.0	3.1	3.2	6.3	2.1	0.7	0.7	0.2
Tyr	3.0	3.9	6.1	1.2	0.9	0.8	0.1	0.02

Figure 1. Protein initiation factors activity in the mammary gland of lactating dairy cows abomasally infused with starch (S) or casein (CN).



Interaction of CN x S on mTOR (P<0.05)
Effect of starch on rpS6 (P<0.03)
Interaction of CN x S on AKT (P<0.14)

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Chapter 5

Overall conclusions

The hypothesis of the first study was that milk protein yield could be maintained and N efficiency improved when reduced dietary MP was combined with high dietary. The first objective of the production study was to assess the efficiency of N utilization under predicted conditions of MP deficiency when dietary energy density was increased. A second objective was to test the NRC predictions of energy and protein requirements for the lactating dairy cow. Results of this investigation indicated that there were no interactions between energy and CP for milk and protein yield. Therefore, these results allow us to confirm the hypothesis that milk protein yield was increased by increasing dietary energy content and promoting duodenal digestion of starch. Additionally, high and low energy in the diet resulted in variable efficiencies of MP utilization. Milk urea N concentrations were low when feeding high energy diets which is an indication of low AA catabolism. The predicted post absorptive efficiency of N utilization was maximal when feeding the combination of high energy and low CP in the diet. Low concentrations of MUN associated with high predicted N efficiency suggests that AA are captured for proteins synthesis in mammary tissues. Therefore, the single limiting nutrient concept used in the NRC should be replaced with a multi-nutrient representation that accommodates at least energy and CP supply to accurate predict milk protein output and N efficiency.

The second study was proposed to investigate general, local, and cellular adaptations during nutritional interventions on the regulation of protein synthesis. The responses on AA metabolism and protein synthesis to AA supply at different levels of

postabsorptive energy supply were tested. Of particular interest was exploration of the molecular mechanisms that regulate protein synthesis yet have not been described in MG. The objectives of this study were to test if MG can regulate blood flow, AA capture, and protein synthesis when AA supply were reduced and energy status increased. We hypothesized that AA and glucogenic substrate could support protein synthesis independently. Results of this investigation support our hypothesis that molecular mechanisms similar to muscle tissue are present and activated during protein synthesis in MG. Infusion of starch increased the activity of mTOR signal factors and protein synthesis in MG. Other results include general adaptations that were driven by greater hormonal concentrations during starch infusions. Insulin and IGF-I could have contributed to the up-regulation of cell signaling factors. However in this study, casein infusions did not elicit a molecular response associated with initiation factors of protein synthesis. Increased insulin likely caused the observed increase MPF which in turn increased supply of AA to MG. Starch infusions increased AA affinity and net uptake when AA supply were limiting. The reduced supply of AA in cows infused with starch did not limit activation of protein synthesis. The supply of AA is a key signal that activates the mTOR signaling however, in our experiment increasing mammary supply of AA did not stimulate the mTOR cascade. Therefore the identification of the mTOR cascade present a potential mechanism that can be manipulated through nutritional and hormonal intervention. However, the precise molecular adaptations and changes in the mTOR cascade remained uncertain in the present study. Increasing mTOR signaling activity could be use to maintain protein synthesis independently form AA supply and increase efficiency of intracellular AA utilization. These results provide valuable information to better understand dynamic of AA and nutrients in lactating cows.

Collectively, this information can be used to empower mechanistic models to accurately predict milk protein production. The identification of mTOR signaling cascade could be included in mathematical models, however, more research is needed to accurately capture the regulatory role in protein synthesis.