Effects of postruminal amino acid supplementation on protein deposition and the mammalian target of rapamycin signaling pathway in growing steers

by

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Abstract

Two experiments were conducted to determine effects of postruminal amino acid (AA) supplementation on protein deposition and signaling of the mammalian target of rapamycin (mTOR) pathway. For both experiments, 7 ruminally cannulated Holstein steers (172.7 ± 3.7 and 201.7 ± 3.8 kg initial BW in Exp. 1 and 2, respectively) were utilized in 6 × 6 Latin square designs with 7 d periods. A basal AA solution containing all essential AA, with the exception of lysine, were provided to all steers in each study in order to meet growth requirements, while making lysine the only limiting AA. Steers were fed 2.8 kg/d of a pelleted soyhull diet designed to be low in ruminally undegradable protein. Glucose was infused abomasally and volatile fatty acids were infused ruminally to prevent energy from being limiting. Steers were housed in metabolism crates to obtain total collection of both urine and feces. Blood and muscle biopsies of the *longissimus lumborum* were collected on the last day of each period. In experiment 1, treatments consisted of 2 levels of lysine (0 or 6 g/d) and 3 levels of leucine (0, 15, or 30 g/d) infused abomasally. Nitrogen retention increased with supplemental lysine. Leucine linearly decreased plasma concentrations of total AA. Plasma urea N (PUN) decreased with supplemental lysine. Total, phosphorylated, and the percent phosphorylated Akt were unaffected by treatments. The percentage of 4E-BP1 phosphorylated decreased linearly when leucine was supplemented. A tendency for a lysine × quadratic leucine effect was observed for the ratio of phosphorylated $RPS6^{240/244}$ in which the intermediate level of leucine led to a decrease in the percent of $RPS6^{240/244}$ phosphorylated when no lysine was supplied but increased when 6 g lysine/d was supplied. No differences were observed in the abundance of total, phosphorylated, or percent phosphorylated mTOR or in total abundance of E3 ubiquitin ligase proteins, MuRF1 or MAFbx. Experiment 2 was conducted similarly as experiment 1. Treatments consisted of 2

levels of lysine (0 or 6 g/d) and 3 mixtures of supplemental essential AA [none (control), 103 g/d essential AA (EAA), or EAA plus 30 g/d leucine (EL)] abomasally infused. Supplementation with essential AA, with or without leucine, increased the percentage of RPS6 phosphorylated, with a greater increase when leucine was included as part of the supplement. A lysine × (control vs. EAA+EL) interaction was observed for N retention in which the EAA and EL treatments did not improve N retention when no lysine was supplemented, but they increased it when 6 g lysine/d was provided. PUN increased above control when EAA or EL was provided, but PUN decreased when lysine was supplied. Supplementation of EAA or EL increased plasma total AA concentrations, but EL led to lower total plasma AA than EAA; however, concentrations were greater for EL than for control. In summary, leucine supplementation alone did not yield effects on whole-body protein deposition or on regulatory factors known to affect muscle protein synthesis, whereas a mixture of excess essential AA improved both lysine utilization and phosphorylation of $RPS6^{240/244}$. These studies demonstrate the effects of essential AA, both limiting and nonlimiting, on protein deposition in growing cattle.

Key words: leucine, amino acids, steer, protein deposition, mTOR

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Dedication

To my parents,

Jeffery and Karen Pearl.

Thank you for your unconditional love

and support

in everything I do.

Chapter 1 - Review of the Literature

INTRODUCTION

The world population continues to increase, putting greater importance on efficient production of food. Animal protein is the main source of protein for many countries in the world, such as the United States (Abbott, 1966). The need for healthy, fast growing animals continues to increase in such places, and in other countries as they continue to develop (World Health Organization). At the same time, it is important to decrease the environmental footprint of food production, which has potential to decrease by increasing efficiency of amino acid (AA) utilization. It should be possible to improve the economics of growing such sources of protein, enabling developing countries to have greater access to these proteins. One possible way to improve production is to feed animals to grow based on their needs at specific developmental stages.

The neonatal period is classified as a high growth period, in which the neonate must adapt to metabolic changes to induce growth. Protein turnover is greatest during the neonatal period and shortly after, particularly in skeletal muscle (Davis et al., 1989), as lean muscle growth drastically decreases with age (Davis et al., 1996). These metabolic changes include increases in organ size, nutrient allocation, hormonal regulation, milk production, and skeletal muscle growth (Escobar et al., 2006; Suryawan and Davis, 2014). One of the major contributors allowing for these adaptations is the mammalian target of rapamycin (mTOR) complex.

mTOR is regulated by the nutrient status of the cell, and particularly by AA availability. Feeding individual AA can allow for a more balanced AA profile for what is required of each animal depending on the developmental stages, which can then decrease the economic cost of feeding high-protein diets. Feeding individual AA allows for alterations in amounts fed, allowing

producers to better target growth depending on developmental stage and metabolic need. Understanding interactions among AA in vivo is critical in order to find the balance for proper growth and development. The objective of this review is to discuss the interactions among essential AA (EAA) and how they work together to affect growth and development on a molecular and whole-body level.

PROTEIN TURNOVER

Protein turnover is protein degradation and protein synthesis that occur as on-going processes in the body. Protein turnover is necessary in order to maintain growth and health of the organism. When protein synthesis is greater than degradation, growth occurs. Body protein loss will take place if protein degradation is greater. Multiple mechanisms affect protein turnover, including hormones, nutrient supplies, and muscle synthesis/degradation pathways. Each of these will be discussed further below.

Nitrogen retention

Over time, there has been increasing concern with the amount of nitrogen entering the soil and air in the environment. A recent article released by the Food and Agriculture Organization reported approximately 29% of greenhouse gases emitted by livestock are in the form of nitrous oxide (FAO, 2018). In addition, they added that nitrous oxide makes up around 29% of all emissions produced by livestock (FAO, 2018). There is an obvious need to decrease nitrogen excretion to benefit both animal efficiency and the environment. One way of achieving this is to increase AA utilization, thereby increasing N-retention. N-retention is defined as the amount of nitrogen deposited in the body, in which more nitrogen is consumed than is excreted by the body. There has been increasing evidence that growing animals may benefit from AA

supplementation to a greater extent than their older counterparts, which results in greater protein deposition during this stage of life.

Additional evidence suggests that among AA, some, primarily leucine, are more effective in stimulating muscle synthesis and N-retention than others (Wilson et al., 2010). A study was conducted in which critically ill human patients were treated with increased doses of BCAA. Those who received moderate (0.5 g/kg/d) to high (0.7 g/kg/d) BCAA maintained greater protein deposition throughout their healing process in comparison to those receiving no additional or low (0.3 g/kg/d) BCAA. The high BCAA treatment group maintained the increased N-retention levels for a greater duration of the high stress period. Additionally, researchers did not observe any differences in plasma 3-methylhistidine concentrations among treatments, indicating that the differences in N-retention were likely due to a greater protein synthesis rather than a decrease in proteolysis (Cerra et al., 1983). Leucine and other BCAA provided to methionine-deficient pigs had a protein sparing effect to alter protein metabolism, ultimately enabling methionine to be directed towards protein synthesis, which increased protein deposition by 8% (Langer and Fuller, 2000).

N-retention can also be influenced by energy sources such as volatile fatty acid (VFA) concentrations in the rumen. Increased proportions of propionate, as would be produced in grain fed ruminants, can improve AA utilization within the rumen to some extent. Propionate is a glucogenic substance, therefore, additional propionate can be used by the liver for gluconeogenesis, thereby sparing AA for potential protein deposition. Sadiq et al. (2008) observed a decrease in protein degradation, which led to an overall increase in N-retention, when calves received jugular infusions of glucose (Sadiq et al., 2008).

MAMMALIAN (MECHANISTIC) TARGET OF RAPAMYCIN

Mammalian target of rapamycin (mTOR) is a major regulator of protein synthesis and plays an important role in skeletal muscle protein synthesis and degradation in mammals. This pathway was originally found in yeast cells, in which it is referred to as target of rapamycin (TOR). mTOR is a phosphoinositide 3-kinase (PI3K)-related protein. Bodine et al. (2001) observed that treating adult rats and mice with rapamycin caused up to a 95% decrease in hypertrophy by blocking mTOR phosphorylation (Bodine et al., 2001). The mTOR pathway can be activated by nutritional means such as AA supplementation or insulin stimulation, by resistance exercise, by a multitude of other factors herein discussed, or by combinations of these factors.

mTOR complex 1 and 2

mTOR is made up of two complexes, mTOR complex (mTORC) 1 and mTORC 2 (Shaw, 2013). mTORC1 is rapamycin sensitive, meaning rapamycin inhibits the phosphorylation of upstream and downstream proteins in the mTOR pathway, causing muscle hypertrophy to cease almost completely; however, mTORC2 is functional in the presence of rapamycin, making it rapamycin insensitive (Bodine et al., 2001; Laplante and Sabatini, 2009). Though mTORC2 still functions in the presence of rapamycin, long term exposure has the ability to dissociate mTORC2, causing suppression of its action in some cell types (Laplante and Sabatini, 2012).

mTORC1 is responsible for activating translation as well as responding to the nutritional status of the cell in order to activate or deactivate the mTOR pathway (Feldman et al., 2009). It is known that mTORC2 plays a role in Akt phosphorylation at both Ser473 and Thr308; however, the extent of its role has not yet been identified and the primary role of mTORC2 has yet to be discovered, as little is known about this complex (Feldman et al., 2009; Laplante and Sabatini,

2009). For this reason, the literature reviewed here will primarily pertain to mTORC1. When rapamycin is absent, a cascade of events must occur in order for AA to initiate mTORC1.

Rag, Rheb, and GATOR complexes

The first step involved in initiating the mTOR pathway requires Ras-related GTP binding (Rag) A and B proteins to bind to GTP and Rag C/D to bind to GDP (Laplante and Sabatini, 2012). The bound Rag/GTP complex then migrates towards the cell surface, where they stimulate the initiation of mTORC1 signaling through regulatory factors increasing phosphorylation (Shaw, 2013). An inhibitor of Ras homolog enriched in brain (Rheb), GAP activity toward Rags (GATOR) complex, is found upstream of mTOR (Shaw, 2013) and is a heterodimer comprised of GATOR1 and 2. GATOR1 is responsible for enhancing mTOR's sensitivity to the cellular environment and acts primarily to shut down mTOR when nutrients are in abundance (Shen et al., 2018). Less is known about GATOR2, thus, at this point in time, the main role of GATOR2 is believed to be to counteract actions of GATOR1. GATOR1 is inhibited by the activation of GATOR2 and is associated with Rag A/B. When GATOR1 is inhibited, it is no longer sensitive to AA concentration, and mTORC1 is unable to be inactivated (Shaw, 2013). The inhibition of GATOR2 leads to the inhibition of mTORC1 (Shaw, 2013). The two GATOR complexes regulate one another, enabling only one complex to be active at a time. Finally, the cascade ends in AA activation of mTOR, which acts as a catalyst to protein synthesis (Hall, 2008).

Specific AA are sometimes able to upregulate mTORC1 better than others. One reason for this is location of the AA and their transporters. Many AA transporters have been found on the lysosomal membrane, however, those not usually found in the lysosomal membrane can be transported there with the help of the Ragulators, though this may take more time and energy

(Wang et al., 2015). For example, leucine uses the vacuolar H^+-ATPase, found on the lysosome, when Rag A/B activity increases due to the Ragulator guanine nucleotide exchange factor (Kimball, 2014). Therefore, when the guanine nucleotide exchange factor increases in activity, leucine transport in and out of the cell may increase. AA transport can then activate downstream events, through a series of phosphorylation and dephosphorylation reactions, that cascade to complete mRNA translation.

Downstream translation initiation factors

The 43S preinitiation complex is formed by eukaryotic initiation factor (eIF) 2B, a translation initiation factor that directs the initiator methionyl-tRNAi to bind onto the 40S ribosomal subunit by converting GDP to GTP (O'Connor et al., 2003). The 48S preinitiation complex is formed by the binding of mRNA to the 43S preinitiation complex with the help of eIF4F (Jackson et al., 2010). Phosphorylation of eIF4B by $p70^{S6K1}$ causes the 5'–untranslated region of mRNA on the 48S preinitiation complex to be scanned, increasing the efficiency of mRNA translation to synthesize more protein (Jackson et al., 2010). These signals continue downstream to phosphorylate 4E-binding protein 1 (4E-BP1), ultimately to render it inactivate. 4E-BP1 is a repressor protein which competes to bind to eukaryotic initiation factor 4E (eIF4E).

Phosphorylation of 4E-BP1 induced by Akt inhibits the ability of 4E-BP1 to bind to eIF4E, freeing the competitive phosphorylation site on eIF4E to bind to eIF4G (Gingras et al., 1998). The eIF4E/ eIF4G complex is then activated, allowing the mTOR cascade to continue downstream (Columbus et al., 2015). Ribosomal protein S6 (RPS6) is activated by the phosphorylation of ribosomal protein S6 kinase 1 (S6K1), a downstream regulator of mTOR responsible for translating mRNA. This event can occur independently or simultaneously to the phosphorylation of 4E-BP1. Phosphorylation of 4E-BP1 activates the protein by releasing it from

the bound 4E-BP1/eIF4E inactive complex. eIF4E is then free to bind to eIF4G, an active complex involved in translation initiation (Columbus et al., 2015).

Upstream regulators

In addition to downstream regulators, the mTOR pathway has additional upstream regulators, for example, Akt, also known as protein kinase B. Akt phosphorylation is specific to Ser^{473} and Thr^{308}, and the phosphorylation of either of these AA at their specific locations activates protein synthesis (Alessi et al., 1996). Ser^{473} and Thr^{308} have additive effects, meaning greater protein synthesis is activated when both sites are phosphorylated (Alessi et al., 1996). Bodine et. al. (2001) observed an increase in phosphorylated Akt when rats were in a state of hypertrophy, as well as an increase in total Akt in the plantaris muscle (Bodine et al., 2001). Akt increases muscle synthesis by activating $p70^{S6K}$, which is an upstream regulator of mTOR. In addition, Bodine et al. (2001) observed that mice injected with a constitutively active form of Akt, had larger muscle cells and atrophy was hindered, even during times of muscle disuse (Bodine et al., 2001).

Additionally, Akt has the ability to phosphorylate proteins such as tuberous sclerosis (TSC) 1 and 2, which keep the Rheb protein bound to GTP rather than GDP, further enabling the mTOR cascade (Parmar and Tamanoi, 2010; Winter et al., 2011). Akt- and PI3K-initiated activation of mTOR, which then led to the release of cellular insulin-like growth factor I (IGF-I) and insulin (Kimball, 2014). Additionally, IGF-I can potentially activate mTORC1 through the phosphorylation of TSC2 at Ser^{664} in the extracellular signal regulated kinase (ERK) 1/2 pathway (Kimball, 2014). Winter et al. (2011) postulated that different phosphorylation sites found on TSC2 are specific to either Akt or ERK phosphorylation; however, Akt and ERK have additive effects to stimulate the mTOR pathway (Winter et al., 2011).

Muscle atrophy mechanisms

Specific downstream proteins such as 4E-BP1 and RPS6 are involved in protein synthesis in the mTOR pathway; however, other proteins are classified as atrogenes, or genes that stimulate muscle atrophy. MAFbx and MuRF1 are muscle-specific E3 ubiquitin ligases and are upregulated in times of nutrient deficiencies, disease, or muscle disuse (Yuan et al., 2015). Examples of atrogenes include forkhead box protein O (FoxO), muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1), which inhibit mTOR signaling. In cases when mTORC2 is low in activity, a decrease in both Akt and S6K1 phosphorylation occurs in response to a decrease in growth factors, thus decreasing the activity of these proteins in addition to the decrease in phosphorylation of FoxO (Sarbassov et al., 2006; Laplante and Sabatini, 2009; Laplante and Sabatini, 2012).

When FoxO is phosphorylated by Akt, it is held in the cytoplasm of the cell; however, once dephosphorylated, the protein migrates to the nucleus to reach target genes (Brunet et al., 1999; Sandri et al., 2004; Greer and Brunet, 2008). The FoxO protein is responsible for apoptosis, which increases muscle atrophy, and also decreases muscle fiber size (Sandri et al., 2004; Laplante and Sabatini, 2009). Sandri et al. (2004) observed an increase in activated FoxO located at the nucleus of starved cells, and an increase in phosphorylated FoxO found in the cytoplasm once Akt was activated (Sandri et al., 2004). FoxO dephosphorylation can be induced by an increase in oxidative stress and acts to increase apoptosis and cell cycle arrest to mitigate the effects of reactive oxygen species produced in response to stress (Greer and Brunet, 2008).

Though E3 ubiquitin ligases are found in muscles throughout the body, they are found in greatest abundance in striated muscle (Bodine et al., 2001; Gomes et al., 2001). Gomes et al. (2001) noted that MuRF1 concentrations increased drastically in skeletal muscle after cells were

exposed to starvation (Gomes et al., 2001). Leucine has been observed to decrease muscle atrophy via the PI3K pathway; however, these results indicate that additional AA may be needed to prevent muscle atrophy rather than just leucine. For example, MuRF-1 mRNA tended to increase in rats fed a protein deficient diet supplemented with excess leucine (Sugawara et al., 2009), suggesting that other AA may be more beneficial to decrease MuRF-1 concentrations.

Amino acid activation of mTOR

Protein synthesis actions of leucine

Growth factors, primarily AA, play a major role in muscle synthesis; however, this effect is greatest in young animals and the response decreases drastically with age (Davis et al., 1996; Davis et al., 1998). When cells sense the presence of AA, the Rag/GTP complex migrates to the lysosomal surface, which then sends a signal to attract and activate the mTOR complex (Shaw, 2013). Columbus et al. (2015) studied neonatal pigs fed an AA restricting diet supplemented with leucine. mTOR phosphorylation and muscle growth in the *longissimus lumborum* were increased by leucine supplementation to levels above those observed for piglets fed restricted protein and not supplemented with leucine, but responses to leucine supplementation remained less than those in piglets fed high protein diets (Columbus et al., 2015).

Yin et al. (2010) found that the liver of weanling pigs was more sensitive to leucine supplementation than skeletal muscle. Increased phosphorylation of S6K1 and 4E-BP1 occurred in the liver when pigs were supplemented with an intermediate level (0.27%) leucine. Skeletal muscle required greater leucine supplementation to reach similar results. This sensitivity continued when observing phosphorylated mTOR (Yin et al., 2010). No differences in mTOR phosphorylation were observed among treatments in skeletal muscle, and only the high level of leucine supplementation was able to stimulate phosphorylation levels of mTOR in the liver (Yin

et al., 2010). Torrazza et al. (2010) observed increases in translational efficiency and muscle synthesis of the *longissimus lumborum* when neonatal pigs were provided leucine in low protein diets (Torrazza et al., 2010). Contradictory to Yin et al. (2010), no differences in liver muscle synthesis or translational efficiency were observed in the study conducted by Torrazza et al. (2010). It is likely that the sensitivity to leucine changes in the liver with age. Yin et al. (2010) utilized weaning aged pigs, whereas Torrazza et al. (2010) conducted their studies on neonatal pigs. These data support the idea that the *longissimus lumborum* is more susceptible to the effects of leucine early in life and decreases with age. It is possible that the liver is more sensitive to leucine later in life when growth is less vital, having more of an effect at age of weaning as opposed to in neonatal life. In contrast, Davis et al. (2002) found that when AA were infused to reach a fed state in 7- and 26-d old pigs, liver protein synthesis decreased with development (Davis et al., 2002).

Wilson et al. (2010) conducted a study using neonatal pigs to determine if leucine had an effect on protein synthesis when EAA were kept at basal levels. Results indicated that leucine supplementation increased translational efficiency of mRNA, but only when EAA remained at basal level (Wilson et al., 2010). Manjarin et al. (2016) detected no differences in muscle synthesis or BW gain in pigs when leucine was supplemented to a protein-limiting diet (Manjarín et al., 2016). This effect may be due in part to a deficiency or imbalance of certain AA, including BCAA when leucine is supplemented. Translational efficiency decreased in the leucine-supplemented, restricted-protein group when compared to pigs fed diets meeting nutritional requirements, but not to the extent of those fed the restricted diet (Manjarín et al., 2016). This could be due to the fact that the added leucine supplementation increased S6K1 and 4E-BP1

phosphorylation, thereby increasing the eIF4E/ eIF4G complex when compared to the nutritionally restricted diet (Manjarín et al., 2016).

Escobar et al. (2006) compared BCAA to observe which was more effective in stimulating mTOR. Valine supplementation led to a decrease in mTOR activation, while isoleucine was similar to saline infusions. Leucine, however, increased protein synthesis primarily in fast twitch muscles and some slow twitch oxidative muscles of 5-d old neonatal pigs (Escobar et al., 2006). Akt in neonatal pigs was unaffected by leucine treatment, possibly indicating that leucine has a greater effect on mTORC1 than on mTORC2 (Wilson et al., 2010). Leucine supplementation in particular is able to increase the phosphorylation of S6K1 and 4E-BP1 in neonatal pigs, regardless of the concentration status of other EAA (Wilson et al., 2010).

Lysine functions in mTOR

Although leucine is known to be the most effective AA to activate mTORC1, other AA, such as lysine, have the ability to stimulate initiation of protein synthesis. A study conducted by Sato et al. (2015) observed that mTOR, Akt, and 4E-BP1 activation increased by 30% when rats fed a low protein diet were supplemented with lysine (Sato et al., 2015). This increase in activation was equivalent to feeding rats a high protein diet with no lysine supplementation. In addition, researchers observed E3 ubiquitin ligase proteins decreased by 45% in rats fed a low protein diet supplemented with lysine (Sato et al., 2015). In support of these results, Tesseraud et al. (2008) observed that when lysine was fed to exceed requirements, phosphorylation of FOXO3 increased, as well as a marked decrease in MuRF1 mRNA levels in the pectoralis major muscles of male broiler chickens when compared to those provided lysine deficient diets (Tesseraud et al., 2008).

PROTEIN SYNTHESIS ACROSS MUSCLE TYPES

Nutrient allocation causes nutrients to be partitioned to critical organs first. Considering skeletal muscle develops and grows at various rates, the mTOR pathway may be more active in specific muscles at certain developmental points. Sato et al. (2013) provided lysine supplementation to Wistar rats weighing between 30 and 50 g. Results indicated that lysine supplementation did not affect the mass of the extensor digitorum longus muscle, but provision did increase the masses of both the gastrocnemius and soleus muscles (Sato et al., 2013). In a different study, Langer et al. (2000) observed branched chain ketoacid dehydrogenase (BCKDH) concentrations in both the adductor muscle and biceps femoris of gilts (30 to 35 kg initial BW) provided methionine-deficient diets with no leucine supplementation, 150% of leucine requirements, or supplementation of all 3 BCAA to meet requirements. Results indicated increased concentrations of BCKDH in the biceps femoris when either leucine or all 3 BCAA were supplied in comparison to the control (Langer et al., 2000). In addition, the adductor muscle was unaffected by dietary treatment (Langer et al., 2000). This indicates that the biceps femoris may be more effective at breaking down BCAA than the adductor muscle in gilts in this particular age group.

HORMONAL FACTORS AFFECTING PROTEIN TURNOVER

Insulin

Insulin, a peptide hormone produced by the pancreas, regulates blood glucose concentrations, and levels can vary depending on dietary energy and AA. When insulin was infused to the fed level, the rate of muscle synthesis increased in skeletal muscle only, whereas hyperaminoacidemia was able to stimulate growth in skeletal muscle, pancreas, liver, and the kidney in both 7- and 26-d old pigs (Davis, 2002). Phosphorylation of translation factors 4E-BP1

and S6K1 increased when AA, particularly BCAA, were increased from basal to fed levels, especially in the presence of insulin (O'Connor et al., 2003). In contrast, Davis et al. (2002) observed no additive effects between insulin and AA supplementation (Davis et al., 2002).

AA and insulin have additive effects on the activation of mTORC1 (Melnik, 2012). Increasing insulin concentrations from a fed state to a hyperinsulinemic state increased the phosphorylation of translation initiation factors (O'Connor et al., 2003). Though O'Connor et al. (2003) did not observe a direct effect of eIF4E/ eIF4G binding, an increase in 4E-BP1 phosphorylation was observed, allowing eIF4E to more easily bind to eIF4G, further enhancing mTOR initiation (O'Connor et al., 2003). Anthony et al. (2002) observed that AA, specifically leucine, can still effectively stimulate muscle synthesis via the mTOR pathway while using somatostatin to keep insulin at fasting levels. However, insulin and leucine had potentiated effects on muscle synthesis, causing a greater increase when both were supplied (Anthony et al., 2002a). AA and insulin appear to increase mTOR initiation in a dose dependent manner, where greater concentrations of both BCAA and insulin allow for an increase in muscle synthesis until maximum nutrient levels have been met (O'Connor et al., 2003).

It has also been observed that when insulin levels drop below basal level, such as in the case of diabetes, leucine is less effective at stimulating downstream factors of the mTOR pathway, potentially decreasing mRNA translation and in turn, protein synthesis (Anthony et al., 2002b). Similar observations have been witnessed when AA concentrations drop below fasting levels in neonatal pigs. Basal requirements of AA for growth are sufficient for insulin to activate mTORC1; however, when AA concentrations drop below fasting levels, insulin is no longer able to initiate mTOR phosphorylation (O'Connor et al., 2003). A recent review (Melnik, 2012) presented concerns in feeding human infants high protein diets. High protein infant formulas

tend to have greater concentrations of leucine than human milk. An increase in dietary leucine potentiates mTOR activation as well as insulin production, which is associated with increased concern of insulin resistance after long term leucine provision (Melnik, 2012).

Insulin-like growth factor-1

Insulin-like growth factor 1 (IGF-1) is a hormone produced in the liver and released from cells when growth hormone (GH) is actively secreted and is involved in a multitude of bodily processes. For example, this protein is important for proper bone growth, to prevent muscle atrophy, and is even involved in cerebral function. This was suggested when elderly participants with dementia had significantly lower IGF-1 concentrations than those without dementia (Arai et al., 2001). In addition, Beck et al. (1995) observed that homogenous IGF-$1^{-/-}$ mice had severely decreased cerebral development at 2 months of age compared to controls. Low doses of IGF-1 increased cerebral recovery when lambs were subjected to brain injury (Johnston et al., 1996). In addition to brain function, IGF-1 is an important protein in bodily growth, especially in that of muscle synthesis. Multiple studies have been conducted to determine the mode of action of IGF-1; whether it be primarily through increased muscle synthesis, a decrease in muscle degradation, or some combination of the two (Latres et al., 2005). Therkildsen et al. (2004) suggested that pigs fed ad libitum prior to slaughter increased in both protein synthesis, due to adequate IGF-1 release, and protein degradation, from increased calpain activity, leading to overall greater protein turnover than those fed restricted diets (Therkildsen et al., 2004).

C2C12 myoblasts were exposed to IGF-1, dexamethasone (DEX), a glucocorticoid involved in muscle atrophy, or IGF-1 + DEX in order to determine if IGF-1 was related to ubiquitin ligase proteins as well as proteins of mTOR. IGF-1 induced the PI3K pathway in C2C12 myoblasts. Cellular exposure to DEX caused myotubes to decrease in size by 37%, and

concentrations of the E3 ubiquitin ligase, muscle atrophy F box (MAFbx), increased. However, when IGF-1 and DEX were added simultaneously, PI3K was activated to a greater extent than the control, indicating cellular growth even in the presence of DEX (Latres et al., 2005).

IGF-1 release is increased by GH as well as by AA stimulation. Breier et al. (1988a) observed a decrease in plasma IGF-1 concentrations in steers fed a protein restricted diet regardless of GH concentrations. Prolonged AA supplementation in the media of cultured ovine hepatocytes drastically increased IGF-1 release (Wheelhouse et al., 1999). AA and GH individually have the ability to stimulate the release of IGF-1; but which of the two is more effective depends on a multitude of factors, including the cellular environment. For example, Breier et al. (1988a) observed a decrease in GH effectiveness when steers were not receiving enough protein. In such case, it is suspected that the liver adapts by becoming insensitive to GH due to a decrease in number of somatotrophic receptors, requiring IGF-1 to be more sensitive to AA supply (Breier et al., 1988a). It is possible that the two may have a symbiotic relationship, in which the supply of each together is more effective in stimulating IGF-1 release than either one alone. In vitro IGF-1 production does not change when cells are provided with a combination of GH and levels of AA below that of physiological values (Wheelhouse et al., 1999), implying that AA concentrations must still be provided to meet maintenance levels.

In addition to cellular environment, age, diet, and species of the animal play roles in IGF-1 secretion. Friesian bull calves had lower plasma IGF-1 concentrations when provided a concentrate/hay diet compared to their counterparts provided a whole milk-based diet (Breier et al., 1988b). IGF-1 was positively correlated with muscle growth throughout the growing period, in which pigs fed restricted diets throughout finishing had lower plasma IGF-1 concentrations (Therkildsen et al., 2004). Low IGF-1 concentrations have been shown in lambs at birth, much

like in cattle. In contrast, lambs tend to reach adult IGF-1 concentrations shortly after birth, whereas cattle gradually increase in concentration (Gluckman et al., 1983; Breier et al., 1988b). IGF-1 release may be mediated by a multitude of mechanisms depending on stage of life and state of digestive development; for example, when young ruminants physically switch from monogastric-like stomachs to that of ruminants (Breier et al., 1988b) or when crossing from the growing phase into the finishing phase (Therkildsen et al., 2004).

Leptin

Leptin is a hormone secreted by chief cells in the stomach, as well as by adipose cells, and it plays a role in energy expenditure, satiety, and has some effects on the immune system and AA absorption (Maya-Monteiro and Bozza, 2008; Fanjul et al., 2015). In addition, evidence suggests leptin can stimulate the mTOR pathway and vice versa depending on the cellular environment (Roh et al., 2002). Rapamycin administered to rat adipocytes inhibited leptin secretion, suggesting that leptin can be, to an extent, controlled by mTORC1 (Roh et al., 2002; Fazolini et al., 2015). Leptin provision to human trophoblastic cells increased the phosphorylation of eIF4E, leading to an overall increase in 4E-BP1 phosphorylation (Pérez-Pérez et al., 2009). An increase in PHAS-1 protein, a translation regulator found in adipocytes, was observed when leucine was added to adipocytes (Roh et al., 2002). Leptin supplementation to adipocytes in vitro increased Akt and $p70^{S6K}$ phosphorylation, suggesting leptin and mTOR work in conjunction (Fazolini et al., 2015).

Carbo et al. (2000) reported that leptin's effects on protein turnover are indirect. After 6 h of leptin infusion, plasma insulin concentrations were greater in obese diabetic mice when compared to those not supplemented with leptin (Burcelin et al., 1999). Increased insulin concentrations may be an indirect method by which leptin can stimulate the mTOR pathway for

protein synthesis. Much like mTOR, Roh et al. (2002) reported that leucine provision increased leptin secretion but had no effect on leptin mRNA numbers (Roh et al., 2002). In contrast to previous research, Carbo et al. (2000) did not observe any differences in protein turnover when leptin was supplemented alongside leucine in vivo (Carbo et al., 2000). These results further support the theory that leptin may activate mTOR indirectly, perhaps via PHAS-1.

Ghrelin

Ghrelin is a hormone secreted by the stomach, and it acts to stimulate hunger and release GH. As previously discussed, GH stimulates the release of IGF-1. Therefore, it is possible that ghrelin may indirectly activate muscle synthesis through the mTOR pathway via IGF-1; however, ghrelin has direct impacts on muscle as well. Release of the hormone stimulated the phosphorylation of Akt, which had direct impact on hindering the action of FoxO, MuRF-1, and atrogin-1 to slow, and possibly prevent, proteolysis of skeletal muscle (Chen et al., 2015). In addition, ghrelin secretion directly activated the hypothalamic mTOR pathway by increasing the phosphorylation of mTOR and S6K1 in rats in vivo (Martins et al., 2012) and in vitro (Xu et al., 2015).

Even though hypothalamic mTOR can be activated by ghrelin secretion, mTOR located in the stomach can act as an inhibitory factor for ghrelin secretion. When nutrients are in abundance, mTOR in the stomach is able to block CB1 receptors (cannabinoid receptors) in order to inhibit the secretion of ghrelin and induce satiety (Senin et al., 2013). In addition, gastric mTOR can block translation of ghrelin (Senin et al., 2013). To further support these results, administration of rapamycin to mice led to an increase in ghrelin production and release due to a decrease in mTOR and downstream signaling (Xu et al., 2010; Stevanovic et al., 2013).

Immune regulation is, in part, controlled by ghrelin and is mediated by mTOR activation. A study conducted by Xu et al. (2015) observed an increase in T-cell numbers, as well as increased mRNA for interleukin (IL) 17 and activated T-helper (Th) 1 and 17 cells via leucine provision, which are otherwise inhibited by ghrelin (Eissa and Ghia, 2015; Xu et al., 2015). Intestinal mTOR phosphorylation increased, which then induced further downstream signaling, particularly in the phosphorylation of S6, in response to tissue damage in mice provided supplemental intraperitoneal ghrelin injections (Zhang et al., 2013). Ghrelin administration to mice inflicted with intestinal damage led to a decrease in inflammation and an increase in tissue repair and survivability via mTOR stimulation (Zhang et al., 2013). It has also been observed that ghrelin can ameliorate muscle wasting in cancer patients by increasing appetite and acting on immune factors (Chen et al., 2015). Tumor necrosis factor (TNF)-α concentrations decreased in the presence of ghrelin and survivability of cancerous mice increased when ghrelin was administered as treatment (Chen et al., 2015).

LEUCINE USE IN ANIMAL DIETS

Leucine is not generally considered a limiting AA for beef cattle (Schwab et al., 2005). However, leucine supplementation has gained interest in recent years after discovering the positive effects for cellular growth. In a recent review, Melnik (2012) observed a positive correlation between growth rate and milk protein concentration among a multitude of species. It was noted that species with greater milk protein concentrations, and particularly those high in leucine, produced offspring that doubled in weight significantly quicker during neonatal life than those with lower milk leucine concentrations (Melnik, 2012). Increased growth rates can be beneficial, for example, in premature births (Gordon et al., 1947); however, an excess of PI3K

activation, as seen during unregulated high growth periods, has potential to lead to long term health issues such as insulin resistance in type 2 diabetes (Melnik, 2011).

Though Melnik (2012) described the dangers of increased leucine supplementation, too little leucine is of concern as well. Low dietary leucine during gestation hindered trophoblast growth in humans, which are vital to fetal growth (Wen et al., 2005). In vitro bovine mammary tissue and mammary alveolar (MAC-T) cells deprived of leucine exhibited a decrease in mTOR and RPS6 phosphorylation, though this effect was reversible when leucine was once again administered to deficient cells (Appuhamy et al., 2012).

Supplemental dietary leucine has a multitude of benefits, as discussed in part above, but an excess can increase potential for adverse metabolic effects. Excess leucine provided to swine decreased plasma isoleucine and valine (Yin et al., 2010). Similar results were observed in a study conducted by Langer et al. (2000), in which gilts were provided diets deficient in methionine and excess in leucine. α-Ketoisovaleric acid and α-keto-β-methyl-n-valeric acid decreased proportionately to plasma valine and isoleucine concentrations, respectively, as dietary leucine concentrations increased (Langer et al., 2000). Additionally, supplementation of all BCAA increased branched-chain keto acid dehydrogenase (BCKDH) in both skeletal muscle and liver (Aftring et al., 1986; Langer et al., 2000), which would increase the oxidation of BCAA.

IMMUNOLOGICAL EFFECTS OF LEUCINE

Leucine, along with the other BCAA, has the ability to greatly alter the status of the immune system. The effects of leucine concentration may be either positive or negative on immune status. When leucine provision is in excess of requirements while dietary protein is low, negative immunological effects take place. When pigs were challenged with an intramuscular injection of keyhole limpet hemocyanin, a T-cell dependent antigen, and a high dose of dietary

leucine (3.12% leucine), IgG numbers decreased after 3 wk in comparison to pigs receiving the control diet (1.56% leucine). An additional group of pigs received an intramuscular injection of DEX, which acts as an immunosuppressant. Liver and kidney weights increased in response to DEX, whereas the spleen weight decreased, indicating a decrease in immune function (Gatnau et al., 1995). In each case stated above, plasma free leucine concentrations increased when leucine provision increased, whereas isoleucine and valine concentrations decreased. In a review on BCAA and immunity, Calder (2006) theorized negative immunological effects associated with increased leucine supplementation may not necessarily be due to the high concentrations of leucine, but rather due to the imbalance among BCAA (Calder, 2006).

Chevalier and Aschkenasy (1977) reported a decrease in immunological factors including serum IgG concentrations when rats were fed a low protein diet (4% casein) plus leucine supplementation (7%) when compared to rats fed a balanced diet (18% casein) or a low protein diet with no leucine supplementation. No differences were observed when excess leucine was supplemented to protein adequate diets, indicating the possibility that leucine-induced valine and isoleucine deficiencies may be a major contributor to the immunological deficiencies brought on by excess leucine provision (Chevalier and Aschkenasy, 1977).

Though leucine's role as a stimulatory agent on the immune system is controversial, there appear to be some differences between species. Nonnecke et al. (1990) observed that human lymphocytes were more responsive to leucine supplementation than bovine lymphocytes, leading to greater lymphocyte synthesis. Additionally, leucine and its catabolite, α-ketoisocaproate (KIC), increased lymphocyte synthesis when induced with mitogens (Nonnecke et al., 1990). These results suggest that KIC has the ability to replace leucine in lymphocyte synthesis within certain ranges. Nonnecke et al. (1990) observed that KIC had a quadratic effect on the synthesis

of lymphocytes, in which synthesis was inhibited when cells were void of or in excess of KIC (Nonnecke et al., 1990). A possible reason for this outcome is due to an imbalance of BCAA resulting from either leucine, or possibly in this case, its catabolite, KIC. This theory was again suggested when pigs were fed one of four diets containing either high leucine, low leucine, KIC, or β-hydroxymethyl butyrate (HMB). Pigs fed the high leucine and KIC diets had lower plasma isoleucine and valine concentrations than those fed the low leucine or HMB diets, indicating an imbalance between BCAA (Gatnau et al., 1995).

Neutral or positive effects of immunological factors may be observed with supplemental leucine. In vitro cloned hepatic stellate cells (cHSC) void of leucine demonstrated increased hepatocyte growth factor (HGF) in a dose dependent manner when exposed to leucine (Tomiya et al., 2002). Similar results were obtained when cHSC were incubated for increasing times in media containing leucine (Tomiya et al., 2002). Receiving steers fed a diet containing supplemental BCAA had greater blood ovalbumin antibody concentrations compared to those not receiving additional BCAA (Carter et al., 2011). These results indicate that supplemental BCAA increases antibody production as well as cellular growth and efficiency.

Delgoffe et al. (2009) deleted Frap1, a gene responsible for encoding mTOR, from T-cells. Naive T-cells continued to proliferate and resulted in increased numbers of regulatory T-cells but could not continue to differentiate into active immune cells (Delgoffe et al., 2009). mTOR void T-cells were unable to produce interferon (IFN)-γ producing cells, which are vital for innate and adaptive immunity (Delgoffe et al., 2009). Leucine is an important factor for mTOR stimulation in many animal models. Therefore, these results indicate that leucine, when supplemented to a protein adequate diet, may be beneficial to the proliferation of vital immune cells.

LYSINE PROVISION IN MAMMALS

Lysine is an EAA that is often found in limiting concentrations in plant products fed to cattle. Due to low lysine concentrations, cattle are sometimes supplemented with additional lysine in order to promote efficient growth and meet genetic potential. In comparison to other EAA, in muscle lysine is found in the greatest concentrations (Bergström et al., 1974; Tesseraud et al., 2008). Therefore, adequate intake of lysine is important in order to maintain efficient growth. Motil et al. (1981) provided adult human males with a diet deficient in protein. Plasma lysine levels were significantly reduced, however N-retention increased numerically during the post-absorptive phase when lysine infusions were provided. Protein accretion significantly increased when infusions were provided to a diet balanced for protein (Motil et al., 1981), indicating other AA may be distributed towards protein synthesis when lysine is no longer limiting.

There is evidence that lysine not only contributes to protein deposition, but it also may decrease protein degradation. Protein autophagy was decreased by 45% when rats fed a low protein diet were supplemented with lysine versus those not supplemented (Sato et al., 2015). Additionally, lysine supplementation to a protein-limiting diet increased protein synthesis by activating the mTOR pathway to the same extent as a high protein diet (Sato et al., 2015). Prolonged feeding of the lysine-rich diet led to an increase in overall muscle mass (Sato et al., 2015). No differences were observed between rats fed a high protein diet and those fed the high protein diet supplemented with additional lysine (Sato et al., 2015). These results indicate that additional lysine does not influence muscle growth or protein deposition when lysine is provided to meet requirements, but growth efficiency increases when provided to protein deficient diets.

In contrast, chickens fed lysine to meet or exceed requirements decreased protein degradation through a decrease in MuRF-1 found in skeletal muscle (Tesseraud et al., 2008).

On the other hand, oral lysine provision to lysine-deficient rats did not affect ubiquitin-related proteins such as MuRF-1 (Sato et al., 2013). A decrease in myofibrillar proteolysis and methylhistidine concentrations were observed in both the extensor digitorum longus and soleus muscle by 40 and 35%, respectively (Sato et al., 2013), and there were no differences in the phosphorylation of mTOR, $p70^{S6K1}$, or 4E-BP1 (Sato et al., 2013). However, these results contradict those of Sato et al. (2014) in which the phosphorylation of Akt, 4E-BP1, and $p70^{S6K1}$ increased when C2C12 myotube cells were supplemented with lysine (Sato et al., 2014). Differences in these results may be due to differences often seen between in vivo versus in vitro studies.

LYSINE REQUIREMENTS OF GROWING CATTLE

Determining lysine requirements in cattle has been an ongoing and difficult process due to a large number of factors that can alter results. Large variation between experiments increases the difficulty. For example, while feeding a corn gluten meal-based diet, Klemesrud et al. (2000) observed maximum average daily gain (ADG) of growing steers (210 kg initial BW) when 0.9 g lysine/d was supplemented. A larger amount was found to be beneficial when Batista et al. (2015) fed pelleted soybean hulls to growing steers (165 initial BW), in which 9 g lysine/d was observed to lead to the greatest protein deposition concentration.

Bulls (373 kg initial BW) fed a maize stalk silage-based diet reached maximum performance in both ADG and feed efficiency when supplemented with 10 g lysine/d (Xue et al., 2011). Differences in animal size and basal lysine supplies should be noted between these studies. Abe et al. (1997) reported work with bull calves with different ages and weights. Those

younger than 3 mo (72 kg average study BW) required more supplemental lysine, as lysine was found to be the first limiting AA, whereas this was not the case for older bulls (160 kg average study BW) fed the same lysine deficient diet (Abe et al., 1997). These results could be attributable to rumen development as the animals age, allowing more microbial protein to be produced, thereby altering the AA composition reaching the small intestine. Results could also be associated with different DMI that led to different basal AA supplies.

Feeding adequate to excess amounts of protein increases ADG by providing potentially limiting AA to animals. Hussein et al. (2016) described increased N retention in steers provided with supplemental lysine and urea when growing animals were fed a soybean hull-based diet; however, protein fed in excess of requirements could lead to increased dietary cost as well as negative effects on the environment through excess nitrogen deposited on the soil. Growing animals are more susceptible to the benefits of high protein diets due to the increased requirements of nutrients for growth (Davis et al., 1996). During the growing period, nutrient requirements tend to increase as body size increases (NRC, 2000).

With these difficulties considered, it is hard to accurately determine specific AA requirements. Greenwood and Titgemeyer (2004) observed a 2.7 g/d decrease in N-retention when lysine was removed from supplemental infusions. Such a depression in growth indicates that lysine may be a limiting AA in their soybean hull-based diet (Greenwood and Titgemeyer, 2000). Additionally, urinary nitrogen concentrations decreased when steers fed a diet consisting primarily of pelleted soyhulls were supplemented abomasally with lysine. This trend continued until it plateaued when steers were provided with 9 g/d supplemental lysine, suggesting that this is the optimal amount to reach peak ADG (Batista et al., 2015).

Further discrepancies to consider when determining specific AA requirements involve the method of calculations. The method utilized in studies discussed above is N-retention. Another method identifies requirements using plasma AA concentrations. This method is under the assumption that a limiting AA will not increase in plasma concentration until it is no longer limiting (Bergen, 1979). However evidence suggests this is not true, because plasma lysine in growing cattle has been observed to increase when steers are deficient in lysine (Batista et al., 2016). Such increases contributed to whole-body protein deposition. Batista et al. (2015) observed increases in N retention from 21.4 to 30.7 g/d when lysine supplementation increased from 0 to 9 g/d, suggesting that growing steers may have a basal requirement of around 9 g/d of lysine.

CONCLUSION

In conclusion, mTOR is a major regulator of cellular growth and is required for a number of bodily processes, including muscle synthesis and degradation, hormonal control, organ development, and disease control. mTOR is regulated by the nutrient status of the cell and is highly sensitive to leucine concentrations. Though mTOR is necessary for the survival of mammals, balance is necessary, as excess or deficiency can lead to disease or death. Maximum production and genetic potential can be reached once this balance is found. Minimal research has been conducted to date on optimizing mTOR in skeletal muscle of cattle, and further research is required to discover optimal nutrient requirements.

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Chapter 2 - Effects of supplemental leucine on nitrogen retention, efficiency of lysine utilization, and mTOR-related regulation of muscle in growing beef cattle

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ABSTRACT

Seven ruminally cannulated Holstein steers (initial body weight 172.7 ± 3.7 kg) were used in a 6 × 6 Latin square design. Factorial treatments consisted of 2 levels of lysine (0 or 6 g/d) and 3 levels of leucine (0, 15, or 30 g/d) infused abomasally. Steers were fed 2.5 kg DM/d with additional nutrients infused ruminally or abomasally. All steers received constant ruminal infusions of 350 g/d of VFA and abomasal infusions of 300 g/d of glucose to provide additional energy to the steers without increasing ruminal microbial protein synthesis, thereby making energy supply less limiting than amino acids (AA). All steers received basal abomasal infusions of all essential amino acids (AA), except lysine, to ensure that lysine was the only limiting AA. Leucine was included in the basal infusions at a rate of 20 g/d to ensure leucine was not limiting. The leucine treatments provided additional leucine to determine the effects of leucine as a regulatory factor. Periods lasted 7 d, with 2 d of adaptation, 4 d for urine and fecal collections, and 1 d for collection of blood and *longissimus* muscle biopsies. Urinary N decreased ($P < 0.01$) with lysine supplementation and linearly increased ($P = 0.03$) with leucine supplementation. No treatment differences ($P \geq 0.20$) were observed for fecal N. Nitrogen retention increased ($P < 0.01$) with supplemental lysine. Plasma urea N decreased ($P < 0.01$) with lysine supplementation. Leucine supplementation linearly decreased ($P < 0.01$) plasma total AA concentrations. No differences ($P \geq 0.19$) were observed among treatments for total mammalian target of rapamycin (mTOR), phosphorylated mTOR, or the percentage of mTOR phosphorylated. Total 4E binding protein 1 (4E-BP1) tended ($P = 0.08$) to decrease as lysine was supplemented. Percentage of 4E-BP1 phosphorylated decreased linearly ($P = 0.02$) with leucine supplementation. Muscle atrophy F box (MAFbx; $P \geq 0.19$) and muscle RING finger protein 1 (MuRF1; $P \geq 0.18$) were unaffected by treatment. Although leucine can play a regulatory role in activating mTOR and interrelated

mechanisms in various species, we did not find evidence of this effect in muscle of cattle. It is possible that the lysine-limiting conditions of our experiment prevented responses to leucine, that the basal supplies of leucine already yielded maximal stimulation of mTOR and related mechanisms, that decreases in plasma concentrations of valine and isoleucine in response to leucine supplementation offset benefits of leucine supplementation, or that leucine alone does not play a major role in regulating protein deposition in cattle.

INTRODUCTION

Amino acids (AA) are used for a wide variety of biological processes, one of which is protein deposition. Though AA are clearly required, the requirements for optimal efficiency of bovine growth have yet to be well defined. As a limiting AA in cattle, lysine delivery and utilization is very important; however, lysine utilization may be affected by dietary conditions. Leucine supplementation could affect the efficiency of AA use due to leucine's role as a key regulator of protein synthesis (Escobar et al., 2006; Torrazza et al., 2010; Yin et al., 2010). Leucine triggers a series of phosphorylation events that activates mammalian target of rapamycin (mTOR), which is a key regulator of muscle protein synthesis in mammals. mTOR senses nutritional status through pathways involving AA and insulin concentrations as well as AMPK.

In research by Awawdeh et al. (2006), supplementation of excess AA improved N retention in steers that were limited by supplies of either methionine or leucine; however, the responses to the excesses of the non-limiting AA were greater for methionine-limited steers than for those limited by leucine supply. Due to the nature of the experiment, the methionine-limited steers received a mixture containing leucine, whereas those limited by leucine received a mixture devoid of leucine. These results suggest that the differences in the responsiveness to the excess AA could be due to the presence of leucine in the treatments. Therefore, leucine may increase performance by playing a regulatory role in muscle (or in other tissues within the body), but little work has been conducted to determine the role leucine has on protein synthesis in growing cattle.

The objective of this study was to determine if leucine supplementation could improve protein deposition and lysine utilization in growing steers.

MATERIALS AND METHODS

Animals and Treatments

The Kansas State University Animal Care and Use Committee approved animal care and use in this study.

Seven Holstein steer calves (initial BW = 172.7 ± 3.7 kg, initial age = 5 mo) were used in an AA metabolism study. Steers were housed indoors in a temperature-controlled room (17.2°C) in individual tie stalls with rubber mats during the adaptation period. After 2 wk of adaptation to the room, calves were ruminally cannulated (Bar Diamond, Parma, ID) by the Kansas State University School of Veterinary Medicine. Steers were allowed to recover for 4 wk in tie stalls before being placed in individual metabolism crates for total collection of urine and feces for the duration of the study. Adaptation to the experimental diet lasted 2 wk and took place prior to placing steers in metabolism crates. Ruminal and abomasal infusion lines (internal diameter = 2.38 mm, outside diameter = 3.97 mm; Tygon S3^{TM} E-3603 flexible tubing; Fisher Scientific, Pittsburgh, PA) were inserted through the ruminal cannula; abomasal lines were anchored into the abomasum with a 10-cm rubber flange. Steers were housed in metabolism crates for the duration of the study and were allowed to adapt for 1 wk prior to the start of the study. This study was a 6 × 6 Latin square design balanced for carry over effects with steers randomly assigned to 1 of 6 treatments in each of 6 periods. The seventh steer was provided a treatment sequence identical to one of the other steers. Each treatment period lasted for 7 d (2 d for treatment adaptation, 4 d for urine and fecal collection, and 1 d for blood and muscle biopsy collections). Short adaptation periods were adequate for this study because cattle adapt quickly to postruminal infusion of AA (Schroeder et al., 2007). The short periods allowed steers to maintain

a consistent physiological state throughout the trial and are important for optimizing animal welfare.

Diet Composition

The experimental diet consisted of soybean hulls, wheat straw, molasses, and a vitamin and mineral premix (Table 2.1) provided as a total mixed ration. Each calf had ad libitum access to water and received 2.5 kg DM/d of feed in 2 equal portions at 12-h intervals (Table 2.1). Urea was provided daily (10 g/d) via continuous ruminal infusion and was considered part of the diet. The diet was designed to be limiting in metabolizable protein supply, but adequate in ruminally degradable protein. Ruminal infusion lines were connected to a perforated bottle inside the rumen to diffuse VFA. Volatile fatty acids (VFA) were infused into the rumen to ensure energy was not as limiting as metabolizable protein. Each animal received continuous daily infusions of 150 g acetic acid, 150 g propionic acid, and 50 g butyric acid into the rumen as well as 300 g/d of dextrose abomasally in order to provide an energy source without increasing ruminal microbial protein production. The VFA solution, which provided 10 g/d urea as described above, was diluted with water to a final weight of 4 kg/d (Table 2.2).

Treatment Allocation

Treatments consisted of 3 levels of leucine (0, 15, and 30 g/d) and 2 levels of lysine (0 and 6 g/d; Table 2.2) provided daily abomasally. Both ruminal and abomasal infusates were infused continuously using a peristaltic pump (Model ISM444A-230V; Cole-Parmer Instrument Company, Vernon Hills, IL) at a rate of 4 L/d. Fresh ruminal and abomasal infusion solutions were placed twice daily at 0700 and 1900 h. Infusion lines were flushed daily with 20 mL water to limit clogs. Steers were allowed to adapt to basal infusions for 1 wk prior to the start of the study.

Infusions

Each animal received a daily infusion of glycine, and glutamate, and all essential AA except lysine to ensure lysine was the only limiting AA. Additional leucine and/or lysine was supplemented depending on treatment. AA were provided in basal amounts according to Lambert et al. (2004). Infusates containing basal AA were prepared daily by dissolving 20 g/d leucine, 15 g/d valine, and 15 g/d isoleucine in 2,593 g hot water containing 71 g 6 *M* HCl. Once dissolved, 40 g glycine, 150 g monosodium glutamate (providing 120 g L-glutamic acid), 10 g L-methionine, 15 g L-arginine, 20 g L-phenylalanine, 8 g L-histidine-HCl-H_2O, 5 g L-tryptophan, and 15 g L-threonine were added to the solution and mixed until dissolved. After AA were fully dissolved, 300 g of dextrose was added. Lastly, 34 g of 50% NaOH was added to the solution (Table 2.5). Folic acid (10 mg/d), pyridoxine-HCl (10 mg/d), and cyanocobalamin (0.1 mg/d) were provided daily in abomasal solutions to prevent deficiencies of these vitamins (Lambert et al., 2004). The AA mixes were refrigerated from mixing until time of use.

Lysine and leucine treatments were mixed separately. The lysine solution was prepared by dissolving 7.5 g lysine-HCl into 92.5 g of hot water. The leucine treatment was prepared by dissolving 30 g leucine in 120 g of hot water containing 50 g of 6 *M* HCl. The AA, lysine, and leucine solutions were mixed together in individual bottles for infusion dependent on treatment. AA treatments were refrigerated once mixed until time of use. Water was added until the infusate weight equaled 4 kg/d.

Sample Collection

Urine and feces from d 3 through 6 of each period were collected and weighed daily for each steer. Urine was collected in containers containing 900 mL of 10% (wt/wt) H_2SO_4 to preserve ammonia. Urine was stirred throughout the day to ensure adequate mixing with the acid.

Feces were collected in metal pans lined with plastic bags and were thoroughly mixed prior to sampling. Representative subsamples of urine and feces were collected daily and stored frozen at -20°C. Representative feed samples from d 2 to 5 of each period were collected, and any orts from d 2 to 5 of each period were collected and weighed for each steer. Urine, feces, orts, and feed samples were representatively pooled within period and stored at -20°C until analyzed.

Jugular blood samples were collected into 10-mL BD Vacutainer sodium heparinized vacuum tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) at 0900 h (2 h after the morning feeding) on d 7 of each period. Blood was immediately inverted several times and placed on ice until centrifuged at 1,200 × g at 4°C for 20 min within 30 min of collection. Plasma was removed and pipetted into 2-mL microcentrifuge tubes and stored at -20°C until analysis.

Muscle biopsy samples were collected on d 7 of each period from the *longissimus lumborum* muscle at 1400 h (7 h after the morning feeding) using an 8-gauge piercing needle to pierce the skin (Precision Needles, Hanover, MD) and a Quick-Core biopsy needle (10-gauge 5 cm, Cook Medical, Bloomington, IN) to collect the sample. Lidocaine (1 mL, 1% solution) was used as a local anesthetic prior to biopsy to prevent pain. Each period, biopsy sites were alternated across each side of the vertebrae and moved 2.5 cm posteriorly to allow for adequate recovery and prevent unrepresentative sampling due to the healing process. Biopsy sites were covered with liquid bandages immediately after biopsies were collected. Sites were monitored until completely healed. No infections or issues were observed. Biopsy samples were immediately frozen using liquid N_2 and stored at -80°C until analysis.

Nutrient Composition Analysis

Feed and orts samples were dried in a 55°C forced-air oven for 48 h, ground through a 4-mm screen, followed by a 1-mm screen using a Thomas Wiley Mill (Thomas Scientific,

Swedesboro, NJ). Samples were dried for 24 h in a 105°C forced-air oven to determine total dry matter (DM) and then heated to 450°C for 18 h to determine organic matter (OM) content. All samples were analyzed in duplicate. Neutral detergent fiber (NDF) using alpha amylase and acid detergent fiber (ADF) were analyzed sequentially in duplicate using an ANKOM Fiber Analyzer (Model 200, ANKOM Technology, Macedon, NY).

Fecal samples were dried in a 105°C oven for 24 h to determine total DM content. Samples were placed in a 450°C oven for 18 h to determine the OM content. Nitrogen content for feed, orts, wet feces, and urine were measured using a LECO Nitrogen Analyzer (LECO Corporation, Saint Joseph, MI). Crude protein (CP) content was determined by multiplying the N content by 6.25. All samples were analyzed in duplicate.

Immunoblot Analysis

Muscle biopsies obtained from the *longissimus lumborum* were homogenized for 10 s using a Fisher brand 850 homogenizer (Thermo Fischer Scientific, Waltham, MA) on low speed in extraction buffer (50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 1, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 μg/mL soybean trypsin inhibitor pH 7.4; Sigma Aldrich, St. Louis, MO) prepared on the day of analysis. Homogenates were centrifuged at 10,000 × *g* for 12 min at 4°C and the supernatant was collected. A Pierce's BCA Protein Assay Kit (Thermo Scientific; Waltham, MA) was utilized to determine protein concentration.

Forty-five micrograms of protein was separated using 7.5 or 10% polyacrylamide gels. Gels for phosphorylated and total proteins of interest were analyzed on gels run concurrently. Proteins were transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL) and membranes were blocked with 5% dry nonfat milk in Tris-buffered saline plus Tween 20 (TBS-

T; 10 mM Tris, 150 mM NaCl, 0.1%, pH 8.0, Tween-20) for 30 min. Blots were incubated for 18 h at 4°C in 1% nonfat dry milk in TBS-T containing primary antibodies at the following dilutions: 1:2,000 P-Akt^{Thr308} (polyclonal rabbit; Cell Signaling) and MuRF-1 (polyclonal rabbit; Santa Cruz), 1:1,000 total Akt (polyclonal rabbit; Cell Signaling), MAFbx (monoclonal mouse; Santa Cruz), P-RPS6$^{240/244}$, P-4E-BP1 (polyclonal rabbit; Cell Signaling), RPS6 (polyclonal rabbit; Cell Signaling) and 4E-BP1 (polyclonal rabbit; Cell Signaling), and 1:500 P-mTOR (polyclonal rabbit; Cell Signaling) and total mTOR (polyclonal rabbit; Cell Signaling). After washing with TBS-T, membranes were incubated in secondary antibodies [anti-rabbit (or anti-mouse for MAFbx only) horseradish peroxidase-linked; Cell Signaling], washed again, and a standardized enhanced chemiluminescence (ECL) kit (GE Healthcare, Little Chalfont, United Kingdom) was used to illuminate proteins during imaging. A ChemiDoc-It Imaging System (UVP) using VisionWorks analysis software (Version 7.1 Beta 1; UVP) was utilized for photo capture and densitometry analysis. Results reported are relative to a pooled sample analyzed on each individual gel.

Plasma Metabolite Analysis

Plasma glucose was measured using a modified technique from Gochman and Schmitz (1972) using an AutoAnalyzer (Technicon Analyzer II; Seal Analytical, Mequon, WI) with standards prepared in 0.2% benzoic acid. Plasma urea nitrogen (PUN) was analyzed using an Auto Analyzer (Technicon Analyzer II; Marsh et al., 1965).

Plasma AA were analyzed using a modified Pickering method on a lithium cation-exchange column (4 mm × 100 mm, Pickering Laboratories Inc., Mountain View, CA) utilizing lithium eluents. Auto sampler temperature was held constant at 4°C, and a 10 µL loop was utilized. Measurements were made using a florescence detector (HP 1046; Hewlett Packard,

Alto, CA) using post-column derivatization with *o*-phthalaldehyde. A column temperature gradient was used in which the column was held at 32°C for 13 min, then increased to 50°C for 49 min, increased once again to 71°C for 39 min, and decreased to 32°C for 24 min for a total run time of 125 min. Four mobile phases were utilized, consisting of A: 1700-1125 Lithium Eluent (Pickering Laboratories Inc., Mountain View, CA); B: Li365 Lithium (Pickering Laboratories Inc.); C: Li375 Lithium (Pickering Laboratories Inc.); D: RG003 Lithium (Pickering Laboratories Inc.). A gradient elution was made by the following: 0-15 min, 100% A; 15-27 min, 100% A – 40% A/60% B; 27-45 min, 40% A/60% B – 100% B; 45-60 min, 100% B; 60-60.1 min, 100% B – 100% C; 60.1-85 min, 100% C; 85-85.1 min, 100% C – 70% C/30% D; 85.1-105 min, 70% C/30% D; 105-105.1 min, 70% C/30% D – 100% A; 105.1-125 min, 100% A, with a flow rate of 0.4 mL/min. The florescence detector was set with excitation and emission wavelengths of 330 nm and 465 nm, respectively, using a response time of 1 s. OPA diluent (Pickering Laboratories Inc.) was mixed with column effluant at a rate of 0.4 mL/min.

Calculations

Retained N was calculated by subtracting N output (fecal + urinary) from N intake. Dry matter and organic matter digestibilities were calculated without consideration for the ruminal and abomasal infusions (i.e., intake was only of dietary origin). Percent phosphorylation of proteins was calculated by dividing the phosphorylated protein value by the total protein; these ratios do not represent actual percentages because both values are arbitrary units. To calculate lysine deposition, we assumed protein deposition equaled 6.25 × N retention and 6.4% of muscle protein deposition is lysine (Ainslie et al., 1993). Efficiency of supplemental lysine utilization was calculated by dividing the increase in lysine deposition by the increase in lysine supply (6 g/d).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NC) as a Latin square design. Treatments (lysine, leucine, and lysine × leucine) and period were fixed effects. Animal was a random effect. Orthogonal polynomial contrasts were used to evaluate effects of leucine and its interactions with lysine. The LSMEANS function was used to determine treatment means. Significance was declared at $P \leq 0.05$ and tendencies when $0.05 < P \leq 0.10$. One data point was removed because the steer had digestive distress during the period. Outliers from the western blot dataset were identified and removed (studentized residual ≤ -3 or ≥ 3). In total, 9 outliers were removed from the dataset.

RESULTS AND DISCUSSION

Nutrient Intake and Digestibility

When some AA are provided in limiting amounts, dietary protein is not utilized as effectively as when all AA are supplied to meet or exceed requirements of the animal. Our experimental model was designed so that lysine would be the only limiting AA, and the supplementation amount of 6 g/d was within the linear response range, which was observed by Batista et al. (2015) to be 9 g/d. Basal supplies of leucine exceeded the steers' requirements for growth (Lambert et al., 2004). Our amounts of supplemental leucine (15 and 30 g/d) were designed to allow leucine to act as a regulatory factor for protein synthesis in growing steers.

Supplementation of lysine at 6 g/d increased ($P < 0.01$; Table 2.3) DM intake from 2,428 g/d to 2,465 g/d when compared to our treatments with no lysine supplementation. No evidence of a lysine × leucine interaction ($P = 0.37$) was observed, though, a tendency ($P = 0.09$) for a linear response to the leucine treatments was observed. On average throughout the study, steers had 20.5 g DM/d of orts. These differences are slight and are likely due to variation in orts.

Considering steers in our study were limit-fed, this response represents a minor difference in feed refusals. In addition, an increase ($P < 0.01$) in organic matter intake was observed with the provision of lysine at 6 g/d. DM digestibility and OM digestibility were 72.7 and 74.6%, respectively. These results are similar to observations of Awawdeh et al. (2006) for a similar diet. No evidence ($P \geq 0.28$) was observed for an effect of treatment on DM or OM digestibilities.

Nitrogen Intake, Excretion, and Retention

As expected, total N intakes were increased when more N was infused as part of the treatment structure, but the greatest increase in total N intake as a result of treatment was 4.5%. There was no evidence ($P \geq 0.43$) of an effect of treatment on fecal N; however, lysine provision decreased ($P < 0.01$) urinary N excretion. Nitrogen retention increased when lysine was infused ($P < 0.01$; Figure 2.1). The effect of lysine on N retention was expected considering lysine was a limiting AA in our model (Batista et al., 2016). An increase in N retention when lysine was supplied indicates our model was successful and more AA were partitioned towards whole-body retention.

No changes in N retention were observed with leucine supplementation. This may suggest the basal amount of leucine provided to steers was enough to maximally stimulate protein accretion. At the levels included in this study, leucine neither directly affected protein deposition nor had an effect on lysine utilization (Figure 2.1). However, in conditions in which lysine is not limiting, it is possible that leucine could yield a benefit by increasing the steers' capacity for protein deposition (i.e., increasing maximal protein deposition). Awawdeh et al. (2006) observed steers provided 4 g/d leucine supplementation had increased N retention from 30.6 to 34.6 g/d, but that was in a model designed to be deficient in leucine.

Providing supplemental leucine to a diet limiting in multiple AA did not have any effects on N retention in pigs (Langer and Fuller, 2000). N retention increased in pigs supplemented with leucine when methionine was the only limiting AA in the diet (Langer and Fuller, 2000). It is possible we did not observe a difference in N retention in response to additional leucine supplementation because leucine was already provided to each animal in the basal AA infusions at 20 g/d, which may have been enough to stimulate N retention at maximal level through a mechanism such as mTOR. It is also possible that leucine is not capable of stimulating protein deposition in cattle.

Lysine Efficiency

Our model was designed for lysine to be the only limiting AA. Batista et al. (2015) determined 9 g/d to be the supplemental lysine requirement for growing cattle to maximize growth. Because we wanted lysine to remain limiting throughout our study, we selected our lysine treatment amount to be 6 g/d. Assuming protein deposition is 6.25 × N retention and 6.4% of protein deposition is lysine (Ainslie et al., 1993), we estimated lysine deposition. From the amounts of lysine deposited, we estimated that, for 0, 15, and 30 g/d of leucine provision, lysine efficiencies were 33, 46, and 42%, respectively (Figure 2.1), without differences among leucine amounts. Averaged cross treatments, lysine efficiency averaged 40% for deposition of whole-body protein, which is identical to the results of Batista et al. (2015). Hussein et al. (2016) observed lysine efficiency to be 51% when experimental conditions were similar to those in our study. Lysine efficiency between the current study and that of Hussein et al. (2016) were only slightly different, and overall, suggests inefficient utilization in cattle. Our estimates for efficiency of lysine utilization were much less than those of NASEM (2016), in which they estimated lysine would be used with 64% efficiency for steers of the size used.

Plasma Metabolites

***Effects of lysine supplementation on plasma metabolites*.** Lysine supplementation increased ($P < 0.05$) plasma concentrations of lysine, isoleucine, glutamate, alanine, methionine, arginine, and glutamine. Decreases were observed ($P < 0.05$) for concentrations of taurine, serine, glycine, and urea N when lysine was supplemented (Table 2.4). Bergen (1979) theorized that when an AA is limiting, plasma concentrations will not increase until that AA is no longer limiting. Although plasma lysine increased statistically in response to lysine supplementation in amounts below the requirement, the increases were not large and thus may not reflect a biologically significant accumulation of lysine in blood. Agreeing with our results, other studies conducted in our lab have demonstrated an increase in circulating lysine in growing cattle when lysine was supplemented in amounts below the requirement (Batista et al., 2015; Hussein et al., 2016).

Effects of leucine supplementation on plasma metabolites. Supplementation of leucine linearly increased plasma leucine concentrations ($P < 0.01$). Leucine provision linearly decreased ($P < 0.05$) circulating concentrations of total AA, isoleucine, valine, aspartate, threonine, glutamine, alanine, methionine, tyrosine, asparagine, glycine, and phenylalanine.

A decrease in plasma AA is often considered to indicate greater cellular uptake of AA. Because no differences were observed for N retention, we can hypothesize that AA uptake was likely not used for deposition. As observed by Yin et al. (2010) plasma concentrations of isoleucine, valine, and threonine decreased with each increasing amount of leucine provided. BCAA have antagonistic effects on each other, thus the decreased plasma concentrations isoleucine and valine was expected when leucine was supplemented (Gatnau et al., 1995). Various other metabolic issues may ensue due to an imbalance between the BCAA, especially

when leucine is exceptionally greater than either valine or isoleucine (Calder, 2006). Yin et al. (2010) reported that a dietary imbalance between leucine and threonine led to a decrease in threonine concentrations when leucine was supplemented. A similar imbalance may have been present in the current study, causing the observed decrease in threonine concentrations when leucine was supplemented. The inability of leucine to affect protein synthesis may have been related to the BCAA antagonism. Wilson et al. (2010) reported that when leucine alone was supplemented, other EAA decreased up to 50% in concentration, and this prevented an extended improvement in leucine-stimulated protein deposition. However, when other AA were supplemented along with leucine to counteract the leucine-induced decrease in plasma AA, the effects of leucine were restored. Furthermore, chronic leucine provision may have deleterious effects on plasma essential AA, and some nonessential AA plasma concentrations (Wilson et al., 2010). Asparagine, glutamine, glycine, alanine, methionine, tyrosine, and phenylalanine each decreased when leucine was supplemented. It is possible that the stimulatory effects of these AA on portions of the mTOR pathway were lost when leucine supplementation led to decreases in their concentrations.

No changes in circulating taurine concentrations were observed when leucine was supplemented. Our results differ from that of Sadri et al. (2017) in which dairy cows supplied with leucine post ruminally had lower taurine concentrations than those not receiving supplemental leucine. Decreases in circulating taurine, aspartate, serine, and glycine when lysine, a limiting AA, was provided could indicate an increase in uptake of these AA, possibly for muscle synthesis or for other metabolic processes. Glutamate, glutamine, alanine, and arginine each increased with lysine provision; reasons for these increases are unknown, but these four AA are important in the shuttling of N through the body, including arginine's role in ureagenesis

(Gouillou-Coustans et al., 2002), which may have changed with increased protein deposition in response to lysine supplementation. Opposite to our results, Batista et al. (2016) observed no differences in circulating methionine with the addition of lysine. PUN concentrations decreased as lysine provision increased, likely due to an increase in the utilization of N. No differences ($P \geq 0.27$) among treatments were observed for plasma glucose concentrations.

mTOR Stimulation in Bovine Skeletal Muscle

A lysine × quadratic leucine interaction ($P < 0.01$) was observed for total Akt because 15 g/d of leucine without lysine supplementation led to a decrease in total Akt abundance, whereas 15 g/d of leucine along with 6 g/d lysine provision increased total Akt. Akt^{Thr308} and the percentage of Akt phosphorylated were not different among treatments ($P \geq 0.20$; Table 2.5). These results are consistent with those of Wilson et al. (2010), in which neonatal pigs infused with leucine, along with other AA to prevent a leucine-induced imbalance, did not demonstrate differences from the control treatment for total and phosphorylated Akt (phosphorylated on Ser^{437}). It has been suggested that Akt responds independently of mTOR stimulation (Wilson et al., 2010) indicating that Akt may respond to stimulants other than those that control mTOR, and it is possible that Akt may be more responsive to other stimulants. However, Akt can be stimulated by factors that also influence mTOR activation, such as insulin and glucose (Alessi et al., 1996; Latres et al., 2005; Columbus et al., 2015). It is unclear why the absence of lysine supplementation would only lower total Akt for steers receiving 15 g/d leucine.

No treatment effects were observed for phosphorylated mTOR ($P \geq 0.19$), total mTOR ($P \geq 0.29$), or the percentage of mTOR phosphorylated ($P \geq 0.19$). In humans, mTOR activation was stimulated by feeding alone (Churchward-Venne et al., 2012) rather than specifically stimulated by leucine (Glynn et al., 2010). Rapamycin, a known mTOR inhibitor, prevented

protein synthesis and prevented phosphorylation of S6K1 and 4E-BP1 when fed to pigs (Kimball et al., 2000). Because rapamycin is known to prevent phosphorylation of the mTOR protein within mTORC1, the results of Kimball et al. (2000) suggest that mTOR phosphorylation decreased, thereby hindering downstream phosphorylation of S6K1 and 4E-BP1. In contrast to our results, Manjarin et al. (2016) observed an increase in phosphorylated 4E-BP1 when supplemental leucine was provided to swine. Though Manjarin et al. (2016) did not specifically measure mTOR phosphorylation, it can be inferred from their results that mTOR phosphorylation increased in response to leucine. Our model differed from those discussed above due to the fact that primarily monogastrics have been used to study the effects of leucine on mTOR in skeletal muscle, whereas we used ruminants. It is possible that ruminants may vary from monogastrics in the nutrient factors that regulate the mTOR pathway; however, when leucine was supplemented to MAC-T cells in bovine mammary tissue in vitro, mTOR phosphorylation increased (Appuhamy et al., 2012). The study conducted by Appuhamy et al. (2012) was conducted *in vitro*, therefore, results may differ biologically in a live animal. It is also important to note that cell type could also lead to variation when comparing results. It is possible that mammary cells may utilize the mTOR pathway more than skeletal muscle in ruminants.

No differences in phosphorylated 4E-BP1 were detected ($P \geq 0.41$) among treatments. However, the phosphorylation percentage of 4E-BP1 decreased linearly ($P = 0.02$) as leucine supplementation increased. Contradictory to our results, we had expected leucine treatments to increase the phosphorylation of 4E-BP1, thereby increasing the observed overall percent of phosphorylated 4E-BP1. Leucine provided to neonatal pigs fed a low protein diet increased 4E-BP1 activation to the same extent as pigs fed a high protein diet (Torrazza et al., 2010).

O'Connor et al. (2003) observed an increase in 4E-BP1 phosphorylation and an increase in the eIF4E/ eIF4G complex, but only when insulin and AA were supplied simultaneously in neonatal pigs. We did not measure insulin concentrations in the current study, however, it is possible that insulin levels were too low to elicit a response. In another study, 4E-BP1 phosphorylation, as well as formation of the eIF4E/ eIF4G complex, increased when leucine was supplemented to neonatal pigs fed a protein restricted diet (Columbus et al., 2015). A leucine-induced decrease of valine and isoleucine was not observed in the study by Columbus et al. (2015) as it was in our study. Therefore, an imbalance among the BCAA could be a potential reason we did not observe treatment differences in 4E-BP1 phosphorylation.

The amount of phosphorylated RPS6 (at $Ser^{240/244}$) was not affected ($P \geq 0.14$) by either lysine or leucine supplementation, and no treatment effect ($P \geq 0.13$) was observed for total RPS6. However, for the percentage of RPS6 phosphorylated, a tendency was observed for a lysine × quadratic leucine ($P = 0.07$) interaction because the intermediate leucine treatment decreased the percentage of phosphorylation when lysine was not supplemented, but 6 g/d of lysine supplementation increased the ratio. Lysine provision tended ($P = 0.08$) to decrease total RPS6 concentrations.

Opposite of our results, Suryawan and Davis (2014) observed that leucine supplemented to neonatal pigs increased RPS6 phosphorylation in skeletal muscle of both 6- and 26-day old pigs. Authors noted that phosphorylation decreased as pigs aged. The lack of response observed in our study may have in part been due to the age and stage of development of our steers; leucine may have been more beneficial closer to birth. In an *in vitro* study conducted using C2C12 myotubes, addition of lysine to the media increased p70 RPS6 kinase 1 ($p70^{S6K1}$) activation, and was associated with an overall increase in protein anabolism (Sato et al., 2014). Another study

noted that lysine provision to rats fed a low protein diet increased $p70^{S6K1}$ activity to that of rats fed a high protein diet (Sato et al., 2015). It is unknown why our low and high leucine treatments, when supplemented alongside lysine, decreased phosphorylation when compared to no lysine provision.

It is possible that we did not observe differences in protein synthesis due to the developmental stage of our cattle and the muscle sampled. Nutrient allocation is used to partition nutrients to parts of the body requiring them most at a particular physiological stage. This changes throughout development. Because of this, it is possible that mTOR may have been more active in other muscles during the physiological state of our steers.

No differences were observed in total MuRF1 ($P \geq 0.18$) or total MAFbx ($P \geq 0.19$), indicating that our treatments did not alter protein degradation. Agreeing with our findings, Suryawan and Davis (2014) observed no response in MAFbx when leucine was provided to pigs. On the other hand, Baptista et al. (2010) observed a decrease in MAFbx activity when immobilized mice were supplemented with leucine before, throughout, and after hindleg immobilization. Sugawara et al. (2009) did not observe any differences in MuRF1 activity when leucine was supplemented to swine fed a protein free diet for 1 wk. In contrast, LPS challenged pigs fed additional leucine had lower MuRF1 activation than control pigs or LPS challenged pigs not supplemented with leucine (Hernandez-García et al., 2016).

The E3 ubiquitin ligase pathway can be triggered by phosphorylation and dephosphorylation of the mTOR pathway. Therefore, considering we did not see any differences among treatments in the other mTOR proteins observed in this study, it is not surprising that MuRF1 and MAFbx expression did not change.

Conclusions

In conclusion, lysine supplementation increased protein deposition in growing steers deficient in lysine, whereas leucine was without effect. Leucine did not affect protein deposition or alter activation of the mTOR pathway. No effects of lysine supplementation were observed for total, phosphorylated, or percent of phosphorylated Akt, mTOR, RPS6, MuRF1, or MAFbx; thus, we can surmise that lysine's effect on N retention was not driven by mTOR-related regulation. It is possible that the lysine-limiting conditions of our experiment prevented responses to leucine, that other conditions of our experiment may have prevented responses to leucine, that decreases in plasma concentrations of valine and isoleucine in response to leucine supplementation offset benefits of leucine supplementation, or that ruminants do not utilize mTOR as a key pathway for regulating muscle synthesis.

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Table 2.1. Composition of experimental diet and chemical composition

Item	% of dietary DM
Ingredient	
Soybean hulls	82.25
Wheat straw	8.63
Molasses	4.00
Vitamin/mineral premix	5.12
Calcium phosphate	1.96
Sodium bicarbonate	1.24
Calcium carbonate	1.03
Magnesium oxide	0.41
Selenium premix (600 mg Se/kg)[1]	0.010
Bovatec-91[2]	0.018
Trace mineralized salt[3]	0.22
Vitamin A (30,000 IU/g)[4]	0.017
Vitamin D (30,000 IU/g)[5]	0.012
Vitamin E (44.1 IU/g)[6]	0.10
Analyzed composition (± SD across 6 periods)	
DM, % as is	88.1 ± 0.52
OM	90.6 ± 0.69
NDF	66.9 ± 2.02
ADF	47.9 ± 2.41
CP[7]	9.6 ± 0.09

[1]Provided 0.06 mg Se/kg diet DM from sodium selenite.
[2]Bovatec-91 = 20% lasalocid (Zoetis, Parsippany, NJ). Provided 36 mg lasalocid/kg diet DM.
[3]Contained > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.
[4]Provided 4,950 IU vitamin A/kg diet DM.
[5]Provided 3,450 IU vitamin D/kg diet DM.
[6]Provided 45 IU vitamin E/kg diet DM.
[7]Steers were fed 2.8 kg/d of diet (as fed basis). Urea (10 g/d) was provided via continuous ruminal infusions, and it was considered part of the basal diet, although not included in this table. The infused urea represented 0.41% of dietary DM. Dietary CP with the infused urea included as part of the diet was 11.3% of DM.

Table 2.2. Daily abomasal and ruminal infusates

Item	Amount provided
Abomasal infusions[1]	g/d
Basal amino acids to all steers	
L-methionine	10
L-threonine	15
L-histidine-HCl-H_2O	8
L-phenylalanine	20
L-tryptophan	5
L-leucine	20
L-isoleucine	15
L-valine	15
L-arginine	15
L-glutamic acid	120
Glycine	40
Dextrose	300
50% (wt/wt) NaOH	34
HCl, 6 M	500
Water	2,593
Basal vitamins to all steers	mg/d
Pyridoxine-HCl[2]	10
Folic acid[3]	10
Cyanocobalamin[2]	0.1
Treatments (added 300 g/d to abomasal infusions)	g/d
L-Lysine-HCl (0 or 6 g/d lysine)[4]	0 or 7.5
L-Leucine[5]	0, 15, or 30
Basal ruminal infusion	g/d
Acetic acid	150
Propionic acid	150
Butyric acid	50
Urea	10
Water	3,640

[1]Infusions were continuously infused.

[2]Pyridoxine-HCl/ cyanocobalamin solution was prepared by mixing 10 mg pyridoxine-HCl and 0.1 mg cyanocobalamin with 2 mL of water. Solution was stored in a refrigerator until time of infusion.

[3]Folic acid solution was prepared by mixing 40 mg NaOH with 2 mL water and allowed to cool. 10 mg folic acid was added and mixed into the mixture. Solution was stored in a refrigerator until time of infusion.

[4]Lysine solution was prepared by dissolving 7.5 g lysine-HCl into 92.5 g of hot water. Solution was stored in a refrigerator until time of infusion.

[5]Leucine treatment was prepared by dissolving 30 g leucine in 120 g of hot water containing 50 g of 6 *M* HCl. 200 g/d leucine solution was used to provided 30 g leucine/d, whereas 100 g/d leucine solution (plus 100 g/d water) was used to provide 15 g leucine/d. Solution was stored in a refrigerator until time of infusion.

Table 2.3. Effects of postruminal lysine and leucine supplementation on intake, digestion and N retention in growing steers

	Leucine, g/d											
	0		15		30							
	Lysine, g/d							*P*-value				
Item	0	6	0	6	0	6	SEM	Lysine	Linear leucine	Quadratic leucine	Lys × linear leucine	Lys × quadratic leucine
n	7	7	7	7	7	6						
Intake, g/d[1]												
DMI	2445	2470	2433	2466	2405	2457	17.8	<0.01	0.09	0.67	0.37	0.80
OMI	2218	2238	2209	2235	2185	2228	14.6	<0.01	0.10	0.69	0.36	0.79
Apparent digestibility, %[2]												
DM	72.1	71.3	74.1	72.5	73.0	73.3	1.65	0.42	0.20	0.36	0.62	0.47
OM	74.0	73.3	76.0	74.4	74.8	75.2	1.69	0.49	0.24	0.37	0.62	0.44
N, g/d												
Feed+urea[3]	44.4	44.2	43.8	44.7	43.8	44.6	0.39	0.10	0.83	0.95	0.19	0.37
Infused[4]	33.8	34.2	35.1	36.2	36.8	37.1	0.35	0.04	<0.01	0.44	0.81	0.23
Total intake	78.2	78.4	78.9	80.9	80.6	81.7	0.52	0.01	<0.01	0.58	0.37	0.10
Urine	33.4	30.8	37.1	30.0	37.6	32.5	1.22	<0.01	0.03	0.99	0.73	0.42
Fecal	22.4	22.3	21.5	22.7	21.7	21.8	0.69	0.44	0.29	0.98	0.89	0.20
Retained	20.4	25.4	20.4	27.2	21.2	27.5	1.59	<0.01	0.22	0.86	0.60	0.55

[1]Amount provided from diet.

[2]Calculated using intake of diet only (i.e., infusions were not considered).

[3]Ruminally infused urea was considered part of the basal diet.

[4]Amount provided from abomasal infusions.

Table 2.4. Effects of postruminal lysine and leucine supplementation on plasma glucose, urea, and amino acid concentrations in growing steers

	Leucine, g/d											
	0		15		30							
	Lysine, g/d							*P*-value				
Item	0	6	0	6	0	6	SEM	Lysine	Linear leucine	Quadratic leucine	Lys × linear leucine	Lys × quadratic leucine
n	7	7	7	7	7	6						
Glucose, m*M*	5.37	5.6	5.36	5.49	5.60	5.56	0.15	0.28	0.27	0.32	0.31	0.73
Urea, m*M*	2.70	2.3	2.78	2.35	2.84	2.35	0.15	<0.01	0.21	0.74	0.63	0.84
Amino acid, µ*M*												
Lys	45.1	67.3	42.3	59.3	38.1	65.6	4.96	<0.01	0.28	0.35	0.51	0.26
Leu	58.3	62.7	78.9	79.0	107.9	103.5	4.56	1.00	<0.01	0.15	0.20	0.97
Ile	78.0	92.9	58.1	62.1	55.4	56.2	3.80	0.01	<0.01	<0.01	0.01	0.41
Val	323	329.2	257.1	239.4	237.8	222.0	12.11	0.15	<0.01	<0.01	0.15	0.32
Tau	43.3	36.2	39.0	34.3	39.69	34.98	3.17	0.01	0.31	0.35	0.60	0.75
Asp	15.2	12.9	13.1	11.9	11.5	12.5	0.68	0.08	<0.01	0.28	0.01	0.58
Thr	157.5	163.9	140.9	143.4	131.9	136.8	9.28	0.25	<0.01	0.20	0.88	0.70
Ser	123.5	85.6	125.6	88.2	116.2	88.4	5.01	<0.01	0.52	0.25	0.16	0.45
Asn	35.4	38.5	33.9	33.8	33.1	32.2	1.81	0.62	0.02	0.53	0.26	0.69
Glu	89.9	97.7	78.4	94.7	80.7	100.5	7.31	<0.01	0.44	0.12	0.16	0.72
Gln	333.9	348.3	314.3	325.8	299.9	321.7	10.08	0.03	<0.01	0.42	0.66	0.65
AAA[1]	2.0	2.8	3.1	1.9	2.8	2.0	1.15	0.44	0.99	0.83	0.24	0.26
Gly	626.5	569.1	621.3	570.6	557.9	529.2	32.89	0.02	0.02	0.20	0.53	0.85
Ala	181.5	209.5	156.8	180.5	147.1	164.6	7.17	<0.01	<0.01	0.12	0.31	0.91
Cit	80.4	74.3	72.6	72.0	74.4	71.6	4.07	0.19	0.14	0.25	0.58	0.44
Met	40.5	48.4	34.4	41.5	34.0	39.3	2.13	<0.01	<0.01	0.10	0.47	0.86
Tyr	93.6	96.2	86.5	79.4	80.5	75.6	5.05	0.38	<0.01	0.35	0.39	0.44
Phe	75.5	84.7	72.9	73.9	74.3	68.9	4.11	0.61	0.03	0.45	0.06	0.89
Trp	59.0	63.5	60.7	55.2	56.9	61.3	3.26	0.64	0.47	0.38	0.98	0.06
Orn	78.4	83.1	75.8	77.06	71.0	84.4	7.11	0.18	0.60	0.58	0.45	0.43
His	101.6	110.8	106.5	103.6	106.0	105.9	3.66	0.48	0.93	0.73	0.19	0.22
Arg	87.5	105.1	85.6	94.2	78.4	94.8	7.84	<0.01	0.10	0.74	0.92	0.39
Total AA	2,723	2,783	2,558	2,522	2,435	2,471	74.23	0.70	<0.01	0.18	0.87	0.40

[1]AAA = α-Aminoadipic acid

Table 2.5. Effects of postruminal lysine and leucine supplementation on regulatory proteins in *longissimus lumborum* muscle of growing steers

	Leucine, g/d											
	0		15		30							
	Lysine, g/d							*P*-value				
Item	0	6	0	6	0	6	SEM	Lysine	Linear leucine	Quadratic leucine	Lys × linear leucine	Lys × quadratic leucine
mTOR pathway signaling[1]												
Akt, AU[2]												
Total	1.24	1.26	0.52	1.71	1.78	1.55	0.17	0.11	0.12	0.17	0.59	0.01
Phosphorylated[3]	1.84	1.37	1.28	2.09	1.99	1.68	0.12	0.98	0.67	0.95	0.87	0.23
Ratio phosphorylated	136.04	120.87	174.97	119.07	109.11	160.34	9.64	0.80	0.86	0.62	0.30	0.20
mTOR, AU												
Total	2.13	2.18	1.62	0.94	2.42	2.12	0.20	0.71	0.91	0.29	0.86	0.75
Phosphorylated[4]	1.20	1.02	2.01	1.05	1.20	0.71	0.16	0.19	0.75	0.25	0.76	0.46
Ratio phosphorylated	162.81	120.85	153.58	195.87	182.69	63.45	17.96	0.37	0.73	0.36	0.48	0.19
4E-BP1, AU												
Total	1.51	0.58	1.27	1.14	1.48	1.14	0.13	0.08	0.40	0.91	0.36	0.34
Phosphorylated[5]	0.93	0.81	0.81	0.87	1.02	0.94	0.03	0.66	0.41	0.46	0.90	0.50
Ratio phosphorylated	105.15	115.01	68.68	90.13	89.33	72.00	6.74	0.63	0.02	0.13	0.25	0.22
RPS6, AU												
Total	3.44	5.34	3.80	3.73	5.02	8.04	0.64	0.16	0.13	0.16	0.69	0.30
Phosphorylated[6]	0.97	0.61	0.48	0.98	1.01	1.32	0.11	0.58	0.14	0.26	0.32	0.23
Ratio phosphorylated	70.24	42.29	43.76	107.56	97.66	77.69	10.04	0.81	0.25	0.87	0.88	0.07
MuRF1, AU	1.10	1.20	1.14	1.19	1.09	0.87	0.04	0.82	0.18	0.34	0.21	0.64
MAFbx, AU	0.86	1.04	1.13	1.15	0.91	1.18	0.05	0.19	0.52	0.25	0.73	0.41

[1]Akt = protein kinase B, mTOR = mammalian target of rapamycin, RPS6 = ribosomal protein S6, 4E-BP1 = eukaryotic translation initiation factor 4E binding protein, MuRF1 = muscle ring finger-1, MAFbx = muscle atrophy F-box

[2]AU = arbitrary unit.

[3]Akt phosphorylated at Thr^{308}.

[4]mTOR phosphorylated at Ser^{2448}.

[5]4E-BP1 phosphorylated at $Thr^{37/46}$.

[6]RPS6 phosphorylated at $Ser^{240/244}$.

Figure 2.1. The effect of leucine supplementation on lysine efficiency in growing steers fed soybean hull-based diets. Lysine retention was calculated assuming protein deposition is 6.25 × nitrogen retention and 6.4% of protein deposition is lysine (Ainslie et al., 1993). Efficiency of supplemental lysine utilization averaged 40% and was not affected by amount of supplemental leucine.

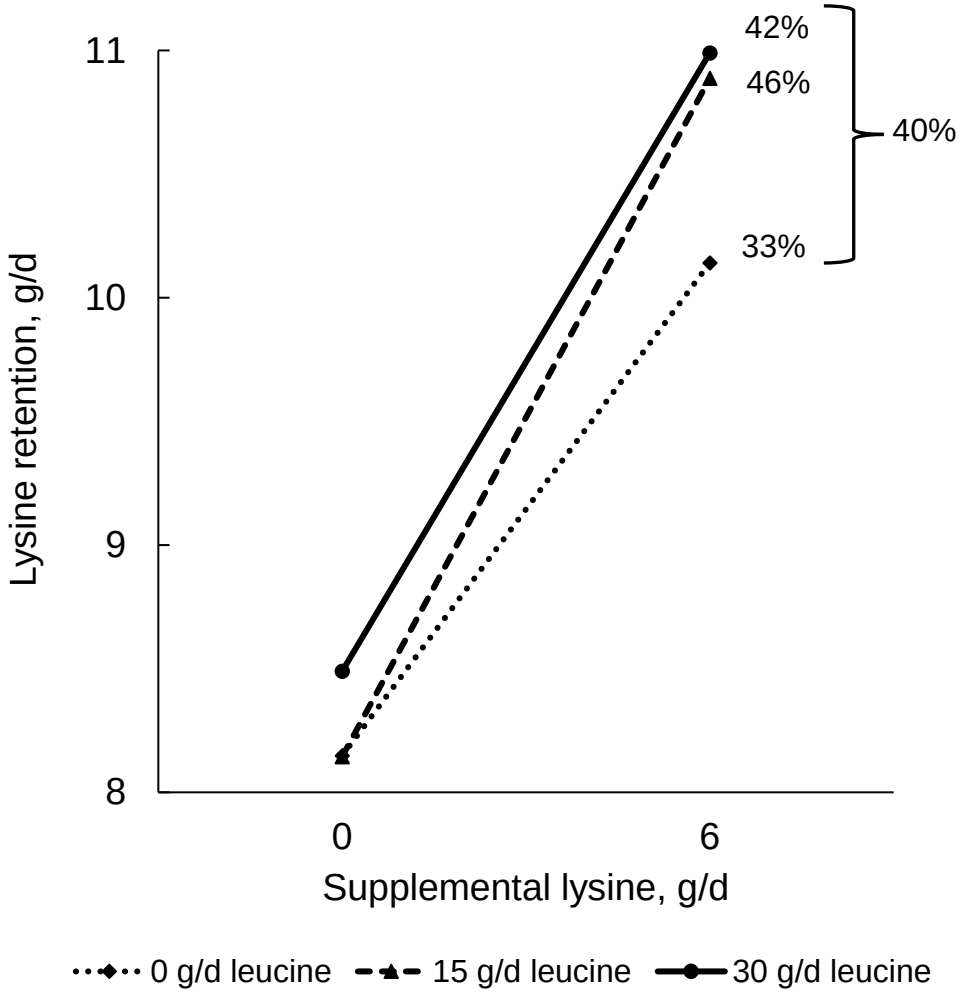
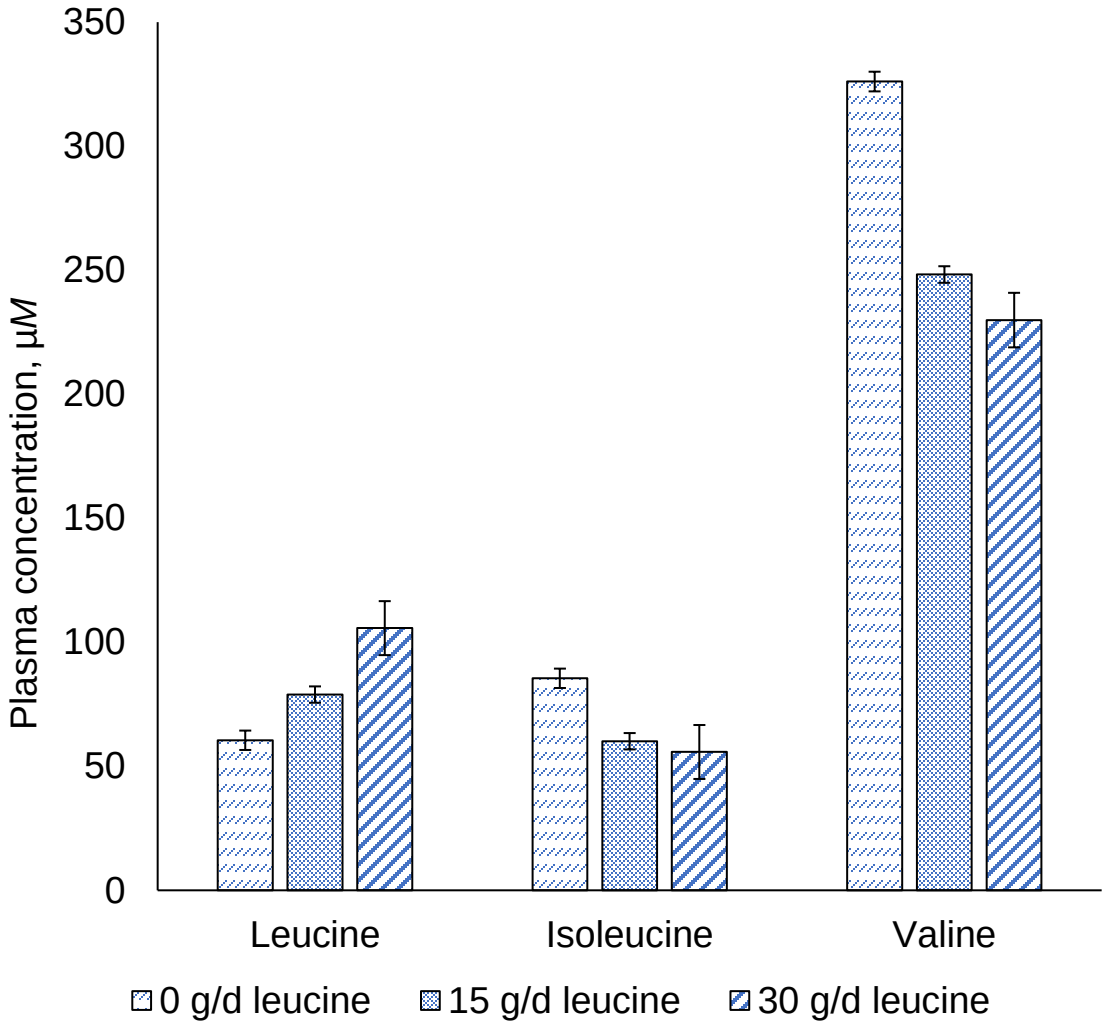


Figure 2.2. Effects of postruminal leucine supplementation on plasma concentrations of branched chain amino acids in growing steers fed soybean hull-based diets. Leucine supplementation linearly ($P < 0.01$) increased plasma leucine and decreased plasma isoleucine and valine concentrations.



Chapter 3 - Effects of excess essential amino acids and leucine on nitrogen retention, efficiency of lysine utilization, and mTOR-related regulation of muscle in growing beef cattle

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ABSTRACT

Seven ruminally cannulated Holstein steers (initial body weight = 202 ± 3.8 kg) were used in an experiment using a 6 × 6 Latin square design. Factorial treatments consisted of 2 levels of lysine (0 or 6 g/d) and 3 levels of amino acid (AA) supplementation [control (no additional AA), essential AA mix (EAA; 103 g/d mix of all essential AA except lysine and leucine), or EAA plus Leu (EL; mix of all essential AA except lysine)] abomasally infused. Steers were limit fed 2.6 kg DM/d of a soybean hull-based diet, and additional nutrients were supplied via ruminal and abomasal infusions. To provide additional energy without altering microbial protein synthesis, all steers were provided 350 g/d of VFA ruminally, as well as abomasal infusions including 300 g/d glucose. Basal infusions of all essential AA except lysine were continuously infused abomasally to ensure lysine was the only limiting AA. Leucine was provided in the basal infusion at a rate of 20 g/d, which exceeded the steers' requirement. The AA supplementation treatments were designed to determine if excess AA with or without leucine served a regulatory role for muscle protein synthesis and ultimately whole-body protein deposition. Treatment periods lasted 7 d, with 2 d of treatment adaptation, 4 d of urine and fecal collection, and 1 d for collection of blood and muscle biopsies from the *longissimus lumborum*. Fecal N excretion was not affected ($P \geq 0.16$) by treatment, so effects on N retention were mediated by changes in N intake and urinary N excretion. Lysine provision increased ($P < 0.01$) N retention, as expected for our lysine-deficient model. Supplementation of EAA or EL tended ($P = 0.06$) to improve N retention responses to Lys supplementation, suggesting an improvement in the efficiency of Lys utilization for growth. Plasma urea N decreased with lysine provision ($P = 0.02$), but EAA and EL ($P < 0.01$) both increased PUN concentrations when compared to control. Supplementation with EAA or EL increased plasma concentration of total AA. No

differences were observed for the muscle concentration of mammalian target of rapamycin (mTOR) or the fraction that was phosphorylated. However, the amount of phosphorylated mTOR tended to decrease ($P = 0.08$) when lysine was supplemented. No differences among treatments ($P \geq 0.12$) were observed for total or phosphorylated protein kinase B (Akt). The fraction of RPS6 phosphorylated at $Ser^{240/244}$ increased ($P = 0.04$) when steers were supplemented with EAA or EL, and the response was greater for EL than for EAA ($P = 0.03$). No differences were observed for the E3 ubiquitin ligases muscle RING finger 1 (MuRF1; $P \geq 0.21$) and muscle atrophy F box (MAFbx; $P \geq 0.11$). Although leucine supplementation to steers receiving EAA did not affect protein deposition, it did further increase the percentage of RPS6 that was phosphorylated in muscle. Supplementation of excess essential AA, with or without leucine, improved the efficiency of lysine utilization and increased the fraction of RPS6 that was phosphorylated in muscle. Leucine may not have affected protein deposition because supplies of AA from the EAA treatment already maximized regulatory stimulation or perhaps negative effects of Leu on Val and Ile concentrations may have limited response.

INTRODUCTION

Amino acids (AA) are required for protein synthesis and deposition to occur, and requirements for cattle have yet to be fully described. The two most commonly limiting AA in most cattle diets are lysine and methionine. Other essential AA have not been extensively researched, because supplementation is not usually necessary. However, there is evidence that the efficiency of AA utilization can be altered by dietary conditions (Chapter 2, Awawdeh et al., 2006; Batista et al., 2016). Some evidence (Awawdeh et al., 2006) suggests that supplemental essential AA may increase the efficiency of methionine and leucine utilization in growing cattle. Leucine, specifically, has been observed to increase protein synthesis by regulating protein synthesis via the mammalian target of rapamycin (mTOR) pathway through a series of phosphorylation events (Columbus et al., 2015). In contrast, Wilson et al. (2010) observed no differences in mTOR phosphorylation when leucine was supplemented to pigs, likely due to a leucine-induced decrease in concentrations of other essential AA. In addition, nitrogen (N) retention was reduced in rats provided branched chain AA (BCAA) in amounts large enough to yield antagonistic effects among BCAA (Blackburn et al., 1979). By providing mixes of essential AA, it may be possible to increase phosphorylation within the mTOR pathway while preventing detrimental effects brought on by excess leucine. Awawdeh et al. (2006) observed an increase in N retention in steers maintained in methionine- and leucine-deficient models when excess AA were provided.

Our objective was to determine the effects of supplemental leucine in addition to excess essential AA on lysine utilization and regulatory features related to muscle protein synthesis in growing steers. We hypothesized that essential AA would increase protein synthesis to

ultimately increase whole-body protein deposition of growing steers and that the response would be greater when leucine was included as part of the supplement.

MATERIALS AND METHODS

Animals and Treatments

The Kansas State University Animal Care and Use Committee approved animal care and use in this study.

Seven Holstein steer calves (initial BW = 201.7 ± 3.8 kg, initial age = 7 mo) were used in an AA infusion study. Steers were housed in a temperature-controlled room (18.1°C) in individual tie stalls with rubber mats during the adaptation period. After 2 wk of adaptation to the room, steers were ruminally cannulated (Bar Diamond, Parma, ID) by the Kansas State University School of Veterinary Medicine. Calves were allowed to recover in tie stalls for 4 wk before being placed in individual metabolism crates for total collection of urine and feces for the duration of the study. Adaptation to the experimental diet lasted 2 wk and took place prior to placing steers in metabolism crates. Ruminal and abomasal infusion lines (internal diameter = 2.38 mm, outside diameter = 3.97 mm; Tygon S3^{TM} E-3603 flexible tubing; Fisher Scientific, Pittsburgh, PA) were inserted through the ruminal cannula. Steers were housed in metabolism crates for the duration of the study and were allowed to adapt for 1 wk prior to the start of the study. This study was a 6 × 6 Latin square design balanced for carry over effects with steers randomly assigned to 1 of 6 treatments in each of 6 periods. The seventh steer was provided a treatment sequence identical to one of the other steers. Each treatment period lasted for 7 d (2 d for treatment adaptation, 4 d for urine and fecal collection, and 1 d for blood and muscle biopsy collections). Short adaptation periods were adequate for this study because cattle adapt quickly to postruminal infusion of AA (Schroeder et al., 2007). The short periods allowed steers to maintain

a consistent physiological state throughout the trial and are important for optimizing animal welfare.

Diet Composition and Basal Infusions

The experimental diet consisted of soybean hulls, wheat straw, molasses, and a vitamin and mineral premix (Table 3.1) provided as a total mixed ration. Animals were provided ad libitum access to water and 2.6 kg DM/d of the experimental diet in equal portions at 12-h intervals. Urea was provided at a rate of 10 g/d through continuous ruminal infusions, and this was considered part of the basal diet (Table 3.3). All steers received a daily infusion of B-vitamins, glycine, glutamate, and all essential AA except lysine to ensure lysine was the only limiting AA. Ruminally degradable protein in the diet was designed to be adequate. To supply additional energy without increasing ruminal microbial protein production, 300 g/d of dextrose was infused into the abomasum of each steer. Steers also received continuous ruminal infusions of VFA; these VFA infusions consisted of 150 g acetic acid, 150 g propionic acid, and 50 g butyric acid daily, and the ruminal infusates contained 10 g/d urea as described above. Daily ruminal infusion solutions were diluted with water to reach a final weight of 4 kg (Table 3.3).

Treatment Allocation

Our study utilized a 6 × 6 Latin square design. Treatments were arranged as a 2 × 3 factorial. The 3 essential AA treatments included a control (no AA supplemented), supplementation of 103 g/d of an essential AA mixture not including lysine or leucine (EAA), and the EAA treatment with the addition of 30 g/d supplemental leucine (EL). Each of these 3 AA treatments were provided with either 0 or 6 g/d lysine supplementation to make our 6 treatments. The EAA and EL treatments only differed in the leucine provision. Treatments were provided continuously along with the basal abomasal infusions.

Abomasal infusions containing both basal infusions as well as the treatments had a total weight of 4 kg/d. All infusion solutions were mixed daily and were continuously infused at a rate of 2.78 mL/min using a peristaltic pump (Model ISM444A-230V; Cole-Parmer Instrument Company, Vernon Hills, IL). Fresh infusates were provided at 0700 and 1900 h. Infusion lines were flushed with 20 mL water once daily to prevent clogging.

Preparation of Abomasal Infusion Solutions

For basal AA solutions (Table 3.3), BCAA were mixed into hot water combined with the HCl. Once dissolved, the remaining AA were mixed into solution according to Chapter 2. Lysine, leucine, and EAA treatments were mixed separately. The lysine solution was prepared by dissolving 7.5 g L-lysine-HCl (providing 6 g/d lysine) into 92.5 g of hot water. The leucine solution was prepared by dissolving 30 g leucine in 120 g of hot water containing 50 g of 6 *M* HCl. The EAA treatment solution (Table 3.2) was mixed by adding 36 g of 6 *M* HCl with 694 g hot water, and 15 g L-valine and 15 g L-isoleucine were dissolved into solution. Once dissolved, 10 g L-methionine, 15 g L-threonine, 8 g L-histidine-HCl-H_2O, 20 g L-phenylalanine, 15 g L-arginine, and 5 g L-tryptophan were mixed into solution until dissolved. Lastly, 17 g of 50% (wt/wt) NaOH was added and mixed until the solution was clear. Treatment solutions were mixed together for infusion dependent on treatment. Water was added until the infusate weight equaled 4 kg. Abomasal infusates were refrigerated until use.

Sample Collection

Samples were collected as described in Chapter 2. In brief, complete collections of urine and feces were made daily on d 3 through 6 of each period. Each was weighed prior to sampling. Urine was collected daily into buckets containing 900 mL of 10% (wt/wt) H_2SO_4 to preserve N. Feces were collected in plastic bags lining metal pans behind the steers. Feed samples and orts

were collected from d 2 through 5 of each period. Feed, orts, urine, and feces were stored at -20°C immediately following collection and pooled appropriately prior to analysis. Jugular blood samples were collected on d 7 of each period, 2 h after the morning feeding. Muscle biopsy samples were collected 7 h after the morning feeding. Chapter 2 provides details on blood and muscle biopsy sample collections. Briefly, muscle biopsy samples were collected from the *longissimus lumborum* muscle, on alternating sides of the spine each week. All steers received a subcutaneous injection of lidocaine (1 mL, 1% solution) prior to muscle biopsy sampling to prevent pain. Once collected, biopsy samples were flash frozen using liquid N_2 and stored at -80°C until analysis. At the conclusion of our study, steers were housed in tie stalls for 1 wk to ensure biopsy sites had healed.

Sample Analyses

Concentrations of DM, OM, and N were analyzed in duplicate for feed, orts, feces, and urine as described in Chapter 2. Western blot analyses were conducted in order to determine the abundance of total and phosphorylated Akt, mTOR, 4E-BP1, and RPS6, as well as total MuRF1 and MAFbx proteins in our muscle biopsy samples. Alpha tubulin (polyclonal rabbit; Cell Signaling) was used as an internal standard and was included in the primary antibody at a dilution of 1:2500. Western blots were relative to the pooled sample as well as alpha tubulin and were otherwise conducted according to Chapter 2. Jugular plasma was analyzed to determine concentrations of AA, BUN, and glucose as described in Chapter 2.

Calculations

Proteins were standardized to gel by dividing each total and phosphorylated protein value by the alpha tubulin value for the corresponding gel, and those alpha tubulin-standardized values were then divided by the alpha tubulin-standardized value for the pooled sample from the same

gel. Percent phosphorylation of proteins was calculated by dividing the phosphorylated protein value by the total protein; these ratios do not represent actual percentages because both values are arbitrary units. All other calculations were conducted as described in Chapter 2.

Statistical Analysis

The MIXED procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NC) was used to analyze data. The study was analyzed as a Latin square design. Fixed effects in the model were period, AA treatment, lysine treatment, and the AA × lysine interaction; animal was a random effect. Orthogonal contrasts used to evaluate AA treatment effects included: control vs. the average of EAA and EL, and EAA vs. EL. Interactions of these contrasts with lysine were also evaluated. Significance was declared at $P \leq 0.05$ and tendencies were identified as $0.05 < P \leq 0.10$. One steer was removed from the study after period 3, as well as two steers removed from periods 1 and 2, and one steer removed from period 3 due to digestive problems. Therefore, 34 of 42 possible observations were collected. Outliers from the western blot dataset were identified and removed (studentized residual ≤ -3 or ≥ 3). In total, 10 outliers were removed from the dataset.

RESULTS AND DISCUSSION

Nutrient Intake and Digestibility

Intakes of DM ($P \geq 0.15$) and OM ($P \geq 0.14$) did not vary depending on treatment (Table 3.4). This response was expected considering our steers were limit fed and only an average of 2.6% of feed was refused during the experiment. Digestibility of DM and OM averaged 71.9% and 73.7%, respectively, and did not differ ($P \geq 0.26$) among treatments. These digestibilities are similar to Chapter 2, as well as to Batista et al. (2015) with growing steers fed a similar diet.

Feed N (including ruminally infused urea) was similar among treatments ($P \geq 0.15$, Table 3.4). By design, the lysine and AA treatments increased N intakes. Urinary N decreased ($P < 0.01$) when lysine was supplemented, and fecal N was unaffected by lysine ($P = 0.47$). A decrease in urine N, along with no changes in fecal N, led to increases in N retention. Previous work within our lab (Chapter 2; Batista et al., 2016) observed similar responses when growing steers were maintained in a similar lysine-deficient model. N retention increased ($P < 0.01$) when lysine was supplemented. Lysine was provided in the current study in concentrations within the linear response range, and clearly below the supplemental requirement of 9 g lysine/d (Batista et al., 2016). Because lysine was a limiting AA in our study, these results were expected. An increase in supply of a limiting AA will increase the deposition of nonlimiting AA, enabling greater protein deposition.

Intake N increased ($P < 0.01$) with the AA treatments (Table 3.4), which was expected because we were supplying the steers with more N. Urinary N increased ($P < 0.01$) when the EAA or EL treatment was administered compared to the control. Fecal N did not change ($P \geq 0.60$) with AA supplementation with or without additional leucine, demonstrating absorption of the infused AA and urine as the mode of excretion for excess N. Nitrogen retention was unaffected ($P \geq 0.27$) by the main effect of AA treatment, but a tendency ($P = 0.06$) for a lysine × (control vs. EAA+EL) interaction was observed, where AA treatments increased N retention when lysine was supplemented but not when lysine was not supplemented. These data indicate that excess AA supplementation improved efficiency of lysine utilization. Li and Jefferson (1978) observed an increase in protein turnover when rats were provided infusions of AA, including BCAA, but was without effect once BCAA were removed from the solution. Freund et al. (1980) provided injured rats jugular infusions containing glucose mixed with 22, 35, or 100%

BCAA. Contrary to our results, injured rats receiving BCAA infusions maintained N equilibrium when provided BCAA in adequate amounts, whereas those fed the BCAA deficient diet had negative N balance (Freund et al., 1980). In addition, Blackburn et al. (1979) fed rats a diet to either meet AA basal requirements or the same diet with excess BCAA. Nitrogen retention did not differ between diets, possibly due to a leucine-induced decrease in valine and isoleucine (Blackburn et al., 1979). Differences between studies may be due to a deficiency in AA to meet basal requirements, whereas in the current study, AA were provided by the basal infusions to meet or exceed requirements for muscle synthesis. The lack of difference between EAA and EL is unlikely due to an antagonism among BCAA, because EL provided enough valine and isoleucine such that plasma concentrations of those 2 AA were not lower than those for control, even though 30 g/d Leu was included.

Lysine Efficiency

Our model was designed to meet all essential AA requirements, with the exception of lysine, which was designed to be below the requirement even when supplemented at 6 g/d. Batista et al. (2015) observed linear increases in N retention when steers were supplemented with up to 9 g/d lysine in the same research model. Assuming that 6.4% of deposited protein is lysine (Ainslie et al., 1993) and protein deposition is 6.25 × N retention, we estimated that the efficiency of lysine utilization was 50, 98, and 98% for control, EAA, and EL treatments, respectively (Figure 3.1). Using these calculations, we can determine that for every 6 g lysine/d supplemented, approximately 3 g lysine/d is retained for protein deposition in the control treatment. Batista et al. (2015) as well as Chapter 2 each determined lysine utilization to be approximately 40% when lysine deficient growing cattle were fed a similar diet and supplied lysine. Hussein et al. (2016) estimated lysine efficiency to be approximately 51% when no urea

was supplemented. This value is similar to our control treatment as well, and not far from the 40% efficiency Batista et al. (2015) observed. Lysine efficiency was estimated to be 59 and 69% when growing steers were ruminally supplemented with 40 and 80 g urea/d, respectively. MuCuistion et al. (2004) provided steers with no supplemental or 1 g histidine/d along with no supplemental AA, 100 g essential AA+nonessential AA, or 200 g essential AA/d. Steers provided additional AA had improved efficiencies of histidine utilization when compared to the control (36, 56, 98% for control, essential AA+ nonessential AA, and essential AA, respectively; McCuistion et al., 2004). These observations support our results, and therefore suggest that regulatory signals from our treatments increased the efficiency of lysine use. Methionine efficiencies were observed to be 16, 50, and 21% when control, nonessential AA+essential AA, and essential AA infusions were provided to growing steers (Awawdeh et al., 2006). In addition, Awawdeh et al. (2006) observed leucine efficiencies to be 49, 34, and 41% when steers were provided control, nonessential AA+essential AA, and essential AA treatments. However, Awawdeh et al. (2006) observed little difference between AA treatments. Differences between studies may have been because steers in the current study received greater amounts of daily leucine in the basal essential AA solution than those in the study conducted by Awawdeh et al. (2006).

It was previously thought (Batista et al., 2016; Hussein et al., 2016) that the NASEM (2016) overestimates lysine efficiency by growing cattle, with an estimate around 65% based on the body weights of our steers. In contrast, lysine utilization results from the current study indicate that excess essential EAA can increase lysine utilization. However, NASEM (2016) suggests that only BW affects the efficiency of AA utilization, whereas our work shows that an increase in supply of AA can enhance the utilization of lysine.

Plasma Metabolites

With the exception of plasma lysine, none of the plasma metabolites demonstrated highly significant interactions between lysine and the AA treatments for which an obvious explanation was available. Thus, predominantly, main effects of treatments on plasma metabolites are discussed.

Effects of lysine supplementation on plasma metabolites. Increasing lysine supplementation from 0 to 6 g/d increased ($P < 0.05$) circulating concentrations of arginine, glutamine, and glutamate, and increased ($P < 0.01$) lysine concentrations (Table 3.5). We are unsure as to why arginine, glutamine, and glutamate increased in response to lysine treatment, but it may be associated with ureagenesis (Gouillou-Coustans et al., 2002) or the movement of N throughout the body for various other metabolic processes. Plasma glycine and serine concentrations decreased ($P < 0.01$) and leucine concentrations tended ($P = 0.08$) to decrease as lysine was supplemented. A decrease in plasma leucine concentrations when our limiting AA was supplemented might indicate an increase in cellular uptake of leucine, although similar responses were not apparent for the other essential AA. In addition, plasma urea N decreased ($P = 0.02$) with lysine supplementation, likely induced by an uptake of N by tissues for protein deposition with a resultant decrease in urea production. Plasma urea N concentrations are highly associated with N uptake, in which decreased plasma urea N typically indicates an increase in N uptake (Xue et al., 2011).

It has been theorized that plasma concentration of a limiting AA does not increase until that AA is provided to minimally meet requirements (Bergen, 1979). Our results and others (Batista et al., 2015; Hussein et al., 2016) are in contrast with this theory. Batista et al. (2016) observed lysine requirements to be 3.12 g/d when estimated using break point analysis on plasma

lysine concentrations. This value is clearly lower than the observed 9 g lysine/d required by growing steers for protein deposition. Although lysine statistically increased plasma lysine concentrations in our experiment, the increases were not so large as to suggest that the lysine requirements were fully met by 6 g/d of lysine supplementation.

A Lys × (control vs. EAA+EL) interaction was observed for plasma lysine ($P < 0.01$) wherein lysine supplementation increased plasma lysine concentration more for steers receiving control than for those receiving either EAA or EL. As discussed above, lysine provision increased N retention, and more so for EAA and EL than for control. Thus, the increased utilization of Lys caused by the EAA and EL treatments may have limited lysine accumulation in plasma by increasing lysine uptake by tissues for protein deposition.

Effects of AA treatments on plasma metabolites. Plasma concentrations of arginine, histidine, isoleucine, leucine, methionine, ornithine, phenylalanine, serine, taurine, threonine, tyrosine, and valine increased ($P \leq 0.05$) when EAA or EL were supplemented, in comparison to the control. These responses were expected because AA supply was increased by those treatments. Typically, leucine has deleterious effects on valine and isoleucine concentrations (Langer and Fuller, 2000). Though isoleucine and valine were both provided in excess by the EAA treatment, the addition of leucine to infusions (EL vs. EAA) decreased Val and Ile concentrations, likely due to increased catabolism of those two AA as a result of leucine antagonism. Wilson et al. (2010) observed a decrease in most essential AA when excess leucine was provided to neonatal pigs, but in that case the leucine increased protein deposition. In our study, leucine supplementation had no effect on protein deposition, and, outside of effects on BCAA, leucine supplementation had little effect on plasma AA with the exception of modest decreases in threonine and alanine. However, Wilson et al. (2010) also observed that a leucine-

induced decrease in essential AA did not occur when excess essential AA were provided with excess leucine (Wilson et al., 2010), which also matches the situation in our experiment.

Glutamine, glutamate, and lysine decreased ($P \leq 0.05$) when our EAA or EL was provided, compared to the control treatment. Plasma urea N increased ($P < 0.01$) when essential AA were supplemented; this response was expected due to the increased supply of N being greater than the effects of EAA or EL on N retention, thus necessitating greater synthesis of urea to dispose of the excess N.

Alanine, arginine, threonine, isoleucine, and valine each decreased ($P \leq 0.05$) in concentration, and glutamine tended ($P = 0.06$) to decrease, when leucine was supplemented to the EAA treatment (EAA vs. EL) (Table 3.5; Figure 3.2). Circulating leucine was increased ($P < 0.01$) when leucine was provided, likely reflecting the increased supply of leucine. Our results are in contrast with those of Awawdeh et al. (2006), in which a decrease of most essential AA was observed in growing steers when supplemental leucine was provided (Awawdeh et al., 2006). Variations between studies can be attributed to a difference in the models. Awawdeh et al. (2006) used a leucine-deficient model where the supplementation of leucine increased protein deposition, whereas we were supplementing steers receiving adequate amounts of leucine and leucine did not affect protein deposition.

Plasma urea N decreased ($P = 0.02$) as lysine supplementation increased from 0 to 6 g/d suggesting that cellular uptake of N increased when lysine was supplemented. However, a control vs. EAA+EL effect ($P < 0.01$) was observed because addition of the AA treatments led to an increase in plasma urea N. A Lys × (EAA vs. EL) interaction ($P = 0.05$) was also observed, because EL led to numerically greater plasma urea than EAA when no lysine was supplemented

but the reverse was true when lysine was supplemented. Glucose concentrations were not affected ($P \geq 0.17$) by any of the treatments.

Supplementation of excess amounts of essential AA have previously been observed to increase plasma concentrations of the corresponding essential AA. Leucine, on the other hand, has been observed to decrease circulating concentrations of AA. Decreases in a range of AA would be expected if leucine increased protein deposition, whereas leucine can induce decreases in Val and Ile through a classic antagonism with increases in catabolism of all BCAA.

Regulatory Proteins in Muscle

Effects of lysine treatment on protein signaling. Total Akt, phosphorylated Akt (phosphorylated at Thr^{308}), and phosphorylated:total Akt were not affected ($P \geq 0.12$) by treatment (Table 3.6). No effects ($P \geq 0.16$) were observed for total mTOR. Lysine administration tended to decrease ($P = 0.08$) phosphorylated mTOR concentrations, whereas our EAA and EL treatments were without effect ($P \geq 0.21$). Phosphorylated:total mTOR was unaffected ($P \geq 0.24$) by treatment. A lysine × (EAA vs. EL) interaction was observed ($P = 0.02$) for the abundance of total 4E-BP1 because lysine supplementation decreased total 4E-BP1 when EAA was provided but it increased it when EL was provided. Phosphorylated 4E-BP1 abundance was unaffected ($P \geq 0.17$) by all treatments. We observed a tendency for an increase ($P = 0.09$) in the percent of phosphorylated 4E-BP1 when the only limiting AA, lysine, was supplemented to the EAA or EL treatments. In contrast, lysine supplementation was ineffective in increasing phosphorylated 4E-BP1 when provided to control steers, and this matches the lower N retention responses to lysine supplementation in the control steers than in those receiving EAA or EL. No differences ($P \geq 0.13$) were observed among treatments for total RPS6 concentration, and lysine supplementation did not affect the amount of RPS6 phosphorylated at $Ser^{240/244}$ or

phosphorylated:total RPS6 ($P \geq 0.20$). However, supplementing mixtures of AA (EAA or EL) tended ($P = 0.10$) to increase the amount of phosphorylated protein. Phosphorylated:total RPS6 increased ($P = 0.04$) when EAA or EL was supplemented, and it was also greater for EL than for EAA ($P = 0.03$).

Effects of AA treatments on protein signaling. We did not observe a difference in N retention from leucine supplementation, therefore, it is not surprising that we did not observe differences in regulatory proteins. However, lysine increased protein deposition in the current study, and we expected an increase in the phosphorylation of regulatory proteins when steers were provided supplemental lysine; Sato et al. (2015) observed that lysine provision to rats fed a low protein diet increased Akt phosphorylation. We observed a decrease in plasma isoleucine and valine when leucine was supplemented to cattle, and this antagonism among the BCAA may have decreased the effects of our leucine treatment on Akt.

The effect lysine had on phosphorylated mTOR concentrations was opposite of what we had expected. By increasing the provision of the limiting AA, we increased protein deposition, which we presume was associated with increases in protein synthesis. In contrast to our results, Sato et al. (2015) observed an increase in mTOR phosphorylation when rats fed a high protein diet and supplemented with lysine. In the current study, the tendency for a decrease in phosphorylated mTOR in response to the supplemental lysine suggests that mTOR phosphorylation may not be a mandatory precursor to improving growth responses to all essential AA. Lysine may simply increase protein deposition through increases in protein synthesis driven by increases in substrate (lysine) availability. However, it is important to note that protein deposition values reported in the current study represent whole-body protein deposition, whereas signaling pathways were only measured in the *longissimus lumborum*.

Churchward-Venne et al. (2012) observed similar results in which no differences were observed in total or phosphorylated mTOR among treatments when humans were fed a high protein diet with supplemental essential AA (without leucine), or the high protein diet plus supplemental leucine only (Churchward-Venne et al., 2012). It is possible that all AA met the requirements, and that maximum protein synthesis was already obtained from the essential AA solutions provided to all steers. Glynn et al. (2010) observed an increase in mTOR phosphorylation when humans were provided an essential AA solution containing 35% leucine when compared to the control solution containing lower concentrations of essential AA and 18% leucine. However, with regard to our study, it is possible that leucine supplementation stimulated antagonistic effects among the BCAA.

Leucine supplementation in piglets fed a low protein diet increased 4E-BP1 phosphorylation to the same extent as those fed the high protein diet (Torrazza et al., 2010). The low protein diet provided in the work of Torrazza et al. (2010) may have been limiting in multiple AA. Therefore, it may be possible that leucine may have had more of an effect on 4E-BP1 phosphorylation than observed in our study.

Several points may be made from the phosphorylated:total RPS6. First, the increases in response to EAA and EL are suggestive that RPS6 phosphorylation may be a key regulatory mechanism by which the increased sensitivity to lysine for N retention was mediated. However, if that is true, the level of phosphorylation achieved by EAA must have been enough to yield a maximal performance response, because the greater increase in phosphorylation of RPS6 by EL than by EAA was not associated with further improvement in N retention of steers receiving supplemental lysine. It is possible we did not observe differences in protein deposition or in other factors within the mTOR pathway when RPS6 increased because RPS6 is involved in various

other pathways in addition to mTOR, including the AMPK signaling pathway and MAPK-Erk (Weizmann Institute of Science, 2018). Our results are similar to those of Escobar et al. (2006), in which an increase in RPS6 phosphorylation was observed when neonatal pigs were supplemented with leucine (Escobar et al., 2006). Non-diabetic rats infused with leucine had increased concentration of phosphorylated RPS6 compared to those infused with saline (Anthony et al., 2002). Manjarin et al. (2016) observed an increase in phosphorylated RPS6 when neonatal pigs were provided a protein adequate diet compared to those provided a protein restricted diet. Leucine was able to mitigate some of the effects brought about by a low protein diet by increasing the phosphorylation of RPS6, but not to the extent of the protein-adequate diet (Manjarín et al., 2016).

The E3 ubiquitin ligases, MuRF-1 and MAFbx, were not affected by treatment ($P \geq 0.21$ and $P \geq 0.11$, respectively), suggesting that treatment differences in protein deposition were not greatly impacted by differences in protein degradation. Sugawara et al. (2009) did not observe differences in MuRF-1 or MAFbx in response to 1.5% leucine supplemented to rats fed a protein free diet. In contrast to our results, both MuRF-1 and MAFbx were reduced in immobilized rats when they were supplemented with leucine (Baptista et al., 2010).

Conclusions

Essential AA supplementation increased the efficiency of lysine utilization in growing steers. Although the amounts of AA that were supplemented to yield this response would not be economically viable, these results demonstrate that regulatory mechanisms can be enacted to improve the efficiency of AA use by growing cattle.

Supplementation of lysine, which was the limiting AA in our experiment, tended to decrease the amount of phosphorylated mTOR in muscle, but it did not appear to influence phosphorylation of Akt or RPS6 in bovine muscle.

Supplementing a mixture of essential AA that provided excess amounts of AA, with or without leucine, was able to increase the fraction of RPS6 in muscle that was phosphorylated, and this effect was greater when leucine was provided as a part of the mixture. Interestingly, the increases in phosphorylation of RPS6 by supplementation of essential AA was not associated with upstream changes in phosphorylation of mTOR or Akt.

Further work will be needed to better characterize the regulatory signaling associated with the improvements in the efficiency of AA use. Identifying methods, such as modifying nutrient supplies, to optimize AA utilization and growth could improve biological and economic efficiency of cattle production.

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Table 3.1. Composition of experimental diet and chemical composition

Item	% of dietary DM
Ingredient	
Soybean hulls	80.02
Wheat straw	8.27
Molasses	5.82
Vitamin/mineral premix	4.98
Calcium phosphate	1.91
Sodium bicarbonate	1.21
Calcium carbonate	1.00
Magnesium oxide	0.39
Selenium premix (600 mg Se/kg)[1]	0.010
Bovatec-91[2]	0.020
Trace mineralized salt[3]	0.21
Vitamin A (30,000 IU/g)[4]	0.016
Vitamin D (30,000 IU/g)[5]	0.011
Vitamin E (44.1 IU/g)[6]	0.100
Analyzed composition (± SD across 6 periods)	
DM, % as is	91.1 ± 0.47
OM	90.9 ± 0.63
NDF	61.4 ± 0.51
ADF	42.7 ± 0.66
CP[7]	8.5 ± 0.07

[1]Provided 0.06 mg Se/kg diet DM from sodium selenite.
[2]Bovatec-91 = 20% lasalocid (Zoetis, Parsippany, NJ). Provided 35 mg lasalocid/kg diet DM.
[3]Contained > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.
[4]Provided 4,819 IU vitamin A/kg diet DM.
[5]Provided 3,364 IU vitamin D/kg diet DM.
[6]Provided 44 IU vitamin E/kg diet DM.
[7]Steers were fed 2.8 kg/d of diet (as fed basis). Urea (10 g/d) was provided via continuous ruminal infusions, and it was considered part of the basal diet, although not included in this table. The infused urea represented 0.41% of dietary DM. Dietary CP with the infused urea included as part of the diet was 10.0% of DM.

Table 3.2. Treatment composition

	AA Treatment					
	Control		EAA		EL	
	Lysine, g/d					
Item[1]	0	6	0	6	0	6
	g/d					
L-Lysine-HCl	0	7.5	0	7.5	0	7.5
L-Leucine	0	0	0	0	30	30
L-Valine	0	0	15	15	15	15
L-Isoleucine	0	0	15	15	15	15
L-Methionine	0	0	10	10	10	10
L-Threonine	0	0	15	15	15	15
L-Histidine-HCl-H_2O	0	0	8	8	8	8
L-Phenylalanine	0	0	20	20	20	20
L-Arginine	0	0	15	15	15	15
L-Tryptophan	0	0	5	5	5	5
50% (wt/wt) NaOH	34	34	34	34	34	34
6 *M* HCl	122	122	122	122	122	122
Water	994	986.5	891	883.5	861	853.5

[1]Treatments were mixed daily and provided to steers through continuous abomasal infusions that had total weights of 4 kg/d.

Table 3.3. Abomasal and ruminal infusates

Item	Amount provided
Basal abomasal infusion[1]	g/d
L-methionine	10
L-threonine	15
L-histidine-HCl-H_2O	8
L-phenylalanine	20
L-tryptophan	5
L-leucine	20
L-isoleucine	15
L-valine	15
L-arginine	15
L-glutamic acid	120
Glycine	40
Dextrose	300
50% NaOH	34
HCl 6 M	68
H_2O	2,165
Vitamins	mg/d
Pyridoxine-HCl[2]	10
Folic acid[3]	10
Cyanocobalamin[2]	0.1
Basal ruminal infusion[1]	g/d
Acetate	150
Propionate	150
Butyrate	50
Urea	10
Water	3,640

[1]Continuously infused. Basal abomasal infusions were mixed with treatments solutions (Table 3.2) to provide final abomasal infusate weights of 4 kg/d.

[2] Stock solution was prepared by mixing 1 g pyridoxine-HCl and 10 mg cyanocobalamin with 200 mL of water. Solution was refrigerated for storage. Steers were provided 2 mL/d mixed with the abomasal infusates.

[3]Folic acid solution was prepared by mixing 8 g NaOH and 1 g folic acid into water to make 200 mL of solution. Solution was refrigerated for storage. Steers received 2 mL/d mixed with the abomasal infusates.

Table 3.4. Effects of supplementation of lysine and excess essential amino acids on intake, digestion and N retention in growing steers

	EAA Treatment											
	Control		EAA[1]		EL[2]							
	Lysine, g/d							*P*-value				
Item	0	6	0	6	0	6	SEM	Lysine	Con vs EAA+EL	EAA vs. EL	Lys × (control vs. EAA+EL)	Lys × (EAA vs. EL)
n	7	4	5	5	6	7						
Intake, g/d[3]												
DM	2495	2471	2385	2521	2367	2479	0.07	0.15	0.41	0.62	0.17	0.84
OM	2269	2250	2170	2294	2157	2260	0.07	0.14	0.42	0.67	0.18	0.84
Apparent digestibility, %[4]												
DM	72.2	72.0	71.1	74.6	70.3	71.4	2.06	0.37	0.87	0.31	0.46	0.52
OM	74.4	74.3	73.0	77.4	72.8	73.3	2.02	0.32	0.89	0.26	0.45	0.30
Nitrogen, g/d												
Feed+urea[5]	39.8	39.5	38.3	40.2	38.0	39.6	1.02	0.15	0.41	0.63	0.18	0.83
Infused[6]	33.9	34.7	48.4	49.4	51.8	52.3	0.22	<0.01	<0.01	<0.01	0.66	0.23
Intake	73.7	74.3	86.7	89.6	89.9	91.9	1.04	0.02	<0.01	0.01	0.24	0.64
Urine	30.5	23.5	44.3	34.1	48.3	34.9	1.79	<0.01	<0.01	0.19	0.14	0.37
Fecal	22.6	22.2	23.4	21.4	22.6	23.2	1.30	0.47	0.74	0.60	0.84	0.16
Retained	20.6	28.2	19.1	33.8	19.2	33.8	2.07	<0.01	0.27	0.97	0.06	0.99

[1]EAA = mixture of 103 g/d essential AA containing no lysine or leucine (Table 3.3).
[2]EL = EAA plus 30 g/d leucine.
[3]Amount provided from diet (ruminal and abomasal infusions were not considered).
[4]Calculated using intake of diet only (i.e., ruminal and abomasal infusions were not considered).
[5]Ruminally infused urea was considered part of the basal diet.
[6]Amount provided from abomasal infusions.

Table 3.5. Effects of supplementation of lysine and excess essential amino acids on plasma glucose, urea, and amino acid concentrations in growing steers

	AA Treatment											
	Control		EAA		EL							
	Lysine, g/d							*P*-value				
Item	0	6	0	6	0	6	SEM	Lysine	Con vs EAA+EL[1]	EAA vs EL[1]	Lys × (control vs EAA+EL)[1]	Lys × (EAA vs EL)[1]
n	7	4	4	5	6	7						
Plasma												
Glucose, m*M*	5.1	5.0	5.4	5.2	5.2	5.1	0.2	0.28	0.17	0.24	0.59	0.75
Urea, m*M*	2.4	2.0	3.0	3.0	3.3	2.8	0.2	0.02	<0.01	0.59	0.44	0.05
AA, µ*M*												
Lys	29.3	49.2	25.6	34.4	23.2	31.8	2.6	<0.01	<0.01	0.25	0.01	0.96
Leu	50.0	41.4	55.2	49.6	91.8	86.2	4.4	0.08	<0.01	<0.01	0.69	0.99
Ile	63.0	65.4	120.8	128.4	77.2	75.6	5.6	0.56	<0.01	<0.01	0.96	0.41
Val	271.2	247.2	527.8	526.2	355.1	331.4	17.4	0.28	<0.01	<0.01	0.70	0.53
Tau	40.2	37.8	50.4	46.9	58.6	46.2	5.0	0.13	0.01	0.42	0.49	0.33
Asp	8.6	9.1	8.9	9.9	9.1	9.1	0.8	0.49	0.58	0.77	0.99	0.54
Thr	126.6	112.0	215.0	214.4	185.8	192.2	18.4	0.77	<0.01	0.04	0.39	0.75
Ser	116.2	78.2	138.2	94.6	128.1	93.9	11.2	<0.01	<0.01	0.36	0.92	0.39
Asn	27.2	24.4	31.4	28.6	28.4	26.9	3.0	0.25	0.16	0.35	0.87	0.79
Glu	84.2	90.6	61.2	71.8	53.4	65.4	7.0	0.05	<0.01	0.21	0.60	0.89
Gln	259.0	275.0	225.6	270.0	219.8	243.6	13.4	<0.01	<0.01	0.06	0.19	0.19
Gly	545.2	499.2	559.0	444.4	497.2	445.0	54.2	<0.01	0.13	0.26	0.41	0.22
Ala	153.6	182.8	170.2	175.6	143.2	140.4	9.6	0.12	0.13	<0.01	0.05	0.59
Cit	56.0	61.8	64.8	67.4	60.0	65.6	4.0	0.19	0.14	0.40	0.81	0.71
Met	30.2	41.8	103.6	99.8	111.2	77.6	17.2	0.46	<0.01	0.61	0.22	0.27
Tyr	69.4	69.6	87.4	90.9	146.2	89.2	7.4	0.72	<0.01	0.84	0.80	0.98
Phe	63.9	64.8	113.6	101.6	96.6	98.1	8.0	0.62	<0.01	0.19	0.64	0.36
Trp	153.4	121.5	165.7	152.8	190.7	165.1	23.4	0.26	0.16	0.43	0.76	0.79
Orn	62.6	61.6	77.4	80.6	68.0	74.4	6.0	0.49	0.01	0.14	0.49	0.74
His	78.2	76.8	88.6	88.8	88.9	88.8	6.2	0.93	0.05	0.99	0.89	0.99
Arg	68.5	82.8	83.6	102.2	72.6	88.6	6.8	<0.01	0.04	0.05	0.77	0.81
Total AA	3350	3271	3956	3857	3645	3527	140.6	0.27	<0.01	<0.01	0.87	0.93

Least square means and associated standard error for plasma metabolite concentrations in growing steers.

Table 3.6. Effects of supplementation of lysine and excess essential amino acids on mammalian target of rapamycin (mTOR) signaling pathway proteins in *longissimus lumborum* of lysine-deficient growing steers

	AA Treatment							*P*-value				
	Control		EAA		EL							
	Lysine, g/d											
Item	0	6	0	6	0	6	SEM	Lysine	Con vs EAA+EL[1]	EAA vs EL[1]	Lys × (control vs EAA+EL)[1]	Lys × (EAA vs EL)[1]
mTOR pathway signaling[1]												
Akt, AU[2]												
Total	1.33	1.12	1.61	0.91	1.21	1.36	0.09	0.25	0.82	0.93	0.89	0.12
Phosphorylated[3]	0.89	1.24	1.80	1.46	1.49	1.45	0.11	0.97	0.15	0.67	0.41	0.67
Ratio phosphorylated	1.65	1.24	0.78	1.52	1.45	1.21	0.11	0.93	0.57	0.65	0.35	0.22
mTOR, AU												
Total	0.70	0.65	0.79	0.65	0.51	0.56	0.04	0.67	0.68	0.16	0.99	0.45
Phosphorylated[4]	2.06	0.92	1.95	1.47	1.47	1.34	0.16	0.08	0.84	0.42	0.21	0.63
Ratio phosphorylated	2.26	1.71	3.05	2.62	2.67	2.67	0.17	0.60	0.24	0.82	0.79	0.76
4E-BP1, AU												
Total	1.37	1.14	1.52	1.12	1.03	1.39	0.07	0.44	0.97	0.53	0.41	0.02
Phosphorylated[5]	1.34	0.86	1.18	1.09	1.10	1.37	0.07	0.62	0.67	0.67	0.17	0.45
Ratio phosphorylated	1.01	0.80	0.87	1.65	1.03	1.33	0.12	0.17	0.15	0.74	0.09	0.32
RPS6, AU												
Total	0.94	1.35	1.22	1.31	0.77	1.18	0.08	0.13	0.92	0.21	0.68	0.47
Phosphorylated[6]	0.70	1.07	1.02	1.08	1.24	1.58	0.11	0.20	0.10	0.13	0.65	0.55
Ratio phosphorylated	0.78	0.68	1.01	0.94	1.60	1.77	0.17	1.00	0.04	0.03	0.78	0.70
E3 ubiquitin ligases												
MuRF1, AU	1.14	0.81	0.10	0.90	1.00	0.99	0.14	0.21	0.86	1.00	0.43	0.55
MAFbx, AU	1.45	1.52	1.44	1.21	1.25	0.99	0.07	0.37	0.11	0.26	0.30	0.93

[1]Akt = protein kinase B, mTOR = mammalian target of rapamycin, RPS6 = ribosomal protein S6, 4E-BP1 = eukaryotic translation initiation factor 4E binding protein, MuRF1 = muscle ring finger-1, MAFbx = muscle atrophy F-box

[2]AU = arbitrary unit.

[3]Akt phosphorylated at Thr^{308}.

[4]mTOR phosphorylated at Ser^{2448}.

[5]4E-BP1 phosphorylated at $Thr^{37/46}$.

[6]RPS6 phosphorylated at $Ser^{240/244}$.

Figure 3.1. The effect of supplementing excess essential amino acids abomasally, with or without leucine, on lysine efficiency in growing steers fed soybean hull-based diets. Steers were supplemented with no supplemental AA (control), 103 g/d supplemental essential AA (EAA), or EAA + 30 g/d supplemental leucine (EL). Lysine retention was calculated from N retention assuming protein deposition is 6.25 × nitrogen retention, and 6.4% of protein deposition is lysine (Ainslie et al., 1993). Slopes tended ($P = 0.06$) to be improved by supplementation of excess essential amino acids (control vs. EAA and EL) but were not affected ($P = 0.97$) by leucine provision (EAA vs. EL).

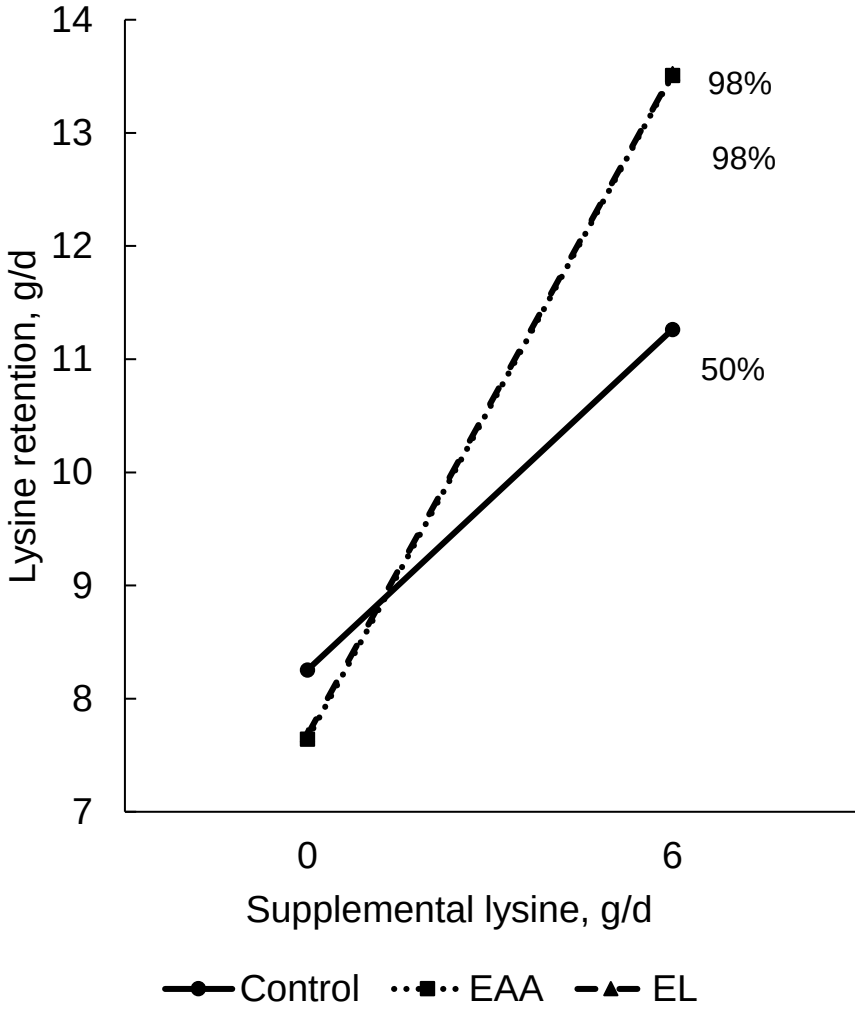

Figure 3.2. Effects of supplementing excess essential amino acids (with or without leucine) abomasally on plasma concentrations of branched chain amino acids in growing steers fed soybean hull-based diets. Steers were supplemented with no supplemental AA (control), 103 g/d supplemental essential AA (EAA), or EAA + 30 g/d supplemental leucine (EL). The addition of supplemental essential AA increased (Con vs. EAA+EL; $P < 0.01$) valine, isoleucine, and leucine concentrations, but the addition of supplemental leucine (EL vs. EAA) decreased ($P < 0.01$) isoleucine and valine concentrations and increased leucine concentrations.