Effect of Orally Administered Branched-chain Amino Acids on Protein Synthesis and Degradation in Rat Skeletal Muscle*

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ABSTRACT : Although amino acids are substrates for the synthesis of proteins and nitrogen-containing compounds, it has become more and more clear over the years that these nutrients are also extremely important as regulators of body protein turnover. The branched-chain amino acids (BCAAs) together or simply leucine alone stimulate protein synthesis and inhibit protein breakdown in skeletal muscle. However, it was only recently that the mechanism(s) involved in the regulation of protein turnover by BCAAs has begun to be defined. The acceleration of protein synthesis by these amino acids seems to occur at the level of peptide chain initiation. Oral administration of leucine to food-deprived rats enhances muscle protein synthesis, in part, through activation of the mRNA binding step of translation initiation. Despite our knowledge of the induction of protein synthesis by BCAAs, there are few studies on the suppression of protein degradation. The recent findings that oral administration of leucine rapidly reduced N^{τ}-methylhistidine (3-methylhistidine; MeHis) release from isolated muscle, an index of myofibrillar protein degradation, indicate that leucine suppresses myofiblilar protein degradation. The details of the molecular mechanism by which leucine inhibits proteolysis is just beginning to be elucidated. The purpose of this report was to review the current understanding of how BCAAs act as regulators of protein turnover.
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Key Words : Branched-chain amino acids, Protein synthesis, mRNA translation, Protein degradation, Skeletal muscle, Rats

INTRODUCTION

Skeletal muscle comprises the majority of protein in the body. Understanding the mechanisms and regulation of protein metabolism in skeletal muscle is essential for increasing the efficiency of protein deposition in meat animals. Changes in muscle protein mass are due to alterations in the balance between protein synthesis and degradation. Thus, both processes determine the efficiency of protein gain. To optimize this efficiency, the mechanisms and regulation of both processes must be known. Both are coordinately regulated in the physiological state, but they have distinct and independent mechanisms.

Recent advances in biomedical research have revealed a key role for amino acids as nutritional signals in the regulation of a number of cellular processes. Amino acids, as well as other nutrients, have attracted growing attention to their regulatory activities on protein synthesis, and proteolysis (Fafournoux et al., 2000; Mordier et al., 2002), and to their signaling mechanisms (van Sluijters et al., 2000). In mammals, amino acids have been shown to stimulate protein synthesis and inhibit proteolysis in skeletal muscle. For example, infusion (Louard et al., 1990, 1995; Watt et al., 1992; Zanetti et al., 1999) or oral administration (Anthony et al., 2000a,b) of amino acids increase muscle protein synthesis. In addition, infusion (Louard et al., 1990;1995) or dietary intake (Goodman and del Pilar Gomez, 1987; Nagasawa et al., 2002) of amino acids decreases muscle protein breakdown.

More specifically, the effect of amino acids on protein synthesis and degradation can, in large part, be attributed to the contribution of branched-chain amino acids (BCAAs). Most of the effects appear to be due to leucine, although valine and isoleucine may also participate (Fulks et al., 1975; Anthony et al., 2000b). In fact, oral administration of leucine to fasted rats mimics the ability of a complete meal to stimulate protein synthesis (Anthony et al., 2000a) and inhibit protein degradation (Nagasawa et al., 2002) in skeletal muscle.

Here, we review our current understanding of how BCAAs act as regulators of protein turnover. In particular, we emphasized studies in which oral administration of leucine to fasted rats has been used as an experimental model.

THE STIMULATORY EFFECT OF LEUCINE ON MUSCLE PROTEIN SYNTHESIS

All of the *in vivo* studies described in this review utilized the same experimental model wherein leucine (135 mg/100 g body weight) was administered to overnight fasted rats by oral gavage. This amount of leucine administered is equivalent to that consumed in a 24 h period when rats are provided free access to food (Anthony et al.,

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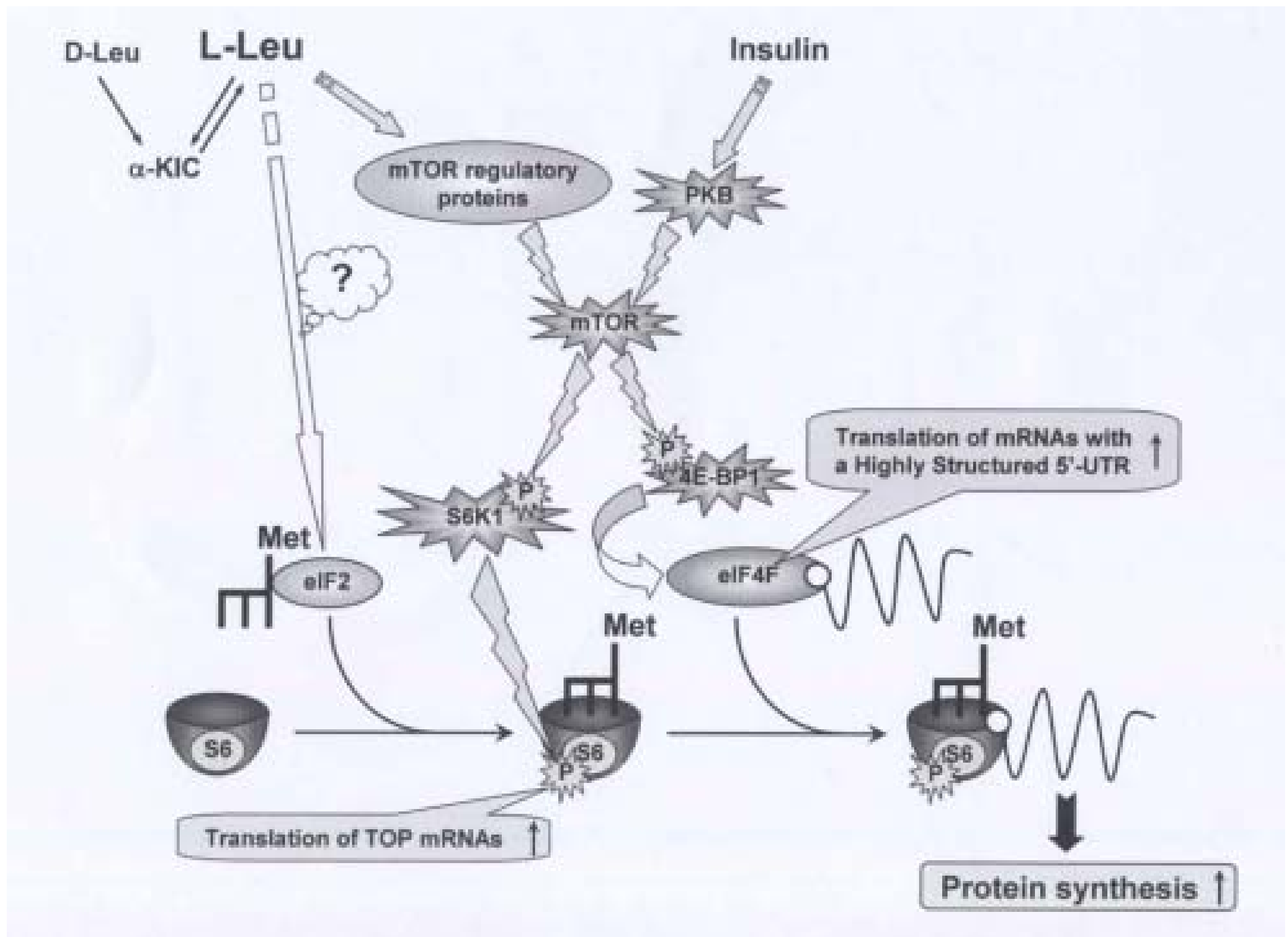

Figure 1. Regulation of protein synthesis through modulation of translation initiation by leucine and insulin. The diagram outlines the mechanisms through which *in vivo* administration of leucine enhances the initiation phase of mRNA translation. The details are discussed in the text. PKB, protein kinase B; mTOR, mammalian target of rapamycin protein kinase; eIF, eukaryotic initiation factor; 4E-BP1, eIF4E binding protein-1; S6K1, 70-kDa ribosomal protein S6 protein kinase; S6, ribosomal protein S6; TOP, terminal oligopyrimidine tract; UTR, untranslated region.

1999). Protein synthesis was measured by the flooding dose technique, which consists of administration of a large bolus of phenylalanine containing radioactive phenylalanine as tracer, and 10 min later measuring the amount of radioactivity incorporated into protein (Garlick et al., 1980). Studies using this approach have shown that oral administration of leucine enhances protein synthesis in skeletal muscle (Anthony et al., 2000a). Furthermore, neither isoleucine nor valine stimulate muscle protein synthesis when administered by oral gavage, indicating that the effect is specific to leucine (Anthony et al., 2000b).

Mechanisms involved in the stimulatory effect of leucine on protein synthesis

The mechanisms involved in the stimulation of protein synthesis by oral administration of leucine have been best characterized in skeletal muscle. In this tissue, leucine administration affects a number of different signaling pathways and mechanisms that regulate the initiation phase of mRNA translation. Two of the many steps involved in translation initiation are particularly important in its regulation.

In the first step of translation initiation, initiator methionyl-tRNA (met-$tRNA_i$) binds to the 40S ribosomal subunit as a ternary complex with eukaryotic initiation factor 2 (eIF2) and GDP (Hinnebusch, 2000). During a subsequent step, the GTP bound to eIF2 is hydrolyzed to GDP and eIF2 is released from the 40S ribosomal subunit as an eIF2-GDP binary complex. For eIF2 to participate in another round of initiation, it must exchange GDP for GTP prior to formation of a new ternary complex. This guanine nucleotide exchange reaction is catalyzed by a second initiation factor, eIF2B. Thus, the activity of eIF2B limits the rate at which met-$tRNA_i$ can bind to the 40S ribosomal subunits. The best characterized mechanism for regulating eIF2B activity is through phosphorylation of α-subunit of eIF2. Phosphorylation of eIF2α converts it from a substrate into a competitive inhibitor of eIF2B, effectively

sequestering eIF2B into an inactive complex. Because translation of essentially all mRNAs begins with met-tRNA$_i$, phosphorylation of eIF2α results in a decline in the synthesis of almost all proteins.

A second regulated step in translation initiation involves the binding of mRNA to the 40S ribosomal subunit, a reaction mediated by a triad of initiation factors collectively referred to as eIF4F (Raught et al., 2000). The eIF4F initiation factors include: eIF4E, a protein that binds to the m^{7}GTP cap structure at the 5' end of mammalian cytoplasmic mRNA; eIF4A, a RNA helicase; and eIF4G, a scaffolding protein that in addition to binding to eIF4A and eIF4E binds to the poly(A) binding protein (PABP) and eIF3. Thus, mRNA binds to the 40S ribosomal subunit through the association of the eIF4F-mRNA complex with the eIF3-40S ribosomal subunit complex. Assembly of the eIF4F complex is regulated, in part, by the association of eIF4E with the eIF4E binding proteins (4E-BP) 4E-BP1, 4E-BP2, and 4E-BP3. Of these proteins, only 4E-BP1 is well characterized. The binding site on eIF4E for 4E-BP1 overlaps with the eIF4G binding site, such that eIF4E can bind to either 4E-BP1 or eIF4G, but not both at the same time (Ptushkina et al., 1999). Thus, the binding of eIF4E to 4E-BP1 precludes the binding of the eIF4E-mRNA complex to the 40S ribosomal subunit. The interaction between eIF4E and 4E-BP1 is regulated by phosphorylation of 4E-BP1: hyperphosphorylation prevents binding, while hypophosphorylation allows binding. Increased eIF4F formation may stimulate global rates of protein synthesis but can preferentially enhance the translation of mRNAs containing 5'-leader sequences that are predicted to form extensive secondary structures.

Phosphorylation of ribosomal protein S6 (rpS6) is another mechanism for the regulation of mRNA binding to 40S ribosomal subunits. rpS6 is located near the mRNA/tRNA binding site on the 40S ribosomal subunit (Fumagalli and Thomas, 2000) and, therefore, may be optimally positioned for a potential role in selecting mRNA to be translated. Phosphorylation of rpS6 occurs on multiple sites and is mediated by a 70 kDa protein kinase termed S6K1 (Fumagalli and Thomas, 2000). Activation of S6K1 is associated with the preferential translation of mRNAs containing a terminal oligopyrimidine tract (TOP), which is an oligopyrimidine tract adjacent to the m^{7}GTP cap. Proteins encoded by such mRNA include the ribosomal proteins, eIF4G, PABP and eukaryotic elongation factor-2; i.e., proteins that are involved in mRNA translation. Thus, activation of S6K1 results in an overall increase in the capacity to synthesize protein.

Administration of BCAAs *in vivo* enhances muscle protein synthesis through activation of the mRNA binding step in translation initiation, and there does not appear to be an effect on the met-tRNA$_i$ binding step. Administration of leucine to rats that have been fasted for 18 h does not cause significant alterations in either the activity of eIF2B or the phosphorylation of eIF2α (Anthony et al., 2000a). This agrees with an earlier study reporting no change in either parameter in response to feeding a complete meal to fasted rats (Yoshizawa et al., 1997,1998). Rather, 4E-BP1 is hyperphosphorylated, resulting in the release of eIF4E from the inactive 4E-BP1-eIF4E complex (Anthony et al., 2000a). The freed eIF4E then associates with eIF4G to form the active eIF4F complex. Leucine administration also stimulates the phosphorylation and, thus, the activation of S6K1. Oral administration of isoleucine is less effective than leucine in activating translation initiation (Anthony et al., 2000b). In rats that have been administered isoleucine, the level of 4E-BP1 and S6K1 phosphorylation is intermediate between fasted and leucine-treated animals, and global rates of protein synthesis are not elevated. Likewise, the binding of eIF4G to eIF4E is enhanced by isoleucine, although at a significantly lower level than in leucine-treated rats. Finally, valine administration has little or no effect on protein synthesis or the phosphorylation of 4E-BP1 or S6K1 in skeletal muscle of fasted rats.

Comparison of the effect of L-leucine, D-leucine and α-ketoisocaproate (α-KIC) on translation initiation

As noted above, oral administration of leucine stimulates global rates of protein synthesis in skeletal muscle concomitant with enhanced phosphorylation of 4E-BP1 and S6K1. However, a number of questions remain unanswered, including whether leucine itself or a leucine metabolite is the activator of protein synthesis. For example, leucine is reversibly interconverted with α-KIC by branched chain amino transferase. In this regard, previous studies show that α-KIC is as effective as L-leucine at stimulating S6K1 activity and that α-KIC potently stimulates 4E-BP1 phosphorylation (Patti et al., 1998; Xu et al., 1998). In our laboratory, we have found that oral administration of α-KIC mimics the stimulatory effect of L-leucine in skeletal muscle (Yoshizawa et al., 2004). Furthermore, the amino transferase inhibitor, (aminooxy) acetic acid, attenuates α-KIC-stimulated phosphorylation of 4E-BP1 in primary cultures of rat adipocytes but has no effect on the response to L-leucine (Fox et al., 1998). Thus, the stimulation of 4E-BP1 and S6K1 phosphorylation by α-KIC can be explained by its conversion to L-leucine.

Another important question is how mammalian cells perceive the leucine supply and relay this information to signaling pathways within the cell. To answer this question, we compared the effect of L- and D- enantiomers of leucine on the phosphorylation of 4E-BP1 and S6K1 (Yoshizawa et al., 2004). L-leucine administration clearly stimulated the phosphorylation of 4E-BP1 and S6K1 in skeletal muscle,

while D-leucine was much less effective. This indicates that the effect of leucine is stereospecific and suggests that the mechanism used by leucine to stimulate the phosphorylation of 4E-BP1 and S6K1 involves the interaction of leucine with specific binding site.

The relative contribution of insulin to leucine-induced protein synthesis

The interpretation of results of studies examining the effect of oral administration of leucine on protein synthesis is complicated by the fact that leucine administration causes a transient increase in plasma insulin concentration (Anthony et al., 2002). Thus, changes in protein synthesis as well as alterations in phosphorylation and association between translation initiation factors could be due to changes in either plasma leucine or insulin, or a combination of both. A variety of studies have attempted to prevent or attenuate an increase in plasma insulin concentration to help define the role of insulin in leucine-enhanced protein synthesis. A recent study shows that leucine retains its ability to induce 4E-BP1 and S6K1 phosphorylation in the skeletal muscle of streptozotocin-induced diabetic rats (Yoshizawa et al., 2002). However, the phosphorylation levels of 4E-BP1 and S6K1 in leucine-fed diabetic rats were significantly lower than those in leucine-fed nondiabetic rats. Serum insulin could not be detected in these diabetic rats by enzyme immunoassay. These results suggest that insulin plays a permissive role in leucine-induced protein synthesis. Specifically, insulin facilitates, but is not required for, leucine stimulation of 4E-BP1 and S6K1 phosphorylation.

Anthony et al. (2002) also addressed the question of whether oral leucine administration stimulates muscle protein synthesis in the absence of an increase in plasma insulin. Rats were infused with somatostatin to maintain plasma insulin at a level equivalent to that observed in fasted rats. They found that leucine-induced phosphorylation of 4E-BP1 and S6K1 was unaffected by somatostatin infusion. Moreover, somatostatin infusion did not prevent the leucine-induced changes in eIF4E association with 4E-BP1 or eIF4G. Together with our results, this demonstrates that an increase in plasma insulin concentration above fasting values is not required for the leucine-induced changes in these parameters. Moreover, their study also showed that, in animals infused with somatostatin, oral leucine administration failed to stimulate protein synthesis in skeletal muscle. This result suggests that neither the phosphorylation of 4E-BP1 or S6K1 nor the enhanced binding of eIF4G to eIF4E associated with oral leucine administration was sufficient to stimulate global rates of protein synthesis. Another unidentified mechanism therefore must be involved in this effect of leucine.

Signaling pathways involved in the stimulatory effect of leucine on protein synthesis

The signal transduction pathway mediating leucine-induced hyperphosphorylation of 4E-BP1 and S6K1 has not been completely defined. In cultured cells and animals *in vivo*, hormones such as insulin and insulin-like growth factor-1 (IGF-1) promote phosphorylation of 4E-BP1 and S6K1 through the phosphatidylinositol-3 kinase (PI-3 kinase)/protein kinase B (PKB)/mammalian target of rapamycin (mTOR) signal transduction pathway (Schmelzle and Hall, 2000). However, although the ultimate effect of oral leucine administration on each of these parameters is the same as that observed in response to treatment with insulin or IGF-1, leucine does not stimulate either PI-3 kinase or PKB (Hara et al., 1998; Patti et al., 1998; Pham et al., 2000), bringing into question the role of this pathway in the leucine-mediated changes *in vivo*. To address the question of whether or not mTOR activity is required for the leucine-induced changes in 4E-BP1 and S6K1 phosphorylation, a specific inhibitor of mTOR, rapamycin, was administered to rats prior to oral administration of leucine (Anthony et al., 2000b). It was found that rapamycin eliminated the leucine-induced increases in 4E-BP1 and S6K1 phosphorylation and thereby implicated mTOR in the response.

The stimulation of mTOR by insulin or IGF-1 is mediated in part by phosphorylation of PKB on Ser^{473}, which results in its activation (Alessi et al., 1996). PKB subsequently phosphorylates a residue (Ser^{2448}) on mTOR within a domain that normally acts to repress mTOR protein kinase activity (Sekulic et al., 2000). Oral administration of leucine does not enhance the phosphorylation of PKB on Ser^{473} in skeletal muscle, but leucine administration does promote the phosphorylation of Ser^{2448} on mTOR (Yoshizawa, unpublished data). Although there is no direct evidence that leucine activates the protein kinase activity of mTOR, mTOR appears to be a point of convergence of signals generated by the action of hormones such as insulin and those generated by the cell's recognition of a sufficiency of leucine (Figure 1). There is also evidence that leucine regulates a step in translation initiation that is independent of rapamycin-sensitive signaling through mTOR (Anthony et al., 2000b). Although rapamycin treatment prevents the leucine-induced changes in 4E-BP1 and S6K1 phosphorylation, this compound attenuates but does not prevent the leucine-induced stimulation of protein synthesis or eIF4F complex assembly. This suggests that mTOR signaling is essential for the leucine-induced stimulation of translation initiation but that signaling through mTOR alone is not sufficient for the stimulation of muscle protein synthesis. Indeed, there appears to be a mTOR-independent pathway for activation of eIF4F

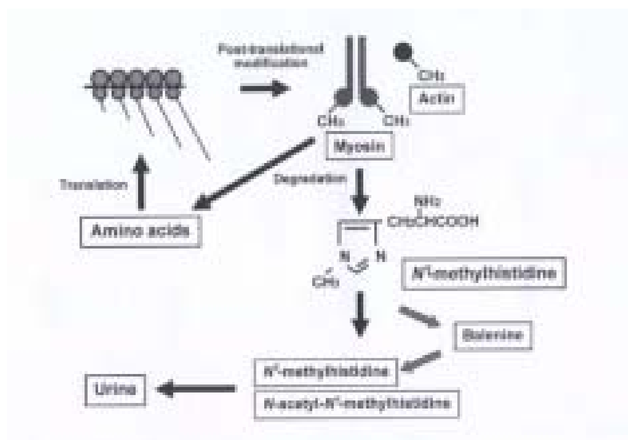

Figure 2. Model of N-methylhistidine metabolism.

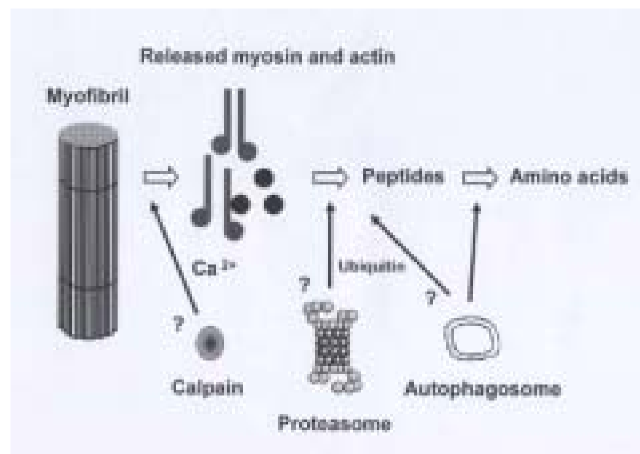

Figure 3. Possible mechanisms of myofibrillar protein degradation.

complex assembly that participates in leucine-induced protein synthesis. Thus, leucine regulates muscle protein synthesis through both an insulin- and mTOR-dependent signaling pathway, which results in 4E-BP1 and S6K1 phosphorylation, and an insulin- and mTOR-independent pathway, which results in enhanced eIF4F complex assembly.

THE INHIBITORY ACTION OF LEUCINE ON PROTEIN DEGRADATION

Muscle protein degradation is also important factor to determine the muscle mass. The decline in skeletal muscle mass following nutritional changes and during aging and disease is due to a decrease in the rate of protein synthesis, an increase in the rate of protein degradation, or both (Lecker et al., 1999). Muscle tissue is the largest tissue and can act as a store of amino acids. Thus, the body can adapt to an amino acid deficiency by increasing muscle protein degradation. However, the accelerated degradation of muscle protein during aging and disease causes severe muscle atrophy, leading to sarcopenia and attenuated muscle function (Doherty, 2003).

We have used N^{τ}-methylhistidine (3-methylhistidine; MeHis) as an index of myofibrillar protein degradation. MeHis is located in myosin and actin, which are main myofibril proteins, and is not reused for protein synthesis (Young and Munro, 1978). Furthermore, in rats and human, MeHis is not metabolized like other amino acids; in humans it is excreted directly into the urine, while in rats, it is acetylated before excretion in the urine (Young and Munro, 1978). Therefore, urinary excretion of MeHis can be an index of the rate of myofibrillar protein degradation (Figure 2). Urinary MeHis is not an indicator of myofibrillar protein degradation in sheep and pig because they convert it to balenine (β-alanyl-N^{τ}-methylhistidine), which is not excreted in the urine (Milne and Harris, 1978; Harris and Milne, 1980). It is noteworthy that MeHis in non-skeletal muscle, such as in the gastrointestinal system, affects amount of MeHis excreted into the urine (Nishizawa et al., 1977, Nagasawa and Funabiki, 1981). We used the released MeHis from isolated muscle tissues (soleus and extensor digitorum longus muscle) in rats and mice (Nagasawa et al., 1998,2004) to directly assess myofibrillar protein degradation.

This method can be used to determine the short-term physiological condition of the host animal. When starved rats were fed on a 20% casein diet, there was a 50% decrease in MeHis release from the isolated muscle 4 h after refeeding (Nagasawa et al., 1998). When dietary protein was replaced with a mixture of amino acids or leucine alone, myofibrillar protein degradation was suppressed to a similar extent as with the casein diet (Nagasawa et al., 2002). Oral administration of leucine alone also suppressed myofibrillar protein degradation (Nagasawa et al., 2002). Thus, these results show that amino acids, particularly leucine, can suppress myofibrillar protein degradation. Several additional reports have confirmed that leucine inhibits muscle protein degradation (Tischler et al., 1982; Busquets et al., 2000). Finally, these findings demonstrate that oral administration of leucine causes rapid changes in not only myofibrillar protein synthesis but also protein degradation.

Insulin, which is a dietary induced hormone, is known to inhibit muscle protein degradation (Rooyackers and Nair, 1997). Some amino acids, including leucine, are capable of stimulating insulin secretion (Malaisse, 1984), which rapidly suppress muscle protein degradation. However, the suppression of myofibrillar protein degradation by dietary protein or amino acids likely occurs independent of insulin action, because myofibrillar protein degradation is not suppressed in rats fed on a protein free diet despite a significant rise in plasma insulin concentration (Nagasawa et al., 1998).

Mechanisms involved in the inhibitory action of Leucine on protein degradation

Presently, there are three known pathways for intracellular proteolysis (Figure 3). The most well-known pathway is the lysosomal system, which is called autophagy. This is an intracellular membrane-mediated process and is thought to participate in the degradation of slow-turnover proteins (Kadowaki and Kanazawa, 2003). The second protein degradation system is mediated by calpain, a calcium-dependent, cytosolic cysteine proteinase. Although calpain has a high substrate specificity (Carafoli and Molinari, 1998), calpain degrades fodrin, which bundles myofilaments (Huang and Forsberg, 1998). When calpain degrades myofibrils, myosin and actin may be released from myofilaments. Therefore, calpain may carry out the first step of myofibrillar protein degradation (Huang and Forsberg, 1998). Finally, recent studies have identified a third degradative pathway mediated by the ATP ubiquitin-dependent proteolytic system (the proteasomal system). The proteasomal system participates in many important cellular functions, such as the cell cycle and antigen presentation (Hershko and Ciechanover, 1998), and it is also connected to the degradation of muscle proteins (Lecker et al., 1999). Increased muscle protein degradation in some diseases, such as sepsis and cancer, is due to increased activity of the proteasomal system (Lecker et al., 1999).

Which proteolytic system is regulated by leucine? Some recent findings indicate that amino acids regulate the proteasomal system or the autophagy/lysosome system. Hamel et al. (2003) showed that leucine directly inhibits proteasomal activity *in vitro*. However, there is no evidence that leucine suppresses proteasomal activity *in vivo*. On the other hand, Kanazawa et al. (2004) found in rat hepatocytes that some amino acids suppress the formation of the autophagosome, possibly via an intracellular signaling pathway. Therefore, amino acids may regulate the autophagy system in skeletal muscle.

CONCLUSION

The biological mechanisms that regulate the synthesis and degradation of skeletal muscle protein are of great importance to animal agriculture. Although some studies have investigated the regulation of protein synthesis and degradation in food animals, the molecular mechanisms involved in these processes are not well defined. Our knowledge of how amino acids regulate cellular functions in rodents has grown considerably during the past decade, but our understanding of protein metabolism in meat animals has not increased accordingly. This is particularly true regarding the role of BCAAs in the regulation of protein turnover. The application of tracer kinetic methods combined with measurements of the activity of components of the cellular signaling pathways involved in protein synthesis and degradation, will afford new insights into the regulation of skeletal muscle protein metabolism in food animals *in vivo*.

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