INVITED REVIEW

Regulation of skeletal muscle protein synthesis by amino acid and resistance exercise

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ABSTRACT

The maintenance of skeletal muscle mass is very important for the prevention of life style-related diseases and the improvement of quality of life. It is well-known that resistance exercise and nutrition (especially amino acids) are the most effective interventions for maintaining skeletal muscle mass. It has been reported that many molecules are involved in the regulation of protein synthesis in response to resistance exercise and nutrition. Understanding the molecular mechanisms regulating muscle protein synthesis is crucial for the development of appropriate interventions. The role of intracellular signaling pathways through the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase in the regulation of muscle protein synthesis, has been extensively investigated for these years. Control of protein synthesis by mTOR is mediated through phosphorylation of downstream targets that modulate translation initiation and elongation step. In contrast, upstream mediators regulating mTOR and protein synthesis in response to resistance exercise and amino acid still needed to be determined. In this brief review, we discuss the current progress of intracellular mechanisms for exercise- and amino acid-induced activation of mTOR pathways and protein synthesis in skeletal muscle.

Keywords: amino acids, exercise, mTOR, skeletal muscle

INTRODUCTION

Aging and physical inactivity leads to the loss of skeletal muscle mass [1]. A decrease in skeletal muscle mass greatly impairs not only movement but also metabolic health since muscles serve as the substrate storage site of energy for other tissue and consume excess energy. Lean muscle mass generally contributes up to 50% of total body weight in young adults but declines with aging to ~25% at approximately 80 years of age [2]. The maintenance of skeletal muscle mass is primarily dictated by the balance between the rates of protein synthesis and protein breakdown. An increase in skeletal muscle mass results when the rate of protein synthesis is higher than the rate of protein degradation. The most widely recognized stimuli to enhance muscle protein synthesis are nutrition and resistance exercise. In this brief review, we will concentrate on recent advancement in the understanding of the cellular mechanisms that regulate muscle protein synthesis

in response to nutrients (amino acid) and resistance exercise.

THE ROLE OF MTOR IN THE REGULATION OF PROTEIN SYNTHESIS

The mammalian target of rapamycin (mTOR) signaling pathway is recognized as the major pathway regulating protein synthesis in skeletal muscle. mTOR is a serine/threonine protein kinase of the phosphatidylinositol kinase-related family and is highly conserved from yeast to mammals. In eukaryotes, mTOR is found in two independently regulated, functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [3]. mTORC1 is the nutrient-responsive mediator of cell growth regulation, and is composed of mTOR, regulatory associated protein of mTOR (Raptor), and Lst8/GβL [4]. mTORC2 is composed of mTOR, which is rapamycin-insensitive companion of

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mTOR (Rictor), SIN1, and Lst8/GβL. Signaling by the mTORC2 complex is not directly inhibited by rapamycin, and it seems unlikely that the mTORC2 complex contributes to growth-regulatory effects [4,5]. For the aim of this review, we will discuss the role of mTORC1 in resistance exercise- and amino acid-induced activation of muscle protein synthesis.

The most established pathway for stimulation of mTORC1 is the growth factor dependent activation of phosphatidylinositol-3 kinase (PI3K)/Akt [6,7]. Binding of growth factors (such as insulin, insulin-like growth factor 1, epidermal growth factor) to its receptor lead to activation of its intrinsic tyrosine kinase and autophosphorylation, which generates docking sites for insulin receptor substrates (IRS). Phosphorylated IRS then acts as docking site to recruit and activate PI3Ks, which generates phosphoinositide-3,4,5-triphosphate (PIP₃). PIP₃ acts as a docking site for phosphoinositide-dependent kinase 1 (PDK1) and Akt, a serine/threonine kinase that is the central mediator of the PI3K pathway. Subsequent phosphorylation of Akt at serine 308 by PDK1 leads to Akt activation. Once activated, Akt stimulates protein synthesis by activating mTORC1 through the intermediary tuberous sclerosis 1/2 complex (TSC1/2). Translational regulatory protein p70 S6 kinase (P70S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP1), which both play important roles in the initiation of mRNA translation initiation, are well-known substrates of the mTORC1 complex and thus can stimulate protein synthesis.

EFFECT OF AMINO ACID ON MUSCLE PROTEIN SYNTHESIS

It is well documented that increased availability of amino acid stimulates muscle protein synthesis in human [8-11]. This stimulatory effect of amino acids on protein synthesis is primarily due to essential amino acids [11-13]. Among the essential amino acids, leucine appears to be the most effective for stimulation of skeletal muscle protein synthesis in human [14,15] and rodent [16,17]. However, the addition of leucine to a high-quality essential amino acid solution did not further increase human muscle protein synthesis, even a higher arterial concentration of leucine was observed [18]. This work suggests that intracellular leucine availability may be a regulator of muscle protein synthesis. In addition, several previous studies showed an anabolic effect of leucine and branched-amino acids (BCAAs) on the reduction of muscle protein breakdown, with no measured effects on muscle protein synthesis in human skeletal muscle, as was reviewed by Matthews [19]. Further investigation is needed to more

clearly define the role of leucine in human muscle protein synthesis.

In contrast to these human studies, clear evidence of the stimulatory effects of BCAAs or leucine alone on protein synthesis has been observed in animal studies. The addition of BCAAs to an isolated rat diaphragm [20] and perfused rat skeletal muscle [21] resulted in stimulation of protein synthesis to the same extent as the addition of a complete mixture of amino acid. Enhanced protein synthesis in rat skeletal muscle, following oral administration of leucine, but not isoleucine and valine, was reported by other investigators [17]. Because leucine is an insulin secretagogue, it was speculated that the anabolic effect of leucine *in vivo* is mediated by insulin. Anthony *et al.* [22] investigated the relationship between the concentration of serum insulin and protein synthesis after oral administration of leucine to food-deprived rats. Their results suggested that the transient increase in serum insulin facilitated a leucine-induced increase in protein synthesis. In another study, leucine-induced stimulation of protein synthesis was demonstrated in rats with experimentally induced diabetes, suggesting that insulin may play a permissive role in leucine-induced protein synthesis [23].

INTRACELLULAR SIGNALS FOR MUSCLE PROTEIN SYNTHESIS BY AMINO ACID (LEUCINE)

One site of leucine action is a kinase in the insulin signaling cascade, which was previously identified as mTOR, a serine/threonine protein kinase [24]. Leucine as well as insulin and growth factors stimulate mTOR kinase activity, which then controls the phosphorylation of translation initiation factors. When mTOR is activated by leucine, at least two downstream molecules for translation initiation are stimulated. First, mTOR kinase regulates the availability of eukaryotic initiation factor 4E (eIF4E) via the phosphorylation of 4E-BP1 [25]. In addition, mTOR phosphorylates p70S6K which, in turn, phosphorylates the S6 ribosomal protein and allows the up-regulation of a subclass of mRNA encoding the translational apparatus [26]. Another potentially important signaling protein in the control of translation is eukaryotic elongation factor 2 (eEF2), which mediates the translocation step of elongation. Activation of eEF2 by dephosphorylation after amino acid ingestion was demonstrated in human muscle [10].

The mechanism responsible for the leucine-induced mTOR signaling has not yet been clearly defined. However, it is likely that two proteins, which are directly associated with

kinase, the regulatory-associated protein of mTOR (raptor) and ras-homolog enriched in the brain (Rheb), are involved in this process, as reviewed by Kimball and Jefferson [27]. Raptor can also bind to mTOR and form a nutrient-sensitive complex, called mTORC1 [4,28]. Over-expression of Rheb results in increased phosphorylation of both 4E-BP1 and p70S6K [29]. The activity of Rheb is controlled, in part, by a tuberous sclerosis complex (TSC). Co-expression of TSC1 and TSC2 results in decreased mTOR function, suggesting that the TSC1-TSC2 complex regulates mTOR signaling by inhibiting Rheb [29]. Deprivation of all amino acids, or just leucine, results in dissociation of Rheb to mTOR [30]. Thus, results from these *in vitro* studies suggest that amino acid signaling through mTOR requires both Rheb and the TSC1-TSC2 complex.

It has been indicated that a class III PI3K, hVps34 (human vacuolar protein sorting-34), could mediate the leucine-induced activation of mTOR and its downstream targets [31]. The activity of hVps34 is inhibited under instances of amino acid starvation, whereas readdition of amino acid increased its activity [32].

Another protein that has been implicated as a mediator to mTORC1 is Rag (Ras-related GTPase) [33]. Rag protein has been shown to interact with the mTORC1 complex via association with Raptor [33]. Overexpression of the active form of Rag activates p70S6K and the activity of the mTORC1 complex pathway becomes insensitive to amino acid deprivation [34]. More recently, a novel role for phospholipase D 1 (PLD1) in an amino acid-sensing pathway was reported [35,36]. It was found that PLD1 is indispensable for amino acid activation of mTORC1 and activation of PLD1 by amino acid requires hVps34. The report concluded that the hVps34-PLD1 pathway acted independently of, and in paralleled to, the Rag pathway in regulating amino acid activation of mTORC1 [35].

EFFECT OF LEUCINE METABOLISM ON LEUCINE-INDUCED MUSCLE PROTEIN SYNTHESIS

Anabolic effects of leucine may be self-limiting, because leucine promotes its own disposal by activating branched-chain α -keto acid dehydrogenase (BCKDH) complex, which is a late-limiting enzyme in the catabolic pathway of the BCAAs. The activity of the BCKDH complex is controlled by covalent modification, in which phosphorylation of the E1 subunit by BCKDH kinase inactivates the complex [37] and dephosphorylation of the E1 subunit by a specific phosphatase reactivates the complex [38]. This regulation is necessary,

because excess BCAAs are toxic. The relationship between the activity of the BCKDH complex and leucine-mediated activation of the protein translation in C2C12 myoblasts was investigated using RNA interference [39]. The phosphorylation of p70S6K by leucine was higher in BCKDH E2 subunit knock-down cells (inactivation of the complex), whereas the effect of leucine was diminished in BCKDH kinase knock-down cells (activation of the complex). In addition, suppression of the BCKDH kinase for 48 h resulted in decreased cell proliferation. These results suggest that the activation of the translational regulators by leucine was partly regulated by the activity of the BCKDH complex. Experiments using BCKDH kinase knock-out mouse [40] suggested that loss of control over the BCKDH complex inhibits growth, presumably by limiting the availability of BCAAs for protein synthesis and also decreasing leucine availability to stimulate protein translation. In addition, pharmacological activation of the BCKDH complex by clofibrate acid resulted in the suppression of leucine-induced activation of translation initiation processes [41]. These findings support the notion that BCAA catabolism, which is mediated by the BCKDH complex, has a significant impact on leucine-induced protein synthesis. In contrast to this, Lynch *et al.* [42] reported a disassociation of BCKDH phosphorylation and activation of mTOR. They also demonstrated that phosphorylation of p70S6K was more sensitive to leucine concentration than BCKDH phosphorylation [42].

EFFECT OF LEUCINE ON MUSCLE PROTEIN BREAKDOWN

In general, leucine supplementation by intravenous infusion, incubation with whole muscle or muscle cells, and/or oral feeding consistently resulted in the suppression of skeletal muscle proteolysis [43]. However, a high dose of leucine can promote suppression of muscle protein breakdown, whereas a low dose of leucine can improve skeletal muscle protein synthesis but had no effect on skeletal muscle proteolysis [43]. In addition, the anti-proteolytic effect of leucine was shown to be mediated by the reduction of autophagy and decreased lysosome-dependent proteolysis in C2C12 myotubes [44]. Another study showed that leucine supplementation led to a decrease in ATP-ubiquitin-dependent proteolytic activity after incubation with isolated muscle [45]. In contrast to the acute effect of leucine on proteolysis, a chronic effect has not been well demonstrated. Ventucci *et al.* [46] showed that feeding a leucine-enriched diet over a period of 20 days reduced protein degradation in skeletal muscle by ~11% and increased the myosin heavy chain content by ~47% in rats with cachexia

tumors. Decreased proteolysis induced by chronic leucine supplementation was also reported by other investigators [47]. However, Sadiq *et al.* [48] reported that 5 days of intravenous infusion of essential amino acid did not attenuate the proteolysis of calf skeletal muscle. These reports suggest that such inconsistency may be caused by a lack of standardization related to leucine supplementation (amount and/or method).

EFFECT OF RESISTANCE EXERCISE ON MUSCLE PROTEIN SYNTHESIS

Muscle protein synthesis was shown to be inhibited during an acute bout of resistance exercise [49]. However, muscle protein synthesis is rapidly reversed during post exercise recovery [50,51]. The increase in muscle protein synthesis occurs within the first hour after exercise and can continue for 24 to 48 hrs [52]. Stimulation of muscle protein synthesis after resistance exercise occurs, even when performed in the fasted state. In addition, older individuals do not seem to have the same magnitude of anabolic responses as younger individuals to resistance exercise [53,54]. However, older adults still have the capacity to increase muscle protein synthesis in response to resistance exercise [55,56], suggesting that proper implementation of resistance exercise may contribute to age related muscle loss.

It has been reported that amino acid ingestion after resistance exercise results in greater increases in muscle protein synthesis rates than when amino acid are ingested at rest or when resistance exercise is performed in the fasted state [57]. Thus, resistance exercise and amino acid ingestion independently stimulate muscle protein synthesis.

INTRACELLULAR SIGNALS FOR MUSCLE PROTEIN SYNTHESIS BY RESISTANCE EXERCISE

In response to an acute bout of resistance exercise, muscle protein synthesis increases and this increased is associated with the activation of mTORC1 signaling in human skeletal muscle (reviewed in [58]). Requirement of mTORC1 signaling for exercise-induced activation of protein synthesis was clearly indicated in a previous study that used a specific mTOR inhibitor, rapamycin [59]. Signaling through downstream targets of mTORC1, p70S6K and 4E-BP1, plays an important role in the hypertrophic response of skeletal muscle to overload [60]. Bodine *et al.* [60] reported that the Akt/mTOR pathway was up-regulated during hypertrophy and down-regulated during muscle atrophy. Furthermore, rapamycin

inhibited hypertrophy in all models tested, without causing atrophy in control muscles. Resistance exercise can also activate the mitogen-activated protein kinase (MAPK) signaling pathway in human skeletal muscle [61]. Williamson *et al.* [61] demonstrated that resistance exercise increased the phosphorylation of extracellular regulated kinase 1 and 2 (ERK1/2) and mitogen-activated protein kinase-interacting kinase 1 (MNK1) in skeletal muscle of young and old humans. More recently, Miyazaki *et al.* [62] reported that mTORC1 activation at the early phase of mechanical overload in skeletal muscle occurs independently of the PI3K/Akt pathway. They provided evidence that the mitogen-activated protein kinase kinase (MEK)/ERK-dependent pathway may contribute to mTORC1 activation through phosphorylation of TSC2 [62].

On the other hand, 5'-AMP-activated protein kinase (AMPK) appears to be a negative regulator of protein synthesis [63]. Dreyer *et al.* [49] have reported that muscle protein synthesis decreased and AMPK activity increased during a bout of resistance exercise in human subjects. Another candidate negative regulator of mTORC1 is the mammalian orthologs, which is named regulated in development and DNA damage responses 1 (REDD1) and REDD2 [64]. It was found that REDD1 and REDD2 inhibit mTOR kinase activity in muscle cell [65]. The role of REDD1/2 in the regulation of mTORC1 activity and protein synthesis in human muscle was also reported [66].

ACTIVATION OF MTOR BY MECHANICAL STIMULI

One of the most recognized mechanisms for controlling muscle mass by resistance exercise involves mechanical tension. Mechanical activation of the mTOR pathway was clearly shown by Hornburger *et al.* [67-69]. Results from these reports suggested that mechanical stimuli activate mTORC1 signaling through a PI3K/Akt-independent mechanism. These studies have also indicated that the synthesis of phosphatidic acid (PA) by PLD is necessary for mechanical activation of mTORC1 signaling [68,70]. PA has been reported to directly activate mTORC1 signaling by binding to mTOR on its FRB domain [71]. The rapamycin-FKBP12 complex can inhibit mTOR by binding to the FRB domain [72]. Therefore, mechanical stimuli enhanced PA binding to mTOR not only activates mTOR but also reduces the inhibitory effects of rapamycin.

We have previously reported that stretch-induced activation of protein translation initiation is blocked by pre-treatment of C2C12 myoblasts with a broad-range tyrosine kinase

Fig. 1. Regulation of signaling through the mammalian target of rapamycin (mTOR) in skeletal muscle. Lines ending in arrowheads denote activation, and lines ending in perpendicular lines denote inhibition. IGF-1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; TSC, tuberous sclerosis complex; AMPK, 5'-AMP-activated protein kinase; p90rsk, p90 ribosomal S6 kinase; MEK, mitogen activated protein kinase kinase; ERK1/2, extra-cellular signal-regulated kinases 1 and 2; MNK1, mitogen-activated protein kinase-interacting kinase 1; Rheb, Ras homolog enriched in brain; Raptor, regulatory-associated protein of mTOR; PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; hVps34, human vacuolar protein sorting 34; Rag, Ras-related GTPase; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; eIF, eukaryotic initiation factor; p70S6K, p70 ribosomal protein S6 kinase; rpS6, ribosomal protein S6; eEF2k, eukaryotic elongation factor 2 kinase.

inhibitors, such as genistein [73]. By using various pharmacological inhibitors, we found that some enzymes containing a tyrosine phosphorylation site for its activation, such as Src kinase and Junus kinase (JAK), were not involved in stretch-induced activation of translation initiation [73]. Phospholipase C (PLC) was shown to mediate stretch-induced activation of protein translation. The specific inhibitor of PLC, U-73122, clearly blocked the stretch-induced activation of p70S6K and eEF2 in C2C12 myoblasts (unpublished observation). The PLC family of cellular proteins plays an important role in the regulation of various intracellular signaling mechanisms. PLC hydrolyzes phosphatidyl inositol-4,5-bisphosphate (PIP₂) to inositol-1,4,5-phosphate (IP₃) and diacylglycerol (DAG), both of which act as secondary messengers. Eleven distinct

isoforms of PLC, which are grouped into four subfamilies (β , γ , δ and ϵ), have been identified in mammals. Among the four subfamilies, the effects of PLC β and γ on cell growth and differentiation have been intensively investigated [74]. In C2C12 cells, PLC β 1 and PLC γ 1 are required for myogenin expression and activation of cycline D3 during differentiation [75]. It has also been demonstrated that mechanical stretch-induced IL-6 secretion from endothelial cells requires PLC γ [76]. We have also demonstrated that mechanical-stretching increasing PLC γ 1 phosphorylation of tyrosine783 residue in membrane compartments, which suggests that stretch-induced activation of protein translation initiation is mediated, in part, by PLC γ 1 (unpublished observation). Moreover, PIP₂, which is a PCL substrate, has signaling capacity by itself and can, by direct interaction, affect the activity and subcellular localization of PLD. Since stimulation of PLC isoforms caused PLD activation, it was assumed that PLD activation might be secondary to PLC-initiated increase in cytosolic Ca²⁺ and activation of protein kinase C (PKC) isoforms (reviewed in [77]). In fact, PKC inhibitors and down regulation of DAG-dependent PKC reduced receptor-induced PLD responses [78]. Thus, the PLC-PKC-PLD pathway was proposed to be involved in the mechanical activation of mTORC1. However, further studies are needed to elucidate the molecular mechanisms of the mechanical activation of mTORC1 via lipid second messengers that are produced by PLC and PLD.

CONCLUSIONS

It is clear that amino acid and resistance exercise (mechanical stimuli) play an important role in the regulation of muscle mass. Current studies on human and rodents suggest that anabolic signaling in response to amino acid and resistance exercise is mediated through mTOR signaling in skeletal muscle. Moreover, recent progress has significantly expanded our understanding of the upstream molecules that regulate mTOR in response to anabolic stimuli. As mentioned in this review, resistance exercise and essential amino acid independently stimulate muscle protein synthesis. Thus, when amino acid ingestion is combined with resistance exercise, additive effects on protein synthesis can be obtained. Future findings may lead to the development of novel interventions for improving skeletal muscle mass.

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