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REGULATION OF PROTEIN SYNTHESIS IN HUMAN SKELETAL MUSCLE – SEPARATE AND COMBINED EFFECTS OF EXERCISE AND AMINO ACIDS

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Regulation of protein synthesis in human skeletal muscle – separate and combined effects of exercise and amino acids

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Skeletal muscle is a highly plastic tissue which has the ability to adapt to various forms of external stimuli such as diverse modes of contractile activity. Thus, performance of endurance exercise over several of weeks results in increased oxidative capacity. In contrast, prolonged performance of resistance exercise ultimately results in increased muscle mass. These adaptations are brought about by transient alterations in gene expression and mRNA translation which result in altered protein turnover, i.e. the balance between protein synthesis and protein breakdown. Protein synthesis is the major determinant of muscle growth, which at the molecular level, is regulated by the mTORC1 pathway. This pathway is potently activated by resistance exercise and amino acids, but the stimulatory role of individual amino acids in human skeletal muscle is unclear. Muscle adaptations in response to endurance exercise are largely dependent on the PGC-1α pathway, which regulates mitochondrial biogenesis. Given the different training adaptations after resistance and endurance exercise, it has been suggested that these exercise modalities may be incompatible when combined. Such potential interference could be exerted at the molecular level between the pathways responsible for each adaptive response. AMPK, an enzyme usually activated by endurance exercise and, when pharmacologically activated in cell culture and rodent models, has been shown to inhibit mTORC1 and protein synthesis. However, it is not known if activation of AMPK by endurance exercise inhibits resistance exercise induced signaling through the mTORC1 pathway in human skeletal muscle.

Thus, the main objective of this thesis was to examine the molecular mechanisms regulating protein synthesis in response to amino acids and various modes of exercise in human skeletal muscle.

In study I, the role of BCAAs in stimulating the mTORC1 pathway was examined in both resting and exercising muscle. BCAA increased mTORC1 activity, as assessed by S6K1 phosphorylation, in both resting and exercising muscle, but more so when exercise and BCAA were combined. In study II, the effect of leucine was compared to that of essential amino acids with or without leucine. It was found that when leucine was combined with the remaining essential amino acids, S6K1 phosphorylation was more pronounced than when leucine was provided alone. Furthermore, when leucine was removed from the essential amino acids, the effect was equal to that of placebo. In study III, the impact of endurance exercise on resistance exercise induced mTORC1 signaling was examined. When performed after resistance exercise, endurance exercise did not inhibit S6K1 phosphorylation compared to when single mode resistance exercise was performed. In study IV, performance of high intensity endurance exercise prior to resistance exercise did not inhibit S6K1 phosphorylation compared to single mode resistance exercise, despite prior activation of AMPK.

In conclusion, amino acids and resistance exercise activate mTORC1 signaling, as assessed by S6K1 phosphorylation, in a synergistic manner. Leucine is crucial in mediating the amino acid response, however, additional amino acids appear to be required to induce a maximal

response downstream of mTORC1. Activation of the mTORC1 pathway in response to heavy resistance exercise is robust and this activation does not appear to be inhibited by prior or by subsequent endurance exercise. As such, these results do not lend support to the existence of molecular interference when resistance and endurance exercise are combined acutely.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the four papers listed below and they will be referred to throughout the text by their Roman numerals.

I. **Apró W** and Blomstrand E. Influence of supplementation with branched-chain amino acids in combination with resistance exercise on p70S6 kinase phosphorylation in resting and exercising human skeletal muscle. *Acta Physiol (Oxf).* 2010 Nov;200(3):237-48

II. **Apró W**, Moberg M, Hamilton L, Ekblom B, Rooyackers O, Holmberg HC and Blomstrand E. Leucine does not affect mTORC1 assembly but is required for maximal S6K1 activity in human skeletal muscle following resistance exercise. *Manuscript.*

III. **Apró W**, Wang L, Pontén M, Blomstrand E and Sahlin K. Resistance exercise induced mTORC1 signaling is not impaired by subsequent endurance exercise in human skeletal muscle. *Am J Physiol Endocrinol Metab. 2013 Jul 1;305(1):E22-32.*

IV. **Apró W**, Moberg M, Hamilton L, Ekblom B, van Hall G, Holmberg HC and Blomstrand E Resistance exercise induced S6K1 kinase activity is not inhibited in human skeletal muscle despite prior activation of AMPK by high intensity interval cycling. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

mRNA	Messenger ribonucleic acid
Thr	Threonine
Ser	Serine
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
TBC1D7	Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
p38 MAPK	p38 mitogen-activated protein kinase
CAMK	Ca^{2+}/calmodulin-dependent protein kinase
VO_{2max}	Maximum oxygen uptake
RM	Repetition maximum
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
SSA	Sulphosalicylic acid
GC-MS	Gas chromatography–mass spectrometry
EGTA	Ethylene glycol tetraacetic acid
$MgCl_2$	Magnesium chloride
Na_3VO_4	Sodium orthovanadate
Tris base	Tris(hydroxymethyl)aminomethane
NaCl	Sodium chloride
Hepes	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
NaF	Sodium fluoride
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
cDNA	Complementary deoxyribonucleic acid
PCR	Polymerase chain reaction
CT	Cycle threshold
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
NaOH	Sodium hydroxide
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
MuRF1	Muscle Ring-finger protein 1
MAFbx	Muscle atrophy F-box

1 INTRODUCTION

Skeletal muscle is a highly malleable tissue which exhibits a remarkable ability to adapt to different external stimuli such as the many various factors that constitute physical exercise. The plastic nature of muscle allows general and distinct adaptations in response to the specific stimulus imposed on the tissue. Traditionally, the most diverse forms of exercise are termed resistance and endurance exercise. Endurance exercise is usually defined as repetitive submaximal contractions that can be sustained over prolonged periods of time. When performed over several weeks, endurance exercise ultimately results in increased capillarization (1) and mitochondrial biogenesis (2, 3), thus promoting increased oxidative metabolism and capacity (4). In contrast, repeated performance of high intensity, short duration contractions, i.e. resistance exercise, eventually results in increased muscle mass (5) and improvements in maximal strength (6) but has little effect on oxygen uptake (7). These adaptations are presumed to be brought about by transient but repeated alterations in gene expression and mRNA translation which ultimately result in altered protein turnover. Protein turnover collectively refers to the rates of amino acid exchange between tissue proteins and the free amino acid pool by the processes of protein synthesis and breakdown. For a net increase in muscle protein to occur, protein synthesis must exceed breakdown and if the opposite is true, i.e. breakdown exceeds synthesis, there is a negative net balance and consequently, a loss of protein. In adult human skeletal muscle, the protein turnover rate is relatively low, approximately 1-2% per day, but this rate is subject to change in response to various physiological stimuli such as fasting, feeding and exercise.

1.1 PROTEIN TURNOVER – EFFECTS OF FASTING, FEEDING AND EXERCISE

Since the development of amino acid tracers labelled with stable isotopes, numerous studies have been undertaken to examine how external stimuli such as feeding and exercise influence the turnover rate of skeletal muscle. Protein synthesis and protein breakdown are dynamic processes that are simultaneously active, but to various degrees in relation to each other, dependent on the presiding circumstances. In the postabsorptive state under resting conditions, the rate of protein breakdown is higher than that of protein synthesis, resulting in a negative net balance (8, 9) and thus a loss of muscle protein. The net balance remains negative until amino acids are provided, at which the rate of protein synthesis surpasses the rate of breakdown (8, 10) and protein accretion occurs. During the course of a day, the

alterations in net balance in response to feeding, and lack thereof, constitute the steady state of protein turnover at which there is no net gain or loss of muscle mass. However, this steady state can be greatly offset by exercise, especially resistance exercise, which when repeated over a longer period of time, ultimately results in skeletal muscle hypertrophy (5, 11, 12). As muscle accretion requires protein synthesis to be larger than protein breakdown, one might intuitively assume that muscle growth following resistance training is a result of exercise induced elevations in muscle protein synthesis. This assumption would however be correct, only in part. Resistance exercise does in fact induce a robust increase in protein synthesis during the acute recovery period, resulting in approximately 2-fold higher values compared to rest in untrained subjects (9, 13, 14). However, resistance exercise also exerts a stimulatory effect on protein breakdown which, in the postabsorptive state, still remains higher than protein synthesis (9, 13, 14). In comparison to the synthetic response, the extent of the increase in protein breakdown appears to be substantially less, reaching only around 30-50% higher rates compared to rest (9, 13, 14). Thus, as a consequence of the differential increase in the synthetic and proteolytic response, resistance exercise results in an improved, albeit still negative, net protein balance (9, 13, 14). Similar findings have been reported during recovery from endurance exercise in the post absorptive state (15, 16). In contrast, when amino acids are provided, net protein balance becomes positive, both during resting conditions (8, 10) and during recovery from resistance (10, 17) as well as endurance (15, 18) exercise.

1.2 PROTEIN FRACTIONAL SYNTHETIC RATE

As noted above, for muscle protein accumulation to occur, protein synthesis must exceed protein breakdown, and for this circumstance to take place, exogenous amino acids must be provided. The necessary alterations in protein turnover may be achieved in several ways, i.e. through an increase in protein synthesis, a depression of protein breakdown or a combination of both. However, amino acids appear to alter protein turnover primarily by stimulating protein synthesis in contrast to attenuating protein breakdown (10). Thus, provision of amino acids produces a substantial increase in protein synthesis but has only a minor effect on proteolysis (10). Therefore, the protein synthetic response is believed to be the major determinant of muscle growth (19). As a consequence, coupled with the difficulty of accurately measuring protein breakdown (20), many studies only measure protein synthesis to determine the anabolic response following a certain intervention. A common practice

involves measuring the protein *fractional synthetic rate* (FSR) which represents the synthesis rate of a *fraction* of the total protein pool in a unit of time (21). The FSR is calculated from the rate of tracer incorporation into protein over time, and is independent of the total protein pool size. This trait of the FSR measurement makes it ideal as it can be determined without knowing the size of the protein pool, which in turn could be difficult to assess accurately. When FSR measurements are performed on whole muscle tissue, the synthetic rate is determined for all muscle proteins combined, which results in estimates of mixed muscle protein synthesis (9, 13). However, by prior isolation, whole muscle tissue may be separated into various subfractions (myofibrillar, mitochondrial and sarcoplasmic), thereby enabling fraction-specific FSR measurements (22, 23). Such fractionation of muscle tissue may be important if the aim is to study exercise specific adaptations. As evidenced from several studies, FSR of mixed muscle proteins have been shown to increase in response to resistance exercise (9, 10, 13, 14) as well as endurance exercise (16, 24-26), yet long term training adaptations differ vastly between these two modes of exercise. It seems logical that increases in mixed muscle FSR would reflect alterations in those subfractions that are associated with mode specific training adaptations, i.e. an increase in myofibrillar protein synthesis following resistance exercise and elevated synthetic rates of mitochondrial proteins after endurance exercise. However, this may not always be the case, as mitochondrial FSR has been found to be upregulated in response to resistance exercise (27, 28) and conversely, increases in myofibrillar FSR have been detected following endurance exercise (29, 30). Thus, caution is warranted when interpreting acute changes in mixed muscle protein synthesis. This notion is further supported by findings showing that the acute FSR response in the untrained state may be altered following long term training (22). In this study, in the untrained state, resistance exercise resulted in elevations in both myofibrillar and mitochondrial FSR but after ten weeks of training, increases in FSR were only evident in the myofibrillar fraction (22). It therefore appears as if the acute response in untrained or unaccustomed subjects is less specific, thus extending the need for careful interpretations of the FSR response in particular subfractions as well.

1.2.1 Effects of amino acid provision

The positive effect of feeding on the protein synthetic response is well known and was first established in a seminal study by Rennie and co-workers (31) who showed that feeding a mixed meal to fasted subjects induced a two-fold increase in mixed muscle FSR. It was later

recognized that the main stimulatory constituents responsible for the increase in protein synthesis are the amino acids (8, 10, 32, 33). As a group, they can be divided into essential (EAA) and non essential (NEAA) amino acids and are categorized on the basis of the human body's ability and inability to acquire each amino acid through *de novo* synthesis. Essential amino acids are those that fall in the former category and as a result, they must be provided through the diet. With regard to the anabolic effects of amino acids, it appears as if only the essential amino acids are required to induce a stimulatory effect on protein synthesis (34-36). These findings indicate that certain groups of amino acids, and perhaps individual amino acids, are more potent than others. Indeed, within the group of essential amino acids, the branched-chain amino acids (BCAA; leucine, valine and isoleucine) have received much attention for their role in skeletal muscle metabolism. The BCAAs have the ability to largely bypass splanchnic extraction, thus making them highly available for muscle uptake (37-39). Within muscle, in addition to serving as building blocks for protein synthesis, BCAAs can be oxidized and thus be utilized as substrates to support aerobic energy production, an ability that is unique amongst the essential amino acids (40). Given that BCAAs are predominantly taken up by muscle, and that muscle is equipped with degradative metabolic pathways for these amino acids, it would not be unreasonable to assume that one or more of the BCAAs may also have a regulatory role in muscle protein turnover. Indeed, such a role was indicated in an early *in vitro* study in which rat diaphragm was incubated with all three BCAAs simultaneously as well as with each individual BCAA separately (41). The researchers found that addition of all BCAAs to the incubation medium stimulated protein synthesis. Interestingly, addition of just leucine resulted in a protein synthetic response of a similar magnitude. In contrast, neither valine nor isoleucine had this effect, suggesting that only leucine possesses anabolic properties. This notion was further supported by the finding that leucine alone produced a similar increase in protein synthesis in perfused rat skeletal muscle, as did a complete mixture of amino acids (42). Since then, several studies in experimental animals have strengthened the view that leucine holds the highest stimulatory potential amongst the amino acids (43, 44). In human muscle, relatively few studies have been undertaken to examine the anabolic properties of leucine. Early studies in which intravenous infusion was used as means of delivery, found that leucine reduced the plasma and muscle levels of several essential amino acids without reducing the rate of release from the leg (37, 45, 46). The absence of labelled tracers did not permit assessment of net protein synthesis, however, the results indicated that the decline in amino acid concentrations reflected an increase in protein synthesis. The stimulatory role of leucine on the protein synthetic response in human muscle was later confirmed following a large bolus infusion (47) as well as after

oral intake of leucine (48). In addition to leucine, several other essential amino acids such as valine, phenylalanine and threonine have been shown to stimulate protein synthesis in resting muscle following large dose bolus infusions (49, 50). However, whether these results are due to the route of administration, i.e. large dose infusion, or an effect of the amino acids *per se* is unknown as no study has examined the effects of oral intake of these amino acids. Furthermore, in contrast to animal data (42), the individual impact of leucine on the protein synthetic response in human muscle, compared to that of a complete mixture of essential amino acids, remains to be determined.

1.3 PROTEIN SYNTHESIS AT THE MOLECULAR LEVEL

As made apparent by the presentation above, protein synthesis is a highly dynamic and reactive process. It is therefore reasonable to assume that this process is under strict regulation, as evolutionary logic would dictate that cellular growth would only occur under favourable conditions. This is indeed the case in all living organisms and as a result, highly complex signaling pathways have evolved to ensure the proper response to various environmental cues. At the centre of these regulatory pathways controlling protein synthesis is the evolutionarily conserved serine/threonine protein kinase called the mechanistic target of rapamycin (mTOR; formerly known as the mammalian target of rapamycin). In mammalian cells, mTOR exists in two functionally and structurally distinct multiprotein complexes; mTOR complex 1 (mTORC1) and complex 2 (mTORC2), of which mTORC1 is responsible for regulating cell growth (51). In addition to the catalytic component mTOR, mTORC1 is composed of several other proteins, two of which are unique for complex 1; Raptor (regulatory-associated protein of mTOR) which is the defining component and has both regulatory and scaffolding functions (52), and PRAS40 (proline-rich Akt substrate 40 kDa) which is an insulin-regulated mTORC1 inhibitor (53). mTORC1 exerts control over cellular growth by sensing and integrating a variety of signals emanating from growth factors, nutrients, energy status and cellular stresses.

1.3.1 Downstream of mTORC1

Upon activation by various stimuli, mTORC1 stimulates translation initiation by phosphorylating various downstream targets within the translational machinery (54). Translation initiation refers to the complicated and multistep assembly of the small and large

ribosomal subunits with the mRNA transcript that is to be translated into a polypeptide. All cellular mRNAs contain a 7-methylguanosine cap structure at their 5'end which is used to recruit the mRNA to the small ribosome subunit. For the interaction between the ribosomal subunit and the mRNA transcript to occur, a complex composed of three different eukaryotic initiation factors (eIFs; eIF4E, eIF4G and eIF4A) must be assembled at the 5'cap (54). To assemble this eIF4F complex, eIF4E binds to the cap and subsequently recruits the other two initiation factors. However, the interaction between eIF4E and eIF4G is inhibited by the translational repressor 4EBP1 (eIF4E binding protein 1), which in a hypophosphorylated state tightly binds eIF4E, thereby preventing formation of the eIF4F complex and consequently, cap-dependent mRNA translation (54). One of the best characterized mechanisms by which mTORC1 stimulates translation initiation involves phosphorylation of 4EBP1 at multiple serine and threonine residues in a sequential manner (55). Upon being hyperphosphorylated by mTORC1, 4EBP1 is released from eIF4E which can then recruit eIF4G, thus allowing the assembly of the eIF4F complex and subsequent mRNA translation (54). The various 4EBP1 phosphorylation sites include Thr^{37}, Thr^{46}, Ser^{65} and Thr^{70} of which $Thr^{37/46}$ are phosphorylated first in this sequence (55). It is therefore generally held that phosphorylation of $Thr^{37/46}$ functions as a priming event for subsequent phosphorylation of the Thr^{70} and Ser^{65} residues, in that order (55, 56). It has been shown that mTORC1 directly phosphorylates 4EBP1 at the $Thr^{37/46}$ residues *in vitro* (57) and as a consequence, phosphorylation status of these residues is often used as a readout of mTORC1 activity *in vivo*. The other well characterized target of mTORC1 is the ribosomal protein S6 kinase 1 (S6K1) which upon activation stimulates mRNA translation by mechanisms completely distinct from those of 4EBP1. Being a protein kinase, S6K1 phosphorylates several downstream targets of which most, if not all, are involved in regulating cell growth (54). The most well studied mechanisms of S6K1 mediated stimulation of mRNA translation involve increasing the helicase activity of the eIF4F complex and stimulating peptide elongation. Several mRNAs contain inhibitory secondary structures at their 5'end which suppresses their translation efficiency. Thus, for efficient translation of the mRNA, this secondary structure must be unwound and this is achieved by the eIF4A helicase within the eIF4F complex (54). When active, S6K1 stimulates helicase activity through two distinct mechanisms. First, S6K1 phosphorylates eIF4B which results in the recruitment of this cofactor to eIF4A which in turn promotes helicase activity (54). A second mechanism involves phosphorylation of PDCD4 (programmed cell death 4) which also binds eIF4A, but in contrast to eIF4B, functions as an inhibitor of eIF4A helicase activity. When phosphorylated by S6K1, PDCD4 becomes ubiquitinated and subsequently degraded, thus relieving the inhibition exerted on eIF4A (54).

Collectively, S6K1 mediated stimulation of helicase activity results in enhanced translation efficiency. S6K1 also stimulates mRNA translation by enhancing peptide elongation. The principal mediator of peptide elongation is eEF2 (eukaryotic elongation factor 2), which in a hypophosphorylated state is active and responsible for the translocation of the assembled ribosome along the mRNA construct (58, 59). Phosphorylation of eEF2 by the upstream negative regulator eEF2 kinase (eEF2k) results in the inhibition of eEF2 and consequently, peptide elongation (59). Upon activation by mTORC1, S6K1 stimulates elongation by inhibiting eEF2k through direct phosphorylation, thus relieving the inhibitory effect on eEF2 (60). Activation of S6K1 involves phosphorylation of the mTORC1 specific residue at Thr^{389} (57, 61, 62). Although additional phosphorylation events are required for maximal activation of S6K1 (63, 64), phosphorylation of Thr^{389} is most closely related to the activity of the kinase (65). Consequently, phosphorylation status of the Thr^{389} residue is a widely used marker for both mTORC1 and S6K1 activity *in vivo*. As outlined above, mTORC1 controls protein synthesis by increasing cap-dependent translation initiation and translation efficiency as well as translation elongation.

1.3.2 Upstream of mTORC1

Both growth factors and amino acids have the ability to activate mTORC1, but they appear to do so through different, yet cooperative mechanisms. The ultimate activator of mTORC1 is believed to be the small GTPase Rheb (ras homolog enriched in brain) which resides at various membrane compartments within cells, such as the lysosomal membrane (51). Rheb has been shown to bind directly to mTORC1 *in vitro* which in turn results in the activation of the complex (66, 67). As Rheb is only active when bound to GTP (68, 69), mechanisms directed towards regulating the nucleotide state of Rheb also regulate mTORC1 activity. Indeed, the GTPase activating protein (GAP) TSC2 (tuberous sclerosis 2) has been shown to inhibit mTORC1 signaling by promoting GTP hydrolysis of Rheb, thus converting it to its inactive GDP bound state (68, 69). TSC2 functions as part of a heterotrimeric complex together with TSC1 and TBC1D7 (70) and several environmental inputs converge on this complex to regulate its GAP activity towards Rheb, and consequently, mTORC1 signaling. One such input is growth factor signaling, which usually originates from the plasma membrane in response to activation of tyrosine kinase receptors (RTKs) by extracellular protein hormones such as insulin (71). Stimulation of the RTKs by insulin and related growth factors results in the activation of a serine/threonine protein kinase called Akt (or PKB;

protein kinase B) (72). Once active, Akt phosphorylates the inhibitor protein PRAS40 which results in its dissociation from mTORC1, thereby relieving the inhibitory effect (53). Akt also phosphorylates TSC2 on several residues which ultimately inhibits the GAP activity towards Rheb (73, 74). Amino acid induced activation of mTORC1 also involves Rheb but in contrast to growth factors, amino acids do not engage Akt/TSC2 signaling. The amino acid pathway instead appears to involve the Rag family of small GTPases which is composed of four members (RagA, RagB, RagC and RagD). The Rags function as stabile heterodimers in which RagA or RagB interacts with RagC or RagD leading to four possible combinations. When activated by amino acids, RagA and B become loaded with GTP while RagC and D are loaded with GDP (75). These nucleotide states result in the recruitment of mTORC1 through direct interaction between the Rags and Raptor and subsequent translocation of the complex to the lysosomal membrane (75, 76). There, the Rag-mTORC1 complex interacts with the pentameric complex called the Ragulator, which is tethered to the membrane (76, 77). Through these interactions, mTORC1 becomes activated as it is anchored to the membrane in close proximity of Rheb (76, 77). The signaling events described above are a result of stimulatory inputs during nutrient-rich conditions. As anabolic processes are energetically expensive, cellular mechanisms have evolved which inhibit the stimulatory effect on anabolism during nutrient deficiency and instead activate energy and nutrient producing pathways in order to ensure survival. A key component in this system is the adenosine-monophosphate activated protein kinase (AMPK). AMPK is a serine/threonine protein kinase that is composed of one catalytic (α) and two regulatory (β and γ) subunits and functions as a cellular energy gauge by sensing fluctuations in cellular AMP/ATP ratios (78). AMPK activity is regulated through phosphorylation of its catalytic α subunit at Thr^{172} and by binding of AMP. Maximal activation of AMPK occurs when AMP levels rise as this results in a structural change which prevents the Thr^{172} residue from being dephosphorylated (78). When activated in response to energy deprivation, AMPK signals to inhibit the costly process of protein synthesis through several parallel mechanisms. First, AMPK has been shown to phosphorylate TSC2 which, in contrast to Akt mediated inhibition, results in increased GAP activity towards Rheb and subsequent inhibition of mTORC1 signaling (79-81). AMPK also has the ability to phosphorylate Raptor within mTORC1 which results in loss of kinase activity (82). Lastly, AMPK has been reported to inhibit mTORC1 by direct phosphorylation of the Thr^{2446} residue of mTOR itself (83).

1.4 MTORC1 AND MUSCLE GROWTH

As outlined above, a considerable amount of mechanistic evidence indicates that mTORC1 is a major regulator of protein synthesis and cell growth. It should however be noted that a vast majority of studies undertaken to delineate the role of mTORC1 have been performed on cell cultures, often using immature and transformed cell lines of non muscle origin. Therefore, it must be recognized that these models may not be fully representative of the regulatory mechanisms acting on the protein synthetic machinery in skeletal muscle *in vivo*. As a consequence, several studies have been conducted to examine the role of mTORC1 in muscle growth. The first connection between muscle hypertrophy and mTORC1 signaling was provided by Baar and Esser (84), who showed that an acute increase in S6K1 phosphorylation was highly correlated with changes in muscle mass after six weeks of high frequency electrical stimulation. Subsequent studies in experimental animals found that acute changes in mTORC1 signaling in response to resistance exercise as well as amino acids were accompanied by an increase in protein synthesis (85-87), thus indicating that long term muscle growth was related to acute changes in protein synthesis. Further support for the specific role of mTORC1 in skeletal muscle growth was provided in a pioneering study by Bodine *et al.* (88) who showed that muscle hypertrophy was prevented when animals were injected with the mTORC1 specific inhibitor rapamycin. Definitive proof of mTORC1's involvement in regulating muscle mass was recently provided in a series of elegantly designed genetic mouse models which showed that activation of mTORC1 is sufficient to induce muscle hypertrophy (89) and that load-induced muscle growth is fully dependent on mTORC1 signaling (90). The first study to examine mTORC1 signaling in human skeletal muscle in response to exercise and amino acids was performed by Karlsson *et al.* (91). They found that provision of BCAAs in connection with resistance exercise increased S6K1 phosphorylation and later studies demonstrated that exercise also induced mTORC1 signaling in the absence of nutritional supply (92-94). In parity with data from experimental animals, the relationship between long term muscle growth and S6K1 phosphorylation is also present in human muscle (95). Lastly, recent studies have confirmed the role of mTORC1 in human muscle by the use of the specific inhibitor rapamycin. In these studies it was shown that rapamycin treatment prevented the increase in mTORC1 signaling and protein synthesis in response to resistance exercise as well as amino acids (96-98). Collectively, there is abundant evidence from various experimental models, ranging over a wide array of species, which clearly defines mTORC1 as a major regulator of protein synthesis and cellular growth.

1.5 MITOCHONDRIAL BIOGENESIS

The most prominent peripheral adaptive response following long term endurance training is an enhanced oxidative capacity as a result of increased mitochondrial content (4). A key component in the regulation of mitochondrial biogenesis is the peroxisome proliferator-activated receptor co-activator 1-alpha (PGC-1α) (99). Expression of PGC-1α mRNA is usually seen after acute endurance type exercise in both rodent and human muscle (100, 101) and overexpression of PGC-1α in transgenic mice is associated with increased mitochondrial enzyme activity and fatigue resistance (99). Several signaling molecules have been implicated in the activation of PGC-1α, including AMPK, p38 MAPK and CAMKs (99), all of which are typically activated by endurance exercise (102-104).

1.6 POTENTIAL MOLECULAR INTERFERENCE BETWEEN RESISTANCE AND ENDURANCE EXERCISE

The apparent difference in muscular adaptations following endurance and resistance training (4, 5) places these exercise modalities in contrasting ends of the training adaptation continuum. As such, the opposing phenotypes are likely dependent on highly specific adaptations which may be incompatible when different exercise modes are performed simultaneously (105). The first evidence in support of such incompatibility was provided more than thirty years ago by Hickson (7). The results of that study demonstrated that when high volume strength and endurance training were performed concurrently for ten weeks, strength development was attenuated compared to single mode resistance exercise (7). The seminal findings of Hickson were subsequently confirmed in several investigations showing detrimental effects on the development of strength and power (7, 106-108) when both modes of exercise were performed concurrently over longer periods of time. In contrast, several other studies were unable to confirm the existence of this interference effect (109-116). The reasons for these discrepancies are not readily apparent, but may be related to experimental variables such as intensity, volume, sequence and nutritional status. The differences in experimental protocols utilized do not allow for a decisive conclusion regarding the existence of an interference effect, yet, it has been suggested that attenuation of strength development in some cases may be due to a blunted growth response following concurrent training. While muscle hypertrophy was not affected in the original study by Hickson (7), some studies have indeed found that muscle growth may be negatively affected when combining resistance and endurance exercise (106, 117, 118). Consequently, several molecular mechanisms have been

implicated in mediating the negative effect of endurance exercise on muscle growth. These mechanisms involve AMPK signaling. As noted previously, AMPK is activated in response to increased energy turnover and cellular stress, such as that exerted by exercise (78). During such conditions, the cellular response is to inhibit energetically expensive processes such as protein synthesis and to stimulate energy producing pathways which generate ATP (78). Thus, AMPK is situated perfectly within the signaling network to co-ordinately regulate training adaptations in a mode specific manner. Consequently, activation of AMPK is purported to inhibit growth related adaptations by repressing mTORC1 signaling. Support for such negative regulation of mTORC1 comes from cell culture and rodent studies in which pharmacological activation of AMPK has been shown to inhibit mTORC1 signaling as well as protein synthesis (79-81, 119-121). From these studies, one might infer that performing endurance exercise, which is known to potently activate AMPK (104), would inhibit mTORC1 signaling if performed in connection with resistance exercise. Few studies have investigated the interaction between AMPK and mTORC1 in human muscle. When performed under postabsorptive conditions and in close proximity to each other, endurance type exercise performed prior to resistance exercise have been shown to have a minor impact on mTORC1 signaling compared to when resistance exercise was performed first (122, 123). Whereas these studies did not include single mode resistance exercise for comparison, one study which did, could not detect any inhibitory effect on mTORC1 signaling when endurance exercise was performed after resistance exercise (92). When performed in the fed state (28, 124) and with ample recovery time between sessions (124), mTORC1 signaling was similar between concurrent exercise and single mode resistance exercise. Thus, at present and based on the available data, it is difficult to fully reconcile the existence of molecular interference in human muscle.

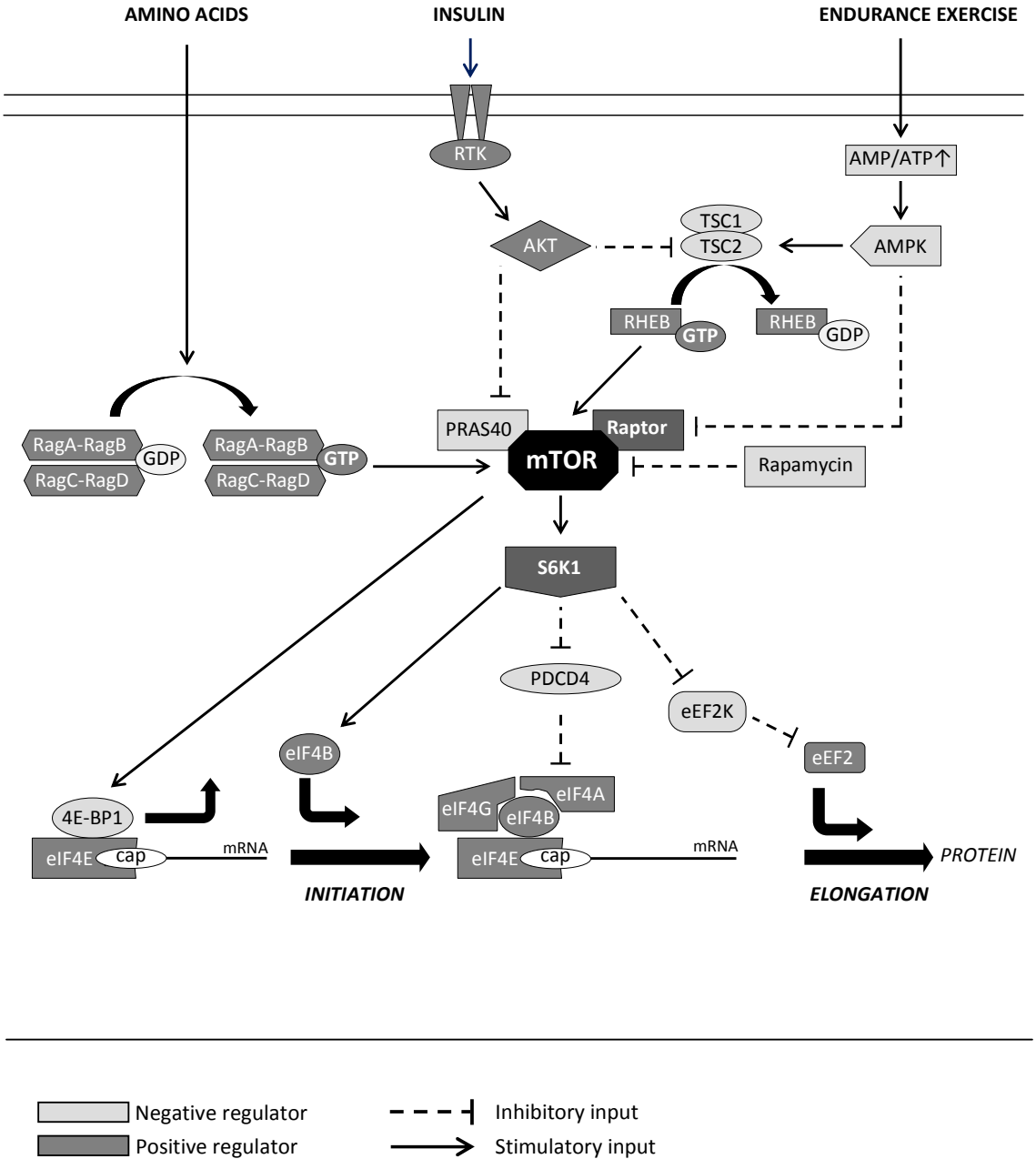

Figure 1. *Simplified illustration of the mTORC1 pathway. Courtesy of Marcus Moberg.*

2 AIMS

The overall aim of this thesis was to study the molecular mechanisms regulating protein synthesis in response to amino acids and various modes of exercise in human skeletal muscle. The specific aims were:

1. To distinguish between the effects of resistance exercise and BCAA on mTORC1 signaling.

2. To examine the particular role of leucine in the amino acid induced mTORC1 signaling response and to gain further insight into the molecular mechanisms responsible for mediating the amino acid effect on this pathway.

3. To examine whether resistance exercise induced mTORC1 signaling would be repressed by subsequent performance of endurance exercise in comparison to single mode resistance exercise.

4. To examine if prior performance of high intensity interval cycling would inhibit resistance exercise induced mTORC1 signaling and protein synthesis compared to single mode resistance exercise and to gain further mechanistic insight into inhibitory AMPK signaling.

3 MATERIALS AND METHODS

3.1 SUBJECT CHARACTERISTICS

All subjects enrolled in the studies were healthy volunteers which after being informed of the purposes and of all associated risks, gave oral or written consent. Each study was approved by the Regional Ethical Review Board in Stockholm and performed in accordance with the principles outlined in the Declaration of Helsinki. Subjects in Study I were recreationally active but did not perform resistance exercise on a regular basis. For study II, subjects with a training history of at least twelve months of structured resistance exercise at least four times a week were recruited. For study III and IV, subjects were required to have performed resistance exercise 2-3 times per week and endurance exercise 1-2 times per week for the last six months. For study I there was no lower limit for leg strength, but for studies II-IV, subjects were required to have a maximal leg strength equaling four times their bodyweight, or more. For more details, see table 1. In all studies, subjects were instructed to refrain from any type of vigorous physical activity for a minimum of two days prior to each experiment and in studies I and II, subjects were also instructed to follow a standardized diet during these same two days. In studies III and IV, subjects were instead instructed to follow their habitual diets but to record and duplicate their food intake before the first and second trials, respectively. For each trial in each study, subjects reported to the laboratory early in the morning after an overnight fast from 9.00 PM the evening before.

	Gender	Number	Age (yr)	Height (cm)	Weight (kg)	$VO_{2\,max}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)
Study I	female	5	24 ± 2	162 ± 2	51 ± 2	42.6 ± 1.5
	male	4	27 ± 1	180 ± 4	73 ± 7	43.7 ± 1.3
Study II	male	9	26 ± 1	180 ± 3	89 ± 4	42.1 ± 2.8
Study III	male	10	26 ± 2	179 ± 2	85 ± 3	50.8 ± 1.6
Study IV	male	8	26 ± 2	183 ± 2	85 ± 2	54.8 ± 1.8
Mean			26 ± 1	178 ± 2	80 ± 3	47.7 ± 1.2

Table 1. *Subject characteristics for all four studies.*

3.2 INTERVENTION PROTOCOLS

3.2.1 Study I

After warming up on a cycle ergometer, subjects performed unilateral resistance exercise on two separate occasions separated by approximately four weeks. Each exercise session consisted of three warm up sets followed by 4 sets of 10 repetitions at 80% of 1RM and 4 sets of 15 repetitions at 65% of 1RM with 5 min of rest between each set. Tissue samples were collected before, immediately after and 1 hour after exercise in both the exercising and resting leg. Blood samples were collected at rest before warm-up, immediately before resistance exercise, after the fifth set (following approx. 25 min of exercise) and immediately after resistance exercise and following 15, 30 and 60 min of recovery. In a randomized, double-blind and cross-over fashion, subjects ingested 150 ml of a solution containing either a mixture of the three BCAA (45% leucine, 30% valine and 25% isoleucine) or flavoured water at rest before warm-up, immediately before resistance exercise and after the fourth set (following approx. 20 min of exercise), and immediately after exercise and following 15 and 45 min of recovery. The subjects were provided with a total of 85 mg BCAA · kg^{-1} body weight in 900 ml of flavoured water.

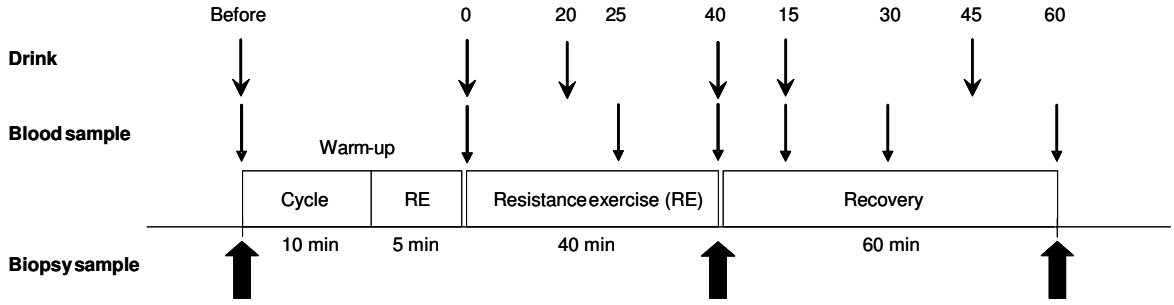

Figure 2. *Schematic overview of the experimental protocol in study I.*

3.2.2 Study II

Subjects performed heavy resistance exercise on four separate occasions, each being separated by approximately one week. Each exercise session began with a 5 min warm up on a cycle ergometer. After cycling, each subject performed four warm up sets after which 5 sets of 6 repetitions at 90% of 1RM followed by 5 sets of 10 repetitions at 75% of 1RM were

performed, with 5 min of rest allowed between each set. Muscle biopsies were sampled before, 60 and 90 min after exercise in all four trials. In each trial and in a randomized, double-blind and cross-over fashion, subjects were supplemented with one of four drinks: flavoured water (Placebo; Pla), leucine (Leu) or essential amino acids with (EAA) or without leucine (EAA-leu). The EAA solution was composed of eight essential amino acids (Ajinomoto, Kanagawa, Japan) in the following proportions: histidine, 14%; isoleucine, 9%; leucine, 17%; lysine, 18%; methionine, 3%; phenylalanine, 14%; threonine, 14%; and valine 11%. In the Leu trial, subjects were provided with 42 mg/kg body weight of leucine, which is the same as in the EAA mixture and similar to the dosage provided in Study I. In the EAA-leu drink, leucine was replaced with equal amounts of the non-essential amino acid glycine in order to keep the solution isonitrogenous compared to the EAA drink. Glycine was chosen as a substitute since it has been shown that large doses of this amino acid do not stimulate protein synthesis in human muscle (49). The total amount of amino acids in the EAA and EAA-leu trials was 240 mg amino acids/kg body weight. A total of 1050 ml solution was ingested in 150 ml boluses at rest prior to warm-up, immediately before performing the resistance exercise and after the fourth and seventh sets (following approximately 20 and 35 min of exercise, respectively), and immediately after termination of exercise and following 15 and 30 min of recovery. FSR was measured following intravenous administration of a flooding dose of L-[$^2H_5$] phenylalanine. Immediately after resistance exercise, the tracer infusion was initiated and completed within 10 minutes. Blood samples were collected at 5, 10, 15, 30, 40, 50, 70 and 90 min after the start of the tracer infusion for determination of L-[$^2H_5$] phenylalanine enrichment in plasma. Blood was also drawn at rest, before resistance exercise, after the sixth set (following approximately 30 min of exercise) and immediately after termination of the resistance exercise, and following 15, 30, 60 and 90 min of recovery for insulin and amino acid measurements.

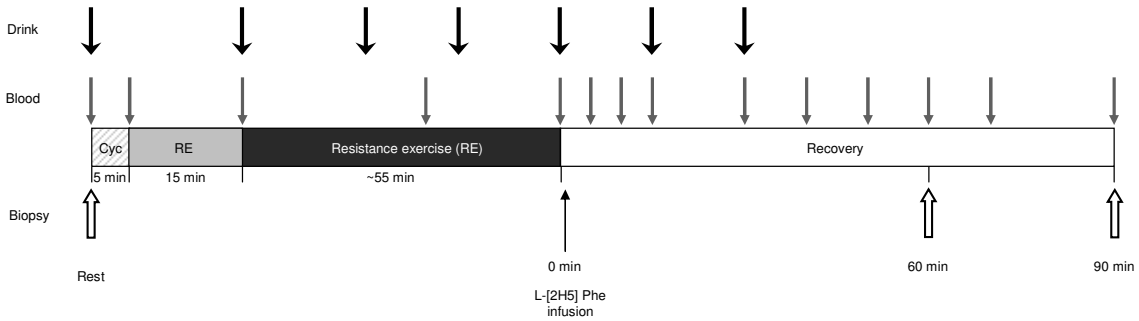

Figure 3. *Schematic overview of the experimental protocol in study II.*

3.2.3 Study III

In a randomized and cross-over fashion, subjects performed one session of resistance exercise (R) and another session of resistance exercise followed by endurance exercise (RE), approximately two weeks apart. Each subject began with three warm-up sets after which the subjects performed 10 sets of heavy resistance exercise. The resistance exercise protocol consisted of 4 sets of 8-10 repetitions at 85% of 1RM, 4 sets of 10-12 repetitions at 75% of 1RM and lastly 2 sets to volitional fatigue at 65% of 1RM with three min of recovery allowed between each set. After resistance exercise in the RE trial, subjects rested for 15 min and then performed 30 min of cycling at an intensity corresponding to 70% of each subjects' maximal oxygen consumption. Muscle and blood samples were collected before, 60 and 180 min after resistance exercise.

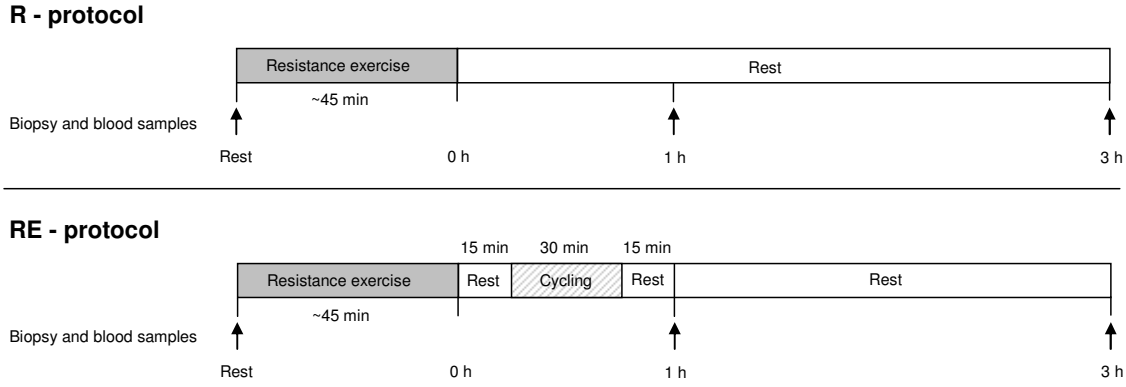

Figure 4. *Schematic overview of the experimental protocols in study III. R-protocol, resistance exercise; RE-protocol, resistance exercise followed by endurance exercise.*

3.2.4 Study IV

Study IV employed a randomized cross-over design in which each subject performed one session of high intensity interval cycling followed by resistance exercise (ER) and another session of resistance exercise only (R), approximately two weeks apart. During both trials, a primed constant infusion of L-[ring-$^{13}C_6$]-phenylalanine was initiated and maintained for the duration of each experiment (~ 10 h). Two hours after the initiation of the tracer infusion, the first resting biopsy was collected and three hours later a second resting biopsy was obtained. After the second resting biopsy, subjects in the ER-trial warmed up on a cycle ergometer for a total of 15 min, after which they performed five 4 min intervals at a work rate corresponding to 85% of each subjects' maximal oxygen uptake. Each high intensity interval was interspersed with 3 min of low intensity cycling. Immediately after the last interval, a third

muscle biopsy was taken after which subjects continued to cycle for an additional 10 minutes which was then followed by five minutes of complete rest. Next, the subjects carried out three warm-up sets and then performed 10 sets of heavy resistance consisting of 4 sets of 8-10 repetitions at 80% of 1RM, 4 sets of 10-12 repetitions at 70% of 1RM and lastly 2 sets to volitional fatigue at 60% of 1RM, with 3 min of recovery allowed between each set. In the R-trial, the cycling was replaced by rest with tissue sampling performed at the same time points as in the ER-trial. In both trials, immediately after resistance exercise, a fourth muscle biopsy was taken and after that, two additional biopsies were collected 90 and 180 min post resistance exercise. During the initial five hours of rest, blood was collected at 30 min interavals. During cycling, blood was drawn after warm up and after the third and fifth intervals. These time points were also used to collect blood in the R-trial in which cycling was replaced by supine rest. During resistance exercise, blood was collected prior to warm-up and following the third, seventh, tenth and thirteenth set. During recovery, blood was sampled 15 and 30 min after resistance exercise and then at 30-min intervals throughout the remainder of the trial.

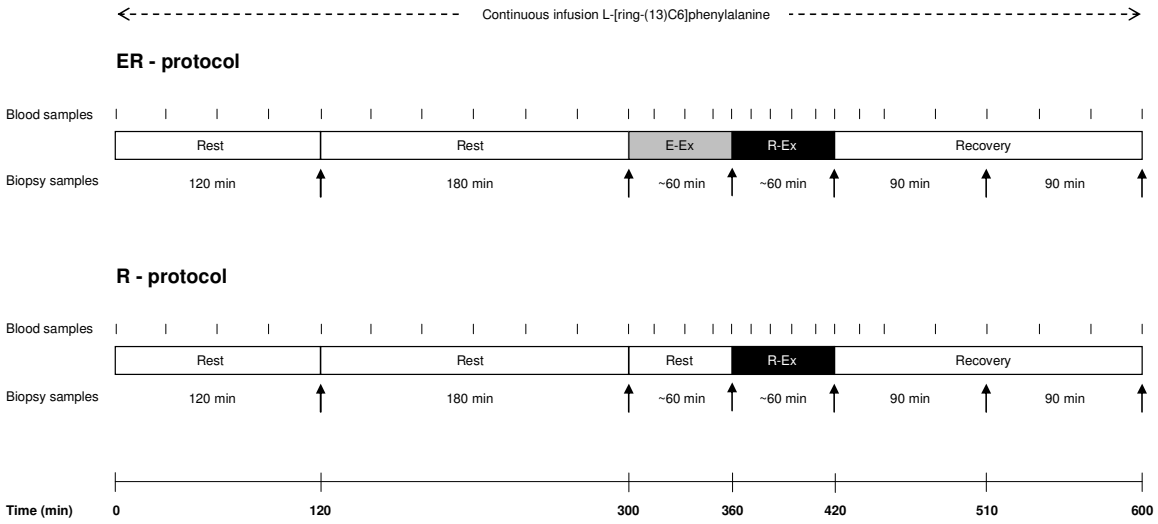

Figure 5*. Schematic overview of the experimental protocols in study IV. ER-protocol, interval cycling followed by resistance exercise; R-protocol, resistance exercise only. Arrows indicate sampling time points for muscle biopsies and vertical lines indicate sampling time points for blood.*

3.3 BIOPSY SAMPLING

Local anaesthesia was applied to the skin and muscle fascia of the vastus lateralis and after numbing, a small incision of approximately 0.5-1 cm was made through these tissue layers

for subsequent sampling. In studies I, II and IV, muscle extraction was performed with a Weil-Blakesley conchotome and in Study III muscle was sampled using a Bergström needle with manually applied suction. For every new time point, a new incision was made approximately 2-4 cm proximal to the previous one. Immediately after sampling, biopsies were blotted free of blood and quickly frozen in liquid nitrogen and stored at -80°C for later analysis. In study I, sampling always started in the exercising leg which in turn was randomly assigned in each subject. In study II, all biopsies were taken from the same muscle of each subject in each trial. Sampling was alternated between both legs throughout the four trials, beginning with the right leg in the first trial. In study III, the resting biopsy during the first trial was sampled from a randomly assigned leg and the two biopsies obtained during recovery were collected from the contra lateral leg. The opposite sampling pattern was used in the second trial. In study IV, sampling was alternated between legs throughout both trials, beginning with the right leg in the first trial.

3.4 PLASMA ANALYSIS

3.4.1 Glucose, lactate and insulin

To obtain plasma, blood was collected in heparinized and/or EDTA tubes and centrifuged at 9,000 *g* at 4°C for three min. Analysis of glucose and lactate was performed on plasma from heparinized tubes in all studies according to Bergmeyer (125). In study I, insulin was measured on heparinized plasma using a radioimmunoassay kit and in Study II plasma samples from EDTA-tubes were used for insulin measurements with an ELISA kit, both according to the manufacturers' instructions.

3.4.2 Amino acids

For amino acid measurements, heparinized plasma samples were deproteinized by precipitation with 5% trichloroacetic acid (TCA; 1:5) after which they were centrifuged at 9,000 *g* at 4°C for three min and the supernatant stored at -80°C. The concentration of amino acids in the supernatants from plasma was measured by reversed-phase high performance liquid chromatography (HPLC) according to Pfeifer *et al.* (126), with orthophthalaldehyde (OPA) as the derivatizing agent.

3.4.3 L-[$^{2}H_5$] phenylalanine enrichment in Study II

Equal volumes of plasma and 15% SSA were combined to precipitate proteins. After precipitation, samples were centrifuged at 16,600 *g* at 4°C for 10 min and the resulting supernatant was purified on a resin column and subsequently dried by vacuum centrifugation. After drying, samples were derivatized and plasma enrichment as well as enrichment of the standard curve was measured using GC–MS by selective ion monitoring for 336 and 341 m/z.

3.4.4 L-[ring-$^{13}C_6$]-phenylalanine in Study IV

200 µl of plasma was combined with 100 µl of internal standard (L-[ring-$^{13}C_9$]-phenylalanine, 50 µmol · L^{-1}) and then precipitated with 500 µl of acetic acid (50%) before being purified on a resin column, dried under a stream of N_2 and derivatized. Plasma enrichment as well as enrichment of the internal standard was measured using gas GC–MS/MS by selective ion monitoring for 336, 342, and 345 m/z.

3.5 MUSCLE ANALYSIS

3.5.1 Tissue processing prior to analysis

Muscle samples were freeze dried and thoroughly dissected clean from blood and connective tissue under a light microscope, leaving only very small fibre bundles intact. The fibre bundles were then extensively mixed, resulting in a homogenous sample pool free of non muscle contaminants. This mixed sample was then divided into aliquots for each subsequent analysis.

3.5.2 General western blot protocol

Cleaned muscle samples were homogenized in ice-cold buffer containing 2 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1% TritonX-100, 1 mM Na_3VO_4, 2 mM dithiothreitol (DTT), 1% phosphatase inhibitor cocktail and 1% (v/v) protease inhibitor cocktail. Homogenates were then cleared by centrifugation at 10,000 *g* for 10 min at 4°C and the protein concentration of the resulting supernatant was determined. Samples were diluted in Laemmli sample buffer, heated at 95°C for 5 min and

20-30 µg of protein was loaded on acrylamide gels for size dependent separation. Electrophoresis was performed on ice at 200-300 V for 40-90 min after which gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 10-20% methanol) for 30 min. After equilibration, proteins were transferred to polyvinylidine fluoride membranes at a constant current of 300 mA for 3 h at 4ºC. To confirm equal loading after transfer, membranes were stained with a total protein staining kit. For each set of target proteins, all samples from each subject were loaded on the same gel and all gels were run simultaneously. Membranes were then blocked for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM Tris base, 137 mM NaCl, pH 7.6) containing 5% non-fat dry milk and 0.1% Tween-20. After blocking, membranes were incubated overnight with commercially available primary antibodies diluted in TBS supplemented with 0.1% Tween-20 containing 2.5% non-fat dry milk (TBS-TM). Following overnight incubation, membranes were washed with TBS-TM and incubated for 1 h at room temperature with secondary antibodies conjugated with horseradish peroxidise. Next, the membranes were washed with TBS-TM and TBS. Finally, membranes with the antibodies bound to the target proteins were visualized by chemiluminescent detection. To standardize the immunoblotting procedure, prior to blocking, membranes were cut and assembled so that for each target protein, all membranes with samples from each subject would be exposed to the same conditions. In study II and IV, following image capture of phosphorylated proteins, membranes were stripped of the phosphospecific antibodies after which the membranes were re-probed with primary antibodies for each respective total protein as described above. All phospho-proteins were normalised to their corresponding total protein. When only total protein was measured, these values were normalized against values obtained with the total protein staining kit. In study III, phosphorylated and total proteins were normalized against total levels of α-tubulin.

3.5.3 Immunoprecipitation

In Study II, the interactions between mTORC1 related proteins were investigated by immunoprecipitating (IP) Raptor from tissue samples homogenized in ice-cold IP-lysis buffer containing 40 mM Hepes (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 50 mM NaF, 0.5 mM Na_3VO_4, 10 mM β-glycerophosphate, 1% (v/v) protease inhibitor cocktail and 0.3% (w/v) CHAPS detergent. Following homogenization, samples were centrifuged at 10,000 *g* for 10 min at 4°C after which an aliquot of 250 µg of protein was incubated with 2.5 µg of sheep anti-Raptor antibody and rotated over night at

4°C. The next morning, each sample was incubated with 12.5 µl of protein G magnetic beads and rotated for an additional hour. Following incubation, beads containing the Raptor-immune-complexes were washed four times with ice cold IP-lysis buffer after which the beads were combined with Laemmli sample buffer, boiled for five min and immunoblotted as described above. The amount of Co-IP targets were normalized against the amount of Raptor in the immunoprecipitate.

In study IV, the interactions between TSC1 and TSC2 was examined in tissue samples homogenized in ice-cold IP-lysis buffer containing 50 mM Hepes (pH 7.5), 0.1 mM EGTA, 1 mM EDTA, 1% (v/v) TritonX-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 0.27 M sucrose, 0.1% (v/v) β-mercaptoethanol (βME) and 1% (v/v) protease inhibitor cocktail. After centrifugation and IP of S6K1 (see below), TSC1 was immunoprecipitated from an aliquot of 175 µg of protein with 1 µg of goat anti-TSC1 antibody and 10 µl of protein G magnetic beads, incubated over night at 4°C. Following incubation, IPs of TSC1 were washed four times in IP lysis buffer after which the beads were combined with Laemmli sample buffer, boiled for five min and immunoblotted for TSC1 and TSC2 as described above. The amount of TSC2 was normalized against the amount of TSC1 in the immunoprecipitate.

In both studies, IP's were also performed for subsequent kinase assays; kinase activity of S6K1 was measured in both Study II and IV, while AMPK activity was assessed only in Study IV. Two different IP-lysis buffers were used in Study II and IV (see above) and these buffers were used to IP S6K1 in each respective study. In both studies, 750 µg of protein was combined with 7.2 µg of rabbit anti-S6K1 antibody and 10 µl of protein G sepharose beads per sample and rotated for 3 hours at 4°C. Immunoprecipitation of the α1 and α2 isoforms of AMPK in Study IV were performed on two aliquots of 225 µg of protein each, that were incubated with 4 µg of AMPKα1 and AMPKα2 antibodies, respectively, and 10 µl of protein G sepharose beads. The AMPK IP samples were also combined with 800 µl of AMPK lysis buffer (50 mM TrisHCl (pH 7.25), 150 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% (v/v) TritonX-100 and 1% (v/v) protease inhibitor cocktail) to adjust for the slightly higher pH in the IP lysis buffer, and incubated over night at 4°C. Following IP, the beads with the S6K1- and AMPK-immune-complexes were washed twice in their respective high salt lysis buffer (i.e. respective IP-lysis

buffers and AMPK lysis buffer; both with 0.5 M NaCl) and once in kinase specific assay buffer (see below).

3.5.4 Kinase assays

Following the last wash in kinase specific assay buffer (S6K1, 50 mM TrisHCl at pH 7.5, 0.03% BrijL23, 0.1% βME; AMPK, 50 mM HEPES at pH 7.4, 1 mM DTT, 0.03% BrijL23), the beads from each sample were suspended in assay buffer and divided into three assays of 20 µl each. Two of the assays were run with a kinase specific substrate and the third assay was run without the substrate, thus serving as a blank. Kinase assays were initiated by the addition of 30 µl of a hot (radiolabeled) kinase specific reaction mix every 20 sec and terminated at 20 sec intervals by the addition of 50 µl phosphoric acid (1% v/v) to each assay. For the S6K1 activity assay, the final reaction mix (50 µl) consisted of 100 µM ATP, 10 mM $MgCl_2$, $^{32}\gamma$-ATP (specific activity: Study II, 1.1 x 10^6; Study IV, ~ 2.5 x 10^6 cpm/nmol), 30 µM synthetic S6K1 substrate (Study II, AKRRRLSSLRA; Study IV, KRRRLASLR) and was carried out at 30°C for 45 min in Study II and 60 min in Study IV. The AMPK activity assays were performed for 30 min at the same temperature and final volume, however, in a reaction mix consisting of 200 µM ATP, 200 µM AMP, 5 mM $MgCl_2$, $^{32}\gamma$-ATP (specific activity: ~ 0.2 x 10^6 cpm/nmol) and 200 µM synthetic AMPK substrate ("AMARA"; AMARRAASAAALARRR). After termination of the assay reactions, assays were spotted onto squares of p81 filter paper and washed three times in phosphoric acid and once in acetone. When the p81 squares had dried they were immersed in scintillation fluid and counted on a liquid scintillation counter. The average values from the duplicate assays with substrate were corrected for background noise by subtraction of the blank (no substrate) and values were expressed as pmol/min/mg protein.

3.5.5 mRNA analysis

Total RNA was extracted from approximately 2-3 mg lyophilized and cleaned tissue which was homogenized in RNA isolation reagent according to the manufacturers´ instructions. The concentration and purity of the RNA was determined by spectrofotometry and 2 µg RNA was used for reverse transcription of 40 µl cDNA using a cDNA synthesis kit. The concentration of cDNA, annealing temperature and PCR cycle protocol was determined for each primer pair to ensure optimal conditions for amplification. Samples were run in triplicate and all

samples from each subject were run on the same plate to allow direct relative comparisons. Relative changes in mRNA levels were analyzed by the $2^{-\Delta CT}$ method with GAPDH used as the reference gene.

3.5.6 Amino acids

For analysis of free amino acids, freeze dried tissue samples were extracted with 5% TCA (30µl/mg), centrifuged at 9,000 *g* for 3 min and the resulting supernatant was measured by reversed-phase high performance liquid chromatography (HPLC) according to Pfeifer *et al.*(126) , with orthophthalaldehyde (OPA) as the derivatizing agent.

3.5.7 Muscle glycogen

Muscle glycogen was determined in approximately 2 mg lyophilized and cleaned muscle tissue according to the method described by Leighton *et al.* (127).

3.5.8 L-[$^2H_5$] phenylalanine enrichment in Study II

Approximately 10 mg of muscle tissue was homogenized in 1 ml of 4% SSA and then rotated for 30 min at 4°C. The precipitated proteins were then pelleted by centrifugation and the pellets washed in 4% SSA and subsequently dissolved in 1 ml of 0.3 M NaOH. To precipitate proteins once again, 130 µl of 40% SSA was added to all samples which were then kept on ice for 10 min. Proteins were pelleted once more by centrifugation and the pellets washed with 4% SSA before being hydrolyzed for 24 h in 1 ml of 6 M HCl at 110°C. Hydrolyzed samples were dried and subsequently dissolved in 450 µl of 0.5 M trisodium citrate and passed through filter tubes after which each sample was combined with a suspension containing 2 mg of tyrosine decarboxylase and 0.25 mg pyridoxal phosphate and incubated over night at 50°C to decarboxylate phenylalanine into phenyl ethylamine. Next morning, 100 µl of 6 M NaOH was added to each vial and samples were pelleted by centrifugation. The supernatants were combined with 500 µl of ether to extract phenyl ethylamine. Samples were shaken vigorously and then placed in an ethanol bath with dry ice. When the bottom layers had frozen, the liquid ether phase was transferred to new tubes containing 100 µl of 0.1 M HCl by which the phenyl ethylamine was back-extracted to the aqueous phase from the

ether phase. The new tubes were shaken and again placed in the ethanol bath and when the bottom layer containing the phenyl ethylamine had frozen, the liquid ether phase was discarded. Samples were then transferred to GC-MS vials and dried after which they were derivatized by the addition of 25 µl of N-Methyl-N- (Tert Butyldimetylsilyl) trifluouroacetamide and ethyl acetate in a ratio of 1:1 and incubated for 1 h at 60°C. The ratio of isotopically labelled and unlabelled phenylalanine was obtained by selective ion monitoring for 180 (m+2) and 183 (m+5) m/z. Protein enrichment was obtained by relating the ratio of labelled and unlabeled phenylalanine in each sample to a standard curve containing 0-0.267 atom percent excess (APE) of L-[$^{2}H_5$] phenylalanine, that was run together with all samples. FSR was calculated as follows:

$$FSR = (E_m/A) \times 60 \times 100$$

Where E_m is the delta enrichment of L-[$^{2}H_5$] phenylalanine in muscle protein between biopsies taken after 90 min of recovery and at rest, and A is the area under the curve for L-[$^{2}H_5$] phenylalanine enrichment in plasma during 90 min of recovery. Values are multiplied by a factor of 60 and 100 to express FSR in percent per hour (%/h).

3.5.9 L-[ring-$^{13}C_6$]-phenylalanine in Study IV

Approximately 7 mg of muscle tissue was combined with 100 µl of internal standard (L-[ring-$^{13}C_9$]-phenylalanine, 5 µmol · L^{-1}) after which samples were pelleted and extracted twice with 500 µl of 2% perchloric acid. To determine intracellular enrichment of free phenylalanine, supernatants were combined and dried, after which they were dissolved in 500 µl of 50% acetic acid before being passed through a cation exchange resin column. Amino acids were then eluted with 2 ml of 2 M NaOH, dried under a stream of N_2 and derivatized by the addition of 50 µl of N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide and acetonitrile (1:1) and heated at 70°C for 1 h. Intracellular enrichment as well as enrichment of the internal standard was measured using GC–MS/MS with electron impact ionization and selective ion monitoring for 336, 342, and 345 m/z. The remaining pellet was washed twice with 70% ethanol and then hydrolyzed over night in 1 ml of 6 M HCl heated to 110°C. The hydrolyzed proteins were then dissolved in 500 µl of acetic acid (50%) and passed through a cation exchange column. To determine protein bound phenylalanine enrichment, the purified

pellet derived amino acids were eluted with 2 ml of 2 M NaOH, dried under a stream of N_2 and converted to their N-acetyl-n-propyl amino acid esters and analyzed by GC–C–IRMS. FSR was calculated using the standard precursor–product method:

$$FSR = \Delta E_{p\ \mathrm{phe}} / (E_{ic\ \mathrm{phe}} \times t) \times 100$$

Where $\Delta E_{p\ \mathrm{phe}}$ is the difference in protein bound phenylalanine enrichment between two biopsies, $E_{ic\ \mathrm{phe}}$ is the intracellular phenylalanine enrichment in the second biopsy, and t is the time period for tracer incorporation in hours. To express FSR in percent per hour (%/h), values were multiplied by 100.

4 RESULTS

4.1 STUDY I

In study I, the effects of BCAA ingestion on mTORC1 signaling in both resting and exercising human skeletal muscle were investigated. It was found that ingestion of BCAA increased phosphorylation of S6K1 at the mTORC1 specific site Thr^{389} in both legs while placebo ingestion had no effect in either leg. Although not quite significant, phosphorylation of S6K1 was higher in the exercising leg of all subjects in the BCAA trial, indicating the existence of a synergistic effect of BCAA and exercise on mTORC1 signaling in human muscle. Phosphorylation of Akt, an upstream effector of mTORC1 was unaffected by both exercise and amino acid supplementation. Downstream of S6K1, phosphorylation of rpS6 increased to a larger extent in the exercising leg in both trials, with no difference between the two. At the end of recovery, phosphorylation of eEF2 decreased to a similar extent in both legs in both trials.

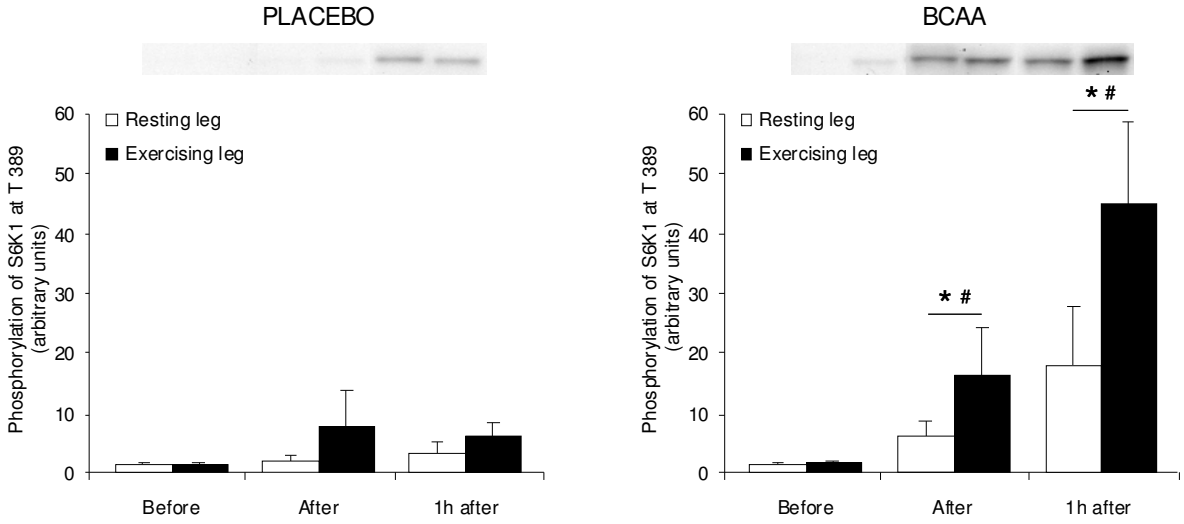

Figure 6. *Phosphorylation of S6K1 at Thr^{389} in resting and exercising muscle during placebo and BCAA trials. Representative immunoblots from one subject are shown above each graph. Bands have been rearranged to fit the illustrated bars. Values in graphs are arbitrary units (means ± SE for 9 subjects). Symbols above lines denote differences revealed by a post-hoc test when a main effect was observed.* $^{*}P < 0.05$ *vs. before exercise;* $^{\#}P < 0.05$ *vs. placebo.*

4.2 STUDY II

In study II, we examined the particular role of leucine in mTORC1 signaling, complex assembly, S6K1 kinase activity as well as protein synthesis in human skeletal muscle. At 60 and 90 min of recovery, supplementation of leucine (Leu) increased S6K1 phosphorylation at Thr^{389} ~170 and ~190% more compared to placebo (Pla) and essential amino acids supplied

without leucine (EAA-leu) , respectively. When essential amino acids were supplied with leucine (EAA), S6K1 phosphorylation was further increased at both time points, resulting in ~340 and ~400% higher values, respectively, compared to Pla and EAA-leu. Compared to Leu, supplementation with EAA resulted in ~60 and ~75% higher S6K1 phosphorylation at the 60 and 90 min time points, respectively.

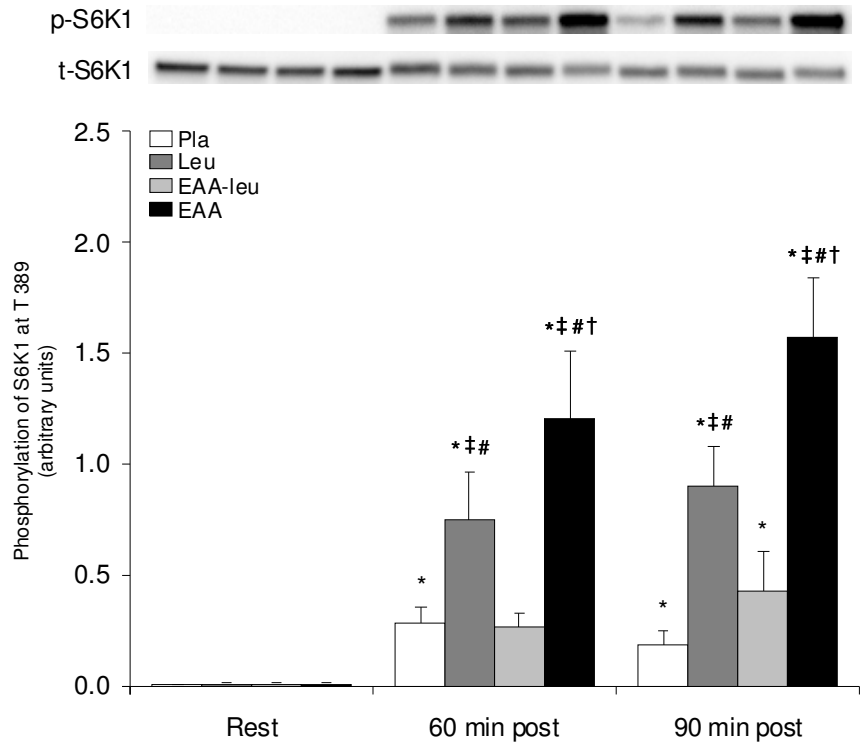

Figure 7. *Phosphorylation of S6K1 at Thr^{389} before, 60 and 90 min after exercise in the four trials; placebo (Pla), leucine (Leu) and essential amino acids with (EAA) and without leucine (EAA-leu). Representative immunoblots from one subject are shown above each graph. Values in graphs are arbitrary units (means ± SE for 9 subjects). Symbols denote differences revealed by a post-hoc test when an interaction effect was observed.* $^{*}P < 0.05$ *vs. before exercise;* $^{‡}P < 0.05$ *vs. Pla;* $^{\#}P < 0.05$ *vs. EAA-leu;* $^{†}P < 0.05$ *vs. Leu.*

Phosphorylation of 4EBP1at Thr$^{37/46}$ was increased approximately 50% at 60 min post resistance exercise with no difference between trials. At the 90 min time point, phosphorylation of 4EBP1 remained elevated at a similar level, again with no difference between trials.

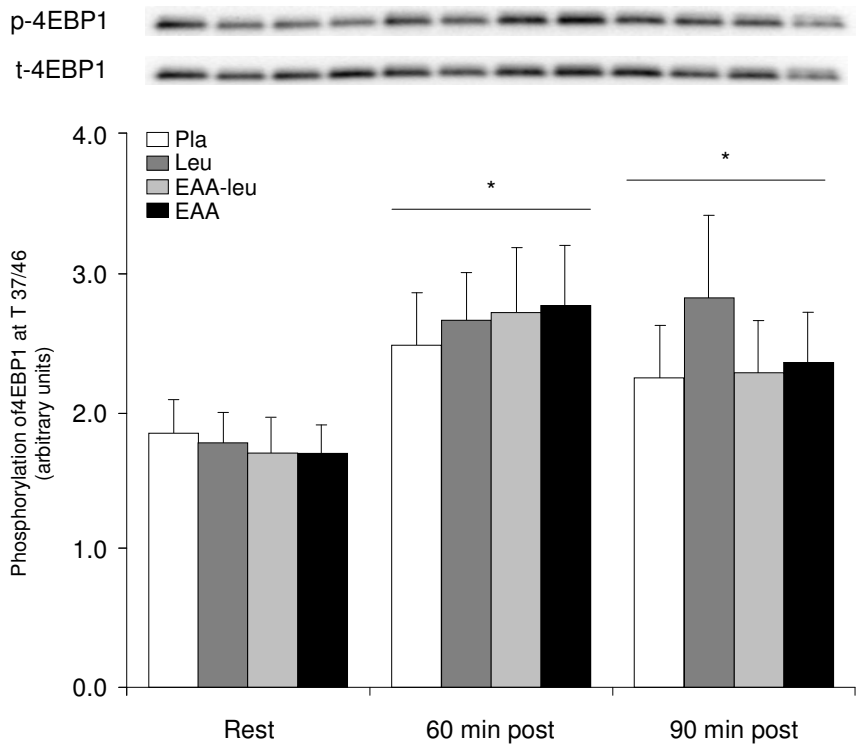

Figure 8. *Phosphorylation of 4EBP1 at Thr$^{37/46}$ before, 60 and 90 min after exercise in the four trials; placebo (Pla), leucine (Leu) and essential amino acids with (EAA) and without leucine (EAA-leu). Representative immunoblots from one subject are shown above each graph. Values in graphs are arbitrary units (means ± SE for 9 subjects). Symbols above lines denote differences revealed by a post-hoc test when a main effect was observed.* $^{*}P < 0.05$ *vs. before exercise.*

Kinase activity of S6K1 was higher in the Leu trial compared to Pla (54%), and tended to be higher compared EAA-leu (36%). S6K1 activity following EAA supplementation was significantly higher compared to both Pla and EAA-leu (97 and 73%, respectively) and tended to be higher compared to Leu (28%). There was a strong correlation between S6K1 phosphorylation at Thr^{389} and S6K1 activity (r=0.72).

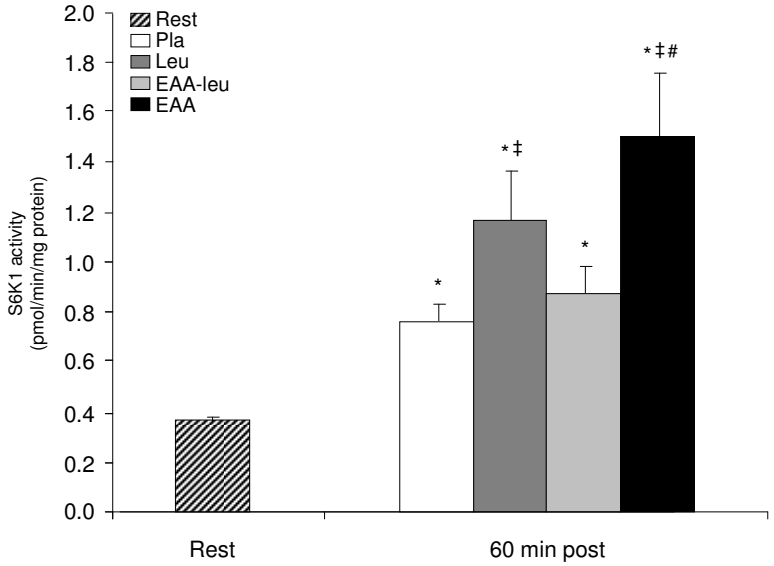

Figure 9. *Kinase activity of S6K1 before and 60 min after exercise in the four trials; placebo (Pla), leucine (Leu) and essential amino acids with (EAA) and without leucine (EAA-leu). Values in graph are presented as means ± SE for 9 subjects.* $^{*}P < 0.05$ *vs. before exercise;* $^{‡}P < 0.05$ *vs. Pla;* $^{\#}P < 0.05$ *vs. EAA-leu.*

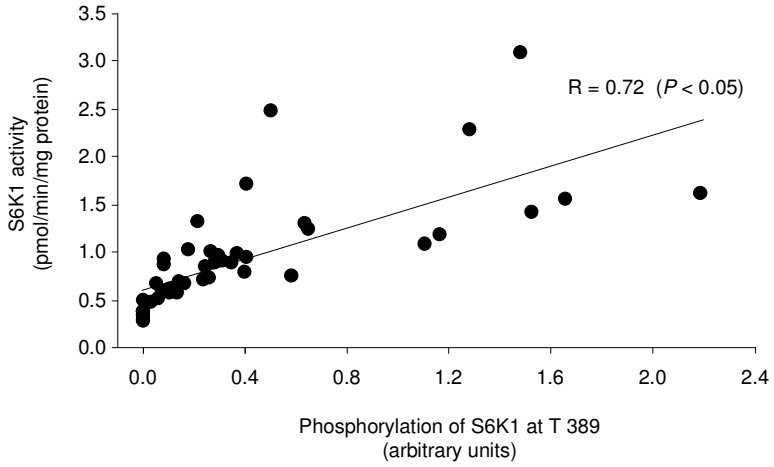

Figure 10. *Correlation between S6K1 activity and S6K1 phosphorylation before and 60 min after exercise in the four trials.*

Complex assembly of mTORC1 was unaltered by exercise as well as amino acid supplementation in all four trials.

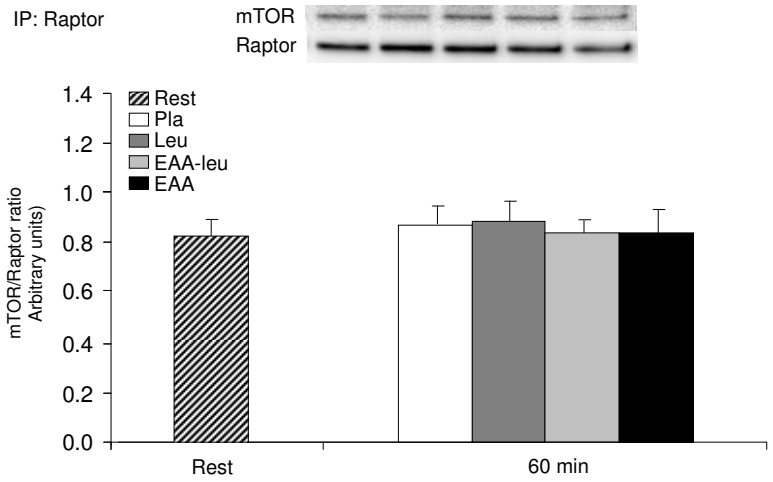

Figure 11. *mTORC1 assembly. The amount of recovered mTOR was related the amount of Raptor present in the immunoprecipitate. Values are means ± SE for 9 subjects.*

Protein synthesis was highly similar in both the Leu and EAA trials (0.091 ± 0.007%/h in both) as well as in the Pla and EAA-leu trials (0.075 ± 0.011 and 0.073 ± 0.008%/h, respectively). Despite the numerically higher FSR in the Leu/EAA trials (26%) compared to the Pla/EAA-leu trials, these differences did not quite reach statistical significance (P = 0.15).

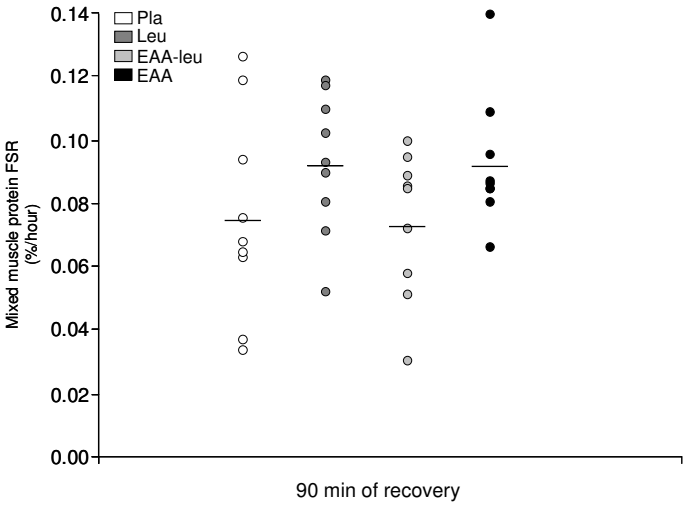

Figure 12. *Individual values for mixed skeletal muscle protein FSR during 90 min of recovery after exercise in the four trials; placebo (Pla), leucine (Leu) and essential amino acids with (EAA) and without leucine (EAA-leu). Values in graph are presented for 9 subjects. Horizontal lines represent mean values.*

4.3 STUDY III

In study III, mTORC1 signaling in human skeletal muscle was examined in response to single mode resistance exercise (R) and resistance exercise followed by continuous cycling (ER). Both exercise protocols induced pronounced increases in mTORC1 signaling during recovery as assessed by S6K1 phosphorylation of the Thr^{389} residue. More specifically, phosphorylation of S6K1 increased ~5-fold at 60 min post resistance exercise and continued to increase until the 180 time point, reaching ~14-fold higher values compared to before exercise, with no difference between trials. In contrast, phosphorylation of 4EBP1, another downstream target of mTORC1, was largely unaffected by either exercise protocol.

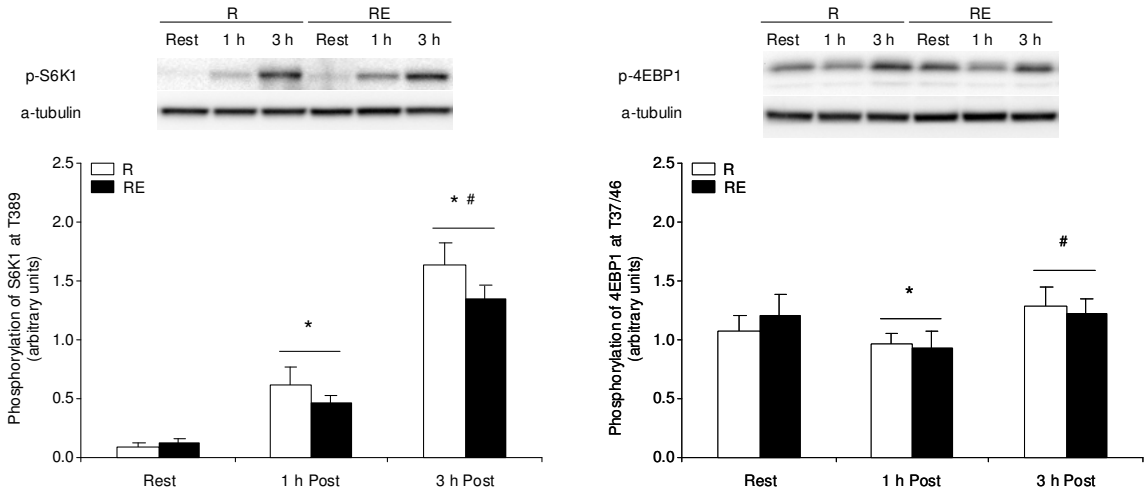

Figure 13. *Phosphorylation levels of S6K1 at Thr^{389} and 4EBP1 at $Thr^{37/46}$ before, 1 and 3 h post resistance exercise in both trials. Representative immunoblots from one subject are shown above each graph. Values are normalized to α-tubulin and presented as means ± SE for 10 subjects (n = 9 for 3 h Post). R, resistance exercise only; RE, resistance exercise followed by cycling. Symbols above lines denote differences revealed by a post-hoc test when a main effect was observed.* $^{*}P < 0.05$ *vs. Rest;* $^{\#}P < 0.05$ *vs. 1h Post.*

Phosphorylation of eEF2 was reduced by approximately 70% at both time points during recovery with no difference between trials. Phosphorylation of AMPK at Thr^{172} and its downstream target ACC at Ser^{79} was reduced 180 min into recovery by ~30 and ~50%, respectively, again with no difference between trials.

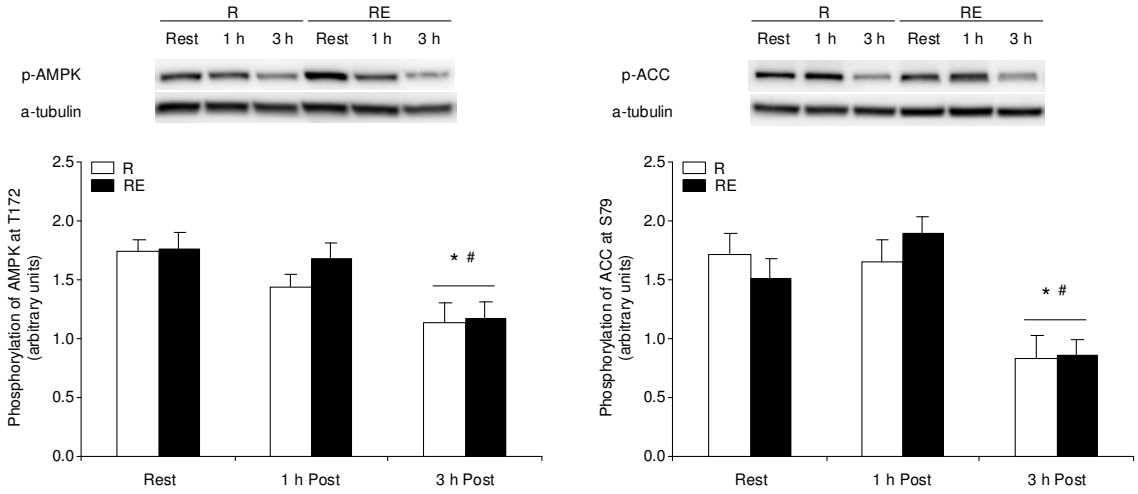

Figure 14. *Phosphorylation levels of AMPK at Thr^{172} and ACC at Ser^{79} before, 1 and 3 h post resistance exercise in both trials. Representative immunoblots from one subject are shown above each graph. Values are normalized to α-tubulin and presented as means ± SE for 10 subjects (n = 9 for 3 h Post). R, resistance exercise only; RE, resistance exercise followed by cycling. Symbols above lines denote differences revealed by a post-hoc test when a main effect was observed.* $^{*}P < 0.05$ *vs. Rest;* $^{\#}P < 0.05$ *vs. 1h Post.*

4.4 STUDY IV

In study IV, mTORC1 and AMPK signaling was examined in response to resistance exercise with (ER) or without (R) prior high intensity interval cycling. Immediately after cycling, phosphorylation of S6K1 and eEF2 increased significantly, but 4EBP1 phosphorylation decreased, compared to before exercise as well as compared to the same time point in the R trial. This phosphorylation pattern was maintained immediately after resistance exercise, but was now evident in both trials with no difference between the two. During recovery at 90 and 180 min post resistance exercise, phosphorylation of S6K1 at Thr^{389} increased ~9 and ~12-fold, respectively, while phosphorylation of eEF2 was reduced by ~55% at both time points. During recovery, phosphorylation of 4EBP1 had largely returned to baseline values. Kinase activity of S6K1 tended to increase at the 90 min time point but did not quite reach statistical significance until 180 min post resistance exercise at which kinase activity was elevated ~125% in both trials, compared to before exercise.

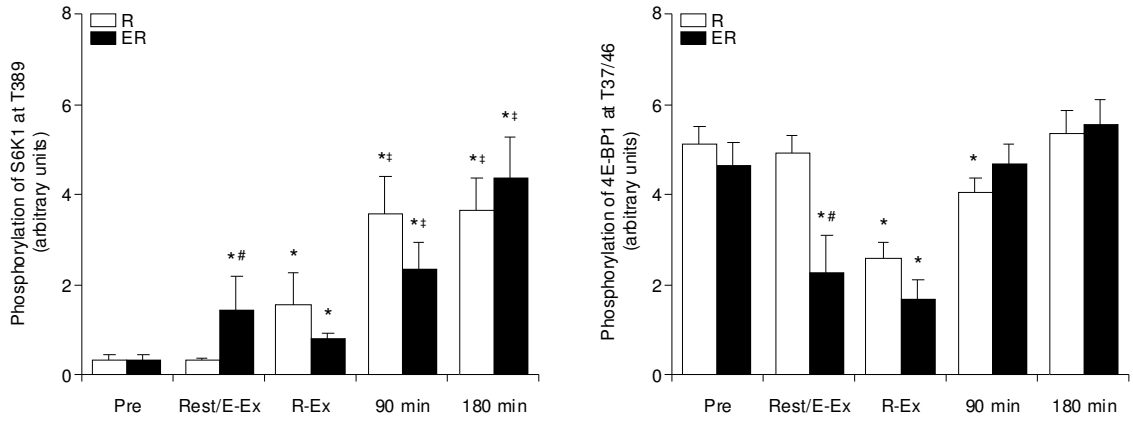

Figure 15. *Phosphorylation levels of S6K1 at Thr^{389} and 4EPB1 at Thr$^{37/46}$ before and after exercise. ER, interval cycling followed by resistance exercise; R, resistance exercise only. Phosphorylation values are normalized to the corresponding total values for each protein and presented as means ± SE for 8 subjects. Symbols denote differences revealed by a post-hoc test when an interaction effect was observed. $^{*}P < 0.05$ vs. Rest; $^{\#}P < 0.05$ vs. R-trial; $^{\ddagger}P < 0.05$ vs. R-Ex.*

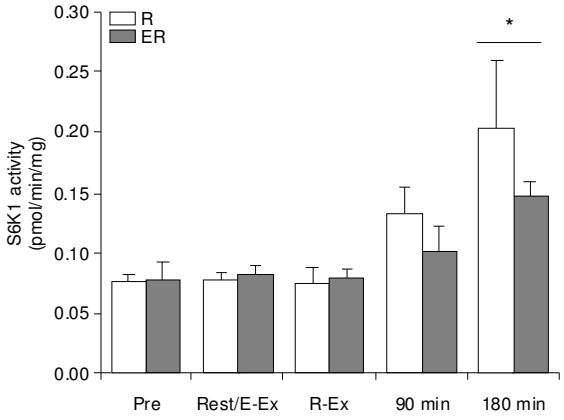

Figure 16. Kinase activity of S6K1. ER, interval cycling followed by resistance exercise; R, resistance exercise only. All values are presented as means ± SE for 8 subjects. Symbols above lines denote differences revealed by a post-hoc test when a main effect was observed. $^{*}P < 0.05$ vs. Rest.

Kinase activity of AMPK α1 was unaffected by either exercise protocol. In contrast, AMPK α2 activity increased ~90% immediately after cycling and this elevation was maintained after resistance exercise in the same trial, but had returned to baseline values during recovery. In the R trial, AMPK α2 activity was unaffected by resistance exercise at all time points. Phosphorylation of TSC2 at Thr^{1387}, downstream of AMPK, increased ~40% after cycling as well as after resistance exercise in both trials. Phosphorylation of Raptor at Ser^{792}, another downstream target of AMPK, was unaffected by cycling but increased after resistance exercise to the same extent in both trials.

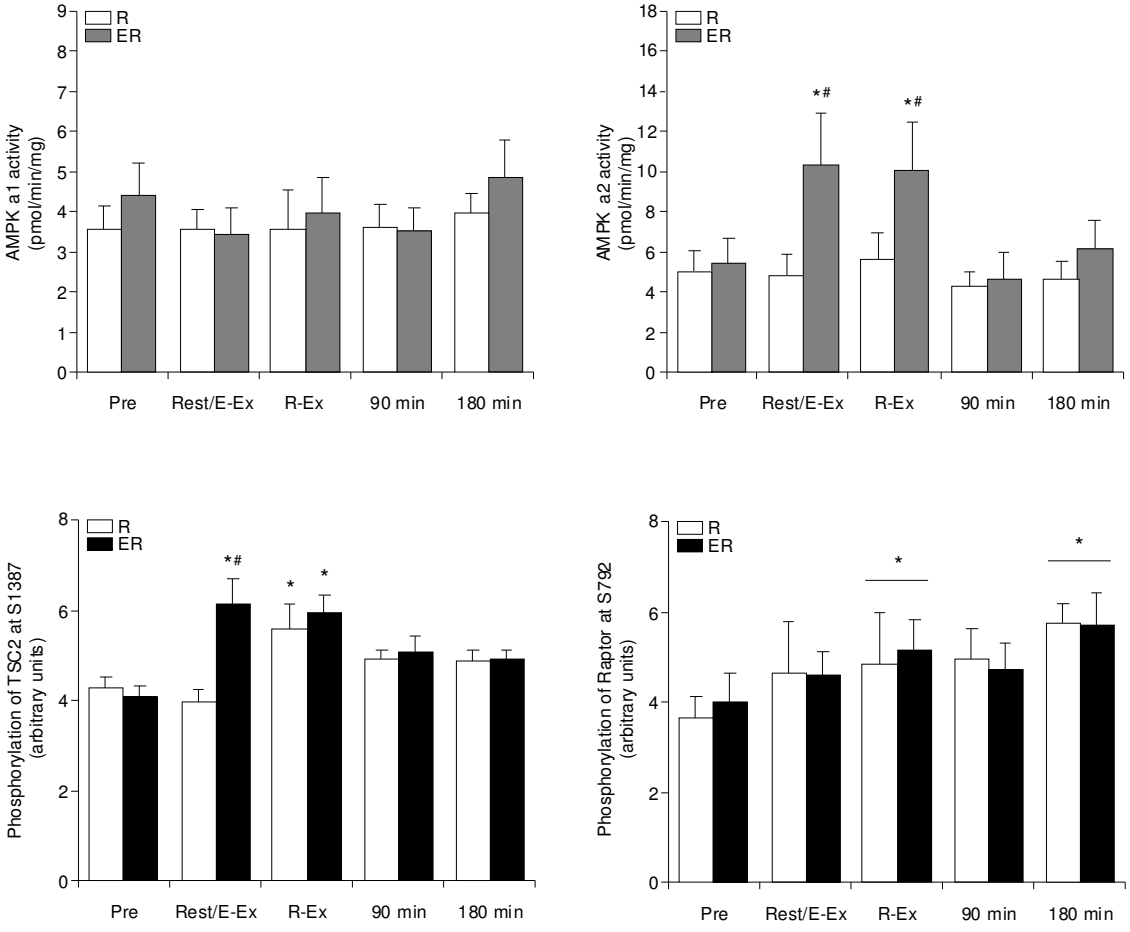

Figure 17. *Kinase activity of AMPK isoforms α1 and α2 and phosphorylation levels of TSC2 at Ser^{1387} and Raptor at Ser^{792} before and after exercise. ER, interval cycling followed by resistance exercise; R, resistance exercise only. All values are presented as means ± SE for 8 subjects. Symbols above lines denote differences revealed by a post-hoc test when a main effect was observed. Symbols without lines denote differences revealed by a post-hoc test when an interaction effect was observed.* $^{*}P < 0.05$ *vs. Rest;* $^{\#}P < 0.05$ *vs. R-trial.*

Furthermore, assembly of the TSC1/2 complex was unaffected by both single mode resistance exercise as well as concurrent exercise. Skeletal muscle protein synthesis during rest was 0.056±0.005%/h. During 180 min of recovery in the R-trial, the FSR value was 0.069±0.012%/h. In the ER-trial, this value was 0.082±0.015%/h. While numerically higher in both trials compared to rest, these increases were not statistically significant (P = 0.18). Expression of MuRF1 mRNA was unchanged in the R-trial at all time points but increased 2.2 and 1.6-fold at 90 and 180 min after resistance exercise in the ER-trial. Protein expression of MuRF1 did not change in the R-trial but increased in the ER-trial at 180 min during recovery by approximately 15% compared to before exercise as well as compared the R-trial. In contrast, at 180 min post exercise, mRNA expression of MAFbx decreased by approximately 50% the R-trial while it tended to increase at the same time point in the ER-

trial. Protein expression of MAFbx decreased by approximately 10% at the 180 min time point during recovery in both trials, with no difference between the two.

5 METHODOLOGICAL CONSIDERATIONS

5.1 RAPTOR IP AND MTORC1 ASSEMBLY

In study II, immunoprecipitation of Raptor was performed to examine mTORC1 assembly in response to amino acid supplementation and resistance exercise. The aim was to measure the interaction of Raptor with mTOR, RagC, Rheb and Lamtor1. However, only mTOR was detected in the immunoprecipitates, indicating that the remaining proteins may not be present in human skeletal muscle at detectable levels. As many previous studies have used HEK293 cells to examine theses interactions (52, 53, 75-77), we chose to compare the abundance of RagC, Rheb and Lamtor1 in these cells with that of human skeletal muscle. As can been seen in figure 18, when equal amounts of protein are loaded, abundance of all proteins of interest is much higher in HEK293 cells compared to that of muscle. Furthermore, while Raptor and mTOR are readily detectable in skeletal muscle, RagC is undetectable even in 50 µg of protein. In contrast, faint bands of Lamtor1 could be detected when 25 and 50 µg protein was loaded. For Rheb, a very faint band was detected only in the 50 µg lane. Thus, the lack of these proteins detected in the immunoprecipitates is likely explained by the very low abundance in skeletal muscle. These results indicate that experiments performed in transformed cell lines such as HEK293 may not be representative of those performed on adult skeletal muscle. Thus, caution should be exercised when extrapolating data from cell lines and applying these on skeletal muscle.

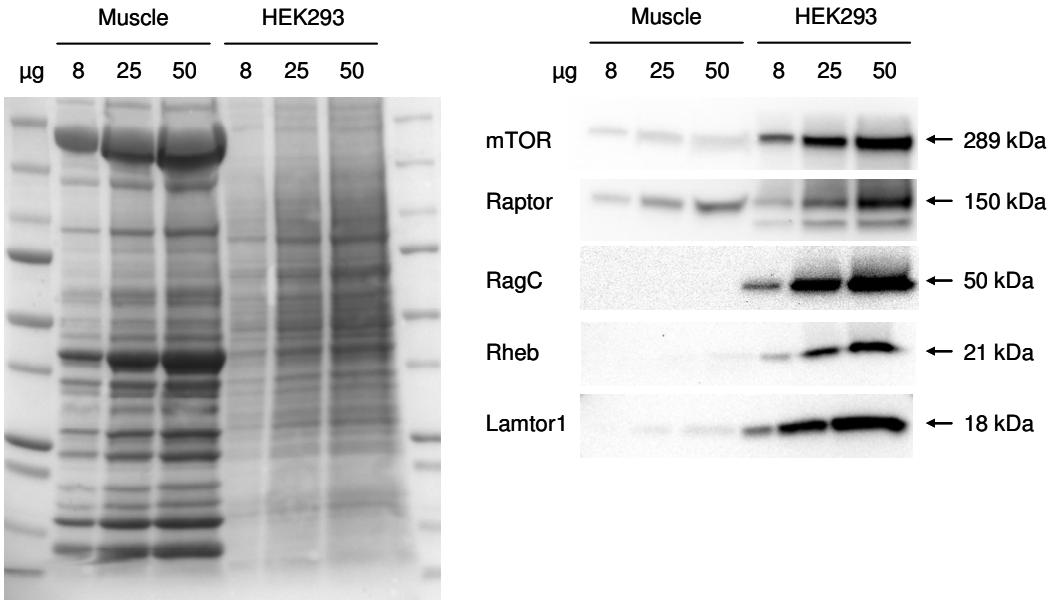

***Figure 18**. Left: image of total protein staining in various amounts of loaded protein in adult human skeletal muscle and HEK293 cells. Right: blots following incubation with target specific antibodies at a dilution of 1:1000 as described in the general western blot protocol.*

5.2 S6K1 KINASE ACTIVITY

In studies II and IV, kinase activity of S6K1 was assessed by measuring the *in vitro* rate of incorporation of radiolabeled phosphate onto a peptide substrate. In order to determine the appropriate incubation length, a time dependent saturation assay was performed on pooled muscle samples. The sample was treated as in study IV. Briefly, after homogenization and incubation, bead-immune-complexes were split into nine assays. Three time points were chosen and three assays were used for each time point. For each time point, two of the assays were run with substrate and the third assay was run without substrate, thus serving as a blank. The average values from the duplicate assays with substrate were corrected for background noise by subtraction of the blank (no substrate) and values were expressed as pmol/min/mg protein. Time points chosen were 15, 30 and 60 minutes.

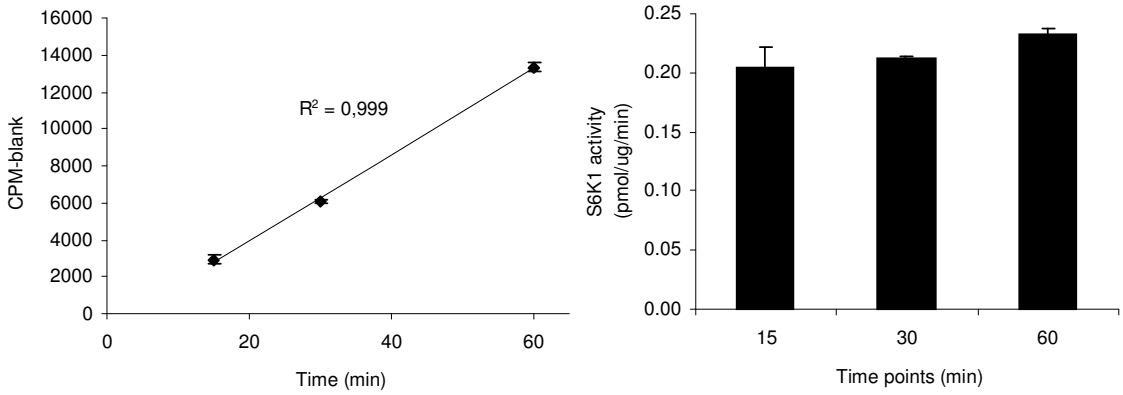

Figure 19. *Left: Time dependent saturation curve for the S6K1 kinase assay. CPM, counts per minute. Right: Kinase activity of S6K1 at each time point measured in the saturation curve. Values are means ± SD for blank corrected duplicates.*

As can be seen in figure 19, each time point measured was within the linear range of the assay, thus kinase activity was highly similar at all time points. Based on these experiments, it can be concluded that the incubation times chosen in studies II (45 min) and IV (60) were appropriate for this assay.

In study II, tissue limitations prevented us from measuring kinase activity in the resting state in all subjects in all trials. Therefore, resting values for S6K1 activity were only obtained from a single pre-exercise biopsy from all subjects. The rationale behind this choice was that the pre-exercise values were always obtained in the postabsorptive state prior to exercise and

amino acid supplementation in each trial. Thus, all pre-exercise values should be equally representative of the resting state. However, in three subjects there was sufficient biopsy material from each trial and these were therefore measured to validate our previous assumption.

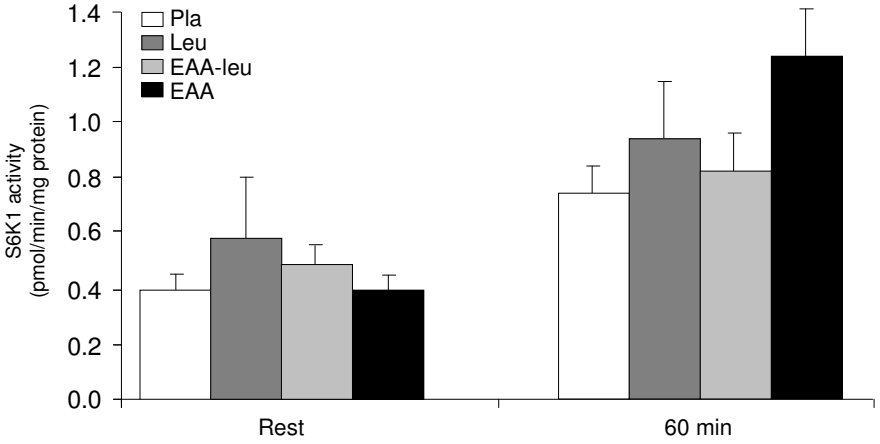

Figure 20. *Kinase activity of S6K1 at rest and 60 min after resistance exercise. Values are means ±SE for three subjects.*

As evident from figure 20, resting values were very similar between trials in the three subjects and they corresponded well with mean values obtained from the single biopsies in the complete set of subjects (see figure 9). At rest in the Leu trial, one subject had approximately two fold higher kinase activity compared to the other trials as well as compared to the two other subjects, thus explaining the numerically higher mean and standard error on this trial.

6 DISCUSSION

Since the discovery of mTOR almost twenty years ago (128), our understanding of cellular growth regulation has increased immensely, yet several of the mechanisms regulating this pathway remain to be fully determined. Thus, the aim of this thesis was to study the molecular mechanisms involved in the regulation of protein synthesis in response to amino acids and various modes of exercise in human skeletal muscle.

In the first study examining the mTORC1 pathway in response to amino acids and resistance exercise in human muscle, Karlsson *et al.* (91) found that S6K1 phosphorylation increased only when resistance exercise was combined with BCAA ingestion. The results indicated that the BCAA were solely responsible for this effect as phosphorylation of S6K1 was unaltered after exercise alone. However, due to the experimental design, it was not possible to distinguish the separate effects of each independent stimulus. To address this question, study I was undertaken using a unilateral exercise model in which one leg performed resistance exercise and the other served as a resting control. This model allowed us to examine the impact of BCAA in both resting and exercising muscle in contrast to only the latter as in the study by Karlsson *et al.* (91). In line with their results, we found that the most potent increase in S6K1 phosphorylation occurred when exercise was combined with BCAA intake, while resistance exercise alone did not alter Thr^{389} phosphorylation. Interestingly, there was a significant difference between BCAA and placebo, showing that amino acid provision increased S6K1 phosphorylation in both resting and exercising muscle. These results suggested that BCAA intake is more potent than exercise alone as S6K1 phosphorylation remained unchanged in response to the latter. Although mean differences were not significant, further examination of individual data showed that S6K1 phosphorylation was more pronounced in the exercising leg compared to the resting leg in all subjects. Nevertheless, the combined effect of resistance exercise and BCAA intake increased S6K1 phosphorylation to a greater extent than did the sum of the two when exerted separately, indicating the existence of a synergistic effect. Whether these data are reflective of the anabolic potential of each stimulus, separate and combined, could not be determined due to the lack of protein synthesis measurements. However, such an assumption would be supported by previous studies showing that resistance exercise alone is unable to induce a positive net balance without the presence of amino acids and that theses two stimuli are additive with regard to the protein synthetic response (9, 10).

The many animal studies identifying leucine as a potent stimulator of mTORC1 would suggest that the observed effects in study I might not have been mediated by the three BCAA combined, but rather by leucine alone. Consequently, study II was undertaken to examine the stimulatory role of leucine using an unconventional study design. Previous studies examining the role of leucine in human muscle used study designs in which additional leucine was provided to an already fully potent protein or amino acid supplement, with no added benefit on the mTORC1 signaling (129) or protein synthesis in young subjects (129-132). While ample evidence exists identifying the potency of leucine, no previous study had compared the effects of all EAA to that of leucine alone. Furthermore, the observation that several other essential amino acids (49), including the BCAA valine (50), were capable of stimulating protein synthesis suggested that these amino acids could have separate or synergistic effects when combined with leucine. Therefore, in addition to comparing leucine and EAA, we also included a trial in which EAA were provided without leucine. This study design would allow us to examine not only the individual contribution of leucine, but also that of the remaining EAA and thus the potentially synergistic effect of them, when combined. In contrast to our hypothesis, provision of leucine alone resulted in a substantially less pronounced signaling response of mTORC1, as assessed by S6K1 phosphorylation, compared to when leucine was provided in combination with the other EAA. This finding suggested that the remaining essential amino acids were indeed important in mediating a stimulatory effect on mTORC1 signaling. Surprisingly, when provided without leucine, the EAA response was no greater than that of flavored water, clearly showing that the individual effect of leucine on mTORC1 is synergistically enhanced by these amino acids. The molecular mechanisms responsible for the amino acid induced mTORC1 response in human muscle still remain unclear as neither of the previously implicated mediators (RagC, Lamtor1 and Rheb) we set out to examine were detected in the immunoprecipitates. It could however be concluded that it did not involve alterations in mTOR and Raptor association which is in contrast to some previous reports using cell culture and non muscle tissue models (52, 133, 134). Whether the synergistic effect seen after combined intake of leucine and the remaining essential amino acids was mediated by the BCAAs valine and isoleucine remains to be determined. Nevertheless, the data gathered from studies I and II clearly indicate that leucine has a major role in mediating the amino acid signal to mTORC1 in human skeletal muscle.

Diverse modes of exercise result in vastly divergent training adaptations which are believed to be mediated by different and potentially opposing molecular pathways. At the centre of

muscle growth adaptations following resistance exercise lies the mTORC1 pathway while a corresponding role in the oxidative adaptations seen after endurance exercise has been identified for the PGC-1α pathway. Interactions between these pathways have been linked to the energy sensing protein AMPK which, in various cell and animal models, has been shown to both stimulate and inhibit signaling through the PGC-1α and mTORC1 pathways, respectively (119-121, 135). The frequent finding that AMPK is robustly activated by acute endurance exercise (104, 136, 137) has implicated AMPK as a potential mediator of the interference effect observed in some long term concurrent training studies with regard to muscle hypertrophy in humans (106, 117, 118). As previous human studies on molecular interference either did not include a resistance exercise only comparison (122, 123) or were performed in the fed state (28, 124), decisive conclusions could not be made regarding the influence of mode specific contractions *per se*.

In an attempt to fill this gap, study III was undertaken to examine the mTORC1 signaling response induced by resistance exercise alone and resistance exercise followed by endurance exercise, performed in the postabsorptive state. The major finding of study III was that both exercise protocols induced robust increases in mTORC1 signaling during recovery, as assessed by S6K1 phosphorylation, and that the response was highly similar between trials. Thus, in contrast to our hypothesis, we could not detect an inhibitory effect on mTORC1 signaling. The reason for a lack of interference is unclear but is likely explained by the lack of increase in phosphorylation, and consequent activation, of AMPK following both exercise protocols. While the AMPK response following resistance exercise appears to be quite variable (93, 138-140), acute endurance exercise has consistently been shown to increase AMPK signaling (104, 136, 137, 141-144), and as such, the lack of increase in AMPK phosphorylation in the concurrent trial was quite surprising. Even more so was the finding that AMPK phosphorylation was actually depressed below baseline values three hours after exercise in both trials. The observation that S6K1 phosphorylation was largely elevated at the same time point suggests that this kinase may be involved in the repression of AMPK phosphorylation at the Thr^{172} residue. Support for this notion is provided by Dagon *et al.* (145) who recently demonstrated that S6K1 can phosphorylate AMPK at the inhibitory Ser^{491} residue in hypothalamic cells of mice. Additional support for S6K1 mediated control of AMPK comes from the study by Aguilar *et al.* (146) in which it was shown that genetic ablation of S6K1 in mouse myotubes resulted in increased AMPK phosphorylation at Thr^{172}.

While not yet confirmed, these studies lend support to the idea that the reduced AMPK phosphorylation observed in study III is indeed a result of increased S6K1 phosphorylation.

While it could be concluded from study III that endurance exercise performed after resistance exercise does not appear to inhibit resistance exercise induced mTORC1 activity, the lack of AMPK activation prevented us from drawing any decisive conclusions regarding the purported role of AMPK as an inhibitor of growth related signaling. Thus, to further explore the potential mechanisms of AMPK mediated inhibition of the mTORC1 pathway, study IV was undertaken. We identified three possible reasons for the lack of AMPK activation in study III. First of all, the fact that endurance exercise was performed after resistance exercise suggests that the exercise sequence may be of importance. Second, while the intensity employed in study III has previously been shown to activate AMPK, it might not have been high enough to elicit a response in our subject population. Lastly, sample collection was performed 15 minutes after endurance exercise in the RE trial which may have been to late, as most studies have noted increased AMPK signaling in muscle collected immediately after exercise. Thus, to maximize the probability of detecting an increased AMPK response in study IV we designed the concurrent protocol so that endurance exercise would not only be performed prior to resistance exercise, but would also be of greater intensity. In addition, biopsy sampling was performed immediately after cessation of muscle contractions in both trials and after both modes of exercise in the ER trial.

As a result of the modified exercise protocol in study IV, we could indeed detect a robust AMPK response elicited by the high intensity interval cycling. However in contrast to study III in which we measured AMPK phosphorylation, in study IV we chose to focus on kinase activity of the two catalytic α subunits instead of AMPK phosphorylation. There are several rationales for this change in analytical endpoints; one is the fact that the two isoforms of the α subunit appear to have diverse metabolic functions and respond differently to endurance exercise (104, 142-144). Another is that the activating Thr^{172} residue is present in both isoforms which does not allow them to be distinguished from one another using available phospho-specific antibodies without prior isolation of each isoform specific subunit. Lastly, phosphorylation status of the Thr^{172} residue does not always reflect *in vivo* AMPK activity (147), which is the most relevant biological function of interest in human muscle.

High intensity cycling induced an almost two-fold increase in AMPK α2 kinase activity, which was maintained at the same elevated level immediately after resistance exercise in the same trial. Concomitant with these elevations, phosphorylation of S6K1 at Thr^{389} was also significantly elevated, reflecting an increase in mTORC1 activity. Interestingly, performance of resistance exercise without prior cycling did not affect AMPK activity but did increase S6K1 phosphorylation to the same extent as after resistance exercise in the ER-trial. These findings indicate that mTORC1 activity is not inhibited despite prior activation of AMPK. As the mechanisms mediating the inhibitory effect of AMPK involve both direct and indirect actions of this kinase, we also measured several of the downstream targets which have been identified as requisite for the inhibition to occur. One indirect mechanism involves the phosphorylation of TSC2 at Ser^{1387} which is believed to result in increased GAP activity towards Rheb, the essential and proximal activator of mTORC1 (68, 69). While the GAP activity of TSC2 was not measured in study IV, phosphorylation of the AMPK site did in fact increase after cycling as well as after resistance exercise, surprisingly, in both trials. The increase in Ser^{1387} phosphorylation after resistance exercise alone was unexpected since AMPK activity did not increase in the R-trial. This suggests that this site may also be targeted by other kinases independent of AMPK. Nevertheless, the phosphorylation status of TSC2 at Ser^{1387} indicates that mTORC1 signaling should have been inhibited after either form of exercise, yet it was not. A direct mechanism by which AMPK is said to inhibit mTORC1 is by phosphorylating Raptor at Ser^{792} (82). Interestingly, phosphorylation of Raptor did not differ between trials despite marked elevations in AMPK activity induced by the cycling protocol. A second direct inhibitory mechanism involves phosphorylation of the catalytic component of mTORC1, namely mTOR itself. When activated, AMPK directly phosphorylates the Thr^{2446} residue which results in blunted phosphorylation of S6K1 downstream of this complex. This blunting coincides with decreased phosphorylation of mTOR at Ser^{2448} by upstream signals such as insulin, indicating that this site is important for the catalytic activity of mTORC1 (83). While the precise function of Ser^{2448} in mTOR remains elusive, phosphorylation of this site is often increased following amino acid provision and exercise (93, 148, 149). In study IV, Ser^{2448} phosphorylation was increased immediately after cycling and continued to increase after resistance exercise in the ER-trial, despite increased AMPK activity. In the R-trial, mTOR phosphorylation increased after resistance exercise to the same extent as in the ER-trial. Thus, while the Thr^{2446} residue was not measured in study IV, the dissimilar AMPK response between trials, yet highly similar and elevated mTOR phosphorylation in both trials, indicates that Thr^{2446} was likely not phosphorylated by AMPK. Briefly, out of the three inhibitory mechanisms that mediate

AMPK derived inhibition of mTORC1, only one appeared to have been activated in response to high intensity cycling. The lack of a differential inhibitory phosphorylation of Raptor at Ser^{792} between trials, and the potential lack of Thr^{2446} phosphorylation may therefore explain the absence of mTORC1 inhibition despite increased AMPK activity. Indeed, in the original study by Gwinn *et al.* (82), in which the inhibitory residue on Raptor was found to be phosphorylated by AMPK, the authors noted that Raptor phosphorylation was required for complete inhibition of mTORC1, thus lending support to the idea that AMPK mediated phosphorylation of TSC2 alone is not sufficient to inhibit mTORC1.

The results discussed above beg the question as to why the robust increase in AMPK activity did not result in increased Raptor phosphorylation. While data obtained from this thesis does not allow for this question to be answered, an interesting observation in study IV was that AMPK activity increased in an isoform specific manner. More specifically, the increase was specific to the α2 isoform while α1 activity remained unchanged at all time points in both trials. This finding may have some bearing on the reason as to why mTORC1 was not inhibited. As noted previously, the two catalytic α isoforms seem to have diverse cellular functions and, consequently, some studies have suggested that the negative regulation of mTORC1, and thus muscle growth, is under the influence of the α1 isoform, not α2. For instance, in a study by McGee *et al.* (150) it was shown that genetic knockout of LKB1, the primary upstream kinase of AMPK in skeletal muscle, reduced AMPK α2 activity to barely detectable levels while the activity of the α1 isoform was unaffected by this manipulation. Interestingly, overload induced muscle hypertrophy increased to a similar extent in wild type mice and in mice with ablated AMPK α2 activity, suggesting that inhibition of this isoform does not affect muscle hypertrophy. Another interesting finding was that the activity of AMPK α1 increased robustly in response to overload in both strains of mice, yet muscle hypertrophy was not only similar between groups but also quite substantial. Thus, muscle growth occurred despite increased α1 activity. These results led the authors to conclude that the α2 isoform is not involved in the regulation of muscle growth and that the α1 isoform may function to limit hypertrophy (150). In subsequent studies, it was shown that the knockout of either AMPK α1 alone (151) or double knock out of both isoforms (152) in cultured myotubes and *in vivo* muscle of mice was associated with an increase in cell and muscle size as a result of increased mTORC1 signaling. In contrast, in the same experimental model, α2 specific knockout had no impact on muscle hypertrophy (153). Collectively, these studies identify the α1 isoform as the primary mediator of AMPK induced inhibition of mTORC1

signaling, thus, potentially explaining the lack of inhibition found in study IV. However, based on additional experimental findings, there may be several objections to this conclusion.

As noted previously, several studies have shown substantial inhibition of mTORC1 signaling and protein synthesis in response to pharmacological activation of AMPK (119-121). In this context, it is important to note that the pharmacological agent used in these studies, the AMP mimetic AICAR, appears to specifically activate the α2 isoform of AMPK (119, 135, 154, 155), which in contrast to the genetic knockout models, instead identifies AMPK α2 as the mTORC1 inhibiting isoform. Furthermore, in human muscle, acute endurance exercise predominantly activates the α2 isoform (104, 136, 141-144), which given this association, is a far more likely candidate to exert inhibition over mTORC1 than AMPK α1. Regardless, there appears to be substantial disagreement as to which isoform is the principal mediator of the inhibitory effect. The reasons for these conflicting results are not clear but may be related to the various experimental models used. It is therefore not unlikely that results based on genetic and pharmacological models in rodents are not representative of the molecular interactions in response to a physiological stimulus such as exercise, in human muscle. The overall interpretations are further complicated by additional studies in humans showing simultaneous increases in AMPK and mTORC1 signaling following endurance (22, 26, 156) and resistance exercise (22, 93) as well as after concurrent exercise (149). Evidently, despite the large number of studies investigating the role of AMPK induced inhibition of mTORC1, the elucidation of the precise interactions and mechanisms by which they are exerted, as well to what extent, requires further study. Nevertheless, from studies III and IV it can be concluded that prior or subsequent endurance exercise does not inhibit resistance exercise induced mTORC1 signaling in human skeletal muscle.

As noted previously, regulatory control of protein synthesis by mTORC1 is exerted mainly through phosphorylation of its downstream targets S6K1 and 4EBP1 (54). One might therefore assume that the mTORC1 specific phosphorylation sites of each downstream target would be affected in a similar manner and extent upon activation of mTORC1 by upstream signals. However, as noted in studies II, III and IV in which phosphorylation of both targets was measured, this is not necessarily the case. In response to each stimulus in each study, i.e. amino acids in studies I and II and exercise in studies III and IV, phosphorylation of S6K1 at Thr^{389} increased dramatically during recovery. In contrast, phosphorylation of the $Thr^{37/46}$

residues of 4EBP1 was more or less unaffected by amino acids in study II as well as by exercise in study III. This differential phosphorylation response was even more explicit in study IV, in which the pattern of S6K1 phosphorylation was completely opposite that of 4EBP1, i.e. an increase was seen in the former and a decrease in the latter immediately after exercise. While somewhat confounding, similar results have been obtained in various experimental models using the mTORC1 inhibitor rapamycin. For a long time, rapamycin was believed to be a specific inhibitor of mTORC1, and consequently, an inhibitor of its downstream targets (157). However, recent studies have shown that treatment of cells with rapamycin may also inhibit mTORC2 signaling under certain conditions (158), but more importantly, that rapamycin differentially regulates S6K1 and 4EBP1. More specifically, S6K1 phosphorylation at Thr^{389} is highly sensitive to rapamycin treatment, whereas phosphorylation of the $Thr^{37/46}$ residues of 4EBP1 appears to be rapamycin resistant in some cell types (159, 160). The mechanisms responsible for these differential effects are unclear but recent studies have provided some insight. The interaction between mTOR and its targets is mediated by Raptor within the complex and both S6K1 and 4EBP1 bind to Raptor through their TOS (TOR signaling) motifs (161). The TOS motifs are conserved amino acid sequences within both targets which are absolutely required for phosphorylation by mTORC1 (162). Interestingly, the TOS motifs differ between S6K1 and 4EBP1 and, moreover, the affinity of 4EBP1 for Raptor is much stronger than that of S6K1 (161, 162). Therefore, in response to upstream signals, even minor modifications of the mTORC1 and S6K1 interaction could potentially result in large fluctuations of the phosphorylation status of S6K1. In contrast, the stronger affinity of 4EBP1 for Raptor may render this interaction insensitive to upstream signals and consequently, changes in phosphorylation status (157). With regard to the findings presented in this thesis, it is noteworthy that in study II, there was only a small increase in 4EBP1 phosphorylation, but more importantly, the increase was amino acid independent. In contrast, phosphorylation of Thr^{389} was substantial and highly amino acid dependent. Interestingly, it was recently shown that the specific sensitivity towards rapamycin is mimicked in response to nutrient and growth factor availability (163). Thus, being sensitive to rapamycin, Thr^{389} is also highly sensitive to amino acids in contrast to 4EBP1 which is rapamycin, and therefore nutrient resistant. These findings may therefore explain the difference in response between S6K1 and 4EBP1 phosphorylation observed in study II. An interesting observation in study IV was that 4EBP1 phosphorylation decreased immediately after exercise. This finding may be interpreted in several ways. First, it does suggest that mTORC1 activity was inhibited, although, the fact that S6K1 phosphorylation increased simultaneously suggests otherwise. While it is difficult to stipulate the direction of

mTORC1 activity, the higher sensitivity of S6K1 phosphorylation suggests that mTORC1 activity was indeed increased. Furthermore, the decrease in phosphorylation of 4EBP1 may also have been a result of increased phosphatase activity towards $Thr^{37/46}$. However, this notion is purely speculative. Nevertheless, the lack of increase above baseline values in studies IV, as well as in study III, suggests that in the basal state, 4EBP1 is highly phosphorylated by mTORC1 *in vivo*. This in turn suggests that S6K1 modulation is the primary mechanism by which amino acids and exercise potentiate the increase in protein synthesis. It should however be noted that the relevant outcome of the 4EBP1 phosphorylation, i.e. the degree of interaction with eIF4E, was not measured in any of the studies presented in this thesis. Interestingly, some studies have found that phosphorylation of the $Thr^{37/46}$ residues results in dissociation from eIF4E (57), while others have found that neither phosphorylation of $Thr^{37/46}$ (55, 56) nor that of Thr^{70} or Ser^{65}, alone or in combination, has any impact of 4EBP1 and eIF4E association (55). Thus, the significance of the $Thr^{37/46}$ residues, and the extent to which they were phosphorylated here, is difficult to appreciate. Nevertheless, the finding that protein synthesis is inhibited by rapamycin in response to amino acids as well as resistance exercise (96-98), coupled with the high sensitivity of S6K1 to rapamycin treatment, supports the idea that S6K1 has a more prominent role in mediating the protein synthetic response. Thus, while not significant, protein synthesis measurements in studies II and IV tended to increase compared to control values, and these patterns were overall reflected by the S6K1 phosphorylation status at the Thr^{389} residue.

In conclusion, amino acids and resistance exercise activate mTORC1 signaling, as assessed by S6K1 phosphorylation, in a synergistic manner. Leucine is crucial in mediating the amino acid response, however, additional amino acids appear to be required to induce a maximal response downstream of mTORC1. Activation of the mTORC1 pathway in response to heavy resistance exercise is robust and this activation does not appear to be inhibited by prior or by subsequent endurance exercise. High intensity interval cycling increases kinase activity of the AMPK α2 isoform but does not inhibit mTORC1 mediated S6K1 phosphorylation, likely due to insufficient phosphorylation of Raptor. As such, these results do not lend support in favour of the molecular interference hypothesis and suggest that resistance exercise and endurance exercise may be combined without detrimental effects on muscle protein synthesis. However, it must be acknowledged that molecular responses during acute interventions may not necessarily reflect long term training adaptations.

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